Telomerase activity pattern in somatic tissues of the bumblebee (*B.terrestris*)

Bachelor's thesis

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Annotation

In this thesis it was determined if the telomerase activity pattern of primitive eusocial insects, like *Bombus terrestris* (bumblebee), differs from the one already previously found in advanced eusocial insects, like for example *Apis mellifera* (honeybee). The telomerase activity of the somatic fat body tissue and the telomerase activity of larvae of *B.terrestris* were tested using TRAP assay. Possible influences of the life cycle of primitive eusocial insects, the mating process of queens and the endocrine regulation on the observed telomerase activity patterns are discussed.

Declaration

Acknowledgements

List of abbreviations

BCA – bicinchoninic acid

CAT - catalase

Ct - cycle treshold

DNA – desoxyribonucleic acid

DSB – double strand break

EdU - 5-ethynyl-2´-deoxyuridine

FOXO – forkhead box

JH – juvenile hormone

L1-5 – larvae of stage one to five

NLaz - Neural Lazarillo

PCR – polymerase chain reaction

Q 10d – 10-day-old queen

Q 1d – one-day-old queen

Q 1y – one-year-old queen

RNA – ribonucleic acid

RNAi – RNA interference

SD – standard deviation

SOD – superoxide dismutase

TER – Telomerase RNA Domain

TERT – Telomerase reverse transcriptase

TOR – target of rapamycin

TORC1 – TOR complex 1

TORC2 – TOR complex 2

TRAP – telomeric repeat amplification protocol

Vg – vitellogenin

W 2 wks – two-week-old worker

Abstract

The length of the ends of eukaryotic chromosomes, the telomeres, and the activity of telomerase, which maintains the telomere length, have been generally accepted to be an indicator of lifespan. Eusocial insects are suitable model organisms for the investigation of the biology of aging because of the caste-dependent lifespan differences found in these species. In advanced eusocial insects, such as honeybees or termites, colonies are large and perennial with thousands of individuals in the sterile castes (workers, soldiers) that are in many aspects different from the reproductives (queens, kings). Reproductives were found to generally have a profoundly longer lifespan than sterile individuals in the given species, and this feature is likely linked to increased telomerase activity in the somatic tissues of reproductives. In contrast to advanced eusocial insects, primitive eusocial insects, such as bumblebees (*Bombus terrestris)*, have colonies of smaller size, with an annual life cycle and a long diapause period of the reproductive queens. The main aim of this thesis was to determine if the telomerase activity pattern in the primitive eusocial insect species might resemble the one in advanced eusocial insects. Therefore, the telomerase activity was tested in the somatic tissues of *B.terrestris* using TRAP assay. No up-regulated levels of telomerase activity were found in the somatic tissues of *B. terrestris* queens, except of the fat body of very young, pre-diapause queens. This finding is discussed with the results of other experiments on *B. terrestris* collaterally performed in our laboratory.

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1. Introduction

1.1. Insect eusociality

Eusocial animals are characterised by an overlap of adult generations and a reproductive division of labour, where some individuals in colony are specialized on reproduction, whereas others are usually sterile and providing nonreproductive tasks such as foraging, defence or cooperative care for the brood. In eusocial animals usually only one female and several males are reproducing in order to raise the total offspring. In evolution eusociality has been evolved as an advanced concept, and being observed in several animal classes including mammals. Most eusocial species are found in the insects, such as several hymenopteran species (honeybees, wasps, ants) or termites. Insect species are the best known eusocial organisms (Danforth, 2001; Engel, 2012; Plowes, 2010), but eusociality is also found in other Arthropoda species, e.g. the snapping shrimps, and even in the phylum Chordata, such as the naked mole rat *(Heterocephalus glaber)*. Nevertheless, insects display eusociality in its best developed form (Plowes, 2010).

