Sexual cooperation in two male-diphenic *Cardiocondyla* species

DISSERTATION ZUR ERLANGUNG DES

DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.)

DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN

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vorgelegt von

Marion Füßl

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If you watch animals objectively for any length of time, you're driven to the conclusion that their main aim in life is to pass on their genes to the next generation.

– David Attenborough –

Manuscripts that contributed to this Thesis and Author's contribution

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Author's contribution: JH and AS supervised the study. PJO, JR and AS contributed to the manuscript. JR performed the nano-LC-MS/MS-analysis and helped with evaluation of results. Dissection, sample preparation, IEF, 2D-SDS-PAGE, silver-staining and writing the draft of the manuscript was done by MF.

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Author's contribution: AS designed and supervised the study. Data was collected by MF. AS, JH and MF evaluated the data. MF wrote the first draft of the manuscript, on which JH developed the final manuscript.

Contents

Abbreviations (except units, constants and chemical elements)

Name of a species: first letter of the genus in capital letter plus name of the species in lower-case, both in italics. Example: Cardiocondyla (genus) obscurior (species) = *C. obscurior*

DROME	*Drosophila melanogaster* (exclusively Chapter 2, otherwise *D. melanogaster*)
CAROB	*Cardiocondyla* obscurior (exclusively Chapter 2, otherwise *C. obscurior*)
APIME	*Apis mellifera* (exclusively Chapter 2, otherwise *A. mellifera*)

2D-SDS-PAGE	two-dimensional-sodiumdodecylsulphate-polyacrylmide gel electrophoresis
ACP	accessory gland protein
ANOVA	analysis of variance
ATP	adenosine triphosphate
blast	basic logical alignement search tool
bp	basepair
cDNA	complemetary DNA
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
C_q	quantification cycle (= treashold cycle C_t)
CRISP	cysteine rich secretory proteins
DNA	deoxyribonucleic acid
DTT	DL dithiothreitol
EBSP	see PEB-me
ER	endoplasmatic reticulum

EST	expressed sequence tag
fcv	fold change value
Ghitm	Growth-hormone inducible transmembrane protein
IAA	iodoacetamide
IEF	isoelectric focusing
IPG	immobilized pH gradient
IPTG	isoproyl β-D-1-thiogalactopyranoside
LB	lysogeny broth
LC-MS/MS-analysis	liquid chromatography-mass spectrometry/mass spectrometry-analysis
LRR	Leucin rich repeat
MAG	male accessory gland
mRNA	messenger RNA
NCBI	national center of biotechnology information
NHERF	Na^+/H^+ exchange regulatory cofactor
NL	non-linear
nr	non-redundant
PBS	protein biosynthesis
PCR	polymerase chain reaction
PDIA3	protein disulfide isomerase A3
PEB-me	ejaculatory bulb specific protein (of *Drosophila melanogaster*)
PSA	prostate specific antigen
ptuf	see SPS
qPCR	quantitative real-time PCR
RDA	representational difference analysis
RNA	ribonucleic acid

ROS	reactive oxygen species
SECIS	selenocysteine insertion sequence
SELA-D	cofactors in bacterial selenoproteine-biosynthesis
SERPIN	serine protease inhibitor
SFP	seminal fluid protein
SOD	superoxide dismutase
SP	sex peptide
SPS, *sps*, *ptuf*	selenophosphate synthetase
SRY	sex-determining region Y
Tris-HCl	tris(hydroxymethyl)-aminomethane
US	unique sequence
UTR	untranslated region
v/v	volume per volume
w/v	weight per volume

Chapter 1

General Introduction

1.1 Early theories of the evolution of eusociality

When Darwin first published his book "on the origin of species by means of natural selection" in the year 1859 [Darwin, 1859], he wouldn't obscure that his theory of natural selection still posed him an enormous riddle: the sterile female worker caste of social insects. Why would an individual forgo its own reproduction, or even die for the sake of the colony? And how could these workers be so different from fertile males and females concerning "morphology and instinct"? He was puzzled by "how such correlated modifications of structure could have been slowly accumulated by natural selection", as sterile workers "could never have transmitted successively acquired modifications of structure or instinct to its progeny" ([Darwin, 1859],Chapter 7). Despite Gregor Mendel being a contemporary of Darwin, the latter had probably no knowledge of the discoveries of the former [Galton, 2009, Sander, 1988]. Mendel's idea of factors (genes) transmitting genetic information from one generation to the next [Mendel, 1866] came to popularity and great acceptance only several years after the death of Darwin in the year 1882. At Darwin's time the modern knowledge of different gene expression or genomic imprinting as means to shape different morphologies were still unknown. Nevertheless, Darwin's suggestion to resolve his major problem was that slight changes in a sterile worker could be passed to the next generations via its fertile parents ([Darwin, 1859],Chapter 7). Thus, he was the first to think of a transfer of genetic information via closely related kin, even though he could not term it like this. It was W. D. Hamilton who emphasized this thought more detailed in his famous publications "the genetic evolution of social behaviour" one century later [Hamilton, 1964]. Hamilton's theory of kin selection referred to close relationship among nest mates as basis for the evolution of eusociality. The crucial point in his theory was that some individuals could forgo reproduction if they would help therefore closely related kin [Maynard Smith, 1972]. According to the theory, an individual may be naturally selected for being an altruist, if the benefit of an altruistic behaviour outweighs the costs of this behaviour to the donor times the relationship to the recipient [Hamilton, 1964, Trivers & Hare, 1976, West *et al.*, 2002]. Even though in 2010 Nowak and colleagues provoked new discussion as they published an article where they strongly rejected this theory [Nowak *et al.*, 2010, Nowak *et al.*, 2011], kin selection is still accepted by many other scientists (e.g. [Abbot *et al.*, 2011]). In the next section I will present the normal life-cycle of a eusocial insect society.

1.2 Eusocial insect life history

In eusocial Hymenoptera (ants, bees and wasps), mating takes place only during a short period early in a queen's life, most commonly during a brief nuptial flight. Usually queens mate only once or a few times, and effective mate number remains close to one, which means that only one male fathers the entire offspring ([Boomsma & Ratnieks, 1996, Foster *et al.*, 1999, Strassmann, 2001] but see [Barth *et al.*, 2014, Kronauer *et al.*, 2004, Villesen *et al.*, 2002]). Only a few exceptions are known, where queens mate excessively, like in the honey bee, leaf cutter ants or army ants [Barth *et al.*, 2014, Boomsma & Ratnieks, 1996, Kronauer *et al.*, 2004, Strassmann, 2001, Villesen *et al.*, 2002]. After the mating period young queens store the received sperm, in a special organ, called spermatheca, for the rest of their life [Boomsma *et al.*, 2005, Hölldobler & Wilson, 1990, Wilson, 1971].

Males normally die after having inseminated one to several females and persist only as stored sperm [Baer, 2011, Boomsma *et al.*, 2005, Hölldobler & Wilson, 1990, Wilson, 1971]. The father of an insect society plays a minor role and does not take part in the establishment of a new colony. It is the role of the queen to found a new colony by either founding alone, founding in groups with other freshly inseminated young queens, or by budding which means that the young queen leaves her maternal nest with some workers [Heinze *et al.*, 2006]. In any of these cases, she has to rear some worker progeny first, before she is able to rear sexual progeny, a phenomenon that is commonly known as late reproduction. In social Hymenoptera arrhenotokous parthenogenesis (haplodiploidy) as sex determining mechanism is prevalent [Trivers & Hare, 1976], meaning that fertilized (diploid) eggs develop into females and unfertilized (haploid) eggs into males [Cook, 1993, Heimpel & de Boer, 2008, Van Wilgenburg *et al.*, 2006]. A colony's father's genes are transferred to the next generations only via female sexual offspring, the young queens [Baer, 2003], as workers normally do not reproduce and males do not have a father. The males' fitness is directly correlated to the queens' lifespan, as the longer she lives, the more offspring, including female reproductive offspring, she will produce. Due to this life history and the lifelong pair bonding sexual cooperation is expected, as it is deleterious for both genders to harm the other [Baer & Boomsma, 2004, Bourke & Franks, 1995, Greeff & Schmid-Hempel, 2008, Rice, 2000, Schrempf *et al.*, 2005]. In the next section I would like to demonstrate the situation in solitary insects, with special emphasis on the importance of accessory gland proteins in general and their influence on females' longevity in particular. I highlight that a promiscuous lifestyle may lead to sexual conflict and outline the assumptions for eusocial insects.

1.3 Seminal fluid, accessory gland proteins and longevity

During mating, accessory gland proteins (ACPs) are transferred to the female alongside with sperm [Bairati, 1968, Chapman, 1992, Findlay *et al.*, 2008, Ravi Ram *et al.*, 2005, Terranova *et al.*, 1972, Wolfner, 1997]. These ACPs are a major component of the seminal fluid of animals which is a varying composition of different substances: fatty acids, sugars, carbohydrates, pro-hormone precursors, glycoproteins, proteases, protease inhibitors, lipases, antimicrobial proteins, thioredoxins, oxidoreductases, lectins and cysteine rich secretory proteins (CRISPs) [Andrés *et al.*, 2006, Avila *et al.*, 2011, Baer *et al.*, 2009b, Braswell *et al.*, 2006, Chapman & Davies, 2004, Collins *et al.*, 2006, Dottorini *et al.*, 2007, Findlay *et al.*, 2008, Fung *et al.*, 2004, Gillott, 2003, Poiani, 2006, Ravi Ram *et al.*, 2007, Walters & Harrison, 2010, Wolfner, 2002]. Its composition varies between species and within species [Baer *et al.*, 2009b, Braswell *et al.*, 2006, Chen, 1976, Cornwallis & O'Connor, 2009, den Boer *et al.*, 2008].

In many insect species it has been shown that ACPs greatly influence a females post mating behaviour and physiology in various manners [Avila *et al.*, 2011, Chapman & Davies, 2004, Chen, 1984, Wolfner, 1997, Wolfner *et al.*, 1997]. They can influence the females' remating behaviour [Andersson *et al.*, 2000, Chen *et al.*, 1988, Craig, 1967, Fuchs *et al.*, 1969, Manning, 1962, Sauter *et al.*, 2001], oviposition stimulation [Chen, 1984, Chen & Bühler, 1970, Herndon & Wolfner, 1995, Lange & Loughton, 1985, Leopold, 1976, Loher *et al.*, 1981, Maklakov *et al.*, 2005, Ramalingam & Craig Jr, 1978], the sperm storage process [Chapman *et al.*, 2000, Neubaum & Wolfner, 1999, Tram & Wolfner, 1999], sperm viability and sperm competition [Chapman *et al.*, 2000, den Boer *et al.*, 2010, den Boer *et al.*, 2008], lifetime fecundity and life-expectancy [Chapman *et al.*, 1995, Lung *et al.*, 2002, Poiani, 2006] and may cause a juvenile hormone induced down regulation of the immune system [Rolff & Siva-Jothy, 2002].

The ACPs of insects are best studied in the solitary and promiscuous fruit fly *Drosophila melanogaster*. At least 128 different ACPs have been identified in the last few decades [Findlay *et al.*, 2008]. Since its genome was released in 2000 [Adams *et al.*, 2000] new genes for ACPs could be identified and ascribed to a function more easily. In this insect the male ACPs influence the lifespan of a female negatively in a dose dependent manner [Chapman, 1992, Chapman *et al.*, 1995, Chapman & Partridge, 1996, Fowler & Partridge, 1989, Rice, 1996], but a female has to remate to replenish her sperm supply as she - different to social insects - cannot store sperm for her whole lifetime. It is not in the interest of the male, that the female remates, because the sooner she remates, the less offspring will be sired by the first male as the female would use the "new" sperm [Rice, 2000]. So the male tries to manipulate the female via his seminal fluid proteins (SFPs) and the females tries to escape these manipulations.

Generally, when sexual interests between the genders diverge, a circle of adaption and counter adaption is often the consequence. This may culminate in sexual conflict [Arnqvist *et al.*, 2000, Arnqvist & Rowe, 2002, Dawkins, 1976, Findlay *et al.*, 2008, Morrow & Arnqvist, 2003, Parker *et al.*, 1979, Rowe *et al.*, 1994, Stutt & Siva-Jothy, 2001] which may even promote speciation ([Aguadé *et al.*, 1992, Andrés *et al.*, 2006, Arnqvist *et al.*, 2000, Chapman *et al.*, 2003a, Clark & Swanson, 2005, Marshall *et al.*, 2011, Rice, 2000] but see [Cordero & Eberhard, 2003]). Among ACPs and female reproductive proteins are genes that are evolving very rapidly, suggesting that they are under positive Darwinian selection [Civetta & Singh, 1995, Coulthart & Singh, 1988a, Coulthart & Singh, 1988b, Ramm *et al.*, 2008, Swanson *et al.*, 2001a, Swanson *et al.*, 2001b, Swanson *et al.*, 2004, Torgerson *et al.*, 2002]. This may specially account for genes that are involved in this sexual conflict. For example in *Drosophila*, only about 50 % of the ACPs have a homologue with non-*Drosophila* sequences [Swanson *et al.*, 2001a]. Other ACPs are not evolving so fast or are even conserved, suggesting some functional constraint [Mueller *et al.*, 2004, Mueller *et al.*, 2005, Ravi Ram & Wolfner, 2007a], which is probably the case for genes that code proteins which are necessary to maintain successful reproduction.

The composition of the ACPs influences the lifespan in many insects (e.g. fruit flies, bumble bees, mosquitoes, field crickets, butterflies) but not always in the same manner [Greeff & Schmid-Hempel, 2008, Liles, 1965, Taylor *et al.*, 2008]. While ACPs negatively influence the lifespan of some solitary insects such as e.g. female fruit flies [Chapman *et al.*, 1995, Lung *et al.*, 2002, Poiani, 2006, Rice, 2000], they may positively affect females' fitness and lifespan in spermatophore transmitting butterflies and crickets [Karlsson, 1998, Wagner *et al.*, 2001, Wiklund *et al.*, 2001]. Spermatophores or nuptial gifts are absorbed by the female's genital tract and may serve as nutrients. While it seems logical that a female lives longer when mating more often and thus receiving more of these substances, Wagner and colleagues could show that the life-prolongation in crickets is independent from receiving a nutritious spermatophore [Wagner *et al.*, 2001]. Similarly, in mosquitoes (*Aedes*) without nuptial feeding [Clifton *et al.*, 2014], mated females show an enhanced lifespan [Liles, 1965].

In social insects, a positive effect of mating has been shown in *Cardiocondyla* ants, where mated queens live longer than virgin queens [Schrempf *et al.*, 2005]. In social Hymenoptera we do not expect a sexual arms race, as the extraordinary life-cycle with late reproduction predicts sexual cooperation instead of conflict [Rice, 2000]. Thus we expect the ACPs to evolve more slowly than in insects with a perpetual antagonistic coevolution, or to be even conserved among species.

The next section deals with the presentation of the genus *Cardiocondyla* and our study species *C. obscurior* and *C. tjibodana* with special emphasis on the two different male phenes.

1.4 The study species *Cardiocondyla obscurior* and *Cardiocondyla tjibodana*: their life-cycle and its male phenes

Our two study species, *C. obscurior* ([Wheeler, 1929]; previously referred to as *C. wroughtonii*, Forel, 1890) and *C. tjibodana* Karavaiev, 1935 belong to the ant genus *Cardiocondyla*. These species are both polygynous [Heinze & Delabie, 2005, Heinze *et al.*, 2005] which means, that multiple queens co-occur in one nest (see Figure 1.1a). Colonies are usually small [Heinze *et al.*, 2006]. Some members of this genus are tramp species which have become established widely across the globe [Heinze *et al.*, 2006, Kugler, 1983, Seifert, 2003, Wetterer, 2012], and show the typical adaptions as polygyny, intranidal mating, worker sterility and colony founding via budding [Heinze *et al.*, 2006, Kinomura & Yamauchi, 1987]. While *C. obscurior* is one of these species with a distribution in the tropics and subtropics, *C. tjibodana* occurs predominantly in South East Asia and has not yet been described as tramp species [Clouse, 2007, Pfeiffer *et al.*, 2011, Wetterer, 2014], even though it shows all mentioned adaptations (see Figure 1.1a and 1.1b).

One reason why I have chosen members of this ant genus for my studies is the occurrence of a fascinating and striking male diphenism (see Figure 1.1c). Beside the peaceful "normal" winged males of other insect species, wingless ergatoid (worker-like) fighter males occur [Heinze *et al.*, 2004, Kugler, 1983, Stuart *et al.*, 1987]. These wingless fighter males are a rare phenomenon in insects and do co-occur with peaceful winged males only in some fig wasps [Cook *et al.*, 1997, Greeff *et al.*, 2003, Hamilton, 1979, Murray, 1990] and bee species [Danforth, 1991, Kukuk & Schwarz, 1988]. Some *Cardiocondyla* species do only have ergatoid males, which is the derived trait, but in *C. obscurior* and *C. tjibodana*, both male types co-occur, which is the ancestral form [Heinze *et al.*, 2005] (see Figure 1.1b). In both species the winged male phene appears like the typical disperser phene of other ant species, with wings on a bulky thorax, well developed eyes and long antennae [Kugler, 1983, Stuart *et al.*, 1987] (see Figure 1.1c). As their testes degenerate within a few days after eclosion (Heinze et al, 1998), they have a limited sperm supply as usual for Hymenoptera males, which is sufficient for some matings, but cannot be replenished [Heinze *et al.*, 1998, Heinze *et al.*, 2005, Hölldobler & Wilson,

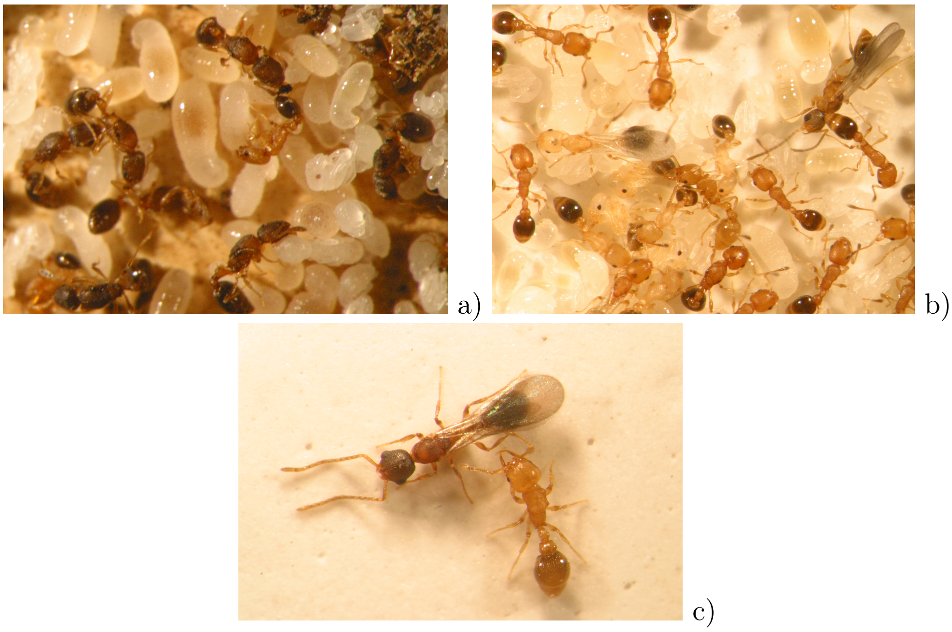

Figure 1.1: a) Nest of *C. tjibodana* with multiple queens. b) Nest of *C. obscurior* with an old queen, a young queen (with wings), an ergatoid male (in the middle), a winged male (right upper corner), workers and brood c) Ergatoid male (amber-coloured) and winged male of *C. obscurior*.

1990, Hölldobler & Bartz, 1985]. Even though winged males appear almost the same in these two species, they differ in terms of behaviour and occurrence. While winged males occur regularly in colonies of *C. tjibodana* (pers. observation), they are rare in *C. obscurior*. In the laboratory, their production can be induced by changing temperature, colony splitting [Cremer & Heinze, 2003, Heinze *et al.*, 2004] and permanent light (pers. observation). In *C. obscurior*, they engage in intranidal mating, before they leave their mother's nest about ten days after eclosion, presumably to mate outside with a queen that founds a new colony elsewhere [Heinze *et al.*, 1998, Kinomura & Yamauchi, 1987]. Under stable "normal" conditions, only one ergatoid male is present.

Generally, ergatoid males have a lifelong ongoing spermatogenesis which is unique in eusocial insect males, and their testes remain fully developed during their whole adult life [Heinze & Hölldobler, 1993, Heinze *et al.*, 1998] (see Figure 1.2). They stay livelong inside the nest, mate intranidal with young queens and engage in lethal fighting [Heinze *et al.*, 1998, Kinomura & Yamauchi, 1987, Stuart *et al.*, 1987]. In *C. obscurior* and *C. tjibodana* they have in common, that they appear worker-like, with worker-like eyes, antennae and a not specially developed thorax [Kugler, 1983, Stuart *et al.*, 1987]. A

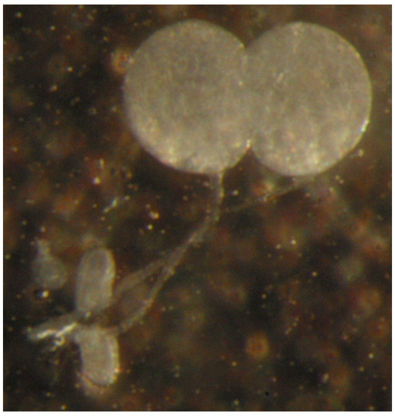

Figure 1.2: Reproductive organs of an ergatoid *C. obscurior* male with accessory glands (left lower corner) and testes

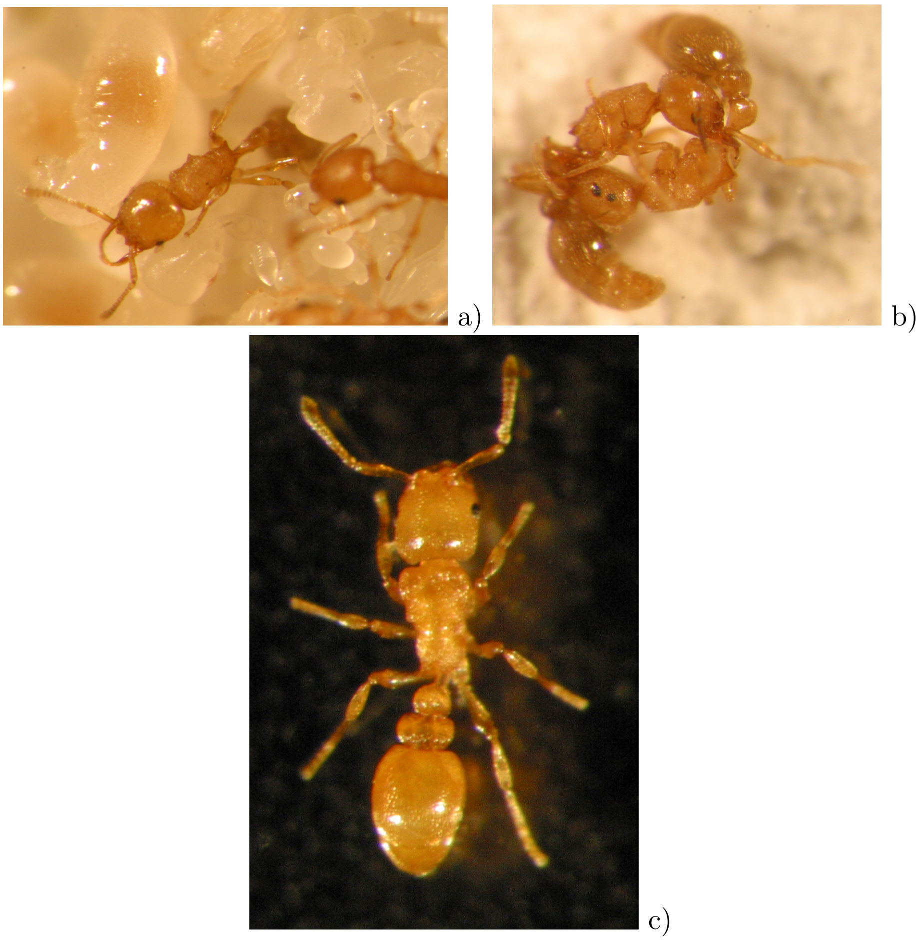

Figure 1.3: a) ergatoid male of *C. obscurior* searching the brood pile for a rival male pupae. b) adult ergatoid *C. obscurior* males, engaged in fighting. c) ergatoid male of *C. tjibodana*

notable difference between the ergatoid males of *C. obscurior* and *C. tjibodana* lies in the shape of the mandibles and the general behaviour. The mandibles of *C. obscurior* are sickle-shaped and that one's of *C tjibodana* are shear-shaped [Oettler *et al.*, 2010]. In both species ergatoid males do not tolerate rivals. Wingless males of *C. obscurior* patrol through the brood pile (see Figure 1.3a) and search for ergatoid pupae or other newly eclosed wingless males to engage in a lethal fight, where the rival is killed (see Figure 1.3b). Older males with a hardened cuticle cannot be killed easily, and if two rivals meat each other they try to grab the other with the mandibles and to besmear the rival. Subsequently, workers kill the besmeared male [Kinomura & Yamauchi, 1987, Stuart *et al.*, 1987, Yamauchi & Kawase, 1992]. As a consequence, there is normally only one male in the colony. Winged males are save from the attacks of ergatoid males as they mimic the odour of workers for about the first ten days of their lifes [Cremer *et al.*, 2002]. The ergatoid male phene occurs regularly in *C. obscurior*, but is rare in *C. tjibodana.* In the latter species the wingless males are – like e.g. in *C. nuda* [Heinze *et al.*, 1993] – not able to kill adult males as their mandibles are not strong enough and they do not besmear each other (see Figure 1.3c). Males search the brood pile for pupae of rivals, which they kill by disrupting their soft shell. In case they have overlooked one pupae, there may be more than one male per colony [Heinze *et al.*, 1993], which is a very seldom case, as ergatoid males are unfrequent anyway in this species. Winged males are tolerated by ergatoid in males *C. tjibodana.*

The genus *Cardiocondyla* is very well suited to investigate fecundity, ageing and fitness of queens, as they live only up to one year and hence can be observed during their whole lifetime. According to their intranidal mating, it is easy to get them mated in the laboratory. In *C. obscurior* it has been shown, that queens mated to a winged male live significantly longer than queens mated to an ergatoid male, and ACPs are presumed to play a role thereby [Schrempf & Heinze, 2008]. For *C. tjibodana* data on longevity of males and females were lacking (see Chapter 5).