In termites the castes are divided into three main categories: reproductives, workers and soldiers. The reproductives are usually a female queen and a male king. They are further differentiated into primary reproductives and neotenic reproductive. Primary reproductives develop into winged sexuals (alates), which loose their wings (becoming dealates) and start a new colony after a nuptial dispersal flight. Neotenic reproductive are wingless and develop in the natal colony. The worker caste comprises most of the individuals in a colony. Workers can be distinguished as false workers in species of one-piece nesting termites and as true workers of multiple-piece nesting termites. In contrast to true workers the false workers are totipotent larvae that can develop into sexuals or sterile soldiers. The soldier caste is specialized by their morphology and behaviour to defend the colony against predators and competitors (Korb and Hartfelder, 2008).

The caste division in Hymenoptera is similar to that in termites, displaying the reproductive caste of queens (gynes) and drones, the sterile caste of workers, and in some species, like in ants, the sterile caste of soldiers. But in contrast to termites, in Hymenoptera eusocial species only adult females can comprise the worker or soldier caste (Lee et al., 2017).

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The various castes are morphologically different even though they can develop from one genotype. This phenomenon is called polyphenism. In honeybees caste differentiation is regulated by the diet provided to larvae; worker larvae are fed with a mixture of royal jelly, pollen and honey, while developing queen larvae are fed with royal jelly only. When individuals within colony differ in behaviour, but not in morphology it is called polyethism. Polyethism is regulated by environmental and social stimuli, leading to a division of labour. For instance, in honeybees young workers stay inside the colony performing tasks there, while old workers are specialized in foraging (Korb and Hartfelder, 2008; Simpson et al., 2011).

1.2. Primitive versus advanced eusociality

It is distinguished between advanced and primitive eusociality. Both examples of eusociality can be observed in the Apidae, a taxon, which the honeybees (*Apis mellifera*) and bumblebees (*Bombus terrestris*) belong to. In honeybees colonies are large and perennial with thousands of individuals comprising the sterile worker caste that can be morphologically, physiologically and behaviourally differentiated from the queen. Task specializations among workers make the colony efficient in foraging and mobilizing colony defences. In comparison to honeybees, the primitive eusocial bumblebee colonies are smaller with only a few hundred workers and only slight morphological differences between bumblebee queen and workers (Danforth, 2001; Engel, 2012; Libbrecht and Keller, 2015). Bumblebees have an annual cycle in which the queen goes through a solitary phase with winter diapause and a (eu)social phase. Only one generation is raised during a year. During the solitary phase the queen mates and enters a diapause period of six to nine months. When emerging from diapause the queen forages, activates her ovaries and lays the first brood, starting a new colony. The solitary phase is followed by the social phase when the first worker emerges. The queen is the only reproducer and the colony members cooperatively work together. At the end of the season competition and conflict arise because females aggressively contest for male production. At the so-called switch-point the queen switches from the diploid to haploid eggs, and males are produced. Ecological conditions probably determine the time of the switch. At the end of the colony cycle gynes are made and the emerging queens leave the colony (Amsalem et al., 2015). Before hibernation fat and glycogen are stored and saved in the fat bodies of queens (Alford, 1969).

1.3. Cost of reproduction and lifespan differences in eusocial insects

Within eusocial insect species, extraordinary lifespan differences are observed between individuals of different castes. Although individuals in the colony have similar genetic information, it was found that reproductives live significantly longer than sterile individuals. For instance, a honeybee queen can live up to 60 times longer than workers (Blacher et al., 2017). This contradicts the phenomenon called "the cost of reproduction".