The question how the ACPs might causally influence the longevity is discussed in the next section of the introduction. I will give a short overview over the most important theories of ageing in different fields.

1.5 Ageing theories, a very brief overview

The question arose why and how do we age and die at all [Kirkwood & Austad, 2000]? Up to date there are countless theories of ageing [Gavrilov & Gavrilova, 2002, Le Bourg, 2001, Ljubuncic & Reznick, 2009, Rose *et al.*, 2008], which are compiled in an own encyclopedia [Ekerdt, 2002]. Weinert and Timiras differentiated four classes of ageing theories in a review upon this topic [Weinert & Timiras, 2003]: a) Evolutionary theories (e.g. theory of programmed death, mutation accumulation theory, theory of antagonistic pleiotropy and disposable soma theory) and mechanistic theories as b) cellular theories (e.g. free radical theory), c) molecular theories (e.g. theory of error catastrophe), and d) systemic theories (e.g. rate-of-living theory). Within this section, I would like to give a brief impression over the mentioned theories of ageing.

Not even a decade after Charles Darwin's death, in 1891 August Weisman published his "Essays upon heredity and kindred biological problems" [Weismann *et al.*, 1891]. In this early theory he postulated that senescence and death in particular is a part of evolution and might be necessary to literally make place for new and younger individuals [Weismann *et al.*, 1891]. He further suggested that an individual would have only a limited number of cell divisions for somatic cells as kind of a "death mechanism". Ageing and death was to help the species and not necessarily a single individual [Weismann *et al.*, 1891]. His ideas were the cornerstone for later theories as e.g. the rate-of-living-theory [Pearl, 1928], which is also one of the earliest theories of ageing and was published in 1928 by Pearl [Pearl, 1928]. The idea of this theory was, that organisms might have a fix amount of energy for their life. If this energy is spent, the organism would die. "In short, the length of life depends inversely on the rate of living" ([Pearl, 1928] p. 151). For Pearl this was a "working hypothesis" and not a final truth, as he states quite modest with: "I am the last person in the world to hold these conclusions to be at this stage final and incapable of change" ([Pearl, 1928] p. 151).

A similar theory is the disposable soma theory, proposed by Kirkwood in the year 1977 [Kirkwood, 1977]. He emphasizes a strong trade-off between the germ-line and soma of an organism, and states that after reproduction the soma becomes "disposable". An individual should seek an optimal balance of energy investment between soma maintenance and reproduction, as energy invested for the one would take off this energy from the other.

The mutation accumulation theory, published by Medawar in 1946 [Medawar, 1946], considers an accumulation of late acting harmful genes. Genes, that show a negative effect at old age only, are not selected against by natural selection, because they are detrimental only in an individual's post reproductive period, and may thus accumulate passively [Charlesworth, 2000,Medawar, 1946]. In 1957 Georges C. Williams came up with a theory, that was later on termed "antagonistic pleiotropy" by Rose [Rose, 1982,Williams, 1957], which was an extrapolation of the idea of "mutation accumulation" [Charlesworth, 2000]. In particular, Williams suggested that a gene might bring some fitness benefits to an organism in youth, but the same gene would bring negative effects at old age [Williams, 1957]. The main difference between these two theories is that according to the mutation accumulation theory, harmful genes accumulate passively, whereas they are accumulated actively according to the pleiotropic genes theory.

In 1955 Harman wrote the article: "Aging: A theory based on free radical and radiation chemistry", where he states that ageing may be a result of accumulation of oxidative damage to cells and tissues [Harman, 1955]. Free radicals occur as a by-product of aerobic metabolism and have the potential do inflict great harm to biological macromolecules as lipids, DNA and proteins. Oxidized or cross linked proteins and seriously damaged DNA-molecules may be the consequence [Berlett & Stadtman, 1997]. Enhanced antioxidant expression or decreased production of free radicals may influence a species lifespan. Reduced calorie intake and slower metabolism may reduce the ROS production and thus increase lifespan [Finkel & Holbrook, 2000,Schulz *et al.*, 2007,Spriggs *et al.*, 2010,Zuin *et al.*, 2010].

In the error catastrophe theory it is supposed that, similarly to the mutation accumulation theory, errors occur and accumulate with time, but on a molecular level. The decreasing fidelity of gene expression with age would lead to aberrant proteins and thus lead finally to the death of the cell. So the loss of accuracy for cellar mechanisms as DNA replication and protein synthesis with age would be responsible for cellular senescence and death [Orgel, 1963].

A lot of ageing studies were performed in short lived animals such as *C. elegans* and *D. melanogaster* [Keller & Jemielity, 2006]. Only recently scientists started to have a closer look to queens of long living eusocial insects such as ants, bees and wasps (e.g. [Heinze & Schrempf, 2008, Keller & Genoud, 1997]). It is necessary to mention that in social insects there is no trade-off between reproduction and lifespan [De Loof, 2011, Hartmann & Heinze (2003)]. Following the ageing theories – specially rate-of-living theory and disposable soma theory – one would predict, that an individual should die the sooner the more offspring it has. Reproductive individuals in a social insect society should die earlier than not reproducing individuals, but the contrary is the case. Queens and workers are genetically identical but differ greatly in lifespan. Some ant queens may live for some decades [Keller, 1998, Keller & Genoud, 1997] while their worker- sisters or -daughters live only for a few weeks. Even more demonstrative is it with males, which are 100 % related to the queen (if they did not develop from worker-laid eggs) but live only for a few days in most of social Hymenoptera.

Epigenetic mechanisms such as chromatin remodelling by DNA-methylation or genetic imprinting are just at the very beginning to be considered as possible reasons for differences in lifespan and polyphenism among insect castes, but may help to further understand the mechanisms underlying such remarkable difference in lifetime [De Loof, 2011, Wedell *et al.*, 2006].

1.6 Aims of the Thesis

With my research I wanted to clarify the role of the male accessory gland proteins in the life-prolonging effect of queens in the male-diphenic ant species *C. obscurior*. A comparison of the protein pattern of *C. obscurior* with *C. tjibodana* should lead to a further comprehension of the possible function of these male derived proteins. Data on queen longevity of *C. tjibodana* should be collected, for a comparison with *C. obscurior*, and to allow me to speculate on a relation between varying protein patterns, sexual cooperation and reproductive tactic in these two *Cardiocondyla* species.

In **Chapter 2**, I present the data on queen longevity, egg laying rate and male survival rate of *C. tjibodana* and compare them with *C. obscurior* and other *Cardiocondyla* species.

In **Chapters 3** and **4** the result of the analysis of the male accessory glands on the protein level by two-dimensional-SDS-polyacrylamide gel electrophoresis (2D-SDS-PAGE) are shown. The protein pattern of the male phenes of *C. obscurior* and the male phenes of

C. tjibodana are compared with each other on an intra- and inter-species level (Chapter 3). Chapter 4 shows that selenophosphate synthetase 1 is present as a protein in *C. obscurior* and emphasizes the possible role of this protein with regard to oxidative stress and ageing.

To determine differences in the gene expression level of the accessory glands of winged males compared to ergatoid males, I did a representational difference analysis (RDA). The results are discussed in **Chapter 5**.

Chapter 2

Suppression subtractive hybridization reveals differentially expressed genes in the accessory glands of two ant male phenes with different life histories*

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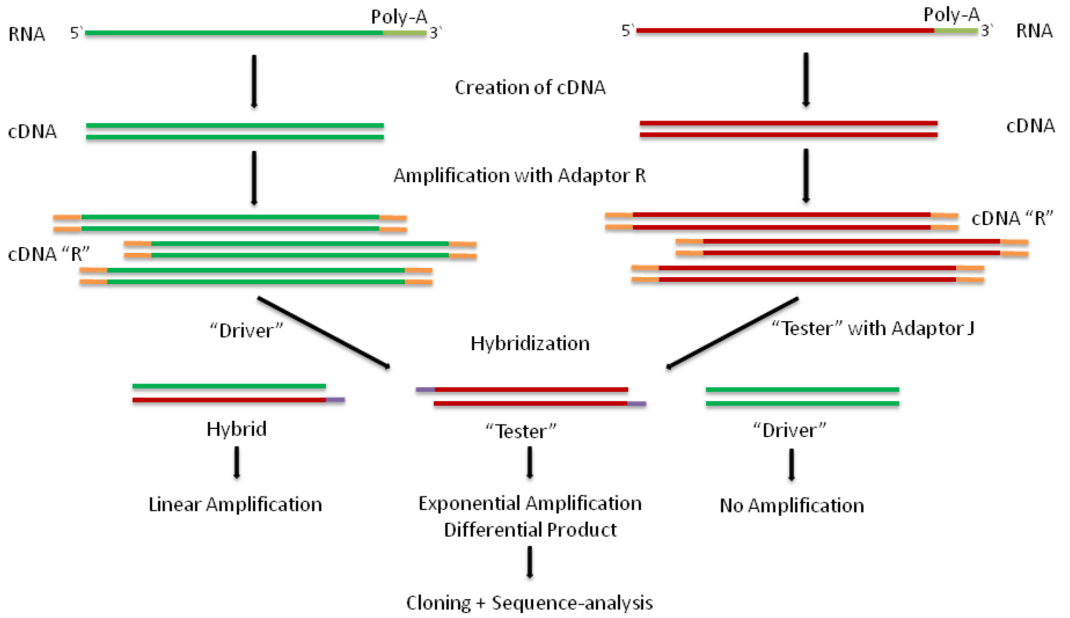

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Abstract

Seminal fluid proteins (SFPs) are transferred alongside with sperm during mating. These proteins are known to induce various changes in the female's physiology. For example, they can affect the remating rate, oviposition and lifetime fecundity of females, and may even influence the lifespan of the mating partner. In the promiscuous fruit fly *Drosophila melanogaster*, it has been shown that life of mated females is shortened in dependence of the received lifetime dose of some peculiar accessory gland proteins (e.g. Acp62F). On the other hand, life can also be prolonged in species where females receive nutritious nuptial gifts from their mating partners. In our study species, the ant *Cardiocondyla obscurior*, data on the composition of the seminal fluid are lacking so far. This ant species shows a male diphenism with winged disperser males and wingless fighter males which stay inside the maternal nest. Male phene affects queen lifespan differently, as a queen that mated with a winged male lives longer than a queen that mated with a wingless male, while both live longer than virgin queens. Hence, seminal fluids seem to have a positive effect on queen lifespan, and even more beneficial in the winged phene. In this study, we analysed the accessory gland proteins (ACPs) of the two male phenes by a suppression subtractive hybridization analysis and found several genes to be differentially expressed, i. a. proteins that are discussed with regard to oxidative stress defence and sperm production.

Keywords: representational difference analysis, cDNA-library, serpin, ejaculatory bulb specific protein

2.1 Introduction

During copulation, seminal fluids are usually transferred to the female, alongside with sperm [Bairati, 1968, Findlay *et al.*, 2008, Terranova *et al.*, 1972]. This fluid consists of fatty acids, sugars, carbohydrates, prohormone precursors, glycoproteins, proteases, protease inhibitors, lipases, antimicrobial proteins, thioredoxins, oxidoreductases, lectins and cysteine rich secretory proteins (CRISPs) [Andrés *et al.*, 2006, Avila *et al.*, 2011, Baer *et al.*, 2009b, Braswell *et al.*, 2006, Chapman & Davies, 2004, Collins *et al.*, 2006, Dottorini *et al.*, 2007, Fung *et al.*, 2004, Gillott, 2003, Poiani, 2006, Ravi Ram *et al.*, 2007, Walters & Harrison, 2010, Wolfner, 2002]. In insects the vast majority of the seminal fluid proteins (SFPs) are synthesized and secreted in the male accessory glands (MAGs) [Chapman, 2001, Chapman & Davies, 2004, Ingman-Baker & Candido, 1980] and are also named accessory gland proteins (ACPs). It has been shown that these ACPs influence the females' behaviour and physiology in various manners [Chapman & Davies, 2004, Chen, 1984, Colonello & Hartfelder, 2005, Gillott, 2003, Wolfner, 1997, Wolfner, 2002]. They can e.g. influence the females' remating behaviour [Chen *et al.*, 1988, Craig, 1967, Fuchs *et al.*, 1969, Manning, 1962, Sauter *et al.*, 2001], oviposition stimulation [Chen, 1984, Chen & Bühler, 1970, Herndon & Wolfner, 1995, Leopold, 1976, Maklakov *et al.*, 2005, Ramalingam & Craig Jr, 1978], the sperm storage process [Neubaum & Wolfner, 1999, Tram & Wolfner, 1999], sperm viability and sperm competition [Chapman *et al.*, 2000, den Boer *et al.*, 2010], lifetime fecundity and life-expectancy [Chapman *et al.*, 1995, Lung *et al.*, 2002, Poiani, 2006, Rice, 2000].

In the well-studied promiscuous fruit fly *Drosophila melanogaster*, more than 100 ACPs are described until today [Avila *et al.*, 2011, Chapman, 2001, Ravi Ram *et al.*, 2007]. In polygamous mating systems interests between the genders diverge. Clear evidence exists that after having received these proteins, females are less attractive to males and they also show a reduced receptivity to further matings [Chapman *et al.*, 2003b] and an enhanced egg-laying rate [Chen, 1976, Wolfner, 1997]. As a deleterious side-effect, the females suffer from a reduced lifespan compared to virgins or females that had mated less often [Chapman *et al.*, 1995, Fowler & Partridge, 1989, Lung *et al.*, 2002]. A female has to mate from time to time to get enough sperm to fertilize her eggs, and during each mating act she receives ACPs [Chapman *et al.*, 1995]. For males it is advantageous to manipulate the females in favour of their own offspring, as the later the female remates, the more offspring will be fertilized with one male's sperm. As a matter of fact, an arms race with perpetual sexual antagonistic coevolution [Holland & Rice, Rice, 2000] and a rapid evolution of the ACPs is the consequence [Aguadé *et al.*, 1992, Civetta & Singh, 1995, Coulthart & Singh, 1988a, Coulthart & Singh, 1988b, Ramm *et al.*, 2008, Rice, 1996, Swanson *et al.*, 2001a], which may even promote speciation events [Andrés *et al.*, 2006, Arnqvist *et al.*, 2000, Chapman *et al.*, 2003a, Clark & Swanson, 2005, Marshall *et al.*, 2011, Swanson & Vacquier, 2002, Swanson *et al.*, 2001b]. Contrary, the functional classes of SFPs are pretty conserved among different insect taxa [Avila *et al.*, 2011, Braswell *et al.*, 2006, Coulthart & Singh, 1988a, Dorus *et al.*, 2006, Findlay *et al.*, 2008, Gillott, 2003, Haerty *et al.*, 2007, Mueller *et al.*, 2005, Mueller *et al.*, 2004, Ravi Ram *et al.*, 2007, Swanson *et al.*, 2001a, Swanson & Vacquier, 2002, Walters & Harrison, 2010].

In other solitary insects with nuptial feeding (via vagina absorbed high energetic ejaculates) like field crickets and butterflies, females may even benefit from multiple mating [Andersson *et al.*, 2000, Andrés *et al.*, 2006, Arnqvist & Nilsson, 2000, Wagner *et al.*, 2001]. They show a higher lifetime fecundity [Arnqvist & Nilsson, 2000, South & Lewis, 2011, Wagner *et al.*, 2001, Wedell & Karlsson, 2003] and a prolonged lifespan [Karlsson, 1998, Wagner *et al.*, 2001], even when spermatophores have been removed after mating [Wagner *et al.*, 2001]. Still, rapid evolution of the reproductive proteins was found (Andrés et al, 2006; Walters & Harrison, 2010), which led Wagner and colleagues to assume that there is mutualistic instead of antagonistic coevolution (Braswell et al, 2006; Wagner et al, 2001), suggesting that sexual conflict is not exclusively the driving force for a rapid evolution of ACPs [Andrés *et al.*, 2006, Walters & Harrison, 2010].

Eusocial insects (e.g. ants, bees, wasps) have a different life history. Queens mate once or several times during a brief period early in life [Strassmann, 2001]. A queen does not remate later in life and stores the received sperm in a special storage organ, the spermatheca, from which she takes the sperm to fertilize the eggs for the rest of her life [Wilson, 1971]. Hence, although sperm competition and conflict between different males might occur, this lasts only for the short period directly after mating [Baer & Boomsma, 2004, Boomsma, 1996]. After storage, the sperm is protected by the spermathecal fluid [Baer *et al.*, 2009a]. In case a queen founds a new colony independently (i.e. without the help of workers) she will have to raise some worker brood before she produces sexual brood such as queen and male pupae [Hölldobler & Wilson, 1990]. Due to this late reproduction and converging interests of males and females, sexual cooperation instead of conflict is expected in social insects [Bourke & Franks, 1995, Greeff & Schmid-Hempel, 2008, Rice, 2000, Schrempf *et al.*, 2005]. Nevertheless, also in monogamous species with relaxed sexual conflict, SFPs are discussed with regard to speciation processes [Haerty *et al.*, 2007, Torgerson *et al.*, 2002].

Males of eusocial insects usually have a limited sperm supply and die shortly after one or a few matings [Boomsma *et al.*, 2005, Hölldobler & Wilson, 1990, Hölldobler & Bartz, 1985, Wilson, 1971]. Exceptions are the "worker-like" (ergatoid) and wingless ant males of many *Cardiocondyla species.* They, have a lifelong ongoing spermatogenesis, which allows them to mate with all available females [Boomsma *et al.*, 2005, Heinze & Hölldobler, 1993, Heinze *et al.*, 1998].

In our study species *C. obscurior*, these ergatoid males patrol through the brood pile to find young eclosing male pupae. They grab them and kill them with their sharp, sabre-shaped mandibles [Stuart *et al.*, 1987]. In case one male escapes this early attacks and has already a hardened cuticule, the adversaries will fight as soon as they meet each other and try to besmear the rival male, which leads the workers to kill the besmeared male [Kinomura & Yamauchi, 1987, Stuart *et al.*, 1987]. As a result there is usually only one wingless male in the nest, which normally stays inside the colony were it mates with young nestmate queens [Heinze *et al.*, 1998, Kinomura & Yamauchi, 1987, Stuart *et al.*, 1987]. This male may occasionally co-occur with several winged males, which are like "normal" social insect males as they have limited sperm supply and die soon after mating [Heinze *et al.*, 1998]. They are the disperser phene and occur when environmental conditions change [Cremer & Heinze, 2003]. For about the first 10 days they perform an odour mimicry as they smell like young queens, which keeps them save from the attacks of fighter males [Cremer *et al.*, 2002]. After this period they are forced to leave their maternal colony and they may mate outside with dispersing young queens [Kinomura & Yamauchi, 1987].

As *C. obscurior* is facultatively polygynous (i.e. may have multiple queens) [Heinze *et al.*, 2006], a queen mated to an ergatoid male usually starts a colony in a dependent way, i.e. lay eggs in the safety of the maternal nest [Schrempf & Heinze, 2008]. Differently, a queen mated to a winged male probably has to found a colony on her own or together with other founder queens, as under deteriorating environmental conditions it might be better to disperse and find a new nest site [Schrempf & Heinze, 2008]. It will last probably several weeks until the first sexual offspring is produced under these conditions.

Schrempf and Heinze (2008) could show that queens, mated to a winged male live significantly longer than queens mated to an ergatoid male. As ACPs have a great influence on females' physiology, we suggest that this life-prolonging effect is mediated through a different composition of the ACPs of the two male phenes. Ultimately, the winged male might assist the queen to found a new colony under changing conditions posthumously. In a recent study it has been shown that on the protein level, 56 proteins of the ACPs are differently expressed between these two phenes (Fuessl et al. in prep.).

The aim of this study was to compare the gene expression of MAGs of the two male phenes of *C. obscurior* by a representational difference analysis (RDA) followed by a quantitative RT-PCR (qPCR) for validation.

2.2 Material and Methods

2.2.1 Colony housing and male dissection

Colonies of *C. obscurior* [Wheeler, 1929] were collected from an experimental coconut plantation in Una, Bahia, Brazil in July 2009. Ants were then housed in artificial nests and nourished twice a week with chopped cockroaches, fruit flies and a drop of honey. Environmental conditions of incubators were set to a day/night- and temperature-cycle of 12 h 28 °C / 12 h 23 °C. To obtain winged males, conditions were changed to permanent light and constant 22 °C. Winged males were collected twice a week and put in a separate nest with some workers, where they were allowed to stay until their dissection seven to ten days later, to ensure that MAGs have been fully developed. As ergatoid males do not tolerate each other in this species, the same procedure was not feasible for this male phene. Instead, we collected wingless males with a minimum time interval of seven days.

Ant males were dissected on a chilled object slide in a drop of beadle solution (128.3 mM NaCl, 4.7 mM KCl, and 2.3 mM $CaCl_2$ [Darrouzet *et al.*, 2002] and MAG samples were stored at -80 °C until RNA extraction.

2.2.2 Representational difference analysis

RNA of both male phenes was extracted with peqGOLD TriFast^{TM} (Peqlab, Erlangen, Germany) according to the manufacturer's standard protocol. We used 1.6 μg RNA of MAGs of winged males and 0.760 μg RNA of MAGs of ergatoid males as no more was available, to generate the cDNA library. Reverse transcription and long distance PCR were carried out by employing the SMART PCR cDNA Synthesis Kit (Clontech Laboratories, CA, USA), resulting in double stranded cDNA. For suppression subtractive hybridisation we employed a cDNA RDA [Pastorian *et al.*, 2000] adapted for applications in insects [Judice *et al.*, 2006]. This strategy removes transcripts shared by the driver and the tester populations and enriches the tester library for differentially expressed genes. An accessory gland library of winged males was generated using cDNA of their MAGs as tester population, which was then hybridised against cDNA of the MAGs of ergatoid males as driver population. The same was done the other way round, with ergatoid males' glands as tester population and winged males' glands serving as driver population. Double stranded cDNAs (1 μg) were restriction digested with MboI (New England BioLabs, MA, USA), ligated to R-adapters [Judice *et al.*, 2006] and amplified via PCR following the protocol of Hubank and Schatz to generate the respective cDNA representations [Hubank & Schatz, 2000]. Enrichment of differentially expressed transcripts was achieved in two successive rounds of PCR amplifications that employed different adapters (J and N) and sequential subtractive hybridizations of the tester to an excess of driver cDNA, first in a 1:100 and then in a 1:800 ratio. After each of these successive steps, cDNAs were purified (Illustra GFX PCR purification kit, GE Healthcare, Little Chalfont, UK).

After the second round of selection, cDNA from the accessory gland library was ligated into the pGEM®-T Easy Vector System (Promega, WI, USA) and then transformed into *E. coli* DH5α cells. Transformants were selected on LB agar plates with 100 μg/mL ampicillin, 80 μg/mL X-Gal and 0.5 nM IPTG by blue-white screening. Positive clones were grown in liquid LB medium containing ampicillin and the plasmids were isolated. The cDNA inserts were sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, CA, USA) with M13 primers on an ABI-PRISM 3100) automated gene analyzer (Applied Biosystems).

2.2.3 Bioinformatic analysis

By means of the E-Gene annotation pipeline, sequencing reads were first filtered to detect and remove ribosomal RNAs and mitochondrial DNA. Subsequently, vector sequences were trimmed using Crossmatch. Read quality was checked and reads were assembled by the Phred-Phrap program module. Reads that had passed the quality check were next submitted to CAP3 assembly to obtain unique sequences (USs) [Huang & Madan *et al.*, 2011]. All contigs, and singlets were dynamically translated and compared to non-redundant (nr) databases. We performed a blastn against the "combined_ants_OGS_transcripts" in Hymenopterabase [Munoz-Torres *et al.*, 2011], the NCBI database [Altschul *et al.*, 1990], the *Cardiocondyla* genome and a blastx against the *Cardiocondyla* transcriptom [Schrader *et al.*, in press].

2.2.4 Differential gene expression analysis by quantitative RT-PCR

For q-PCR analysis, gene-specific primers were designed for all USs which gave a hit with an E-value $< 10^{-10}$ in the bioinformatic analysis. As reference gene we chose the housekeeping gene *rpL32*. Each biological replicate consisted of a least five MAGs of one of the two male phenes. RNA was extracted using peqGOLD TriFast™ (Peqlab). The RNA samples were treated with 0.1 U DNaseI (Invitrogen, CA, USA) for 40 min to eliminate possible DNA contamination. First strand cDNA was produced using the Affinity Script cDNA Synthesis Kit (Agilent Technologies, CA, USA), following the manufacturer's protocol.

Quality and annealing temperatures of the gene-specific primers were tested in a temperature-gradient PCR protocol run in a Mastercycler® ep (Eppendorf AG, Hamburg, Germany). Product length varied from 293 bp for a gene with no known orthologue (TG1 B1004) to 123 bp for the acyl carrier protein (TG6).

q-PCR-amplifications were performed by using the KAPA Sybr Fast Universal (Peqlab) protocol in a CFX Connect Real Time PCR Detection System (Bio-Rad, CA, USA). The amplification protocol was 95 °C for 4 minutes, 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. Subsequently, dissociation curves were acquired to check melting peak quality. For each male phene we ran three biological replicates, and for each biological replicate we ran three technical replicates.

Quantification cycle (Cq) values for each technical replicate were used to calculate 2-△△Cq values [Pfaffl, 2001], using the means of the three replicates of each male phenotype for normalization of the respective gland samples. A deviation of more than 0.35 from the overall mean led to an exclusion of the respective technical replicate, and the remaining two values were used for calculations. Glands of ergatoid males were used as a control to determine overall baseline.

2.3 Results

2.3.1 RDA library characteristics and bioinformatic analyses

In order to detect genes that are expressed exclusively in one of the two male phenes, we sequenced 96 clones from the subtracted MAG library of each male phene. After quality analysis and following CAP3 assembly we obtained a total of 40 unique sequences (USs) for the ergatoid male phene and 69 US for the winged male phene. We searched the NCBI database (blastn), hymenopterabase (blastn, against combined ant transcripts) and the *Cardiocondyla* genome (blastn) and transcriptom (blastx) for matches. For 49 % of all contigs (40.9 % of ergatoid males and 55.5 % of winged males) and 23.3 % of all singlets (27.8 % of ergatoid males and 21.4 % of winged males) we got significant hits (Table 2.1).