Commonly among non-social animals, reproduction comes at the cost of future reproduction and future survival of the organism. According to traditional views this is because of a trade-off between fecundity and longevity, where some of the limited internal energy is used to reproduce (Harshman and Zera, 2007). Due to the investment into reproduction, less energy is available for somatic maintenance. The compromise made for reproduction therefore leads to faster aging (Blacher et al., 2017). There are a few theories explaining the reversal of the conventional cost of reproduction in eusocial insects. One of the possible factors is that the age of first queen reproduction takes place relatively later than the first task of workers. Furthermore, queens stay in the security of the hive, while workers spend a part of their life outside it where face external stress factors cause faster aging. There have been made suggestions that fecundity has a positive effect on longevity in eusocial insects. In studies featuring ants and bumblebees it was found that there is a positive connection between the queen's longevity and egg production or adult productivity (Blacher et al., 2017). This does not only come from the fact that queens are supplied with more resources, because the same superior longevity compared to socially isolated workers was found for socially isolated queens (Rueppell and Schrempf, 2017). Even when the egg laying rate was upregulated in the laboratory the queens did not suffer reduced longevity. This may be caused by a rebuilding of the conserved genetic and endocrine networks, which control aging, reproduction and immunity. The positive connection between fecundity and longevity has been also observed in reproductive workers in eusocial Hymenoptera. These workers can reproduce by arrhenotoky (asexual production of males associated with haplodiploidy) or secondary thelytoky (asexual production of females). Like queens, they also stay in a protected environment (Blacher et al., 2017).

Differences in lifetime can also be observed between different eusocial species. A naked mole rat kept in captivity was found to have a maximum lifespan of over 30 years.

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The prediction for a typical rodent of 40 grams was only about six years, which means 30 years is a remarkable life span for a rodent (Ruby et al., 2018). Regarding the termite species *Cyclotermes* the maximum lifespan of the reproducing caste of queens is 12 years, while in another termite species *Reticulitermes hesperus* it is 30 years (Keller, 1998). The maximum life expectancy of an ant queen reaches 30 years and the one of ant workers three years. Honeybee queens are believed to live up to four to six years. Honeybee drones are said to survive 20 - 40 days on average. The lifespan of honeybee workers depends among other things on the geographic location, demography genotype and season. Workers emerged during summer live 15 - 38 days, while bees emerged in late summer or autumn (winter bees) can live 140 days and more (Page and Peng, 2001). This is because summer bees first go through stages of slow senescence, during which they are mainly nursing, followed by a stage of rapid senescence when guarding and foraging. The reason for the almost negligible senescence of winter honeybee workers is a transition from the nursing stage to a diutinus stage instead of the foraging stage. Exact mechanisms of lifespan differences between summer and winter workers are not completely known, it is speculated about a role of vitellogenin (Vg), a 180-kDa monomeric, zinc-binding glycolipoprotein of adult bees. Vg functions as antioxidant in workers and is a yolk precursor. During the diutinus stage Vg accumulates in the hemolymph of the worker bees and gives them a higher oxidative stress tolerance and presumably longevity (Aurori et al., 2014; Corona et al., 2007).

1.4. Life span regulation

Aging is defined as gradual, time-related, multifactorial process, which leads to the accumulation of cellular degenerative changes. Ultimately the functioning of a biological system decreases, resulting in the death of the organism (Tosato et al., 2007). Several postulated theories for the causes and underlaying processes of aging exist. They can be roughly put into two categories: programmed and damage or error theories. The damage or error theories link the aging process to environmental factors like nutrition and stress (Jin, 2010). Endogenous and exogenous stress factors supposedly lead to accidental random damages. The stress factors can be free radicals or glycation end products of a Schiff base reaction between the amino group of lysine and the aldehyde group of glucose. Glycation end products change the functionality of protein components present everywhere in the

biological system (Harman, 1992; Gkogkolou and Böhm, 2017; Suji and Sivakami, 2004). More recently the programmed theories have gotten more attention. They suggest that there is a biological timetable that regulates growth and development. During the life span certain genes are up- or downregulated as pre-determined by the timetable. This can affect the hormonal regulation or lead to a decline of the immune system, which ultimately changes the maintenance, repair and defence mechanisms of the body (Jin, 2010). Caste differentiation process together with lifespan regulation in eusocial Hymenoptera species involves a crosstalk of several signalling pathways, including the ecdysteroid, juvenile hormone, vitellogenin, epidermal growth factor, insulin/insulin like growth factor 1 and target of rapamycin (TOR) pathways (Mutti et al., 2011; Wang et al., 2013; Patel et al., 2007). In honeybees, the caste determination process is triggered by nutrition during larval development, where the key factor is providing royal jelly. Royal jelly is a nutritious substance made of proteins lipids, carbohydrates and bioactive substances and said to have an anti-aging effect connected to cell regeneration (Buttstedt et al., 2016).