We took only matches into account that had an E-value $< 10^{-10}$ in any of the used databases, a cut-off level frequently used in expressed sequence tag (EST) projects [Swanson *et al.*, 2001a, Walters & Harrison, 2010]. In case of five contigs of ergatoid males and six contigs of winged males, a significant hit in the NCBI database did not result in a hit in the *Cardiocondyla* transcriptom, similar to six USs that had a hit in the *Cardiocondyla* genome, but not the transcriptom (three contigs of each male phene). These results were excluded from further analysis.

Of the 40 USs of the ergatoid males were 22 contigs and 18 singlets, with nine contigs and five singlets yielding a significant hit in the databases. Of the 69 USs of the winged male, were 27 contigs and 42 singlets, with 15 contigs and nine singlets yielding a significant hit in the databases. One contig (TG2) shows a significant hit only in *Cardiocondyla*, but not with any other species for which a genome is available thus far. The same was the case for one of the remaining eight of 42 singlets that had a significant hit. In total 40 USs yielded significant matches.

2.3.2 Quantitative RT-PCR analysis of RDA-library genes

RDA is a high throughput approach, which makes it sensitive for false positives. A gene transcript indicated by RDA as differentially expressed should be further analysed by specific gene-expression analysis methods such as qPCR. For this purpose we designed primers for 31 of the 38 USs with a threshold E-value $< 10^{-10}$ (nine contigs and five singlets of ergatoid males; nine contigs and five singlets of winged males). The remaining seven sequences were not suitable for primer design due to sequence repetition or because the US was too short. Eleven primer pairs yielded specifically the predicted fragments. These primers, their sequences and the predicted function of their corresponding genes are shown in Table 2.5. Eight of these primer pairs finally resulted in qPCR runs of good quality, as e.g. with regard to the melting curve. Results for TG16 (*ejaculatory bulb specific protein 3-like*) and TE2 B0303 (*SERPINB10*) have to be handled with care, as they showed a high variabilty already within the phene. TG16 was three fold up-regulated in ergatoid males, and TE2 B0303 (*SERPINB10*) three-fold up-regulated in winged males. Besides these two genes, three further genes were higher expressed in winged males: TG2 D0107 (hypothetical protein), TG31 (*leucine-rich repeat (LRR) and immunoglobulin-like domaincontaining nogo receptor-interacting protein 2-like*), TG2 (specific to *C. obscurior*) and three in ergatoid males (TG42 (growth hormone-inducible transmembrane protein-like (*ghitm*)), TG6 (*acyl carrier protein*, *mitochondrial-like*, *transcript variant 2*), TG1 B1004 (specific to *C. obscurior*).

Table 2.1: Results of RDA for contigs of MAGs specific for ergatoid males, as well as blastn results against Hymenopterabase, NCBI and the genome of *Cardiocondyla* are shown. 1*: *C. obscurior* genome (blastn) 2*: *C. obscurior* transcriptom (blastx)

			Hymenopterabase		NCBI				1*	2*
Contig	ESTs	bp	Organism and Accession	e-value	Organism	Predicted gene function	Gene ID	e-value	e-value	e-value
TE1	6	388		ns	*Bombus terrestris* and *Bombus impatiens*	Chymotrypsin-1-like and serine protease (SP)	XM003393487.1 and DQ489309	both: 1 E-104	2E-17	8E-17
TE2	2	220	PB15243-RA and Aech13241-RA	9E-42	*Apis florea* and *Bombus terrestris*	Cytosolic carboxypeptidase 6-like	XM003697237.1 and XM003393185.1	both: 5E-24	2E-104	1E-81
TE4	8	184	SINV12645-RA	3E-44	*Bombus impatiens* and *Camponotus floridanus*	Hypothetical protein and UPF0326 protein FAM152B	XM003487751.1 and EFN65714.1	2e-52 and 2E-32	1E-16	1E-35
TE7	3	211	SINV20546-RA	1E-16	*Bombus impatiens*	Homeobox protein homothorax-like	XM003493083.1	1E-17	1E-16	8E-07
TE9	6	311	HSAL18430-RA	7E-22	*Bombus terrestris*	Transmembrane protein C2orf18-like	XM003400675.1	6E-120	ns	2E-36
TE11	18	240	ACEP10788-RA	2E-21	*Bombus impatiens*	Hypothetical protein	XM003491130.1	3E-59	4E-18	1E-43
TE12	20	220	LH11170-RA	2E-67	*Bombus terrestris*	Calcium-transporting ATPasesarcoplasmatic/endoplasmatic reticulum type-like	XM003399810	1E-100	9E-45	2E-78
TE33	3	259		ns	*Bombus impatiens*	Protein Cut A homolog	XM003493847.1	1E-71	ns	2E-27
TE39	4	344		ns	*Apis florea*	15-hydroxyprostalandin-dehydrogenase (NAD+)-like	XM003691537.1	9E-30	1E-13	1E-13

Table 2.2: Results of RDA for contigs of MAGs specific for winged males, as well as blastn results against Hymenopterabase, NCBI and the genome of *Cardiocondyla* are shown. 1*: *C. obscurior* genome (blastn) 2*: *C. obscurior* transcriptom (blastx)

			Hymenopterabase		NCBI				1*	2*
Contig	ESTs	bp	Organism and Accession	e-value	Organism	Predicted gene function	Gene ID	e-value	e-value	e-value
TG1	8	260	HSAL15971-RA		*Bombus impatiens*	Hypothetical protein	XM003492916.1	3E-85	9E-14	4E-25
TG2	6	287		ns				ns	3E-146	3E-44
TG5	2	242	LH23160-RA		*Bombus impatiens*	ATP synthase lipid-binding protein mitochondrial-like	XM003489293.1	7E-92	8E-46	1E-37
TG6	4	242		ns	*Apis florea*	Acyl carrier protein, mitochondrial-like, transcript variant 2	XM003698749	1E-84	7E-10	7E-10
TG7	10	318	HSAL15840-RA		*Bombus impatiens*	Hypothetical protein	XM003488314.1	4E-109	1E-39	6E-68
TG8	7	240	ACEP27471-RA		*Bombus terrestris* and *Bombus impatiens*	Hypothetical protein	XR1356031.1 and XR131815.1	2E-79	4E-56	3E-51
TG9	2	195	HSAL16754-RA		*Bombus terrestris*	26S proteasom non ATPase regulatory subunit 8-like	XM003399049.1	3E-64	5E-57	5E-57
TG12	4	207	PB21395-PA		*Apis florea*	40S ribosomal protein S23-like	XM003692394	2E-73	4E-55	4E-55
TG15	4	297	AECH11137-RA		*Bombus impatiens*	Troponin 1-like, transcript variant 5	XM003490592.1	4E-115	7E-35	5E-75
TG16		243	CFLO19641-RA		*Megachile rotundata*	Ejaculatory bulb-specific protein 3-like	XM003708471	4E-70	1E-11	2E-33
TG30	2	221	CFLO018647-RA		*Bombus terrestris* and *Bombus impatiens*	Serine/threonine-protein kinase KDX1-like and cyclin-dependent kinase 6-like	XM003494391 and XM003400196	both: 3e-77	4E-17	2E-15
TG31	2	343	SINV18925-RA		*Bombus impatiens*	Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 2-like	XM003490631	1E-20	1E-165	1E-165
TG39	2	126		ns	*Bombus impatiens*	Hypothetical protein	XM003491031	5E-39	8E-10	8E-10
TG42	3	223		ns	*Bombus terrestris* and *Bombus impatiens*	Growth hormone-inducible transmembrane protein-like	XM003489800.1 and XM003392880.1	1E-64	6E-22	6E-22
TG46	3	216	HSAL15840-RA		*Bombus terrestris*	Hypothetical protein	XM003398446	3E-90	1E-36	7E-52

Table 2.3: Results of RDA for singlets of MAGs specific for ergatoid males, as well as blastn results against Hymenopterabase, NCBI and the genome of *Cardiocondyla* are shown. 1*: *C. obscurior* genome (blastn) 2*: *C. obscurior* transcriptom (blastx)

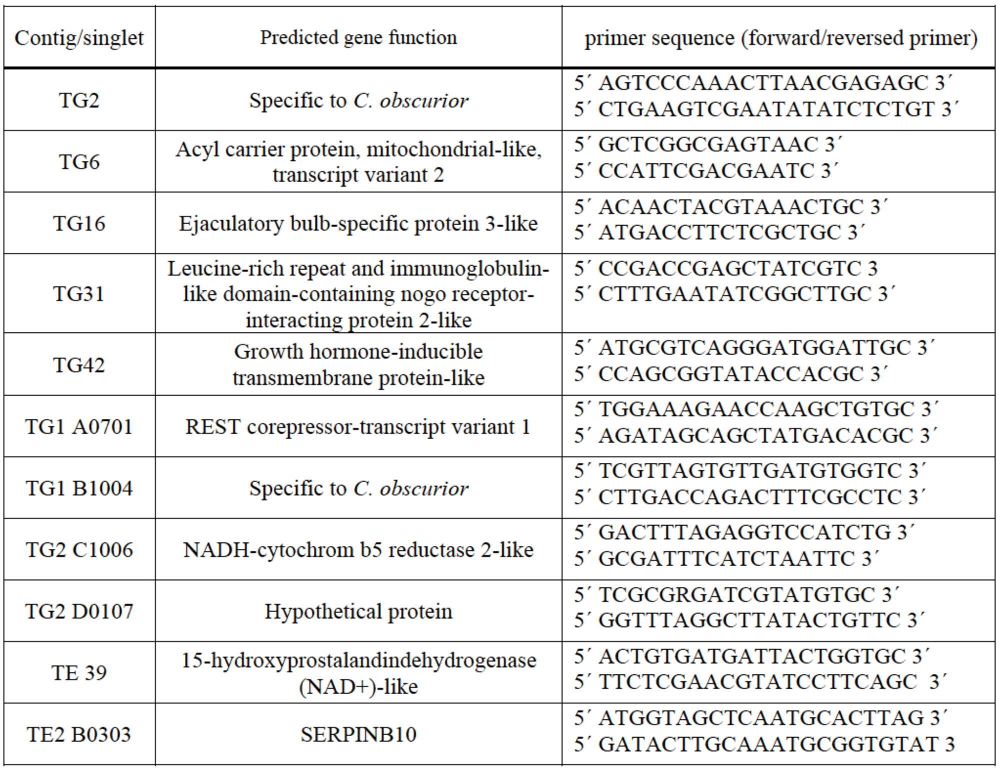
			Hymenopterabase		NCBI				1*	2*
Singlet	ESTs	bp	Organism and Accession	e-value	Organism	Predicted gene function	Gene ID	e-value	e-value	e-value
TE1 H0715	1	195	PB20409-RA	9E-51	*Bombus impatiens*	Troponin I-like, transcript variant-4	XM003490591.1	2E-84	7E-39	6E-46
TE2 B0303	1	193	AECH19678-RA	4E-28	*Acromyrmex echinatior*(*1)	SERPINB10 (*1)	EGI61105.1(*1)	4E-33(*1)	2E-95	4E-42
TE2 D0507	1	193	PB15911-PA	2E-36	*Nasonia vitripennis*	60S ribosomal protein L35 a-like	XM001606828	2E-34	3E-49	1E-93
TE2 E0109	1	193	SINV22792-RA	4E-84	*Apis florea*	cytosolic carboxypeptidase-6-like	XM003697237	2E-21	2E-95	2E-95
TE2 F0812	1	185	ACEP16722-RA	2E-27	*Solenopsiss invicta*	40S ribosomal protein S17	GU471222.1	5E-48	4E-73	3E-31

(*1) blastx results are shown instead of blastn, because blastn did not yield a significant hit. Protein accession instead of gen ID is shown.

Table 2.4: Results of RDA for singlets of MAGs specific for winged males, as well as blastn results against Hymenopterabase, NCBI and the genome of *Cardiocondyla* are shown. 1*: *C. obscurior* genome (blastn) 2*: *C. obscurior* transcriptom (blastx)

			Hymenopterabase		NCBI				1*	2*
Singlet	ESTs	bp	Organism and Accession	e-value	Organism	Predicted gene function	Gene ID	e-value	e-value	e-value
TG1 A0602	1	202	HSAL13640-RA	2E-27	*Apis florea* and *A.pis mellifera*	RING finger protein nhl-1-like and uncharacterized LOC	XM391967 and XM003689856.1	1E-49	3E-38	6E-34
TG1 A0701	1	189	LH24381-RA	3E-35	*Apis mellifera*	REST corepressor-transcript variant 1	XM003250631	1E-31	3E-93	3E-49
TG1 B1004	1	336		ns				ns	4E-171	4E-171
TG1 C0905	1	222		ns				ns	7E-103	ns
TG1 E0909	1	228	see TE2 D0507	7E-46	*Nasonia vitripennis*	see TE2 D0507	XM001606828	1E-48	1E-61	3E-114
TG1 H1115	1	273	PB22370-RA	6E-22	*Bombus terrestris*	Prenylated Rab acceptor protein 1-like	XM003399757.1	5E-107	6E-29	2E-61
TG2 C0406	1	196	HSAL14953-RA	6E-08	*Bombus terrestris* and *Bombus impatiens*	ATP synthase lipid-binding protein, mitochondrial-like	XM003489293.1 and XM003403106.1	5E-34	3E-10	1E-08
TG2 C1006	1	116	AECH25470-RA	1E-11	*Apis mellifera*	NADH-cytochrom b5 reductase 2-like	XM396639.4	1E-20	8E-16	8E-16
TG2 D0107	1	194	CFLO13055-RA	2E-14	*Bombus terrestris* and *Bombus impatiens*	Hypothetical protein	XM003485205.1 and XM003393479.1	1E-63	e-36	e-36

Table 2.5: Contigs/singlets which were suitable for primer-design. The predicted function of the genes and the sequence of the primer pairs are shown



Contig/singlet	Predicted gene function	primer sequence (forward/reversed primer)
TG2	Specific to *C. obscurior*	5′ AGTCCCAAACTTAACGAGAGC 3′ 5′ CTGAAGTCGAATATATCTCTGT 3′
TG6	Acyl carrier protein, mitochondrial-like, transcript variant 2	5′ GCTCGGCGAGTAAC 3′ 5′ CCATTCGACGAATC 3′
TG16	Ejaculatory bulb-specific protein 3-like	5′ ACAACTACGTAAACTGC 3′ 5′ ATGACCTTCTCGCTGC 3′
TG31	Leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 2-like	5′ CCGACCGAGCTATCGTC 3 5′ CTTTGAATATCGGCTTGC 3′
TG42	Growth hormone-inducible transmembrane protein-like	5′ ATGCGTCAGGGATGGATTGC 3′ 5′ CCAGCGGTATACCACGC 3′
TG1 A0701	REST corepressor-transcript variant 1	5′ TGGAAAGAACCAAGCTGTGC 3′ 5′ AGATAGCAGCTATGACACGC 3′
TG1 B1004	Specific to *C. obscurior*	5′ TCGTTAGTGTTGATGTGGTC 3′ 5′ CTTGACCAGACTTTCGCCTC 3′
TG2 C1006	NADH-cytochrom b5 reductase 2-like	5′ GACTTTAGAGGTCCATCTG 3′ 5′ GCGATTTCATCTAATTC 3′
TG2 D0107	Hypothetical protein	5′ TCGCGRGATCGTATGTGC 3′ 5′ GGTTTAGGCTTATACTGTTC 3′
TE 39	15-hydroxyprostalandindehydrogenase (NAD+)-like	5′ ACTGTGATGATTACTGGTGC 3′ 5′ TTCTCGAACGTATCCTTCAGC 3′
TE2 B0303	SERPINB10	5′ ATGGTAGCTCAATGCACTTAG 3′ 5′ GATACTTGCAAATGCGGTGTAT 3

2.4 Discussion

The RDA method is very suitable for systems where genomic or proteomic information is not yet available or not enough developed to use other methods like micro-arrays. With RDAs it is possible to detect known and unknown transcripts which made it the best method for our purposes as the *C. obscurior* genome was published only recently [Schrader *et al.*, in press]. Using this method we found 38 gene transcripts to be differentially expressed. Eight of them could be confirmed in a downstream qPCR to be higher or lower expressed. Four of these eight genes are higher expressed in the MAGs of winged males and four of them in the MAGs of ergatoid males. More abundant in ergatoid males were the genes *ejaculatory bulb-specific protein 3-like* (TG16; fcv: 0.35), *ghitm* (TG42; fcv: 0.95), *acyl carrier protein*, *mitochondrial-like*, (TG6; fcv: 0.51), and a gene for a protein with unknown function that was not found in any other organism than *C. obscurior* (TG1 B1004; fcv: 0.48). In winged males *SERPINB10* (TE2 B0303; fcv: 3.3), *LRR and*

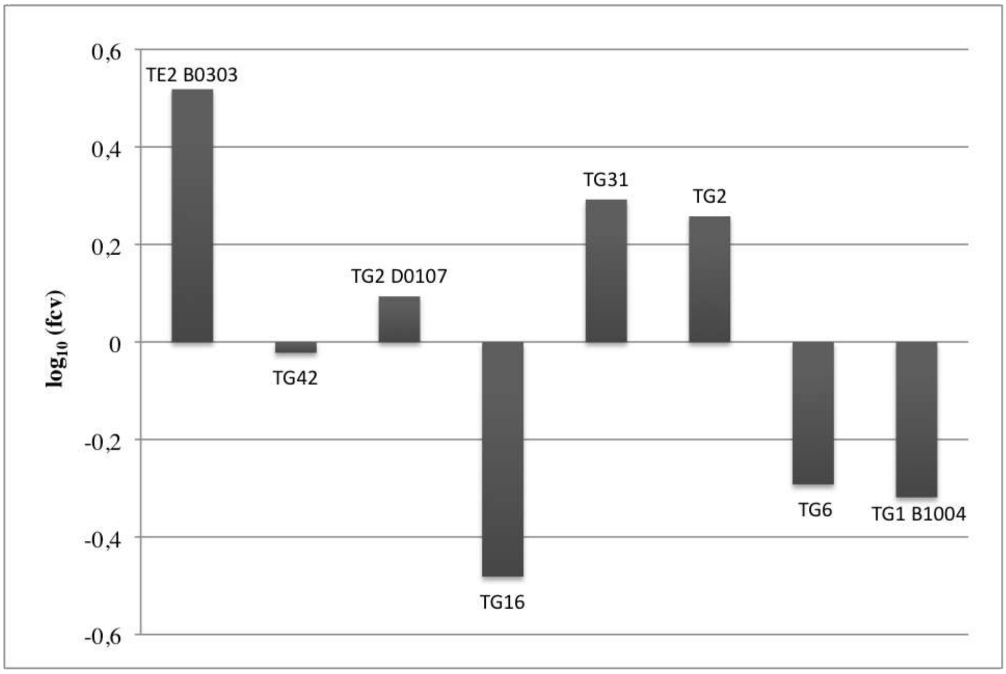


Figure 2.1: Differentially expressed genes shown in $\log_{10}$-scale. Higher expressed in ergatoid males: TG42 (*ghitm*), TG16 (*ejaculatory bulb-specific protein 3-like*), TG6 (*acyl carrier protein, mitochondrial-like, transcript variant 2*), TG1 B1004 (specific to *C. obscurior*). Higher expressed in winged males: TE2 B0303 (*SERPINB10*), TG2 D0107 (hypothetical protein), TG31 (*LRR and immunoglobulin-like domain-containing nogo receptor-interacting protein 2-like*), TG2 (specific to *C. obscurior*).

immunoglobulin-like domain-containing nogo receptor-interacting protein 2-like (TG31; fcv: 1.96), a hypothetical protein (TG2 D0107; fcv: 1.24) and a gene that was unique in *C. obscurior* (TG2 fcv; 1.81) were higher expressed.

Among the differentially expressed genes was a serine protease inhibitor (serpin, fcv: 3.3) which has been shown to be over-expressed in the winged male phene by qPCR. Serpins may be transferred during mating [Lung *et al.*, 2002, Ravi Ram *et al.*, 2007, Reichhart, 2005] and are known to be involved in the Toll signalling in insects, which is an important part of their immune system [De Gregorio *et al.*, 2002, Levashina *et al.*, 1999, Reichhart, 2005, Valanne *et al.*, 2011]. During mating, microbes could be introduced into the females reproductive tract, which certainly would be harmful if they would not be made inoperative by antimicrobial substances [Lung *et al.*, 2001, McGraw *et al.*, 2008, Mueller *et al.*, 2004, Ravi Ram *et al.*, 2007]. It has been shown, that ACPs lead to an up-regulation of twelve genes involved in the immune system after mating in *Drosophila melanogaster* [McGraw *et al.*, 2004, McGraw *et al.*, 2008]. Serpins have a special potential to evolve rapidly and are thus optimal primed to quickly adapt to changing needs [Kanost, 1999, Mangan *et al.*, 2008]. It may be possible that *SERPINB10* (and other proteins) helps the female in general to activate her immune system after mating [Kanost, 1999, Kanost *et al.*, 2004, Ravi Ram *et al.*, 2007].

After copulation, the ACPs may be transferred into the haemolymph [Chapman, 2001, Kubli, 2003, Lung & Wolfner, 1999, Monsma *et al.*, 1990, Ravi Ram *et al.*, 2005, Wolfner, 1997] and thus affect other tissues than the reproductive tract [Chen *et al.*, 1988, Ravi Ram *et al.*, 2007]. They may simply act as transcription factors and e.g. enhance or silence pathways [McGraw *et al.*, 2008, Robertson, 2007] but a long-term effect of ACPs demands multiple ACPs and a *de novo* synthesis of protein [Ravi Ram & Wolfner, 2007a, Ravi Ram *et al.*, 2007]. Directly after mating, more than 30 % of genes are differently regulated in *D. melanogaster* females - most of them less than 2-fold - but after two days only 1.6 % are still differently expressed 2-fold or higher [McGraw *et al.*, 2004, McGraw *et al.*, 2008, Wolfner *et al.*, 1997].

Considering the fact that females mated to a winged male might have to found alone, it is essential that her immune system is activated in the period of her life where she is exposed the most to changing external influences [Baer *et al.*, 2006]. Protease inhibitors may influence the female's lifespan as it has been shown for *D. melanogaster*. The *Drosophila* Acp62F is a trypsine protease inhibitor which increases the females mortality, probably because a low percentage of the transferred protein ends up in the haemolymph [Lung *et al.*, 2002, Lung & Wolfner, 1999, Wolfner, 1997]. The serpin found in our study might act in a similar way, just contrariwise.

Interestingly we found this serpin also higher expressed on protein level (see Chapter 3). This confirms that in this case RNA- and protein-level point to the same direction, (which is not necessarily the case, see discussion below). As mentioned in the introduction, among ACPs are genes that are evolving very rapidly, suggesting that they are under positive Darwinian selection [Swanson *et al.*, 2001b] while others are evolving with moderate speed or are even conserved, suggesting some functional constraint [Andrés *et al.*, 2006, Dorus *et al.*, 2006]. At least the basic reproduction processes - which involve the same classes of proteins from insects to mammalia - have to be assured and genes that operate in more tissues and/or have multiple functions are more conserved [Dorus *et al.*, 2006, Khaitovich *et al.*, 2006, Marshall *et al.*, 2011, Mueller *et al.*, 2005, Pal *et al.*, 2006]. Tissue-specific genes or genes that act species-specific have been predicted to have a higher potential to evolve rapidly [Haerty *et al.*, 2007, Khaitovich *et al.*, 2006, Pal *et al.*, 2006, Swanson *et al.*, 2001a]. Furthermore, it is also important to note that sometimes only a small part of a gene or protein is under strong selection and rapidly evolving, while the rest of the protein may remain unaffected [Kern *et al.*, 2004, Swanson *et al.*, 2001b]. For example it has been shown in Manduca sexta that different serpin proteins are designed from a single gene by using different versions of one exon [Jiang *et al.*, 1996]. Finally, due to the redundancies of the genetic code, differing gene sequences may lead to proteins that are orthologues [Mueller *et al.*, 2004, Ravi Ram *et al.*, 2007, Schrimpf *et al.*, 2009].

Another gene that we found to be among the most differentially expressed genes was predicted as *ejaculatory bulb-specific protein 3-like.* The corresponding protein (PEB-me in *D. melanogaster*) has been intensively studied in *D. melanogaster*, where it has been found exclusively in the ejaculatory bulb, where it is either synthesized or it is transported to this tissue [Ludwig *et al.*, 1991]. It is transferred during mating and has a possible role in mating plug formation in *Drosophila* [Ludwig *et al.*, 1991, Lung & Wolfner, 2001, Wolfner *et al.*, 1997]. Some hours after mating, it cannot be found anymore in the reproductive tract, either because it got lost through the vaginal opening, or because it has been absorbed into the haemolymph. Ludwig and colleagues suggested, that it might be involved in changes in the female physiology after mating [Ludwig *et al.*, 1991]. As *C. obscurior*, to our knowledge, transfer no mating plug, we suggest that the protein is transferred into the haemolymph where it acts in a so far not elucidated manner.

The other three genes that we found to be differentially expressed are: *acyl carrier protein, mitochondrial-like*, *LRR and immunoglobulin-like domain-containing nogo receptorinteracting protein 2-like* and *ghitm.* The latter is expressed in a variety of tissues [Yoshida *et al.*, 2006] and has multiple biological functions [Goodman, 1993, Nagel *et al.*, 2004]. It has been suggested that it might play a role in ageing and immunology [Li *et al.*, 2001, Reimers *et al.*, 2007, Zou *et al.*, 2000]. Generally, transmembrane proteins play an important role in immune defence [Litman *et al.*, 2005]. LRR containing proteins also play a role in immune defence [Litman *et al.*, 2005]. Proteins with a LRR have a short consensus-sequence but are not very conserved in the amino acids around this structure. Substitutions in proteins with a LRR are - similar to serpins [Kanost, 1999] – associated with changes in potential binding partners [Litman *et al.*, 2005], making these proteins perfectly adapted to rapid evolution of proteins involved in immune defence.

In 2010, Oppelt and colleagues performed a similar study in *Leptothorax* ants, where they verified six genes to be differently expressed in MAGs of males in different life-stages [Oppelt *et al.*, 2010]. We found none of these genes in this study. Additionally, in the present study we found two genes to be differentially expressed, which were not yet found in any other organism than *C. obscurior.* These genes may be rapidly evolving ACP genes with highly divergent gene sequences among species which makes it difficult to find an orthologue [Baer *et al.*, 2009b, Braswell *et al.*, 2006, Collins *et al.*, 2006, Dorus *et al.*, 2006, Walters & Harrison, 2010]. The same might be the case for the gene coding for the hypothetical protein. It has been shown for *D. melanogaster* that about 50 % of the ACPs have no homologue outside the drosophilide species complex [Swanson *et al.*, 2001a], and less than 50 % of the ACPs of *D. melanogaster* have homologues outside the *melanogaster/obscura* group [Ravi Ram *et al.*, 2007], probably due to amino acid polymorphism and divergence [Kern *et al.*, 2004].

Most information on the genes, that we found, came from *D. melanogaster*, the best studied insect organism for which good information about possible functions of genes and proteins was available. *D. melanogaster* is a promiscuous solitary insect while *C. obscurior* is a eusocial, effectively monogamous species. So even if the genes for basic reproductive processes are probably conserved throughout organisms with internal fertilization, it remains difficult to find orthologues for fast evolving genes (e.g. two genes in our study). In the last few years, several insect genomes were published, which help to identify genes. However, in these, often only the name, but not its function is known – even in the honey bee *Apis mellifera.* Hence, it is necessary to establish more model organisms from different groups of insects. *C. obscurior* might serve as a good organism for eusocial ant species. Colonies can be easily kept in the laboratory and matings performed under experimental set-ups. Males have seemingly a broad variety of differentially expressed genes as result of different male reproductive tactics. In the future it would be interesting to specifically knock out or knock down the genes that we found to be differentially expressed in this study, to see whether they really affect queens' longevity, whether this effect is dose dependent and whether there may be similarities to humans.

Acknowledgements

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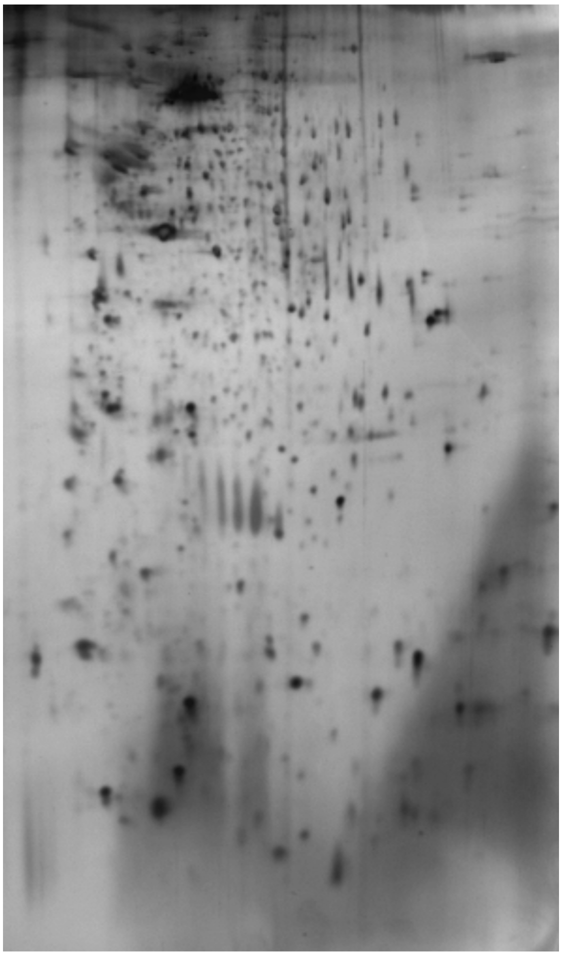
Chapter 3

Comparison of the accessory gland proteins of two male-diphenic ant species and the effect on their mating partners*

Marion Fuessl, Jörg Reinders, Peter J. Oefner, Jürgen Heinze, Alexandra Schrempf



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Abstract

During mating, males transfer seminal fluid to the female, along with sperm. In insects, accessory gland proteins (ACPs) are an important component of seminal fluid and considered to be a major driving force of speciation. They influence a female's post-mating behaviour, physiology, and lifespan. In the ant genus *Cardiocondyla*, many species feature male diphenism with winged and wingless males. For *C. obscurior*, mating with winged males has been shown to increase the lifespan of queens significantly more than mating with wingless males. To date, nothing is known about the mechanism, i.e., the responsible genes or proteins leading to this life prolongation. Here, we compare the accessory gland contents of winged and wingless males. We show that SERPINB10, Na^+/H^+ exchange regulatory cofactor (NHE-RF2), and selenophosphate synthetase 1 (SPS1) are over-expressed in the accessory glands of the winged male phene, while protein disulfide isomerase A3 (PDIA3) is over-expressed in the wingless male phene. Our study makes them candidate proteins to be involved in major physiological changes after being transferred to the female and give a first insight into a possibly lifespan enhancing role of ACPs.

Keywords: seminal fluid, 2D-SDS-PAGE, serpin, protein disulfide isomerase, NHE-RF

3.1 Introduction

Seminal fluid proteins (SFPs) are transferred during mating [Bairati, 1968, Findlay *et al.*, 2008, Terranova *et al.*, 1972] and are known to influence a female's post-mating physiology [Avila *et al.*, 2011, Chapman & Davies, 2004, Chapman *et al.*, 1995, Gillott, 2003, Leopold, 1976, Mueller *et al.*, 2005, Perry *et al.*, 2013, Robertson, 2007]. In insects, these proteins are best studied in the fruit fly *Drosophila melanogaster*, in which females show a polyandrous life style. However, this runs against the male's interest, as fewer eggs may be fertilized with its own sperm. Thus, males have been shown to manipulate the females' physiology through SFPs [Avila *et al.*, 2011, Chapman *et al.*, 2003b, Chapman & Davies, 2004, Chen, 1984, Gillott, 2003, Wolfner, 1997, Wolfner *et al.*, 1997]. In addition, the lifespan of females that mate more often is reduced [Chapman *et al.*, 1995, Fowler & Partridge, 1989]. Females try to escape such effects and a perpetual antagonistic coevolution between the genders accompanied by a rapid evolution of the ACPs, is often the consequence of this conflict over mating [Aguadé *et al.*, 1992, Arnqvist *et al.*, 2000, Dawkins, 1976, Haerty *et al.*, 2007, Parker *et al.*, 1979, Rice, 2000].

By contrast, in eusocial Hymenoptera sexual conflict is not expected [Boomsma *et al.*, 2005, Rice, 2000]. Queens of eusocial insects (ants, bees and wasps) mate only during a short period early in their life and they do not remate later in life [Boomsma *et al.*, 2005, Boomsma, 2009, Wilson, 1971]. The sperm of one or a few matings is stored for the rest of their entire lifes in a special storage organ, the spermatheca, where it is kept alive, sometimes for decades [Boomsma *et al.*, 2005, Boomsma, 2009]. Contrary, males normally die shortly after mating and persist only as stored sperm in the female's spermatheca.

Haplodiploidy as a sex determining mechanism is prevalent, meaning that fertilized (diploid) eggs develop into females and haploid eggs into males [Cook, 1993, Heimpel & de Boer, 2008, Van Wilgenburg *et al.*, 2006]. Thus, the father of a colony benefits only from female sexual offspring, which is produced with some time lag after colony founding. Due to this late reproduction, where males benefit from a prolonged queen's life, sexual cooperation instead of conflict between the sexes is expected as the interests of females and males converge [Boomsma *et al.*, 2005, Bourke & Franks, 1995, Greeff & Schmid-Hempel, 2008]. Consequently, the SFPs of males are unlikely to harm their female mating partners, but rather contribute to keep the colony alive [Boomsma *et al.*, 2005, Schrempf *et al.*, 2005]. Hence, there may be less selection on ACPs to quickly respond to adaptations of the other gender, and a less rapid evolution (in comparison to common polyandrous mating systems as e.g. in *Drosophila*) is expected [Rice, 2000].

In many *Cardiocondyla* species, two different male phenotypes occur [Heinze *et al.*, 2005, Kugler, 1983, Seifert, 2003]. Besides the typical, peaceful, winged males (similar to the males of other ant species), wingless, ergatoid (worker-like) males exist, which are often fighter males that kill each other [Kinomura & Yamauchi, 1987, Stuart *et al.*, 1987]. These wingless *Cardiocondyla* males have several special adaptations that render them very different from normal winged ant males. They have a live-long spermatogenesis, which is exceptional among eusocial insect males [Heinze & Hölldobler, 1993, Heinze *et al.*, 1998]. Due to this, they do not suffer from sperm limitation and are able to mate throughout their entire lifetime, which may last several months [Boomsma *et al.*, 2005, Heinze & Hölldobler, 1993].

In this study, we compare the ACPs of two different *Cardiocondyla* species from different clades (Oettler et al, 2010). In the species *C. obscurior*, winged males are produced in the laboratory when environmental conditions change rapidly [Cremer & Heinze, 2003]. They mate inside the nest with young female nestmates, before they disperse to mate outside [Kinomura & Yamauchi, 1987]. It has been shown that winged males chemically mimic females to avoid being killed by ergatoid males: for about the first ten days of its life, a winged male smells like a young queen and ergatoid males sometimes even attempt to mate with the winged male [Cremer *et al.*, 2002]. If winged males are hindered to leave the nest in the laboratory, they gather together at the edges of the arena, far away from the colony and try to escape, until they are killed or die (pers. observation). In contrast, ergatoid males stay their entire life inside the maternal nest and mate there with young queens [Heinze *et al.*, 1998, Kinomura & Yamauchi, 1987, Stuart *et al.*, 1987]. They are produced regularly in the colony, but they engage in lethal fights as soon as they meet each other. Ergatoid males have sabre-shaped mandibles and always seek the nest and the brood pile for rival males, which they are able to kill if they are freshly eclosed and still with a soft cuticule. In case that a rival male has already a hardened cuticule, the males grab each other and try to besmear the rival with a substance that leads workers to kill the besmeared male [Kinomura & Yamauchi, 1987, Stuart *et al.*, 1987, Yamauchi & Kawase, 1992]. Hence, there is normally only one adult ergatoid male in the colony. *C. obscurior* is a facultatively polygynous ant species [Heinze *et al.*, 2005], and young queens that mated with an ergatoid male usually stay in their maternal nest and start to lay eggs in the safety of the colony.

Contrary, in *C. tjibodana*, wingless males occur only sporadically. These males have worker-like mandibles and kill only other ergatoid pupae during eclosion [Oettler *et al.*, 2010]. In case a male escapes this early attack, two ergatoid males remain inside a colony. Interestingly, winged males of this species can be found regularly inside the colonies (pers. observation). It seems, that they are no more an exclusive disperser phene, but equal to ergatoid males adapted to mating and staying in their maternal nest. They are not forced to leave the nest some days after eclosion, as they are save from attacks of ergatoid males and may stay inside the nest for their entire life, which may last up to at least 41 days, to mate with nestmates (Fuessl *et al.*, accepted, Chapter 5). In this study, none of the winged males seemed to be killed by workers, e.g. had cut off antennae or legs. Contrary to the winged males of *C. obscurior*, no migration behaviour can be observed even after several days, but instead, the males stay in the centre of the colony.

It has been shown that mated *C. obscurior* queens live longer than virgin queens [Schrempf *et al.*, 2005]. Moreover, queens that mated with a winged male live significantly longer than queens that mated with an ergatoid male [Schrempf & Heinze, 2008]. Schrempf and colleagues suggested that substances of the seminal fluid, which are transferred during mating, might account for this life-prolonging effect in *Cardiocondyla* queens [Schrempf & Heinze, 2008, Schrempf *et al.*, 2005]. Ultimately, winged males might assist the queens, with which they mated, to found a new colony alone. In case of changing environmental conditions, a queen probably has to leave the mother nest and found a colony independently, i.e. without the help of workers under impeded conditions [Heinze *et al.*, 2006, Schrempf & Heinze, 2008]. These first weeks are crucial with regard to her future life and reproductive output [Baer *et al.*, 2006].

In *C. tjibodana*, winged males – eventually besides an ergatoid male – and queens can be found regularly inside the colonies. Moreover, as frequently several winged males coexist, queens may easily mate with several males. Multiple mating has been shown for other *Cardiocondyla* species were several males coexist, as e.g. in monogynous *C.*

elegans, *C. batesi* and *C. nigra* [Lenoir *et al.*, 2007, Schrempf, 2005, Schrempf, 2014]. No difference in the life-expectance of queens mated to ergatoid or winged males has been shown (Fuessl *et al.*, accepted, Chapter 5).

The aim of this study was to investigate the ACPs of winged and ergatoid males of the ant species *C. obscurior* based on two-dimensional-SDS-polyacrylamide-gel-electrophoresis (2D-SDSPAGE) in detail [O'Farrell, 1975, Rabilloud *et al.*, 2010]. Furthermore, a comparison with *C. tjibodana*, a species with a different life history, was performed to gain insights into the conservation of ACPs between male phenes as well as among species in the genus.

3.2 Material and Methods

3.2.1 Ant sampling and housing

Colonies of *C. obscurior* (Wheeler, 1929) were collected from an experimental coconut plantation in Una, Bahia, Brazil in July 2009. Lab-colonies of *C. tjibodana* were all split colonies from a single colony that was collected in October 2008 on a parking lot near Gua Londron Cave, Pangkajene, Sulawesi Selantan. Ants were housed in artificial nests and nourished twice a week with chopped cockroaches (*Nauphoeta cinerea*), fruit flies and a drop of honey. Environmental conditions of incubators were set to a day/night- and temperature-cycle of 12 h 27 °C/ 12 h 23 °C. To obtain winged males of *C. obscurior*, conditions were changed to permanent light and constant 22 °C.

3.2.2 Preparation of samples and performance of the first dimension: Isoelectric focusing (IEF)

Winged males of both species were collected twice a week and put in a separate nest with some workers, where they were allowed to stay until their dissection seven to ten days later, to ensure that male accessory glands (MAGs) have been fully developed. As ergatoid males do not tolerate each other in these species, the same procedure was not feasible for this male phene. Instead, we collected wingless males with a minimum time interval of seven days. Ant males were dissected on a chilled object slide in a drop of beadle solution (128.3 mM NaCl, 4.7 mM KCl, and 2.3 mM $CaCl_2$ [Darrouzet *et al.*, 2002]) and nine pairs of MAGs of either ergatoid or winged males were collected in 20 μL of DeStreak Rehydration Solution (GE Healthcare, Little Chalfont, UK) with 2 % IPG (pH 3-11 NL, GE Healthcare). Samples were stored at -23 °C until usage. In total, we dissected 90 males (equivalent to ten samples) of each phene for *C. obscurior*. In *C. tjibodana*, ergatoid males occur only sporadically in the colonies, so that we could only analyze 27 (equivalent to three samples) ergatoid males, aside from 90 winged males (equivalent to ten samples).

The equipment and all solutions for the IEF were prepared following the manufacturer's protocol (Hoefer, MA, USA). Directly before starting the first dimension, the completely thawed samples were treated four times for 15 seconds each with ultrasonic sound and put on ice for 15 seconds between treatments. Then 40 μL of rehydration solution (8 M urea, 1 % CHAPS, 13 mM DTT, 0.5 % (v/v) Servalyt pH 3-10 (Serva)) were added. After thorough mixing, the samples were centrifuged at 16,100 g for 12 minutes to remove insufficiently homogenized tissue. Only the supernatant was applied onto the gels. For the first dimension, we used 11-cm IPG stripes (Serva) with a linear pH-gradient from 3-10. The IEF was performed on an IEF100 (Hoefer) for a total of 15,000 Vhrs using the cuploading method.

One gel was prepared for subsequent mass spectrometric analysis. The sample for this gel contained 30 pairs of MAGs, collected from ergatoid *C. obscurior* males only, in 40 μL DeStreak Rehydration Solution (GE Healthcare). This procedure could not be conducted for *C. tjibodana* due to male limitation, as at that time colonies were not in a good condition anymore.

3.2.3 Sample equilibration and performance of the second dimension (SDS-PAGE)

To reduce disulfide bonds of cysteine side-chains, the IPG strips were incubated for 12 min in 6 mL of equilibration solution (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3 % (v/v) glycerol, 2 % (w/v) SDS), bromophenol blue) containing 1 % (w/v) DTT. In a second equilibration step, for another 12 min, 2.5 % (w/v) iodoacetamide (IAA) were added to 6 mL of equilibration solution to prevent renewed formation of disulfide bonds.

The SDS-PAGE was conducted on a SE900 (Hoefer). Equipment and solutions were prepared following exactly the manufacturer's manual. An Electrophoresis Power Supply-ESP 601 (Amersham Pharmacia Biotech) was employed as power source, which had a maximum of 400 mA instead of the suggested 480 mA (80 mA/gel) for a run with 6 gel plates (settings: 400 mA, 600 V, 100 W). Proteins were separated according to their size in half of a homogeneous home-made 12.5 % SDS-polyacrylamide gels (2500 x 2100 x 1 mm). The Tris-glycine buffer system as described by Laemmli was used [Laemmli, 1970]. First and second dimension were always carried out successively.

3.2.4 Silver staining

After 2D-SDS-PAGE, the gels were removed from the glass plates and put separately into 200 mL of fixation solution (containing 10 % (v/v) acetic acid) for silver staining over night. For quantitative analysis, glutaraldehyde fixation and crosslinking of proteins were used for enhanced silver staining [Slisz & Van Frank, 1985]. Since glutaraldehyde fixation is not compatible with extraction of protein from gels, a less sensitive silver staining protocol using reduced amounts of formaldehyde for maximum protein recovery was employed. Image and statistical analysis of gels were performed with the Progenesis SameSpots software (Nonlinear Dynamics Limited, Newcastle upon Tyne, UK).

3.2.5 Nano-LC-MS/MS-analysis

Seventeen of the 56 significant spots of *C. obscurior* could be excised and subjected to tryptic digestion and subsequent nano-LC-MS/MS as published previously [Thomas *et al.*, 2013]. The MS/MS-spectra were searched against the NCBInr-database using the Mascot Daemon and the Mascot algorithm (version 2.2; Matrix Science Ltd., London, UK) using trypsin as protease with max. one missed cleavage site, carbamidomethylation of cysteines as constant, methionine oxidation as variable modification and 0.2 Da-tolerance for MS- and MS/MS-signals.

3.3 Results

The ACPs of *C. obscurior* and *C. tjibodana* showed a wide distribution over the pHrange of 3-10. There was also a wide distribution in size, with smaller proteins being more numerous. Interestingly, we found less ACPs to be significantly ($p \leq 0.05$) differentially expressed between the male phenes of *C. obscurior*, namely 56 out of 920 (6.1 %), including eleven with a statistical power $q \geq 0.8$ (Figure 3.1, Table 3.1). In comparison, for the *C. tjibodana* phenes, 91 out of 1187 (7.7 %) proteins were significantly differentially regulated ($p \leq 0.05$), 40 of them with a statistical power $q \geq 0.8$ (Figure 3.2, Table 3.2). Unfortunately, the protein patterns of the two species were too different to be compared with each other statistically (see Figure 3.3 for an overlaid picture of the reference gels of *C. obscurior* and *C. tjibodana*). Although some protein-spots were apparently shared by the two species, others had no obviously corresponding homologue.

Four of the total of 17 protein spots that were subjected nano-LC-MS/MS analysis yielded eight proteolytic peptide sequences, with one spot yielding five sequences from three different proteins (for protein assignment, see Table 3.3). Blastp of four of the peptide sequences against the *Cardiocondyla* proteome resulted in four unique hits. A subsequent comparison of the whole *Cardiocondyla* protein sequence with the NCBI database (blastp) resulted in the same hits as before, but with even more significant e-values (see Table 3.3).

During spot picking, it was unclear whether one of the spots of interest (238) was of a very elongated shape or consisted of two or more poorly resolved spots. Hence, we picked this spot twice (238 and 238/2). One of these spots (238/2) yielded peptide sequences from three different proteins (SERPINB10, a hypothetical protein and SPS1), rendering it difficult to conclude which protein was indeed over-expressed in the winged male phene within this spot. However, spot 238 contained only SERPINB10, and we were able to conclude that this protein is indeed over-expressed in the winged male phene. In addition, a western blot analysis (see Chapter 6) confirmed that SPS1 (spot 238/2) is also over-expressed in winged males. The two other spots indicated that NHE-RF2 was also over-expressed in winged males too, while PDIA3 was over-expressed in ergatoid males.

Table 3.1: Results of statistical analysis of the 2D-PAGE of *C. obscurior*: 56 protein spots were significantly differentially regulated ($p \leq 0.05$), eleven of them with a statistical power $q \geq 0.8$ (light grey background colour). Analyzed gel spots that yielded a protein identification result are marked with dark grey background colour. (w = winged, erg = ergatoid)

Number	Anova (p)	Fold	↑
485	0.002	1.4	erg
12	0.002	1.8	erg
371	0.003	2	w
291	0.003	1.6	w
238	0.003	1.4	w
112	0.005	1.4	erg
289	0.006	1.4	w
16	0.007	1.6	erg
605	0.007	1.5	w
356	0.007	1.5	w
15	0.007	1.5	erg
17	0.01	1.7	erg
212	0.01	1.6	w
920	0.01	1.4	w
241	0.01	1.5	erg
67	0.011	1.3	erg
363	0.011	1.4	w
901	0.011	1.7	w
589	0.012	1.2	erg
51	0.012	1.5	erg
7	0.013	1.5	erg
906	0.015	1.6	w
524	0.015	1.3	erg
319	0.016	1.4	w
726	0.017	1.4	w
14	0.019	1.5	erg
353	0.02	1.5	w
204	0.021	1.6	w
25	0.022	1.8	erg
567	0.022	1.7	erg
20	0.023	1.5	erg
355	0.023	1.3	w
549	0.025	1.3	w
200	0.025	1.4	w
926	0.025	1.6	w
619	0.026	1.4	w
568	0.031	1.4	erg
495	0.031	1.3	erg
435	0.034	1.3	w
217	0.034	1.3	erg
245	0.034	1.5	erg
339	0.035	1.3	w
336	0.036	1.4	w
649	0.036	1.4	w
571	0.037	1.6	w
893	0.038	1.2	w
39	0.04	1.4	erg
62	0.04	1.4	erg
33	0.041	1.6	erg
675	0.042	1.4	erg
71	0.043	1.3	erg
695	0.043	1.5	w
384	0.043	1.4	erg
328	0.048	1.6	erg
4	0.048	1.7	erg
685	0.05	1.3	w

Table 3.2: Results of statistical analysis of the 2D-PAGE of *C. tjibodana*: 91 protein spots were significantly differentially regulated ($p \leq 0.05$), 40 of them with a statistical power $q \geq 0.8$ (grey background colour). (w = winged, erg = ergatoid)

Number	Anova (p)	Fold	↑
944	0.0005748	1.9	erg
879	0.001	1.8	w
922	0.001	1.8	w
210	0.001	1.9	erg
683	0.003	2	erg
277	0.003	2.4	erg
1016	0.004	1.6	erg
999	0.006	1.9	w
311	0.006	1.9	w
827	0.007	1.5	w
822	0.007	1.6	w
658	0.008	2.3	erg
151	0.009	2.2	erg
259	0.009	1.9	w
690	0.009	2.4	erg
733	0.009	1.6	erg
675	0.011	1.3	erg
789	0.011	1.5	w
739	0.011	2.1	erg
969	0.012	1.5	w
1068	0.013	1.6	w
802	0.013	1.7	w
1058	0.015	1.2	erg
236	0.015	1.5	w
417	0.015	1.7	erg
791	0.015	1.7	w
964	0.015	1.6	w
810	0.016	1.7	w
353	0.017	2.2	w
260	0.018	1.6	w
375	0.018	1.9	erg
550	0.019	1.6	erg
465	0.019	1.6	erg
498	0.019	1.6	w
544	0.02	1.4	erg
796	0.02	1.7	w
997	0.02	1.8	erg
496	0.022	1.7	w
1008	0.023	1.5	erg
818	0.023	1.4	w
673	0.023	1.4	w
1092	0.023	1.5	w
172	0.023	1.8	w
841	0.024	1.4	erg
1106	0.024	2	w
1050	0.024	1.7	w
750	0.024	1.4	w
251	0.024	1.7	w
36	0.025	1.5	erg
884	0.026	1.6	w
1187	0.026	1.5	w
399	0.026	2	erg
351	0.026	1.6	erg
254	0.027	1.6	erg
198	0.029	1.3	erg
749	0.029	3.5	erg
516	0.03	1.7	w
935	0.031	1.4	w
902	0.031	1.6	w
308	0.031	1.8	erg
926	0.032	1.6	w
738	0.033	1.4	erg
1181	0.033	1.8	w
458	0.034	1.6	w
339	0.034	1.4	erg
261	0.035	1.6	erg
336	0.035	1.4	w
984	0.038	1.5	erg
891	0.038	1.5	w
298	0.038	1.7	erg
437	0.039	2.2	w
295	0.04	1.5	w
689	0.04	2	erg
1035	0.04	1.9	w
1085	0.041	1.4	w
811	0.041	1.4	w
706	0.041	1.8	erg
518	0.042	1.4	w
755	0.042	1.6	erg
1098	0.043	1.8	w
299	0.043	1.5	erg
1076	0.043	1.9	w
974	0.043	1.6	erg
744	0.045	1.3	w
137	0.045	1.5	w
372	0.045	1.9	erg
1091	0.045	1.5	w
961	0.048	1.5	w
769	0.048	1.3	w
461	0.049	1.5	erg
753	0.049	1.2	w

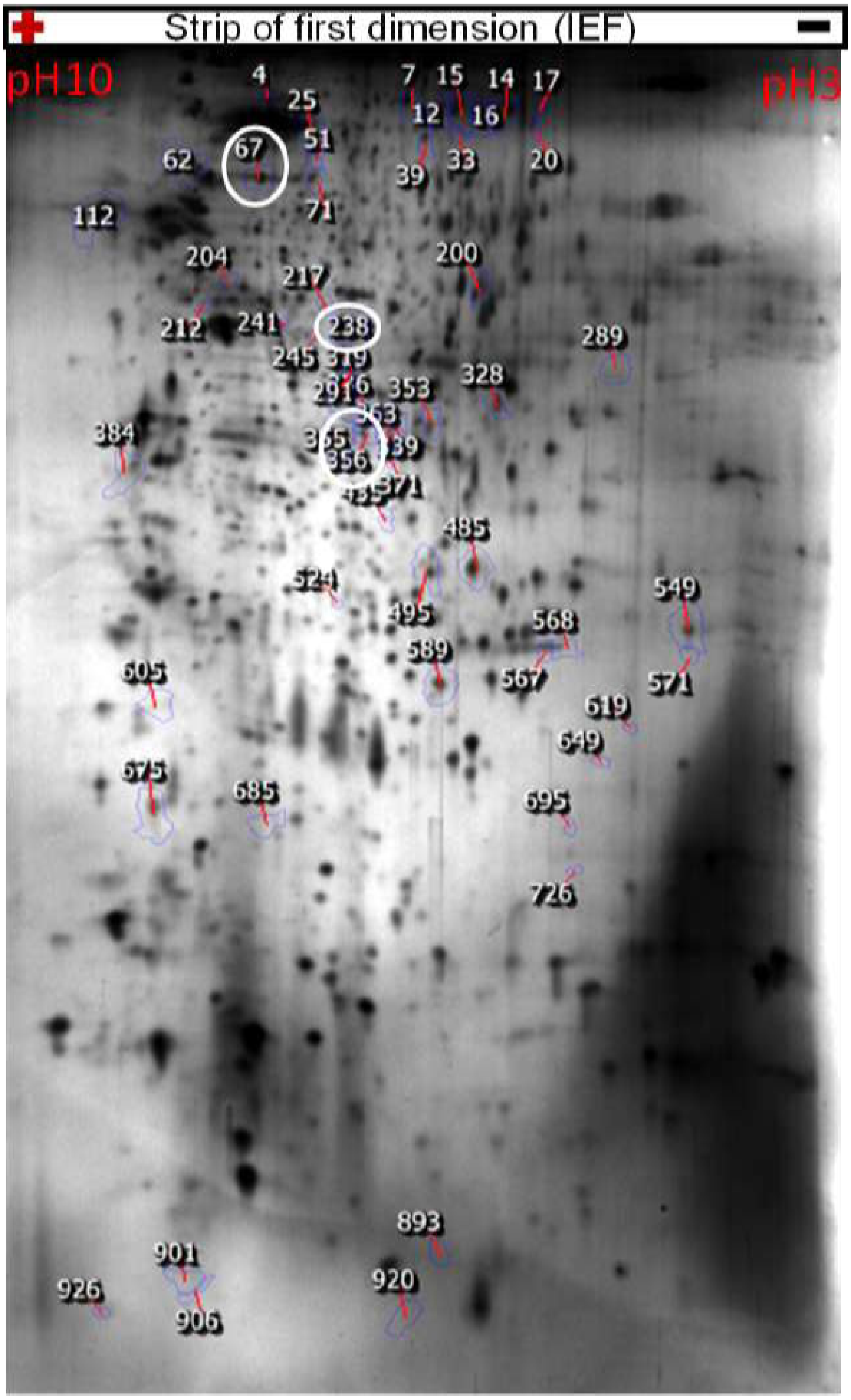


Figure 3.1: Reference gel from *C. obscurior*. Each number marks a protein spot differentially regulated between the two male phenes. White circles indicate protein spots that yielded protein identifications upon nano-LC-MS/MS analysis.