1.5.   Telomeres and telomerase

Telomeres are DNA-protein complexes at the end of eukaryotic chromosomes needed for chromosome stability. The term "telomere" was coined by Hermann Müller, a geneticist who was working with fruit flies in the 1930s. It comes from the Greek words for "end" (telos) and "part" (meros). In pioneering studies Müller and Barbara McClintock, another geneticist who was working in the same time period with maize, suggested that chromosome ends have special structures required for chromosome stability and prevention from the chromosome fusions or chromosome breaking during mitosis. Dysfunction of telomeres results in chromosome instability and harmful effects at the cellular and organismal level (Aubert and Lansdorp, 2008).

Telomeres are simple tandem repeats of short, noncoding DNA sequences, usually containing a high amount of guanine. The telomeric sequence is abbreviated as 5'-Tx-Ay-Gz-3'. To conceal the telomere ends from being identified as double strand breaks (DSB), the 3' single end overhang folds back and invades the duplex region forming a t-loop. This characteristic displacement loop likely acts as a shield from the DSB-recognition and -repair system (Makarov et al., 1997; Williamson et al., 1989).

In vertebrates the telomeric repeat is the hexamer TTAGGG, while TTAGG telomeric sequence has been found in the most insect species. Although the short telomeric repeats are the most common telomeric DNA composition, they are not the universal form. The telomeres of *Drosophila melanogaster* (fruit fly), for instance, consist of telomeric retroelements, while those of midges are created by long satellite sequences (Maeshima et al., 2001).

Telomeres are shortened with each cell cycle. This is because the conventional DNA polymerase cannot completely synthesise the ends of a linear DNA molecule (Hayflick & Moorhead, 1961; Olovnikov, 1973; Blackburn, 1991). In addition to that, telomeres are also shortened by action of free oxygen radicals during oxidative stress (Harman, 1992). There are several compensation mechanisms for the loss of telomere length. The most important one is the action of telomerase, a special reverse transcriptase that adds new telomeric repeats onto chromosome ends. Telomerase is composed of an RNA subunit, the Telomerase RNA Domain (TER) and the Telomerase reverse transcriptase (TERT). The TER serves as RNA template for the TERT to synthesize new telomeric repeats onto chromosome ends (Prowse & Greider, 1995; Blasco et al., 1997; Zhou et al., 2014). After telomerase binding to the 3' end of chromosome end, TERT transcribes the RNA template into new repeats and attaches to the 3' end. After telomerase translocation to the newly synthesized 3'end, the process is repeated. The conventional DNA polymerase α and primase synthesize the 5' end (Wyatt et al., 2010; Cooper, 2000).

The connection between proliferating cells and telomerase activity is well known. According to the Hayflick limit theory of aging, the cell replication stops once the telomeres reach a critical length, resulting into cellular senescence (Jin, 2010). In humans, the highest telomerase activity is observed during human embryonic development, and later, activity of telomerase gradually declines. In most somatic cells of humans, activity of telomerase is absent or very low. High telomerase activity in adult humans is observed in germ cells or cells with a high proliferation rate, such as stem cells (Kim et al., 1994; Harley & Villeponteau, 1995; Yasumoto et al., 1996; Yui et al., 1998; Forsyth et al., 2002; Geserick & Blasco, 2006).

The length of telomeres and the activity of telomerase are biomarkers for aging. In a study on zebra finches it was found that the length of the telomeres at an early life stage of the birds (25 days old) is a very strong predictor of potential lifespan, as the longest living individuals had long telomeres at all measurement points (Heidinger et al., 2012). With advancing age, the attrition of telomeres increases due to the influence of stress

factors in the environment, which include chemicals, psychological stress and disease. The loss of telomeric repeats and absence of telomerase activity ultimately impairs the proliferation of stem cells and thus, the regeneration capacity (Epel et al., 2004; Blasco, 2007; Zhang et al., 2013).