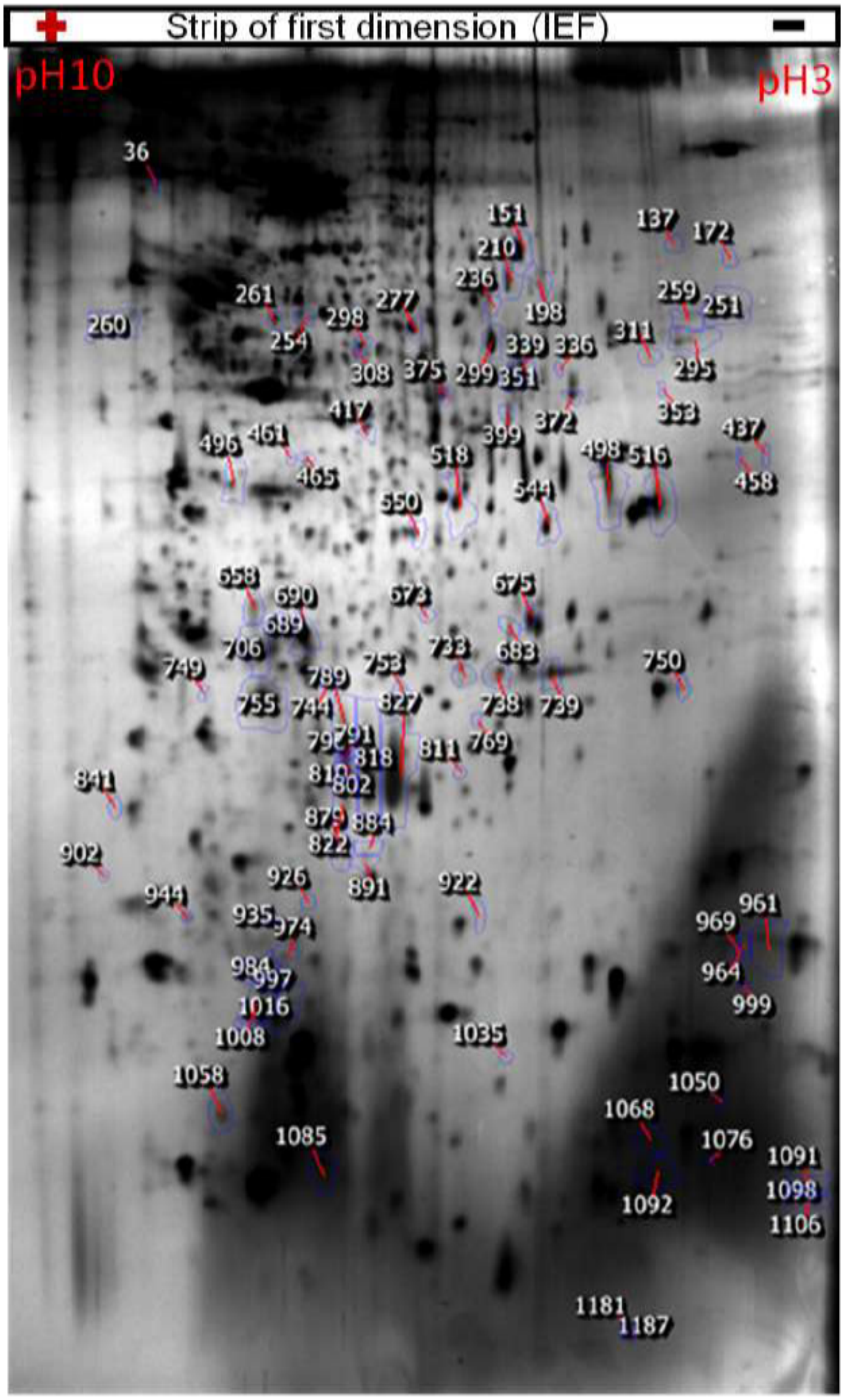


Figure 3.2: Reference gel from *C. tjibodana*. Each number signifies a protein spot that was differentially regulated between the two male phenes.

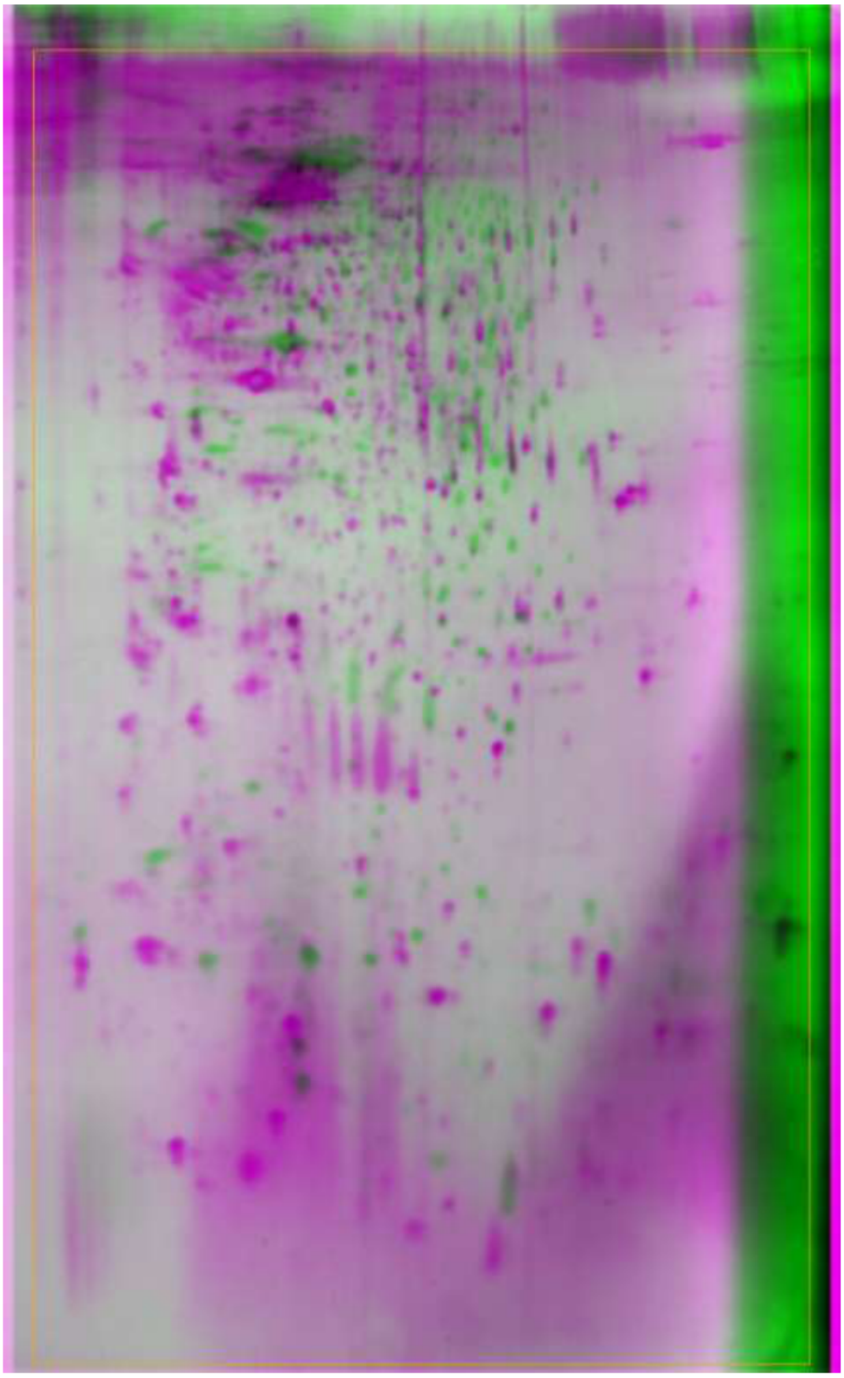
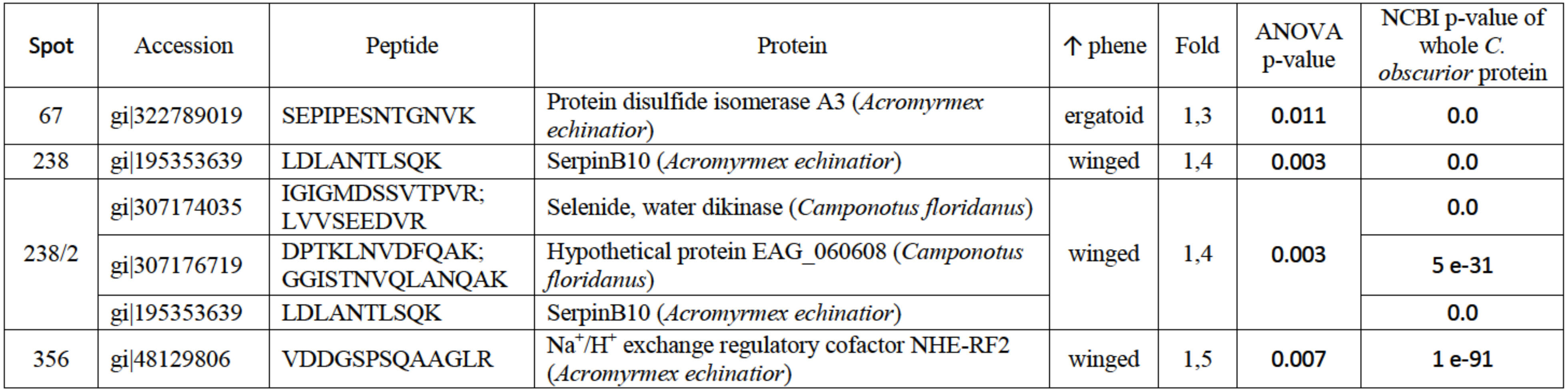


Figure 3.3: An overlaid picture of the reference gels of *C. obscurior* (green) and *C. tjibodana* (purple) is shown. It is obvious that the ACPs of these two species are too different for the identification of spots differentially expressed between these species.

Table 3.3: Protein spots of *C. obscurior* that yielded a result in the nano-LC-MS/MS-analysis. Accession number, peptide sequences and protein identifications are listed. Furthermore, the value of over-expression (fold) and the male phene, in which the protein is over-expressed (↑ phene), is shown. Finally the p-value of the statistical comparison of the male phenes (ANOVA p-value) and the p-value that resulted after a blastp of the whole *Cardiocondyla* protein sequence against the NCBI database (NCBI p-value of whole *C. obscurior* protein) are presented.



Spot	Accession	Peptide	Protein	↑ phene	Fold	ANOVA p-value	NCBI p-value of whole *C. obscurior* protein
67	gi|322789019	SEPIPESNTGNVK	Protein disulfide isomerase A3 (*Acromyrmex echinatior*)	ergatoid	1,3	**0.011**	**0.0**
238	gi|195353639	LDLANTLSQK	SerpinB10 (*Acromyrmex echinatior*)	winged	1,4	**0.003**	**0.0**
238/2	gi|307174035	IGIGMDSSVTPVR; LVVSEEDVR	Selenide, water dikinase (*Camponotus floridanus*)	winged	1,4	**0.003**	**0.0**
	gi|307176719	DPTKLNVDFQAK; GGISTNVQLANQAK	Hypothetical protein EAG_060608 (*Camponotus floridanus*)				**5 e-31**
	gi|195353639	LDLANTLSQK	SerpinB10 (*Acromyrmex echinatior*)				**0.0**
356	gi|48129806	VDDGSPSQAAGLR	Na^+/H^+ exchange regulatory cofactor NHE-RF2 (*Acromyrmex echinatior*)	winged	1,5	**0.007**	**1 e-91**

3.4 Discussion

Our study shows, that in *C. obscurior*, SERPINB10, NHE-RF2, a hypothetical protein SPS1 appear to be over-expressed in the winged phene. Over-expression of SPS1 could be confirmed by western blot analysis [Probst, 2014]. In comparison, of the proteins apparently over-expressed in the ergatoid phene, only PDIA3 could be identified by nano-LC-MS/MS analysis.

Generally, it has been shown that the type of mating system influences greatly the rate of evolution of ACPs [Schumacher *et al.*, 2014]. In polygamous/promiscuous mating systems, rapidly evolving SFPs are even discussed as a driving force of speciation [Aguadé *et al.*, 1992, Arnqvist *et al.*, 2000, Clark & Swanson, 2005, Rice, 1996, Swanson & Vacquier, 2002], e.g. via post-mating prezygotic isolation [Civetta & Singh, 1995, Marshall *et al.*, 2011, Parker & Partridge, 1998, Schumacher *et al.*, 2014]. For example, the *Drosophila* Acp26Aa is rapidly evolving and manipulates females as it induces egg-laying rate [Herndon & Wolfner, 1995, Marshall *et al.*, 2011]. A less rapid evolution is expected in monogamous species with relaxed sexual conflict, but SFPs are still discussed with regard to speciation processes [Torgerson *et al.*, 2002].

Besides the mating system, it has been shown that protein-protein interactions have an impact on the evolution rate of proteins [Clark & Swanson, 2005, Haerty *et al.*, 2007, Schumacher *et al.*, 2014]. A protein that has a broad range of interactions, potentially in more than one tissue, shows a slower evolutionary rate than a more specialized and tissue-specific protein [Dorus *et al.*, 2006, Khaitovich *et al.*, 2006, Schumacher *et al.*, 2014]. Hence, different selection pressures operate on proteins, which led Schumacher and colleagues to suggest "that species-specific levels of sexual selection may have a stronger impact on evolutionary rates of post-mating proteins, whereas functional constraint may particularly restrict sequence evolution for pre-mating sperm proteins" [Schumacher *et al.*, 2014].

As already mentioned, in *C. tjibodana* there is no difference in queen longevity in dependence of the male phene a queen mated with. Therefore, we would have expected a more similar ACP pattern between the male phenes of this species in comparison to the differences in protein pattern of the male phenes of *C. obscurior*, where male phene influences queen longevity [Schrempf & Heinze, 2008]. At the moment, we can only speculate which traits might select for these intraspecies differences in the protein pattern of *C. tjibodana.* For example, as regularly several winged males co-occur, sperm competition might influence the composition of ACPs (see e.g. [den Boer *et al.*, 2010]). Even though we would not expect harmful effects for the females, sperm competition between the ejaculates might occur [den Boer *et al.*, 2008]. In *Atta* leafcutter ants negative effects are opposed by the spermathecal fluid so that effects of post-mating sexual selection and conflict (sperm competition) are limited to a short time after mating [den Boer *et al.*, 2010].

In our study we found a hypothetical protein that seems to be present only in *C. obscurior* and *Camponotus floridanus*, as neither the NCBI- nor the uniprot-database showed a hit with any other species. Hence, this protein seems to be restricted to only a few species and its function is not yet known or described from any other organism. It might be a protein specific to MAGs, as it has been suggested that some ACPs diverge so rapidly that no orthologues can be found [Andrés *et al.*, 2006, Dorus *et al.*, 2006].

SERPINB10 belongs to the family of **ser**ine **p**rotease **in**hibitors (serpins), which consist of more than 1000 members that can be separated into at least 16-17 clades [Irving *et al.*, 2000, Mangan *et al.*, 2008, Silverman *et al.*, 2001].They occur in clusters based rather on their function than on species affiliation [Irving *et al.*, 2000, van Gent *et al.*, 2003]. The clade B serpins, so called ov-serpins, are beside other functions involved in the regulation of cell growth (apoptosis), differentiation, tumor cell invasiveness [Shioji *et al.*, 2005] and motility and angiogenesis [Silverman *et al.*, 2001]. They are mainly intracellular serpins that exhibit a suicide mechanism to scavenge their target [Potempa *et al.*, 1994, Reichhart, 2005, Remold-O'Donnell, 1993, Silverman *et al.*, 2001, van Gent *et al.*, 2003, Ye & Goldsmith, 2001]. In these proteins a single substitution in the reactive centre loop may change the target protease and they are thus perfectly "armed" for the rapid evolution of SFPs [Mangan *et al.*, 2008]. Serpins are common in the seminal fluid and have been found from insects to mammals to be important in the reproductive tract of males and females [Chapman, 2001, Chapman & Davies, 2004, Dottorini *et al.*, 2007, Gillott, 2003, Lung *et al.*, 2002, Marshall *et al.*, 2011, Mueller *et al.*, 2005, Murer *et al.*, 2001, Shioji *et al.*, 2005, Silverman *et al.*, 2001, Silverman *et al.*, 2004, Walters & Harrison, 2010].

Serpins play also a major role in immune response [De Gregorio *et al.*, 2002, Kanost, 1999, Kanost *et al.*, 2004, Mangan *et al.*, 2008]. For example, in *D. melanogaster*, they are involved in the activation of the Toll pathway, which is important for the immune function against fungi and gram positive bacteria [Levashina *et al.*, 1999, Reichhart, 2005, Valanne *et al.*, 2011]. Interestingly, Peng and colleagues [Peng *et al.*, 2005] found that sex peptide - a SFP of *Drosophila* - activates the Toll pathway and the Imd pathway. They suggested that it acts as preventive activation of the immune system, in case that female gets hurt during mating and is then exposed to pathogens [Peng *et al.*, 2005]. In *C. obsurior* SERPINB10 might play a similar role and help the mated female to stimulate its immune system, which is especially important if the young queen has to leave the maternal colony to found a new colony.

Another protein that is over-expressed in the accessory glands of winged in comparison to wingless males is SPS1 (see also [Fuessl *et al.*, 2014], Chapter 4). Usually, this protein is involved in selenocysteine biosynthesis which itself is an important part of selenoprotein biosynthesis [Alsina *et al.*, 1999, Tamura *et al.*, 2004] and needed to defend reactive oxygen species (ROS) [Morey *et al.*, 2003b, Pedersen *et al.*, 2008]. Harmful oxygen-species accumulate under oxidative stress and greatly influence cellular metabolism, damaging RNA, DNA and proteins, which may lead ultimately to cell death [Battin & Brumaghim, 2009, Simon *et al.*, 2000] and thus negatively influence an individual's lifespan [Harman, 1955, Muller *et al.*, 2007]. Apparently social insects lost all selenoproteins but they all still encode the gene for SPS1 [Chapple & Guigó, 2008]. This protein has been suggested to be important for selenoprotein independent ROS defence and is thus a candidate to be involved in the life-prolonging effect of winged males.

A further protein that we found is NHE-RF2. The NHE-RF protein participates in multiple cellular mechanisms such as protein phosphorylation, targeting, and trafficking. Upon alternative splicing, NHE-RF2 mRNA produces a protein that interacts with SRY, which is a nuclear transcription factor that is controlling the expression of testis-specific genes [Poulat *et al.*, 1997, Shenolikar & Weinman, 2001]. How NEH-RF2 influences the female's physiology after mating in *C. obscurior* remains unknown.

A protein that we found to be over-expressed in the ergatoid male phene is PDIA3. This protein is constitutively expressed in most tissues [Wilkinson & Gilbert, 2004] and an essential folding catalyst of the endoplasmatic reticlum [Dun *et al.*, 2012, Noiva & Lennarz, (1992)]. It belongs to the thiol oxidoreductase family with disulfide oxidoreductase and isomerase activity [Freedman *et al.*, 1994], which induces and catalyzes the rearrangement of incorrectly folded disulfide bonds [Wilkinson & Gilbert, 2004]. Its expression can be induced by oxidative stress [Huang *et al.*, 2009, Sharma *et al.*, 2013]. The protein has been detected in the reproductive tract of males and females of different species (including humans), presumably playing a role in sperm egg fusion [Akama *et al.*, 2010, Baer *et al.*, 2009a, Dun *et al.*, 2012, Ellerman *et al.*, 2006, Zhang *et al.*, 2007]. As ergatoid males have an ongoing spermatogenesis, they might produce more of this protein, as it has been shown to abound in various stages of spermatogenesis in sheep [Lv *et al.*, 2011].

Finally, ACPs are apparently less conserved in *C. tjibodana* compared to *C. obscurior*, as we found more ACPs to be differentially expressed between the two male phenes of *C. tjibodana*. At the moment, it is unclear whether these differences are a consequence of the differences in the mating system of these two species. Detailed data on mate number and mode of colony founding in *C. tjibodana* might help to identify possible selection pressures on males and females in *C. tjibodana*. Then, it would be valuable to analyze differentially regulated proteins between these two species in order to determine further possible candidate proteins that account for an enhancement of lifespan in *C. obscurior*, and to get a first hint on the occurrence of sperm competition in *C. tjibodana* respectively.

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Chapter 4

Selenophosphate synthetase in the male accessory glands of an insect without selenoproteins

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Males of *C. obscurior*: left side) ergatoid male, right side) winged male

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Abstract

Selenoproteins (containing the 21st proteinogenic amino acid selenocysteine) play important roles throughout all domains of life. Surprisingly, a number of taxa have small selenoproteomes, and Hymenopteran insects appear to have fully lost selenoproteins. Nevertheless, their genomes contain genes for several proteins of the selenocysteine insertion machinery, including selenophosphate synthetase 1 (SELD/SPS1). At present, it is unknown whether this enzyme has a selenoprotein-independent function, and whether the gene is actually translated into a protein in Hymenoptera. Here, we report that SELD/SPS1 is present as a protein in the accessory glands of males of the ant *Cardiocondyla obscurior*. It appears to be more abundant in the glands of winged disperser males than in those of wingless, local fighter males. Mating increases the lifespan and fecundity of queens in *C. obscurior*, and mating with winged males has a stronger effect on queen fitness than mating with a wingless male. SELD/SPS 1 has been suggested to play an important role in oxidative stress defense, and might therefore be involved in the lifeprolonging effect of mating.

Keywords: Selenoprotein, Selenophosphate synthetase 1, Male accessory gland proteins, Hymenoptera

4.1 Introduction

Selenoproteins occur in almost all life forms, from Archaea and Bacteria to animals including humans. They contain the 21st proteinogenic amino acid selenocysteine in their reactive center and mainly function as redox-enzymes [Hamilton & Tappel, 1963, Stadtman, 1996]. Selenocysteine is incorporated into proteins during translation, but in contrast to most other amino acids is not encoded by its own codon. Instead, an in-frame opal stop codon (UGA) is recoded, and a selenocysteine insertion element (SECIS-element) located at a specific distance from this codon activates several cofactors (e.g. SELA-SELD in Bacteria, [Boeck *et al.*, 1991a, Boeck *et al.*, 1991b, Ehrenreich *et al.*, 1992, Forchhammer *et al.*, 1989]; for Eukaryotes see [Turanov *et al.*, 2011, Xu *et al.*, 2007] that act together as the selenocysteine insertion machinery. As selenium is more reactive than sulfur, proteins with selenocysteine residues show a greater reduction potential in the defense against reactive oxygen species (ROS) than homologous proteins containing cysteine residues [Battin & Brumaghim, 2009, Gladyshev, 2012].

With the increasing availability of insect genomes, it has become obvious that insects have strongly reduced selenoproteomes [Lobanov *et al.*, 2007], probably because their ROS-defense works completely different from that of vertebrates [Kanzok *et al.*, 2001] but see [Morey *et al.*, 2003a, Morey *et al.*, 2003b, Pedersen *et al.*, 2008]. While some fruit fly species still have three functional selenoproteins [Adams *et al.*, 2000, Castellano *et al.*, 2001, Martin-Romero *et al.*, 2001], there is strong evidence that Hymenoptera (ants, bees, wasps etc.) have lost them completely [Chapple & Guigó, 2008]. However, several Hymenoptera still possess different, yet incomplete sets of genes of the selenoprotein biosynthesis machinery. Common to all presently sequenced genomes is the conserved gene selenophosphate synthetase 1 (also referred to as selenide, water dikinase, *sps1*, *selD*, [Persson *et al.*, 1997], *ptuf*, [Alsina *et al.*, 1998, Serras *et al.*, 2001]), which encodes the enzyme that from selenide and ATP synthesizes selenophosphate, the selenium donor for the synthesis of selenocysteine [Chapple & Guigó, 2008]. Aside from selenocysteine biosynthetic processes [Alsina *et al.*, 1999, Tamura *et al.*, 2004] the enzyme has been reported to be – in conjunction with selenoproteins – involved in various physiological processes like cell proliferation, imaginal disc development [Alsina *et al.*, 1998, Serras *et al.*, 2001], glutamine metabolism, mitochondrion organization [Shim *et al.*, 2009, Lee *et al.*, 2011], negative regulation of ROS homeostasis [Alsina *et al.*, 1999, Morey *et al.*, 2003a, Morey *et al.*, 2003b], stress responses [Morey *et al.*, 2003a, Morey *et al.*, 2003b, Pedersen *et al.*, 2008], and neurogenesis [Neumueller *et al.*, 2011]. Recent studies suggest an additional role in selenoprotein-biosynthesis [Alsina *et al.*, 1999, Tamura *et al.*, 2004] and an as yet unknown, selenoprotein-unrelated function [Han *et al.*, 2012, Lobanov *et al.*, 2008, Xu *et al.*, 2006, Xu *et al.*, 2007]. Consequently, the gene, its evolutionary history and its particular function have become the subject of increasing attention. Despite of this growing interest in *selD/sps1* it has remained unclear, whether it is translated into a protein in those insects that do not have selenoproteins, and, if so, in which tissue and with which function. Even in *Drosophila willistoni*, the first animal reported to completely lack selenoproteins [Chapple & Guigó, 2008, Clark *et al.*, 2007], it is not yet clear, whether genes residing in its genome for selenoprotein biosynthesis are translated at all, or which alternative function they might have.

Here we report on the occurrence of SELD/SPS1 in the male accessory glands (MAG) of the ant *Cardiocondyla obscurior*. In solitary insects, peptides and proteins transferred in the seminal fluid of males may harm the female and shorten its life-expectancy. In contrast, *C. obscurior* queens that mated with normal or with sterilized males lived significantly longer than virgin queens, suggesting that compounds transferred in the seminal fluid positively affect their physiology [Schrempf *et al.*, 2005]. *C. obscurior* is characterized by a peculiar diphenism with winged, gracile males, which are produced when environmental conditions change and disperse, and wingless (so called ergatoid, i.e. "worker like") fighter males, which remain in their natal nests throughout their lifes, attempt to kill all rival males with their saber-shaped mandibles and monopolize mating with the young queens [Heinze & Hölldobler, 1993]. The observation that mating with a winged male increases queen lifespan even more than mating with a wingless male [Schrempf & Heinze, 2008] motivated a detailed comparison of the protein compositions of MAGs of the two types of males.

4.2 Material and methods

4.2.1 Ant sampling and housing

Colonies of *C. obscurior* [Wheeler, 1929] were collected from an experimental coconut plantation in Una, Bahia, Brazil in July 2009. Ants were then housed in artificial nests and fed twice a week with chopped cockroaches (*Nauphoeta cinerea*), fruit flies (*Drosophila melanogaster*), and a drop of honey. To obtain winged and wingless males, colonies were transferred from a 12 h/12 h day/night – cycle at 23–27 °C into an incubator with permanent light and a constant temperature of 22 °C. Four to six weeks later the first winged male pupae could be observed and colonies started to produce a cohort of winged males together with wingless males.

4.2.2 Sample preparation and performance of the first dimension: Isoelectric focusing (IEF)

Winged males were collected twice a week and put together with a few workers in a separate nest, where they lived until they were dissected 7–10 days later to ensure that all males had mature accessory glands. Wingless males of *C. obscurior* ants engage in lethal fighting with wingless rivals and we therefore collected them with a minimum time interval of 7 days.

Males were dissected on a cooled object slide in a drop of Beadle-solution (128.3 mM NaCl, 4.7 mM KCl, and 2.3 mM $CaCl_2$ [Darrouzet *et al.*, 2002]) and nine pairs of MAGs were collected in 20 μL DeStreak Rehydration Solution (GE Healthcare) with 2%IPG (pH = 3–11 NL, GE Healthcare) from either wingless or winged males. Samples were stored at –75 °C until usage. For each type of male, a total of ten gels, each loaded with the protein extract of nine pairs of MAGs, were run. The gel prepared for downstream nano-LC–MS/MS-analysis of excised protein spots contained 30 pairs of MAGs of wingless males in 40 μL DeStreak Rehydration Solution.

Equipment and solutions for the IEF were prepared following the manufacturer's protocol (Hoefer). Directly before starting the first dimension, the completely thawed samples were alternately sonicated and put on ice for 15 s each. This procedure was repeated four times, before adding 40 μL of rehydration solution (8 M Urea, 1% CHAPS, 13 mM DTT, 0.5% (v/v) Servalyt pH = 3–10 (Serva)). After thoroughly mixing, the samples were centrifuged at 16,100 for 12 min to avoid contamination with unsolved tissue. Only the supernatant was applied onto the gels-strips (11 cm IPG-strips (Serva) with a linear pH-gradient from 3 to 10). Isoelectric focusing was performed in an IEF100 (Hoefer) apparatus for a total of 15,000 Vhrs using the cup-loading method.

4.2.3 Preparation and performance of the second dimension: SDS–PAGE

For the reduction of disulfide bonds between cysteine residues the IPG-strips were incubated for 12 min in 6 mL of equilibration solution (6 M urea, 75 mM Tris–HCl, pH = 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS; bromophenol blue) containing 1% (w/v) DL dithiothreitol (DTT). To avoid the renewed formation of disulfide bonds, the thiol groups of the cysteine residues were alkylated in a second step by addition of equilibration solution containing 2.5% (w/v) iodoacetamide (IAA) instead of DTT.

The second dimension was conducted on a SE900 (Hoefer). Again equipment and solutions were prepared following the manufacturer's manual. An ESP 601 (Amersham Pharmacia Biotech) power supply was employed as power source. Instead of the suggested 480 mA (80 mA/gel), a maximum of 400 mA was applied for a run with six gel-plates (settings: 400 mA, 600 V, 100 W). Proteins were separated on the basis of their size in homogeneous 2500 x 2100 x 1 mm 12.5% SDS–polyacrylamide gels cast by us. The Tris–glycine buffer as described by Laemmli was used [Laemmli, 1970]. First and second dimension runs were always performed on the same day. After additional manual matching to the reference gel of each phene, statistical analysis was carried out using the Progenesis SameSpots software (Nonlinear Dynamics Limited), which itself does an automatic matching to a previously selected reference gel.

4.2.4 Silver staining

For silver staining SDS–PAGE gels were removed from the glass plates and placed separately overnight into 200 mL of fixationsolution for silverstaining, containing 10% (v/v) acetic acid. For maximum staining sensitivity in the nanogram range, gels were treated after a water wash with a 5% (v/v) glutaraldehyde solution for 30 min prior to silver staining [Blum *et al.*, 1987]. Glutardialdehyde fixation of proteins is not suited for excision and trypsination of protein spots prior to subsequent mass-spectrometric analysis. For that purpose, silver staining without glutardialdehyde was used.

4.2.5 Nano-LC–MS/MS-analysis

Protein spots of interest were excised and subjected to tryptic digestion and subsequent nano-LC–MS/MS as published previously [Thomas *et al.*, 2013]. The MS/MS-spectra were searched against the Uniprot database using the Mascot Daemon and the Mascot algorithm (version 2.2; Matrix Science Ltd., London, UK) using trypsin as protease with max. one missed cleavage site, carbamidomethylation of cysteines as constant, methionine oxidation as variable modification and 0.2 Da-tolerance for MS- and MS/MS signals.

4.2.6 PCR

To prove that the gene-model with 7 exons is due to false automatic annotation of a predicted intron-sequence within an exon, we performed a PCR with primers that spanned the region of interest. The forward primer (5'TGGCGCAGCTTGAGC3') was placed in the middle of the automatically predicted exon 1 and the reverse primer (5'GCACAGGATCACGAGCA3') in the middle of the automatically predicted exon 2. In case that the region of interest (a) is not translated, we expected a PCR product of 127 bp, (b) in case it belongs to the exon, we expected a PCR product of 205 bp. The 100-bp DNA ladder (Peqlab, Erlangen, Germany) was used to size the PCR product.

4.3 Results

We separated and quantified MAG proteins from both winged and wingless males by 2D electrophoresis. Statistical comparison of normalized protein spot intensities revealed that 56 of 920 detected proteins were differentially regulated ($p \leq 0.05$), eleven of them with a statistical power of $q \geq 0.8$. In one of the spots that were significantly higher expressed in the MAGs of winged males (ANOVA: $p = 0.003$, $q > 0.8$), we found the peptide sequences of three proteins; one of them was identified as SELD/SPS1 by nano-LC–MS/MS (Figure 4.1).

The analysis yielded two proteotypic peptide sequences with 13 and nine amino acid residues,

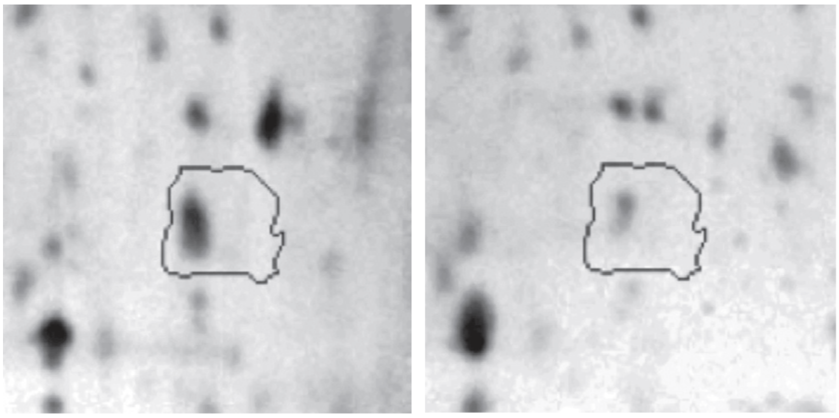

Figure 4.1: Reference gel with the protein spot containing SELD/SPS1 in the electrophoretically separated contents of the male accessory glands of (a) winged males and (b) wingless males of the ant *Cardiocondyla obscurior.* The calculation of differently regulated spots was based on a total of ten gels, each with the extract of nine pairs of MAGs, for each type of male. All gels were matched to a reference gel of the respective phene, and analyzed with the program Progensis SameSpots (Nonlinear Dynamics limited). For spot picking, a single replicate with 30 pairs of glands of wingless males was used.

respectively (see blue letters in Figures 4.2 and 4.3). Both peptide sequences are unique within the proteome of *Cardiocondyla* and do not show similarities to any other locus [Schrader *et al.*, in press]. The same holds true for searches of the databases NCBI (blastp) [Altschul *et al.*, 1990] and UniProtKB [Magrane and Consortium, 2011, Consortium, 2013]. Hence, these two peptides could be assigned unambiguously to SELD/SPS1.

The comparison of the gene and protein sequences of *C. obscurior* with the corresponding sequences of *D. melanogaster*, the only insect in which the SELD/SPS1 protein has been verified so far, showed that both gene and protein sequences are highly conserved (85% and 74% identity, respectively). *D. melanogaster selD/sps1* consists of two exons, one of which contains the 1194-base open reading frame of encoding a protein of 398 amino acid residues [Alsina *et al.*, 1998, Persson *et al.*, 1997]. Automatic annotation of the *C. obscurior* sequence predicted seven instead of six exons due to a falsely annotated intragenic region in exon 1, which actually encodes 26 amino acids after recoding of an inframe UGA codon (green sequence in Figures 4.2 and 4.3). Automatic annotation programs usually read UGA-triplets as stop codons and are not sensitive to the possibility of recoding. Hence, manual annotation is required. We combined the *selD/sps1* gene structure predictions of *D. melanogaster*, *Apis mellifera*, and all available ant genomes to predict the true exon–intron structure for *C. obscurior.* Manual annotation of the *C. obscurior* sequence resulted in six exons, similar to the condition in other Hymenoptera.

Figure 4.2 shows the apportionment of amino acids to the exons of SELD/SPS1. Exons 1

Exon 1:
MAELQGTTVSQEALAVAQLELEGNPNALALRRPFDP**VAHDLDASFRLTRFADLKG**
XGCKVPQEVLGKLLEGLQADDASAQDHEHAHFMH**M**AIPR**IG**

Exon 2:
IGMDSSVTPVRHGGLSLVQTTDFFYPLVDDPYMM

Exon 3:
KIACSNVLSDLYAMGVTDCDNMLMLLGVSTKMTEKERDVVVPLIMRGFKDSALDA
GTTVTGGQTVVNPWCTIGGVATTVCQANEYIV

Exon 4:
PDNAVVGDVLVLTKPLGTQVAVNAHQWLDQPDRWNRIK**LVVSEEDVR**KGYQRAM
DSMARLNRT

Exon 5:
AARLMHKYNAHGATD**VTGFGLLGH**AQNLAKHQKNEVSFVIHNLPVISKMAAVAK
ACGNMFQLLQGHSAETSGGLLICLPREQ

Exon 6: AAAYCKDIEKQEGYQAWIIGIVEKGLRTARIIDKPRVIEVPAKEKDGELW

Figure 4.2: Predicted, manually curated amino acid sequence of SELD/SPS1 of *C. obscurior* with six exons. Green: automatically predicted intron sequence with X replacing an unknown amino acid recoding the UGA codon. Blue: peptide sequences found in the MAGs of *C. obscurior* by mass spectrometry. One sequence spans exons 1 and 2. Red: methionine at position 90, predicted as start of the amino acid sequence in many other Hymenoptera (e.g. *Apis mellifera*, *Camponotus floridanus* and *Acromyrmex echinatior*, [Altschul *et al.*, 1997]). This would completely omit the region with the UGA codon (position 56). Bold: ATP/GTP binding site [Hirosawa-Takamori *et al.*, 2000, Persson *et al.*, 1997]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and 2 predicted by automatic annotation and the separating intron (green area in Figure 4.2, embedded in black areas (exon 1 and 2)) instead form a single large exon (exon 1 in Figure 4.2). The full-length protein comprises 413 amino acids and the first exon incorporates the in-frame UGA codon, which is probably read-through and recoded by a so far unknown amino acid. This amino acid might be an arginine as in the SELD/SPS1 homolog of *D. melanogaster* [Persson *et al.*, 1997], but could be also any other amino acid (as e.g. cysteine, serine and tryptophan have been shown to be incorporated for UGA; [Jungreis *et al.*, 2011]) and here is symbolized by "X" (see Figure 4.3).

A PCR with cDNA and a pair of primers specific to *selD/sps1* resulted in a product of about 205 bp instead of 127 bp (Figure 4.4), proving that the 26 amino acids, which are annotated incorrectly as an intron by automatic annotation, are part of the transcript. Alignment of the amino acid sequences of *D. melanogaster* (DROME), *A. mellifera* (APIME), and *C. obscurior*

```
DROME           M------SYAADVLNSAHLELHGGGDA-ELRRPFDPTAHDLDASFRLTRFADLKGRGCKV
CAROB           MAELQGTTVSQEALAVAQLELEGNPNALALRRPFDPVAHDLDASFRLTRFADLKGXGCKV
APIME           MAELQGTPVAQDALSVAQLELGGNPNALALRRPFDPVAHDLDATFRLTRFADLKGXGCKV
                *      . : :.*  *:*** *. :*  *******.******:*********** ****
                1       10        20        30        40        50        60

DROME           PQDVLSKLVSALQ-QDYSAQDQE-PQFLNVAIPRIGIGLDCSVIPLRHGGLCLVQTTDFF
CAROB           PQEVLGKLLEGLQADDASAQDHEHAHFMHMAIPRIGIGMDSSVTPVRHGGLSLVQTTDFF
APIME           PQEVLGKLLEGLQADDGSAQDHEHAHFMHMAIPRIGIGMDSSVTPLRHGGLSLVQTTDFF
                **:**.**:..** :* ****:* .:*:::********:*.** *:*****.********
                        70        80        90       100       110       120

DROME           YPIVDDPYMMGKIACANVLSDLYAMGVTDCDNMLMLLAVSTKMTEKERDVVIPLIMRGFK
CAROB           YPLVDDPYMMGKIACSNVLSDLYAMGVTDCDNMLMLLGVSTKMTEKERDVVVPLIMRGFK
APIME           YPLVDDPYMMGKIACANVISDLYAMGVTECDNMLMLLGVSTKMTEKERDVVVPLIMRGFK
                **:************:**:*********:********.*************:********
                       130       140       150       160       170       180

DROME           DSALEAGTTVTGGQSVVNPWCTIGGVASTICQPNEYIVPDNAVVGDVLVLTKPLGTQVAV
CAROB           DSALDAGTTVTGGQTVVNPWCTIGGVATTVCQANEYIVPDNAVVGDVLVLTKPLGTQVAV
APIME           DSALEAGTTVTGGQTVVNPWCTIGGVASTVCQPNEYIVPDNAVVGDVLVLTKPLGTQVAV
                ****:*********:************:*:**.***************************
                       190       200       210       220       230       240

DROME           NAHQWIDQPERWNRIKLVVSEEDVRKAYHRAMNSMARLNRVAARLMHKYNAHGATDITGF
CAROB           NAHQWLDQPDRWNRIKLVVSEEDVRKGYQRAMDSMARLNRTAARLMHKYNAHGATDVTGF
APIME           NAHQWLDQPDRWNRIKLVVSEDDVRKAYQRAMDSMARLNRIAARLMHKYNAHGATDVTGF
                *****:***:***********:****.*:***:******* ***************:***
                       250       260       270       280       290       300

DROME           GLLGHAQTLAAHQKKDVSFVIHNLPVIAKMAAVAKACGNMFQLLQGHSAETSGGLLICLP
CAROB           GLLGHAQNLAKHQKNEVSFVIHNLPVISKMAAVAKACGNMFQLLQGHSAETSGGLLICLP
APIME           GLLGHAQNLAKHQKNEVSFVIHNLPVIAKMAAVAKACGNMFQLLQGHSAETSGGLLICLP
                *******.** ***::***********:********************************
                       310       320       330       340       350       360

DROME           REQAAAYCKDIEKQEGYQAWIIGIVEKGNKTARIIDKPRVIEVPAKD------
CAROB           REQAAAYCKDIEKQEGYQAWIIGIVEKGLRTARIIDKPRVIEVPAKEKDGELW
APIME           REQAAAYCKDIEKQEGYQAWIIGIVEKGNRTARIIDKPRVIEVPAKEKDGELW
                **************************** :****************:
                       370       380       390       400       410
```

Figure 4.3: Alignment of the amino acid sequences of *Drosophila melanogaster* (DROME, SwissProt accession number: O18373, *Apis mellifera* (APIME, TrEMBL accession number: H9KC27), and *Cardiocondyla obscurior* (CAROB) based on the multiple sequence alignment program MAFFT [Katoh and Standley, 2013]. Numbers under the underlined letters indicate the amino acid position. Asterisks, colons and dots denote identical as well as strongly and weakly conserved amino acids, respectively. Missing and non-conserved amino acids are indicated by hyphens and gaps. Letters shaded in grey represent the conserved ATP/GTP binding site [Hirosawa-Takamori *et al.*, 2000, Persson *et al.*, 1997].

(CAROB), serving as the general insect model organism, the model organism for Hymenoptera, and a model organism for cooperation between the sexes, respectively, reveals a high degree of sequence similarity between the three species (Figure 4.3).

4.4 Discussion

The major finding of this study is that the *selD/sps1* gene is actually translated in male accessory glands (MAG) of the selenoprotein-less ant *C. obscurior*.

In many metazoan organisms, selenium, integrated in enzymes as selenocysteine, plays an

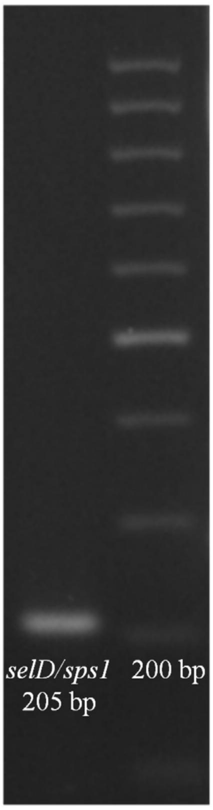

Figure 4.4: The PCR product for the selD/sps1 primers. It is similar in size to the 200 bp band of the DNA ladder, as expected when the falsely annotated intron sequence is actually part of an exon.

important role in the defense against ROS [Battin *et al.*, 2006]. Glutathione peroxidases and superoxide dismutase [Parker *et al.*, 2004] are important examples of such antioxidant enzymes that help to scavenge free radicals and protect cells against damage. These mostly harmful oxygen species accumulate under oxidative stress and greatly influence cellular metabolism, damaging RNA, DNA and proteins, which may ultimately lead to cell death [Battin & Brumaghim, 2009, Simon *et al.*, 2000]. Thus, they are often discussed with regard to aging. For example, "the free radical theory of aging" suggests that longevity can be reduced by an accumulation of harmful oxygen derivatives [Harman, 1955].

However, in fruit flies, which were manipulated so that they could not produce selenoproteins, neither lifespan nor oxidative stress response were negatively affected [Hirosawa-Takamori *et al.*, 2004], indicating that selenoproteins are not essential for ROS defense. Nevertheless, SELD/SPS1 itself appears to play a fundamental role in ROS defense: fruit flies, which lacked SELD/SPS1, were more sensitive to oxidative stress and accumulated more ROS than controls [Morey *et al.*, 2003a, Morey *et al.*, 2003b, Serras *et al.*, 2001]. Excessive intracellular ROS levels may trigger the formation of megamitochondria and apoptosis of a cell [Karbowski *et al.*, 1999, Tandler *et* *al.*, 1968, Teranishi *et al.*, 2000]. SELD/SPS1 might influence megamitochondria formation by inhibiting glutamine synthetase 1 and, thus, the production of glutamine, which at high levels contributes to the formation of megamitochondria [Lee *et al.*, 2011, Shim *et al.*, 2009].

Hence, in selenoprotein-less organisms, SELD/SPS1 might contribute to ROS-defense in a way that does not depend on the generation of selenoproteins [Alsina *et al.*, 1999, Lobanov *et al.*, 2008]. This explains why insects that express SELD/SPS1 but lack selenoproteins, such as *Bombyx mori*, *Tribolium castaneum* [Lobanov *et al.*, 2008], *D. willistoni* (Chapple and Guigo, 2008), and all Hymenoptera, do not suffer from excessive intercellular ROS accumulation, suggesting that they possess a different, selenoprotein-independent system of ROS-defense [Kanzok *et al.*, 2001].

Our data clearly show that SELD/SPS1 is present in the MAGs of *C. obscurior*. We could detect only two peptides of the whole protein, which might be explained (a) by the low amount of protein available for mass spectrometric analysis, and (b) the fact that the database mining software searches sequences with 100% identity. SELD/SPS1 appears to be present in larger quantities in the MAGs of winged than those of wingless *C. obscurior* males (supported by a preliminary western blot analysis). In *Drosophila* and other promiscuous species, males may use MAG proteins to manipulate their mates to increase their own immediate reproductive success at a cost to the females, including a reduction of longevity [Chapman *et al.*, 1995, Chapman, 2001]. Even small amounts of seminal fluid proteins can have dramatic effects in females. For example, sex peptide, a small peptide of the male accessory glands (Acp70A), mediates several profound female postmating responses via a G protein-coupled receptor in *Drosophila* [Yapici *et al.*, 2008]. Such male manipulation of the female's longevity is not expected in social Hymenoptera, as queens mate only during a short period after eclosion and never re-mate later in life. Moreover, queens have to build up a large worker force before they can switch to the production of sexuals. Hence, we expect "sexual cooperation" instead of the "sexual conflict" typical for many solitary insects [Boomsma *et al.*, 2005, Boomsma, 2009]. Mating is indeed beneficial to *C. obscurior* queens and it was suggested that MAG substances transferred in the seminal fluids might positively affect queen physiology in a way that increases their lifespan ([Schrempf *et al.*, 2005], 4.5 months virgin vs. 6.5 months mated). Moreover, the effect of mating with a winged male is significantly larger than that of mating with a wingless male ([Schrempf & Heinze, 2008] 6 months vs. 8 months), and SELD/SPS1 is more abundant in the MAGs of winged males.

Given the fact that SPS1 presumably plays a role in ROS defense, SPS1 is a candidate to be involved in the beneficial effect of mating and in the stronger life-prolonging effect of winged males. It remains to be investigated how and which pathway SELD/SPS1 might activate after its transfer to the young queen during copulation. Further experiments with selenoprotein-less Hymenoptera might help to identify new functions of SELD/SPS1 and in particular how it is involved in the life-prolonging effect of mating in ant queens.

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Chapter 5

Queen and male longevity in the Southeast Asian ant *Cardiocondyla tjibodana* Karavaiev, 1935

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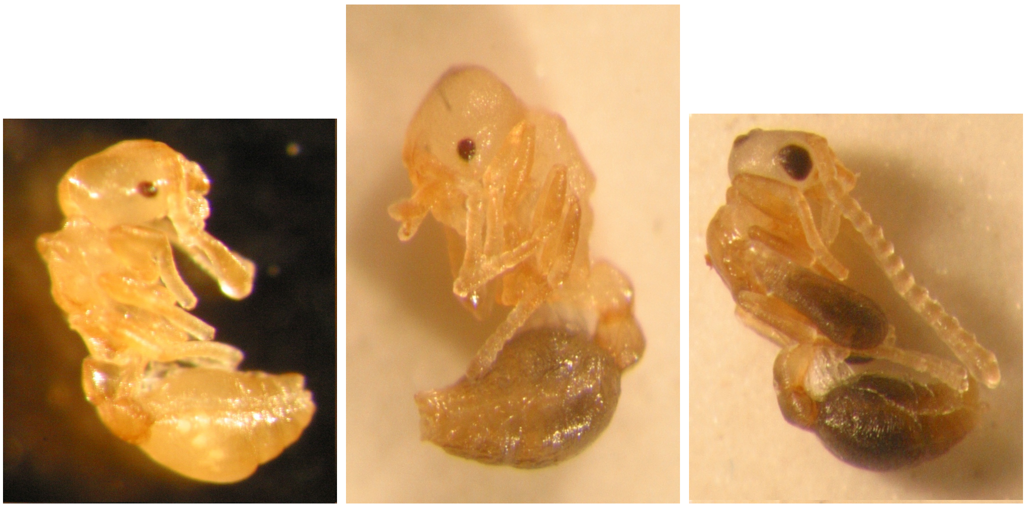

Pupae of *Cardiocondyla* males, from left to right: ergatoid male of *C. tjibodana*, ergatoid male of *C. obscurior*, winged male of *C. obscurior*

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Abstract

Many species of the ant genus *Cardiocondyla* are characterized by a bizarre polyphenism with winged disperser males and wingless males that engage in lethal fighting for access to nestmate queens. In *C. obscurior*, winged males have a stronger life-prolonging and fecundity-enhancing effect for their female mates than wingless males. Here we examine whether the same is true for males of the Southeast Asian *C. tjibodana*. Male phene did not have an effect on queen life expectancy and fecundity. Like the queens of other *Cardiocondyla* species, *C. tjibodana* queens were relatively short-lived, with a maximum life-span of 273 days. Males of both phenotypes survived for a median of 25 days, with one wingless male reaching an age of 194 days. Data from additional species of *Cardiocondyla* are needed to determine how life expectancy of males and queens and the male influence on queen traits have evolved in this genus.