1.6. Telomerase as a clue to lifespan difference in eusocial insects

Social insects are used as model organisms for studies on aging because of their extreme caste-dependent lifespan differences (Keller and Jemielity, 2006). Not only lifespan but also telomerase activity was found to be caste- and development-specific in eusocial insects, as observed in honeybees, where an upregulation of telomerase was found in queens and in long-lived winter bees. ███████████████████████████████ ██ It is hypothesized that the upregulation of telomerase activity in somatic tissues of eusocial insects plays role in the caste determination process and is related to lifespan differences between reproductive and non-reproductive individuals within the colony. The preliminary study to this thesis, performed in our laboratory, was focused on discovering whether telomerase activity in *B. terrestris*, despite its primitive eusocial organization, has the same pattern of telomerase activity as the one observed in the advanced eusocial *A. mellifera*. The upregulated telomerase activity was observed in gonads and in the fat body of young pre-diapause queens. In contrast, telomerase activity in queen somatic tissues such as head or leg muscles shows comparable levels to those found in workers. These finding led us to hypothesis that longevity of *B. terrestris* queens is based on a mechanism different from that in reproductives of advanced eusocial species and that the prolonged lifespan of bumblebee queens is enabled by the long period of diapause.

2. The aims

The aim of thesis was to investigate the telomerase activity in *B. terrestris* during its larval development and in somatic tissues of adult queens of different ages and different mating status using the telomeric repeat amplification protocol (TRAP) assay and including confirmation of the TRAP reaction specificity by cloning and sequencing of the TRAP products. The main goal of thesis was to support or disprove our hypothesis that longevity of *B. terrestris* queens is based on a regulation pathway that is different from the one in reproductives of advanced eusocial species. To fulfil this aim, the experimental data of the thesis was compared to the results of other experiments collaterally performed in our laboratory on *B. terrestris*, as well as to previous studies on advanced eusocial and solitary insect species.

3. Method

3.1. Samples

Samples of *B. terrestris* were obtained from Koppert Biological Systems, Slovakia. The colonies were reared at 28 ± 2 °C and a relative humidity of $50 - 60$ % and supplied with dried pollen and sugar solution (mixture of glucose, fructose and sucrose, 48 °Brix). The following kinds of individuals were used: 24-hour-old virgin queens; 10-day-old queens, which were 24 hours post copulation, and 10-day-old virgin queens; four-week-old queens, which were three weeks post copulation; and one-year-old reproducing queens; one-to-three-week-old workers; two-week-old males, embryos and larvae of stage 1 - 5. Before dissection the individuals were anesthetised by placing them on ice for a few minutes. The tissues obtained from the dissection process were then frozen with liquid nitrogen and stored at -80°C for later use. The following somatic tissues were obtained from workers: head, leg (muscle), fat body, colon, hemolymph, Malpighian tubules. Ovaries were taken from queens and workers and testes were taken from drones.

3.2. Preparation of protein extracts

The dissected tissues were taken from the - 80 °C freezer, thawed on ice, and homogenized in 200 µL of extraction buffer (10 mM Tris/HCl, pH 7.6; 1 mM $MgCl_2$; 1 mM EGTA; 0.1 mM benzamidine (PMSF); 5 mM 2-mercaptoethanol; 0.5 % (w/v) CHAPS; 10 % (v/v) glycerol, and 40 U/ml RNase inhibitor (Promega)). The mixture was incubated on ice for 30 minutes and then centrifuged at 12000 g for 20 minutes at 4 °C. The supernatant was then collected and either frozen in liquid nitrogen and stored at $- 80$ °C or immediately used for analysis. Concentration of total protein was determined using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce). For this 25 µL of each sample were pipetted into a microplate well and 200 µL of reagent were added. The reduction of Cu^{+2} to Cu^{+1} by the present protein in the alkaline solution leads to a purple coloration. The plate was incubated on ice for up 30 minutes. The absorbance was then measured at 595nm on a plate reader.