Keywords: life history trade-off, male polyphenism, longevity

5.1 Introduction

In many animal species, males pursue different approaches to increase their mating success [Oliveira *et al.*, 2008]. Dominant males may provide females with access to their territories or nutritious nuptial gifts in exchange for copulations, while sneaker males do not deliver such direct benefits (e.g., [Brockmann, 2008]). In the male-diphenic ant *Cardiocondyla obscurior*, queens that mate with a winged male have on average a longer life-span, and thereby a larger reproductive success than queens that mate with a wingless fighter male [Schrempf & Heinze, 2008]. The mechanisms of this beneficial effect of mating with winged males are not yet understood but presumably involve seminal fluids [Schrempf *et al.*, 2005], which indeed differ strikingly between winged and wingless males (M. Fuessl, in preperation, Chapter 3). Ultimately it appears to be linked with different colony founding strategies and environmental stress. Female sexuals and wingless males are reared year-round, and most young queens remain in their natal nest after mating and found new colonies assisted by workers in the immediate vicinity ("budding", [Cremer & Heinze, 2003, Heinze & Delabie, 2005]). In contrast, winged males are produced during periods of environmental changes when the natal colony declines [Cremer & Heinze, 2003]. Young queens may then be forced to disperse on the wing and to found solitarily without the help of workers. Independently founding queens need considerably more time before they produce sexuals and the life-prolonging effect of winged males can be seen as a postmating investment into their joint offspring.

Winged and wingless males co-occur also in other species of *Cardiocondyla*, but the production pattern appears to differ considerably among species. In the Southeast Asian endemic *Cardiocondyla tjibodana* Karavaiev, 1935, winged males appear to occur regularly in colonies in the laboratory, and have also been collected in the field. In contrast to *C. obscurior*, where winged males readily emigrate from the natal nest to mate with queens away from it [Kinomura & Yamauchi, 1987], winged males of *C. tjibodana* may stay and mate there throughout their entire lifes (M. Fuessl, pers. observation).

Our study documents that male phene does not differently affect female longevity and fecundity in *C. tjibodana*. In addition, it increases the data base on life expectancies and age-specific fecundity of *Cardiocondyla* queens and thus contributes to insect sociometry, "a field in search of data" [Tschinkel, 1991].

5.2 Material and Methods

C. tjibodana is a species of the *C. minutior* group, which is widely distributed throughout disturbed and ruderal habitats in Southeast Asia [Seifert, 2003]. Several small, polygynous (multi-queen) colonies were excavated from their nests in soil in various places in Indonesia. The study is based on experimental colonies derived from a single stock colony collected on Oct, 19, 2008 from its nest in sandy soil on a parking lot near Gua Londron Cave, Pangkajene, Sulawesi Selantan, Indonesia. Because of intranidal mating and colony budding, populations of many *Cardiocondyla* in such anthropogenically influenced places form interconnected colonies with limited genetic differentiation. Using different stock colonies from the same location would presumably not have resulted in larger genetic diversity and thus not have affected the results of our study. However, we would like to stress that the results of our study can be considered as representative of only the studied supercolony and might depart in other populations.

Winged males of *C. tjibodana* resemble typical ant males in morphology and behaviour – they have long antennae, well-developed eyes, and a bulky thorax. They are dark brown in color, and their mandibles are worker-like. Contrary, but in agreement with many other species of *Cardiocondyla*, wingless males are amber in coloration, have small eyes and strong, shear-shaped mandibles. Their antennae and thorax are worker-like, though the shoulders are more pronounced in comparison to workers. They attempt to monopolize mating with all queens eclosing in their natal nest by eliminating newly emerging wingless male rivals with their strong mandibles but rarely savage adult males. Young males occasionally escape attacks by an older male and in particular large colonies may therefore contain sometimes two or more wingless males.

Following Schrempf et al, (2005) we set up experimental colonies consisting of one female sexual pupa, 20 workers, a few larvae, but no eggs and the pupa of either a winged (n = 26) or a wingless male (n = 23). After eclosion, the sexuals mated in the nest and young queens shed their wings. We checked for the presence of the queen and male as well as that of eggs and pupae twice per week. The number of adult workers was kept constant at 20 to avoid positive feedback between colony size, queen fecundity, and life-span (e.g., see [Heinze & Schrempf, 2012]).

5.3 Results and Discussion

Winged and wingless males did not differ in average life-span (19 wingless males: 4 – 194 days, median, quartiles 25 [11 – 46]; 25 winged males: 7 – 41 days, 25, [16 – 27]; Mann-Whitney U-test, U = 218.5, P = 0.652). However, wingless males reached a considerably higher maximum life-span than winged males (194 vs. 41 days). Male phene did not affect the life-span of queens (23 queens mated with wingless males: 32 – 243 days, median, quartiles 154, [89 – 206]; 26 queens mated with winged males: 31 – 273 days, median, quartiles 112.5, [63 – 200]; U = 237, P = 0.214; all 49 queens: median, quartiles 122, [80 – 205], Figure 5.1). Interestingly, the life-span of wingless males and their mates was positively correlated (n = 19, Spearman rank correlation, $r_S = 0.550$, P = 0.015), whereas those of winged males and their mates was not (n = 25, $r_S = 0.030$, p = 0.888).

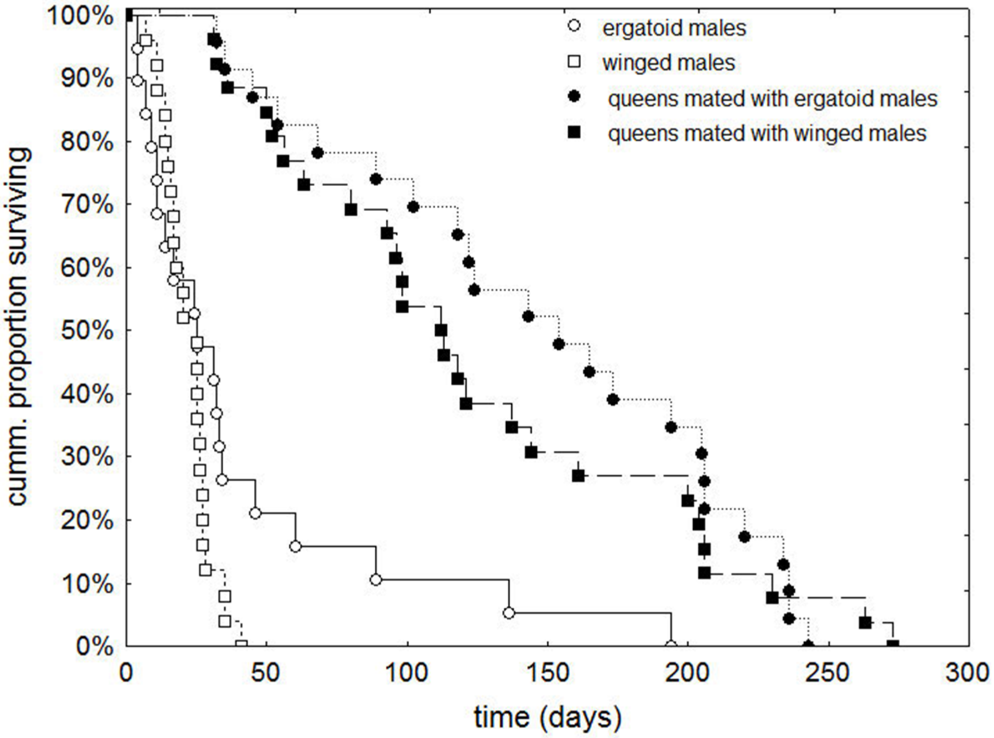

Figure 5.1: Cumulative proportion surviving of the the two male phenes and of queens that mated with either a winged or an ergatoid male (age in days).

Queen longevity was positively associated with the number of eggs observed per scan (n = 49, $r_S = 0.410$, p = 0.0035). Male phene did not have an influence on median egg laying rate (number of eggs per scan: queens mated with wingless males: median, quartiles 5.27, [4.25 – 6.09]; queens mated with winged males: 5.12 [4.14 – 6.49]; U = 245, p = 0.287). Throughout almost the complete lifetime of queens the median number of eggs observed per scan stayed on a plateau of 4 to 6 eggs (Figure 5.2). Brood production was low under our experimental conditions with the reduction of worker numbers to 20, and only 19 of 49 colonies produced worker offspring.

Results from our experiment with *C. tjibodana* in part are in accordance with data from previous studies on the longevity of *Cardiocondyla*. Winged and wingless males of *C. tjibodana* have almost the same median life-spans as those of the close relative *C. minutior*, and like in other *Cardiocondyla*, the maximal life-span obtained by wingless males greatly surpasses that of most winged ant males, including those of *Cardiocondyla* [Heinze *et al.*, 1998, Yamauchi *et al.*, 2006, Schrempf *et al.*, 2007, Shik and Kaspari, 2009, Shik *et al.*, 2013]. With life-spans of less than one year, queens of *C. tjibodana* and other tropical *Cardiocondyla* are ephemeral compared to the notoriously long-lived queens of monogynous ant species [Keller, 1998]. However, this matches their

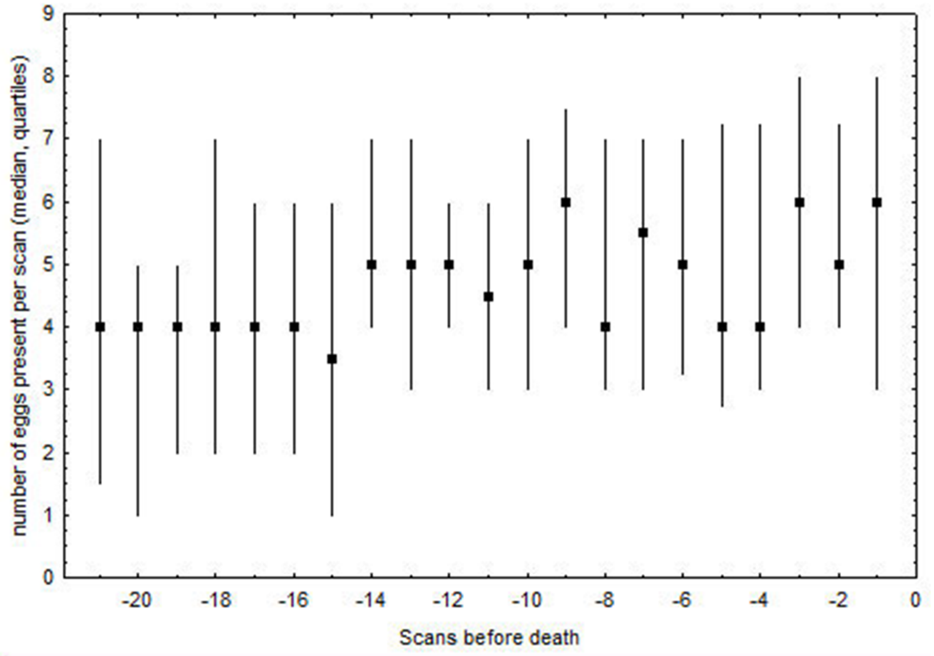

Figure 5.2: Median (± quartiles) egg laying rate of the queens throughout their lifetime, from the beginning till death (0).

polygynous and r-selected life history with frequent queen replacements, colony propagation by budding, and rapid population growth [Tsuji & Tsuji, 1996]. Queens of monogynous *Cardiocondyla* from temperate habitats appear to be more long-lived [Schrempf & Heinze, 2007].

In other aspects, *C. tjibodana* is surprisingly different from other *Cardiocondyla*. First, whereas in *C. obscurior* and *C. kagutsuchi* [Heinze *et al.*, 2013] egg laying rates increased with queen age, egg numbers remained at a rather constant value in our experimental sub-colonies of *C. tjibodana*. The limitation of worker numbers did apparently not have a negative effect on oviposition rates, because in two colonies, which were allowed to increase in size, we also counted only maximally 19 eggs per scan. Furthermore, the two queens in the unmanipulated sub-colonies lived for only 80 and 125 days, respectively, indicating that larger worker numbers do not necessarily prolong the life of queens. Egg laying rates did not considerably decline in the last days before death, indicating negligible reproductive senescence like in *C. obscurior* [Heinze & Schrempf, 2012].

The second difference between *C. obscurior* and *C. tjibodana* is that male phenotype did not affect the life expectancy of *C. tjibodana* queens, while in *C. obscurior* mating with a winged male has a considerably stronger positive effect on queen life-expectancy than mating with a wingless male [Schrempf & Heinze, 2008]. Unfortunately, nothing is known about the influence of mating with winged vs. wingless males in other male-diphenic *Cardiocondyla* and it is thus futile to speculate about possible explanations for this striking difference.

In any case, winged males of *C. tjibodana* seem to have lost their function as an exclusive disperser phene [Cremer & Heinze, 2003], and further studies are needed to elucidate the factors that influence the occurrence and ratio of winged and ergatoid males in the colonies.

Acknowledgements

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Chapter 6

General Discussion

Accessory gland proteins (ACPs) have been studied intensively in solitary insect species (e.g. [Andrés *et al.*, 2006, Braswell *et al.*, 2006, Zareie *et al.*, 2013] and some eusocial insect species (e.g. [Baer *et al.*, 2009b]). Their influence on female lifespan is an intriguing research field, but data on their impact on female's longevity is still scarce. In this thesis I investigated the ACPs of the male-diphenic ant species *Cardiocondyla obscurior* and *Cardiocondyla tjibodana*. In *C. obscurior* I identified several ACPs which are differently expressed in the winged compared to the wingless male phene by conducting a gene expression study (representational difference analysis (RDA)) (Chapter 2), as well as a proteomic study (two-dimensional-SDS-polyacrylamide-gel-electrophoresis (2D-SDS-PAGE) and liquid chromatography-mass spectrometry/mass spectrometry-analysis (nano-LC-MS/MS-analysis) (Chapters 3-4). These ACPs might thus account for the elongation of the female's lifespan, that has been recently shown for winged males of *C. obscurior* [Schrempf & Heinze, 2008], but could not be confirmed for *C. tjibodana* (this study, Chapter 5).

Gene expression studies as for example RDA or microarray studies are RNA-based and give a good overview over known and new genes in a tissue, even when genetic information is lacking, as it was the case when I started my thesis. With the RDA followed by a qPCR I showed that the genes *leucine-rich repeat* (*LRR*) *and immunoglobulin-like domain-containing nogo receptor-interacting protein 2-like*, a hypothetical protein, SERPINB10 and a gene, that is not yet described from any other animal, are expressed higher in winged males, while growth hormone-inducible transmembrane protein-like (*ghitm*), *acyl carrier protein mitochondrial-like*, *ejaculatory bulb-specific protein 3-like* and a gene that has not yet been reported from another organism, are higher expressed in ergatoid males (Chapter 2). The studiex on protein-level (Chapter 3 and 4) revealed Selenide, water dikinase (SPS1), a hypothetical protein, Na^+/H^+-exchange-regulatory-cofactor 2 (NHE-RF2) and SERPINB10 to be over-expressed in the winged male phene while Protein-disulfide-isomerase A3 (PDIA3), was over-expressed in ergatoid males of *C. obscurior*. Unfortunately we could not do a nano-LC-MS/MS-analysis for *C. tjibodana*, as we could not gather enough males, because colonies were running bad at the end.

In the following I am going to give a few more details which were not covered in the respective sections, as this would have been beyond the scope of the single chapters. I will start with stressing differences between the RNA- and the protein-level.

6.1 From the RNA to the protein: a brief overview

While RDA examines the ACPs on the RNA-level, the 2D-SDS-PAGE is a study on the protein-level. RNA approaches do not tell anything about the protein-transfer, as RNA is not transferred at mating, but they may serve as a good hint to identify potential seminal fluid proteins (SFPs) [Perry & Rowe, 2010, Smith *et al.*, 2012]. It is eye-catching, that SERPINB10 occurs in both studies (Chapter 2 and 3). The question might arise why there is only one protein, which is present on both, the RNA- and the protein-level. In the transcription process, DNA is transcribed into RNA and in the translation process the information coded in the mRNA is translated into an aminoacid sequence. In-between, there are several different parameters, such as mRNA-processing, that may influence whether a mRNA is really transcribed into a protein. Alternative splicing results in different mRNAs, which serve as templates for different proteins or isoforms of the same protein. Capping and polyadenylation are further post-transcriptional modifications that affect mRNA stability. As transcription takes place in the nucleus and translation in the cytosol, mRNA localization is another factor that influences gene expression [Curtis *et al.*, 1995, Kim-Ha *et al.*, 1995, Lee & Schedl, 2006, Quenault *et al.*, 2011, Wickens *et al.*, 2002]. In these regulations of protein expression RNA-binding proteins play an important role. They bind to regulatory sequences in the 3'UTR- or 5'UTR-region of the mRNA and act either as repressors that e.g. block the binding sites for enzymes of the protein biosynthesis (PBS), or as activators of expression by recruiting or stimulating other enzymes that enhance PBS [Curtis *et al.*, 1995, Goldstrohm *et al.*, Lee & Schedl, 2006, Quenault *et al.*, 2011, Wickens *et al.*, 2002]. RNA-binding proteins may be the end of a long cascade of a signalling pathway that connects the exterior of a cell with the interior [Spriggs *et al.*, 2010, Wickens *et al.*, 2002]. Finally, epigenetic changes as e.g. DNA-methylation and histone-modifications have an important impact on the protein expression [Tryndyak *et al.*, 2006].

In addition, a lot of different post-translational modifications can occur after PBS, e.g. addition of anorganic and organic groups to the protein, separation of signal peptides (e.g. cleavage of precursor proteins) or proteolysis for protein activation [Walsh *et al.*, 2005]. These and other protein modifications are of great biological relevance but were not in the focus of my study.

As there is a multitude of post-transcriptional and-translational modifications it is in the end not surprising that SERPINB10 was the only gene for which we found the corresponding protein (SERPINB10) in the proteome study (Chapter 3), making it a favourite candidate for playing an important role in the female after mating.

6.2 SERPINB10 a potential factor in the insect immune system (Chapters 2 & 3)

Serpins are a super-family with more than 1000 members, belonging to at least 16-17 different clades with a lot of different functions in a variety of different tissues [Irving *et al.*, 2000, Mangan *et al.*, 2008, Silverman *et al.*, 2004]. Clade B serpins take part e.g. in the regulation of cell growth (apoptosis), differentiation, tumour cell invasiveness [Shioji *et al.*, 2005] and motility and angiogenesis [Silverman *et al.*, 2001]. Serpins are single chain proteins with about 400 amino acids which have an exposed reactive side-loop that interacts with the substrate to form a very stable complex [Kanost, 1999]. After substrate-binding, proteinase inhibitors run, in a suicidal mechanism, through vast and irreversible conformational changes [Huntington *et al.*, 2000, Kanost, 1999, Potempa *et al.*, 1994, Reichhart, 2005, Remold-O'Donnell, 1993, Silverman *et al.*, 2001, van Gent *et al.*, 2003, Ye & Goldsmith, 2001]. In the moth *Manduca sexta* the exon nine of serpin-1, which encodes the side-loop that acts as reactive centre, is encoded by twelve alternative forms, leading to proteinase inhibitors that are specific for different substrates [Jiang *et al.*, 1996, Kanost, 1999, Kanost *et al.*, 2004]. The possibility to create a protein with a changed substrate specificity by changing only one exon gives serpins a high potential for rapid evolution [Mangan *et al.*, 2008].

Proteases and protease inhibitors are in balance in healthy cells. A deviation from this equilibrium has deleterious side-effects as it has been shown e.g. for the serpin α1-Antichymotrypsin which is a component of the amyloid deposits of Alzheimer's disease [Abraham *et al.*, 1988]. An intensively studied serine protease in humans is for example the prostate specific antigen (PSA), which was first purified in 1979 [Wang *et al.*, 1979] and serves as tumour marker, as it shows an elevated serum level in case of prostate cancer [El-Shirbiny, 1994, Stenman *et al.*, 1999]. A very well studied insect protease inhibitor is ACP62F in *D. melanogaster*. This protein is transferred at mating and acts presumably to protect proteins from proteolysis in the genital tract [Wolfner, 2002, Wolfner *et al.*, 1997]. About 10 % of ACP62F enter quickly the haemolymph after mating, which is probably the reason for its poisonous impact on female longevity [Wolfner, 2002]. Together with sex peptide (SP or ACP70A) ACP62F is the main cause for the reduced female lifespan in *D. melanogaster* [Lung *et al.*, 2002, Lung & Wolfner, 1999, Wigby & Chapman, 2005, Wolfner, 1997]. It is assumed that ACP62F leaking into the haemolymph is an undesired side-effect, which is possible because no specific signal sequence is required to enter haemolymph, as for other ACPs it is essential to reach other tissues via this way [Lung & Wolfner, 1999, Wolfner, 1997, Wolfner, 2002].

It is known that insect serpins participate in Toll receptor signalling [Kanost *et al.*, 2004, Levashina *et al.*, 1999], which is important for immune system activation [Levashina *et al.*, 1999, Reichhart, 2005, Valanne *et al.*, 2011], and a preventive or induced activation of the immune system might be necessary in ants to counteract negative effects of mating and reduce immunity costs, a function I suggest for SERPINB10 in *Cardiocondyla*.

6.3 The *ejaculatory bulb-specific protein 3-like* as interesting result of the RDA (Chapter 2)

The ejaculatory bulb-specific protein (PEB) is studied best in *D. melanogaster* were it is called PEB-me and found exclusively in the ejaculatory bulb [Brieger & Butterworth, 1970, Butterworth, 1969, Ludwig *et al.*, 1991]. A transfer at mating has been proven, as well as a function in the mating plug formation [Ludwig *et al.*, 1991, Lung & Wolfner, 2001]. PEB-me shows great similarity to an odorant-binding protein of the moth *Mamestra brassicae* [Bohbot *et al.*, 1998]. The similarity between the odorant-binding protein and PEB-me is probably due to the fact that both bind vaccenyl acetate as ligand. Bohbot and colleagues (1998) hypothesized that PEB-me (here called EBSP-III) is necessary to keep vaccenyl acetate in the seminal fluid in solution. Cis-vaccenyl acetate acts as a pheromone [Bartelt *et al.*, 1985] and occurs only in the ejaculatory bulb of adult males in *Drosophila* [Brieger & Butterworth, 1970, Butterworth, 1969]. It is transferred at mating [Butterworth, 1969] and has a dose dependent effect on both genders: while it is an attractant for both sexes in low doses, it inhibits male courtship in high doses [Bohbot *et al.*, 1998]. What effect this pheromone has in *Cardiocondyla* remains to be elucidated, as well as the exact function of the ejaculatory bulb-specific protein-3like gene. The corresponding protein might enter the haemolymph of the female after mating and serve to regulate female's post-mating physiology, similar to what has been suggested for the mating plug protein of *Drosophila* [Ludwig *et al.*, 1991].

6.4 The evolution of SELD/SPS1 and its possible relation to selenoprotein biosynthesis, ROS & ageing (Chapters 3 & 4)

To get an ample overview of the proteome of a tissue, the 2D-SDS-PAGE is an optimal method as it is possible to cover a broad pH- and size-range of proteins [Anderson & Anderson, 1977, Baer *et al.*, 2009b, Edwards *et al.*, 1981, Diz *et al.*, 2012, Fung *et al.*, 2004, Jonakova *et al.*, 2007, O'Farrell, 1975, Reinhardt *et al.*, 2009, Rabilloud *et al.*, 2010]. Furthermore, proteomic studies are closer to the phenotype than RNA studies as the proteome is less static than the genome and changes with biological conditions [Diz *et al.*, 2012, Schrimpf *et al.*, 2009]. On protein-level it is possible to identify proteins whose genes have been overlooked by automatic annotation [Diz *et al.*, 2012]. Though the gene for the SELD/SPS1 protein has been found by automatic annotation, it was not correct annotated concerning exon/intron allocation as we could show in Chapter 4.

The bacterial selenophosphate synthetase *selD* has two homologues in eukaryotes, *sps1* (*selD* in flies) and *sps2* [Alsina *et al.*, 1998, Persson *et al.*, 1997]. SELD/SPS1 shows high sequence similarities to SPS2, which is essential for selenoprotein biosynthesis and itself a selenoprotein [Alsina *et al.*, 1998, Hirosawa-Takamori *et al.*, 2000, Xu *et al.*, 2007]. A long time SELD/SPS1 was supposed to play - similar to SPS2 and bacterial SELD - a major role in selenoprotein biosynthesis [Alsina *et al.*, 1999, Alsina *et al.*, 1998, Serras *et al.*, 2001], but recent studies strongly suggest a so far unknown, selenoprotein-unrelated function [Han *et al.*, 2012, Lobanov *et al.*, 2008, Xu *et al.*, 2006, Xu *et al.*, 2007], apart from a still discussed, potential role in selenoprotein biosynthesis [Lee *et al.*, 2011, Tamura *et al.*, 2004].

While selenoproteins are very common in all three domains of life, they are completely lacking in social Hymenoptera. They are common in mammals, some fishes and algae [Lobanov *et al.*, 2007, Lobanov *et al.*, 2009]. For example, to date 28 selenoproteins are known in humans [Gladyshev, 2012, Kryukov *et al.*, 2003, Romagné *et al.*, 2013] and at least 30 in fishes [Lobanov *et al.*, 2009]. Typically, genomes of marine organisms such as fish and algae comprise more gene-sequences for selenoproteins than genomes of terrestrial life forms [Lobanov *et al.*, 2007, Lobanov *et al.*, 2009]. Thus it is not surprising that at present, the water-living brown algae *Aureococcus anophagefferens* is with 59 identified selenoproteins, the organism with the highest known number of these proteins [Gobler *et al.*, 2013]. In contrast the land-living roundworm *Caenorhabditis elegans* has only one single selenoprotein [Taskov *et al.*, 2005]. Higher plants and fungi do - like social Hymenoptera - not possess any selenoproteins. My study (see Chapter 4) was the first to show, that the *seID/sps1* gene is not only transcribed, but translated into a protein in eusocial insects. As they do not possess selenoproteins it is an intriguing question which function this protein has in these species.