3.3. Telomeric repeat amplification protocol (TRAP)

Telomerase activity was quantified by real-time polymerase chain reaction (PCR). For the quantification of telomerase modified protocols of Sasaki and Fujiwara (2000) and Wege et al. (2003) were used. An artificial substrate for telomerase, a Ts forward primer, was used to simulate the activity of telomerase. The more repeats were added and thus the longer the elongation product, the higher the telomerase activity. The elongation product was then amplified by thermocycling in a Light Cycler CFX96 BioRad Real-time PCR system for TRAP product detection. The energy transfer primer was labelled with Sybr Green. When Sybr Green is incorporated into the double strand it fluoresces, allowing the detection of the TRAP product. The Real-time PCR was introduced in order to avoid some fundamental problems of end-point PCR that would complicate the exact measurement of telomerase activity. The method also does not require any post-PCR analysis steps. A 25 µL analysis mixture of 5 ng protein extract sample, 5 pmol TS primer (5' – AAGCCATCGAGCAGAGTT – 3'), 5 pmol Bm-CXa primer (5' – GTGTAACCTAACCTAACC – 3') and 12.5 µL Xceed qPCR 2x mix (Institute of Applied Biotechnologies) added into the wells of a 96-well plate. After an incubation period of 60 minutes at room temperature, where the TS primer is elongated by telomerase, real-time PCR was performed with 30 cycles of 30 seconds at 94 °C and 30 seconds at 60 °C. All samples were tested in triplicates.

3.4. Cloning and sequencing of TRAP products

To determine the specificity of the TRAP reaction, the TRAP products were prepared using 25 µL reactions containing 5 ng protein extract sample, 5 pmol TS primer (5' – AAGCCATCGAGCAGAGTT – 3'), 5 pmol Bm-CXa primer (5' – GTGTAACCTAACCTAACC – 3'), 5U DreamTaq polymerase (Thermo Fischer Scientific), 0.2mM dNTP. After an incubation period of 60 minutes at room temperature, PCR was performed with 30 cycles of 30 seconds at 94 °C and 30 seconds at 60 °C using the conventional PCR (Biometra, Professional Trio themocycler). The TRAP products were purified using the PCR Clean-up system (Macherey-Nagel) and cloned into a pGEM®-T easy vector (Promega). For the cloning reaction (10µL) I used 5ng of pGEM

vector, 1 ng of TRAP product and T4 DNA ligase (5U, Promega). The plasmid DNA was isolated using Nucleospin Plasmid Quickpure Kit (Macherey-Nagel) and the inserts were sequenced using the ABI PRISM 3.1 (Applied Biosystems) with T7 and SP6 primers.

3.5. Statistical Evaluation

The data was analysed with the GraphPad Prism 6.0 Software (GraphPad Software, San Diego, CA, USA). The one-way ANOVA and Tukey's multiple comparison test were used. The mean ± standard deviation (SD) was depicted in bar diagrams.

4. Results

4.1. The (TTAGG)n repeats were confirmed by sequencing of the TRAP products

In the first step, the specificity of the TRAP reaction was evaluated in the representative samples from the fat body, ovaries and testes. The samples were analysed with a TRAP assay performed in a conventional PCR cycler, and the TRAP products were cloned into pGEM vector and sequenced. The sequencing of the products revealed arrays of TTAGG repeats and thus, the specificity of the TRAP reactions was confirmed.

4.2. Telomerase activity in adult somatic tissues

Then, the telomerase activity was evaluated using the TRAP assay in a quantitative real-time PCR cycler. The experiment was performed with the extracts prepared from the haemolymph, colon and fat body. As a reference, the levels of telomerase activity in germarium, testes and embryos were tested as well.

My experiments showed no detectable telomerase activity in the haemolymph. Inconsistent data was found in colon samples where the Cycle threshold (Ct) values greatly varied between the tested samples and numerous samples revealed no Ct value (data not shown). The highest telomerase activity was found in the germarium of one-year old

queens, showing approximately 150-times higher levels compared to level in embryos (Figure 1). The relative telomerase activity in testes was up to three-times higher than that in embryos or in the fat body of workers, one-year-old queens or four-week-old pre-diapause queens, but was approximately 120-times lower compared to the germarium of one-year-old queens (Figure 1).

Upregulated telomerase activity was found in the fat body of one-day and 10-day-old pre-diapause queens when compared to the fat body of four-week-old pre-diapause queens, post-diapause queens, or workers. The tested representatives of one-day-old queens had a nearly 10-times higher telomerase activity in their fat body compared to embryos (Figure 1). No statistical significant difference in levels of telomerase activity in the fat body was observed between one-day old and ten-day-old virgin queens. However, a decrease of telomerase in pre-diapause queens was observed after queen mating. 10-day-old queens that were before and after the mating process were compared. Queens that already mated revealed a decrease of approximately 50% in telomerase activity compared to the virgin queens. However, 10-day-old mated queens still showed up to five-fold higher levels of telomerase activity in comparison to older queens or workers. No statistical differences in telomerase activity were found between four-week-old pre-diapause queens, one-year old queens, workers and embryos (Figure 1).
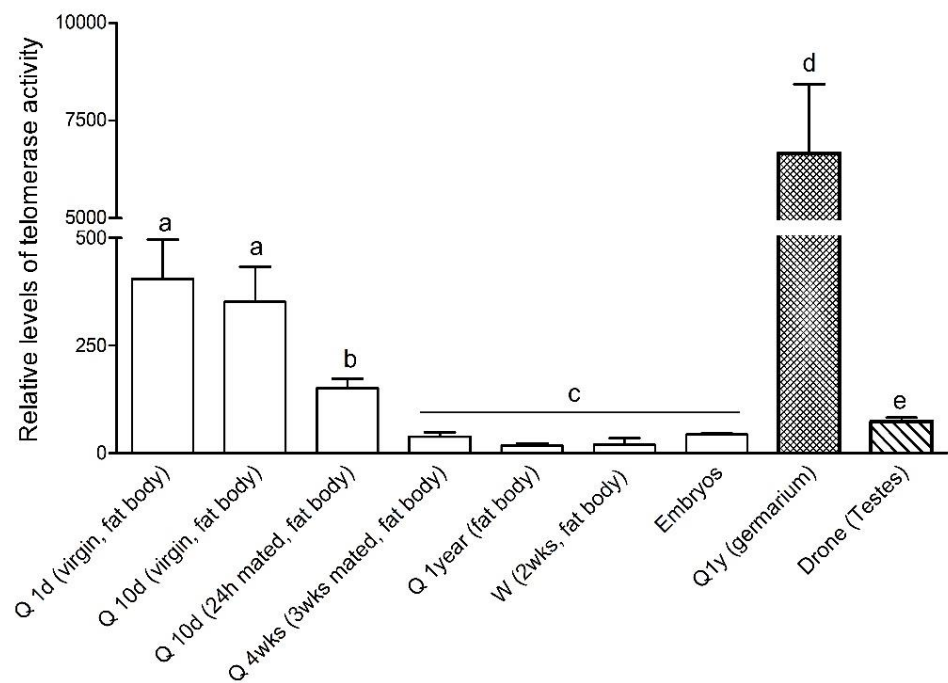

Figure 1. Telomerase activity in the fat body, gonads and in embryos. The relative telomerase activity was determined by TRAP assays of queens of different ages, workers

and males. The embryo samples were used as a reference and were composed of five embryos. Terminology: Q 1d – one-day-old queen; Q 10d virgin – 10-day-old queen before copulation;Q 10d 24h mated – 10-day-old queen 24 hours after copulation; Q 4 wks mated – four-week-old queen three weeks after copulation; Q 1y – one-year-old queen; W 2 wks – two-week-old worker. One-way ANOVA and Tukey's post-hoc tests were used to evaluate the statistical significance; n=3. The mean ± SD is given by the bars of the graph.