In a preliminary Western-blot (Figure 6.1) Probst could show, that the protein is transferred to the female at mating, as a pool of spermathecae from 200 mated females had a higher quantity than a pool of 200 spermathecae of virgin females [Probst, 2014]. Additionally, a former study revealed, that mated queens in general live longer than virgins [Schrempf *et al.*, 2005]. Ageing may be a result of accumulation of oxidative damage to cells and tissues [Harman, 1955]. Free radicals occur as a by-product of aerobic metabolism and have the potential to inflict great harm to biological macromolecules as lipids, DNA and proteins [Berlett & Stadtman, 1997, Hougland *et al.*, 2013]. Oxidized or cross-linked proteins and seriously damaged DNA-molecules may be the

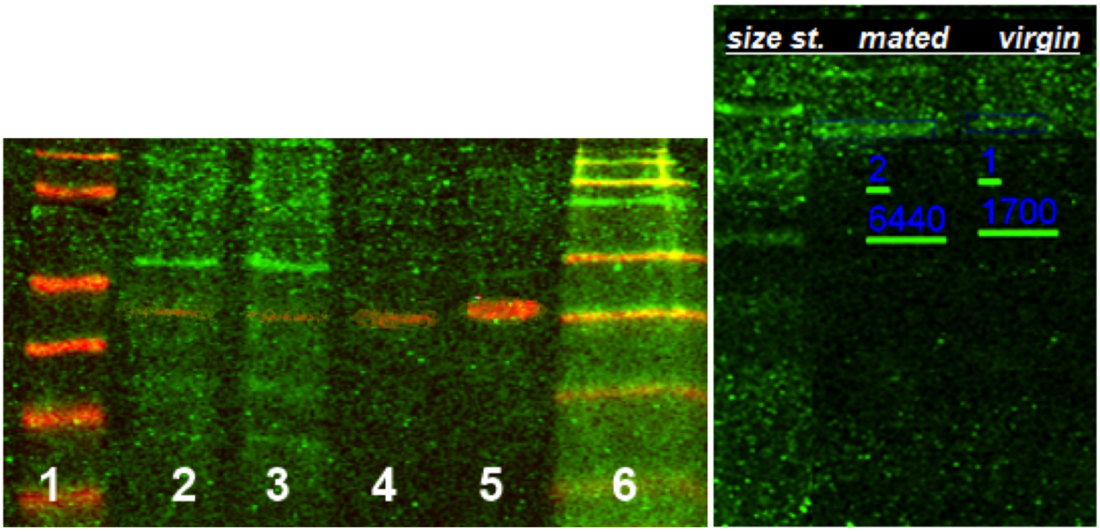

Figure 6.1: Western blot analysis for SPS1/SELD in whole male bodies and 40 male accessory glands (MAGs) of winged and wingless males each, under fluorescent light (left side figure). The molecular weight of the protein is approximately 65 kD (size standard left lane). Tubulin (loading control) is visible in red. Luminous intensities reveal, after normalization for Tubulin, that in the MAGs of ergatoid males, 3.3 times more SPS1/SELD can be detected in comparison to ergatoid males (and 4.25 more in complete males). Lane 2: 10 ergatoid males; lane 3: 10 winged males; lane 4: 40 MAGs of winged males; lane 5: 40 MAGs of ergatoid males. on the right side figure, the signal is only very weak in the pooled spermathecae of 200 virgin queens, but stronger in the pooled spermathecae of 200 mated queens (luminous intensities are given in blue numbers). The Western blot was probed with an antibody against the C-terminal end of human SPS1. Absolute luminous intensities have to be treated with care due to background noise, yet ratios can be assessed as meaningful [Probst, 2014].

consequence that may lead ultimately to cell death [Battin & Brumaghim, 2009,Simon *et al.*, 2000]. Their most common source is oxygen and they are most abundant in mitochondria. Specially the mitochondrial DNA is unprotected and thus exposed to reactive oxygen species (ROS) [Beckman & Ames, 1998]. ROS may be especially high in changing environmental conditions and thus contribute to a reduced lifespan following the "free radical theory of ageing" which is based on the "rate-of living theory" (see general introduction) ([Beckman & Ames, 1998,Muller *et al.*, 2007] but see [Schulz *et al.*, 2007,Zuin *et al.*, 2010]). It has been shown, that ROS play e.g. an important role in human infertility [Agarwal *et al.*, 2003] and they have been demonstrated to be in the semen and spermatheca of honey bees [Weirich *et al.*, 2002]. To protect cells from oxidative damage, antioxidant enzymes, such as superoxiddismutase (SOD) evolved [Weirich *et al.*, 2002,Muller *et al.*, 2007,Weirich *et al.*, 2002] and have been found in the seminal fluid [Collins *et al.*, 2004, Collins *et al.*, 2006]. But there are other, non-enzymatic radical scavengers such as vitamin C or cysteine as well ([Hougland *et al.*, 2013] p.71f.). I suggest SELD/SPS1 to be one of them and to act as a selenocysteine independent ROS-scavenger and thus to reduce the damage induced by these harmful oxygen species.

6.5 PDIA3 a thiol oxidoreductase (Chapter 3)

Another protein possibly related to stress defence is PDIA3. PDIs can be found in many tissues of fungi, plants and animals and it is most abundant in the endoplasmatic reticulum (ER) [Wilkinson & Gilbert, 2004]. PDIA3 has five domains of which two are homologous to thioredoxin and contain two cysteine each, which they need for their oxidoreductase-activity [Freedman *et al.*, 1994, Noiva & Lennarz, (1992), Wilkinson & Gilbert, 2004]. In the salmon (*Salmon salar*) PDIA3 has a high identity with human PDIA3 and its expression in the ER can be induced by oxidative stress [Huang *et al.*, 2009]. Besides other tissues, PDIA3 occurs in the reproductive tract and plays a role in sperm egg fusion [Akama *et al.*, 2010, Baer *et al.*, 2009a, Dun *et al.*, 2012, Ellerman *et al.*, 2006, Zhang *et al.*, 2007]. With its function to repair miss folded disulphide bridges [Freedman *et al.*, 1994] this protein helps to repair malformations which may be ROS induced [Huang *et al.*, 2009, Sharma *et al.*, 2013]. This protein was higher expressed in ergatoid males than in winged males of *C. obscurior*. As both males were kept at the same conditions, I speculate that wingless males are less adapted to react on changing and inhospitable conditions than winged males. However, to speculate how this might influence females's physiology is futile at the moment.

Further proteins that are differentially expressed between the male phenes in *C. obscurior* have been discussed in Chapter 3. As results for the proteome study, I would have expected more peptides, both, from the same proteins as well as from further proteins. The MS/MS-spectra were searched against the Uniprot-database, but only for 100 % homologous sequences, as a prediction of the program we used. This may lead to the rejection of a possible homology as consequence, especially when taking into account, that only 50 % of sequence similarities are still enough to assign proteins to each other with high accuracy by comparative structural models [Baker & Sali, 2001]. Furthermore it is possible, that only parts of a protein are conserved while others underlie strong selection pressures [Findlay & Swanson, 2010, Swanson *et al.*, 2001b], making it especially difficult to find homologues.

6.6 Changing composition of the seminal fluid and how the ACPs might act in *C. obscurior*

The composition of the seminal fluid can be adapted to the mating circumstances [Cornwallis & O'Connor, 2009, Locatello *et al.*, 2013, Perry & Rowe, 2010, Perry *et al.*, 2013, Simmons, 2001, Wedell *et al.*, 2002], e.g. when the male has already mated and is able to replenish his storage (Bissoondath & Wiklund, 1995; Wiklund et al, 2001). For example, males might change the composition of their ejaculate under sperm competition to outcompete previous mates (Perry & Rowe, 2010; Wedell et al, 2002), provided that the male can assess the mating status of the female [LaMunyon & Huffman, 2001, Wiklund *et al.*, 2001]. It has been shown in male fowls that they transfer more seminal fluid proteins (SFPs), which enhance sperm velocity, in case they pair with more attractive females ([Cornwallis & O'Connor, 2009], see [Wedell *et al.*, 2002] for further examples). Ergatoid males of *Cardiocondyla* have a lifelong spermatogenesis, and it would be interesting to investigate, whether they adjust their ejaculate to the mating situation as well, for example transferring more sperm and less SFPs when winged males are around. Winged males, on the other hand, might transfer generally less sperm when mating inside the nest, as they have to economise due to limited sperm reserves [Heinze *et al.*, 1998].

The composition of the SFPs can also be influenced by the mode of reproduction as e.g. monandry and polyandry [Bissoondath & Wiklund, 1995]. For example in the seed beetle *Acanthoscelides obtectus* it has been shown, that the SFPs may enhance a females lifespan if males were selected for late reproduction, meaning that there is a time gap between mating and the occurrence of the first sexuals [Maklakov *et al.*, 2005]. Females of this line were effectively polyandrous and, despite a somewhat elevated base-line mortality, they showed a decelerated rate of senescence. Another line of the same species - selected for early reproduction - showed a shorter lifespan of females, even though they were effectively monandrous [Maklakov *et al.*, 2005]. In my study it became evident, that males of *C. obscurior* and *C. tjibodana* might have different reproductive tactics. In *C. obscurior* queens mated to an ergatoid male, may produce sexual brood earlier than queens mated to a winged male and thus these former queens would show an earlier reproduction than the latter queens, which show a late reproduction that is typical for social insects. Queens of *C. tjibodana* do probably not experience differences in colony founding as males of both types stay inside the nest to mate with queens (pers. observation). Perhaps these differences in mating tactic are one reason for the divergence in the ACPs composition that I found in my study (Chapter 3).

ACPs may act in many different ways. They can e.g. activate a substance which is already present in the female [Loher *et al.*, 1981], or be processed e.g. cleaved after transfer [Chapman, 2001, Lung & Wolfner, 1999, Park & Wolfner, 1995, Ravi Ram *et al.*, 2005]. Proteins transferred to the female at mating may enter the haemolymph, intentional or unintentional, through the vaginal wall to reach the tissues of destination [Lung & Wolfner, 1999, Wolfner, 2002]. Furthermore it is possible that they activate pathways and engage in immunological functions [Robertson, 2007].

To my knowledge, relevant information is lacking about any other than the mentioned proteins or genes that were found. They act probably in one of the described manners, but a final proof remains to be done. As discussed in Chapter 2, additional to SERPINB10, *LRR and immunoglobulin-like domain-containing nogo receptor-interacting protein 2-like* [Litman *et al.*, 2005] and *ghitm* [Li *et al.*, 2001, Reimers *et al.*, 2007, Zou *et al.*, 2000] may play a role in immunology. In Chapter 3, NHE-RF2 is discussed to be possibly involved in a transcription regulating pathway [Poulat *et al.*, 1997, Shenolikar & Weinman, 2001]. The stimulation of the female's immune system after mating might be a crucial point to influence the queens long-term survival. During this time she is exposed the most to pathogens, as a consequence of mating and seeking a new nesting site away from the mother colony. If the winged male is able to support the young queen via his ACPs to defend pathogens effectively during this stressful time, it might be, that the young queen profits from this help for the rest of her life. It has been shown, that stress leads to a changed protein expression pattern which is essential for survival and may even lead to an increased lifespan [Spriggs *et al.*, 2010, Zuin *et al.*, 2010].

6.7 How the ACPs of *C. tjibodana* might be influenced by the mating system (Chapters 3 & 5)

While in *C. obscurior* a significant difference in queens longevity has been shown [Schrempf *et al.*, 2005], in *C. tjibodana*, queens did not show a difference in longevity depending on the male type they had mated with (Chapter 5). However, considering other differences between these species, a simple explanation would be a different mating strategy of the queens. In *C. tjibodana*, queens are - at least theoretically - in the position to easily mate with more than a single male, as commonly several winged males are present, sometimes even contemporaneous with an ergatoid male (pers. observation). Hence, queens might take the opportunity to switch to a more polyandrous lifestyle (in contrast to *C. obscurior*), and to mate regularly more than once, a speculation that remains to be investigated. In this case males would be under sperm competition and SFPs are expected to evolve more rapidly [Ramm *et al.*, 2008, Schumacher *et al.*, 2014] as it has been shown e.g. for primates [Clark & Swanson, 2005, Khaitovich *et al.*, 2006]. Although no harmful effects for the females like in *D. melanogaster* are expected in species with a cooperative life history, sperm competition between the ejaculates can occur (e.g. [den Boer *et al.*, 2008]) . DenBoer and colleagues (2010) showed that the SFPs of *Atta* leafcutter ants had a negative influence on alien sperm survival, but promoted own sperm survival. Furthermore, storage of a certain amount of sperm is more costly in matters of immunity costs when the sperm derives from different males [Baer *et al.*, 2006]. Negative effects were opposed by spermathecal fluid so that effects of post mating sexual selection and conflict (sperm competition) were limited to a short time after mating [den Boer *et al.*, 2010]. However, males in *C. tjibodana* may try to outcompete the rival sperm, as they are often confronted with competitors within the same nest. Perhaps there is besides sexual cooperation some under-estimated conflict over reproduction [Baer, 2014]. An arising sexual conflict is not necessarily connected to antagonistic coevolution, but has the potential for selection [Hosken *et al.*, 2009]. Anyway, a negative influence on the female is unlikely and conflict needs to be ended when sperm reaches the spermatheca [Ratnieks *et al.*, 2006, Sundström & Boomsma, 2001].

However, reliable data concerning the mating system are requested, and then it would be valuable to compare which proteins in *C. tjibodana* show the same expression pattern as in *C. obscurior* and which ones might eventually be related to sperm competition.

6.8 The ageing theories with special emphasis on social insects

Within this last section, I would like to come back to the ageing theories, which I introduced in the general introduction, with special emphasis on social insects, as they have an extraordinary life history. The rate-of-living theory [Pearl, 1928], and the evolutionary theories of ageing (the mutation accumulation theory [Medawar, 1946], the antagonistic pleiotropy theory [Williams, 1957] and the disposable soma theory [Kirkwood, 1977]) seem to be opposed, at least in parts, by the late reproduction in social insects. The rate-of-living theory predicts, that a higher temperature and smaller body size are both, associated with a higher metabolic rate, and a reduced lifespan. This theory is opposed by the fact, that social insect queens live considerably longer then nestmate workers or non-social insects of the same size [Keller & Genoud, 1997]. In fact, evolutionary theories on the other side predict that the lower the risk of extrinsic mortality, the lower is also the rate of ageing and the higher is the lifespan. This is in line with lifespan differences in insect societies [Heinze & Schrempf, 2008, Keller & Genoud, 1997], even though also other factors as e.g. the social environment have been shown to play a role [Schrempf *et al.*, 2011].

The mutation accumulation theory and the antagonistic pleiotropy theory are opposed by the fact that it is unlikely that in social insect queens, which reproduce late in life and even show negative reproductive senescence [Heinze & Schrempf, 2012], a late acting deleterious gene - no matter whether kept in the population passively (mutation accumulation theory) or actively (antagonistic pleiotropy theory) - would not be opposed by a strong selection. Following the disposable soma theory, as more offspring an individual has, the sooner it should die, or, vice versa, an individual that never reproduces, should live longer. While this theory seems to gain some support in humans [Tabatabaie *et al.*, 2011, Westendorp & Kirkwood, 1998], it is again unlikely to be of great importance in social insects as they do not show a trade-off between longevity and reproduction [Heinze & Schrempf, 2008, Heinze & Schrempf, 2012].

ROS have been shown to be an important factor with regard to ageing (discussed in Chapter 4), which may, according to the error catastrophe theory [Orgel, 1963], lead to mutations on a molecular level, that accumulate with time [Ames *et al.*, 1993]. The free radical theory [Harman, 1955] is supported by the fact, that ROS might negatively influence longevity. These negative influences can be opposed in parts by antioxidants (e.g. [Le Bourg, 2001]). SPS1, the protein discussed in Chapter 4, might act as antioxidants in insects and thus counteract a negative impact of ROS in changing conditions ([Finkel & Holbrook, 2000, Le Bourg, 2001, Wickens, 2001] but see [Keller & Jemielity, 2006]).

Chapter 7

Summary

The ant genus *Cardiocondyla* is characterised by a remarkable male diphenism, with "normal" winged males that often co-occur with "worker-like" (ergatoid) males, which is extraordinary in social insects. These male phenes represent different reproductive tactics. The peaceful winged males are the disperser phene, even though they got lost in some *Cardiocondyla* species. Ergatoid males mate intranidal and are in many species aggressive fighters that show a striking adaptation to their behaviour. Young queens usually mate inside their maternal nest.

In my study species *C. obscurior* the winged male phene occurs only when environmental conditions change, while one ergatoid male with sabre-shaped mandibles is regularly in the colony. Queens mated to winged males might have to leave the colony to found a nest elsewhere due to changing conditions and probably need considerably more time until they produce the first sexual offspring. They live longer than queens mated to an ergatoid male. In my second study species *C. tjibodana* on the other side the winged male phene is common, while ergatoid males with shear-shaped mandibles are rare. Data on queen longevity is lacking. The fact that females mate only during a short period and store the sperm lifelong, as well as the production of the sterile worker offspring before the production of sexual offspring in social insects favours sexual cooperation over sexual conflict.

The aim of this thesis was to study the male accessory glands of the male phenes of *C. obscurior* as potential proximate cause of the life-prolonging effect. I investigated the protein pattern and the gene expression pattern of these glands by using two-dimensional-SDS-polyacrylamide-gel-electrophoresis (2D-SDS-PAGE) and a representational difference analysis (RDA), respectively. Additionally, I examined the protein pattern of the accessory glands of the male phenes of *C. tjibodana* and analysed queen longevity based on the male phene they had mated with.

Chapter 2 "Suppression subtractive hybridization reveals differentially expressed genes in the accessory glands of two ant male phenes with different life histories" is based on the result from a study on RNA-level to examine differences in the gene expression in the accessory glands of the male phenes of *C. obscurior*. 38 US could be assigned to predicted genes and eight of these could be analyzed in detail in a quantitative real-time PCR (qPCR) analysis.

In **Chapter 3** "Comparison of the accessory gland proteins of two male-diphenic ant species and the effect on their mating partners" the accessory gland proteins were investigated by

2D-SDS-PAGE. I compared the protein pattern of the two male phenes of *C. obscurior* and found 56 proteins to be differently regulated. A comparison in *C. tjibodana* revealed 91 proteins to be up- or down-regulated. The results of a subsequent nano-LC-MS/MS-analysis of some selected protein spots of *C. obscurior* revealed that SERPINB10, Protein disulfide isomerase A3, Na^+/H^+ exchange regulatory cofactor, Selenophosphate synthetase 1 and a hypothetical protein are differentially expressed between the two male phenes.

Chapter 4 "Selenophosphate synthetase in the male accessory glands of an insect without selenoproteins" demonstrated that Selenophosphate synthetase 1 is transcribed into a protein in *C. obscurior*, which has previously not been shown for any social Hymenoptera. Besides details on predicted gene structure, I discussed the possible role of selenophosphate synthetase (SPS1) in relation to oxidative stress defence in social insects.

Chapter 5 "Queen and male longevity in the Southeast Asian ant *Cardiocondyla tjibodana* Karavaiev, 1935" presented the results of the study on queen and male longevity in *C. tjibodana*. Contrary to *C. obscurior*, queens in *C. tjibodana* live on average equally long, independent of the male phene they mated with. Moreover, queens do not show an increase of egg laying rate with age, as it has been shown in *C. obscurior* and *C. kagutsuchi*.

Interestingly we found SERPINB10 on the RNA-level, as well as on the protein level. While a possible function of Na^+/H^+ exchange regulatory cofactor 2 (NHE-RF2), a hypothetical protein and *ejaculatory bulb specific protein 3-like* remains unknown, SPS1 might help in reactive oxygen species defence. Growth hormone-inducible transmembrane protein-like (*ghitm*), SERPINB10 and *leucine-rich repeat (LRR) and immunoglobulin-like domain-containing nogo receptor-interacting protein 2-like* might function in an immune system related manner.

In summary, my research provides very good candidate genes and proteins to be related to queen longevity, which can serve as an informative basis that can be built upon in future research on this subject.

Chapter 8

Zusammenfassung

Die Ameisengattung *Cardiocondyla* ist durch einen bemerkenswerten Männchendiphänismus gekennzeichnet. Es ist außergewöhnlich für soziale Insekten, dass "normale" geflügelte Männchen zusammen mit "arbeiterinnenähnlichen" (ergatoiden) Männchen vorkommen. Die beiden Männchenphänen weisen verschiedene reproduktive Taktiken auf. Die friedlichen geflügelten Männchen sind die Verbreitungsphäne, obwohl sie in einigen Arten von *Cardiocondyla* nicht mehr vorkommen. Ergatoide Männchen paaren sich im Nest und sind in vielen Arten aggressive Kämpfer, mit auffallenden, an ihr Verhalten angepassten Adaptierungen. Jungköniginnen paaren sich oft innerhalb des Nestes.

In der von mir untersuchten Art *C. obscurior* tritt die geflügelte Männchenphäne nur auf, wenn sich die Umgebungsbedingungen ändern. Normalerweise ist ein ergatoides Männchen mit säbelförmigen Mandibeln in der Kolonie. Königinnen, welche sich mit einem geflügelten Männchen gepaart haben, müssen wegen der sich ändernden Umweltbedingungen vermutlich das Nest verlassen und anderorts eine neue Kolonie grünend. Deswegen brauchen sie vermutlich deutlich mehr Zeit, bis sie die ersten Geschlechtstiere produzieren. Sie leben länger, als Königinnen die sich mit einem ergatoiden Männchen gepaart haben. In *C. tjibodana*, einer weiteren Art, die von mir untersucht wurde, ist andererseits die geflügelte Phäne üblich, wohingegen ergatoide Männchen, die hier scherenförmige Mandibeln haben, selten sind. Daten zur Langlebigkeit der Königinnen fehlen. Weibchen paare sich nur während einer kurzen Zeit und speichern das Sperma lebenslang. Zusammen mit der Tatsache, dass in sozialen Insekten sterile Arbeiterinnen vor Geschlechtstieren produziert werden, führt das dazu, dass sexuelle Kooperation gegenüber sexuellem Konflikt begünstigt ist.

Ziel dieser Arbeit war es die akzessorischen Drüsen der Männchenphänen von *C. obscurior* als mögliche, unmittelbare Ursache des lebensverlängernden Effekts zu untersuchen. Ich habe das Proteinmuster dieser Drüsen mit einer zweidimensionalen Polyacrylamid-Gel-Elektrophorese (2D-SDS-PAGE) und das Genexpressionsmuster mit einer Untersuchung repräsentativer Unterschiede (RDA) erforscht. Zusätzlich habe ich das Proteinmuster der akzessorischen Drüsen der Männchenphänen von *C. tjibodana* untersucht, ebenso wie die Langlebigkeit der Königinnen in Abhängigkeit von der Männchenphäne mit der sie sich gepaart haben.

Kapitel 2 "Suppression subtractive hybridization reveals differentially expressed genes in the accessory glands of two ant male phenes with different life histories" basiert auf den Ergebnissen einer Studie auf RNA-Niveau, welche die Unterschiede in der Genexpression der akzessorischen Drüsen der Männchentypen in *C. obscurior* untersucht. Es konnten 38 einzigartige Sequenzen vorhergesagten Genen zugeordnet werden und acht davon konnten in einer quantitativen Echtzeit-PCR (qPCR)-Analyse detaillierter untersucht werden.

In **Kapitel 3** "Comparison of the accessory gland proteins of two male-diphenic ant species and the effect on their mating partners" wurden die Proteine der akzessorischen Drüsen mittels 2D-SDS-PAGE untersucht. Ich habe die Proteinmuster der zwei Männchenphänen in *C. obscurior* verglichen und dabei 56 unterschiedlich regulierte Proteine gefunden. Ein Vergleich in *C. tjibodana* brachte 91 Proteine welche höher oder niedriger exprimiert waren. Eine nachfolgende nano-LC-MS/MS-Analyse einiger ausgewählter Proteinpunkte zeigte, dass SERPINB10, Protein disulfide isomerase A3, Na^+/H^+ exchange regulatory cofactor, Selenophosphate-synthetase 1 (SPS1) und ein hypothetisches Protein zwischen den beiden Männchenphänen unterschiedlich reguliert sind.

Kapitel 4 "Selenophosphate synthetase in the male accessory glands of an insect without selenoproteins" bewies, dass Selenophosphate synthetase in *C. obscurior* tatsächlich in ein Protein umgeschrieben wird. Bisher wurde das in sozialen Hymenopteren noch nicht gezeigt. Neben einigen Details zur Genstruktur diskutierte ich eine mögliche Rolle von SPS1 im Zusammenhang mit einem Schutz gegen oxydativen Stress in sozialen Insekten.

Kapitel 5 "Queen and male longevity in the Southeast Asian ant *Cardiocondyla tjibodana* Karavaiev, 1935" präsentierte die Ergebnisse der Langlebigkeitsstudie von Männchen und Königinnen von *C. tjibodana*. Im Gegensatz zu *C. obscurior* ist die Lebenserwartung von Königinnen von *C. tjibodana* unabhängig von der Männchenphäne mit der sie gepaart haben. Des Weiteren zeigen Königinnen keinen Anstieg der Eilegerate mit dem Alter, wie es für *C. obscurior* und *C. kagutsuchi* gezeigt wurde.

Interessanterweise fanden wir SERPINB10 auf RNA-Ebene und auch auf Proteinebene. Während die Funktion des Na^+/H^+ exchange regulatory cofactor 2 (NHE-RF2), eines hypothetischen Proteins und des *ejaculatory bulb specific protein 3-like* unbekannt bleibt, könnte SPS1 in der Verteidigung gegen reaktive Sauerstoffarten helfen. Growth hormone-inducible transmembrane protein-like (*ghitm*), SERPINB10 und *leucine-rich repeat (LRR) and immunoglobulin-like domain-containing nogo receptor-interacting protein 2-like* könnten eine Immunsystem verwandte Funktion haben.

Zusammenfassend liefert meine Forschung sehr gute Kandidaten, sowohl Gene als auch Proteine, die mit der Langlebigkeit der Königin zusammenhängen könnten. Diese können als Ausgangspunt für weitere Forschung auf diesem Gebiet dienen.

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Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Marion Füßl
19. Dezember 2014