4.3. Telomerase activity in larval tissues

The second set of experiments was focused on telomerase activity during larval development. The first three larval stages all showed about the same relative levels of telomerase activity and revealed approximately six-fold higher levels compared to embryos (Figure 2). During the fourth larval instar telomerase activity was increased and reached the level of telomerase activity found in germarium. In comparison to embryos the increase in the fourth larval instar was 600-fold.
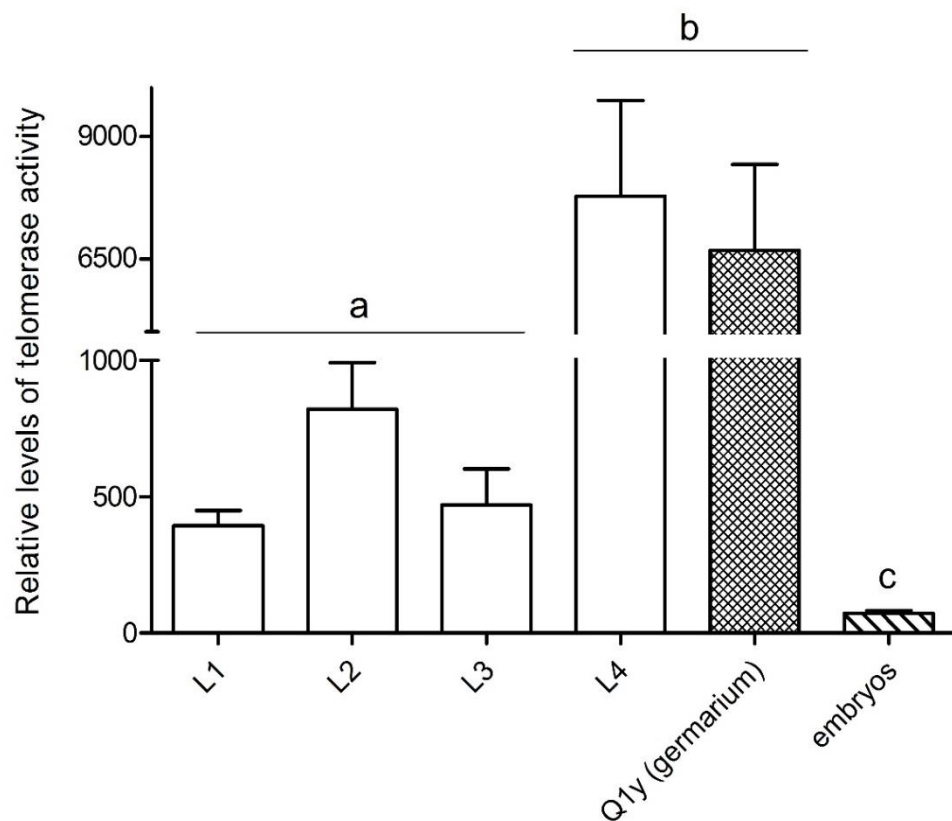

Figure 2. Telomerase activity in larvae. The relative telomerase activity was determined by TRAP assays of larvae of the five different stages. The embryo samples, composed of

five embryos, and the germarium of a one-year-old queen were used as a reference. Terminology: L1 – first larval stage;L2 – second larval stage; L3 – third larval stage; L4 – fourth larval stage; Q 1y – one-year-old queen. One-way ANOVA and Tukey's post-hoc tests were used to evaluate the statistical significance; n=3. The mean ± SD is given by the bars of the graph.

5. Discussion

Previous studies consistently showed elevated levels of telomerase activity in the somatic tissues of reproductives of advanced eusocial insect species such as *A. mellifera* (Korandová and Frydrychová, 2015) ▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮
▮▮▮▮ This lead to the hypothesis, that telomerase activity is associated with the caste determination process of eusocial insects and is part of a mechanism leading to lifespan extension in reproductives of eusocial insects. *B. terrestris* is a primitive social insect species, where queens have prolonged lifespan compared to workers. However, most of the lifetime of queens is spent in diapause. The question arose whether prolonged lifespan of a *B. terrestris* queen is truly based on a special longevity-related program, as could be speculated in reproductives of advanced eusocial insects, or is simply related to the long diapause. A connection of the extended queen lifespan to the diapause would mean that the differences in lifetime regulation between *B. terrestris* castes cannot be directly compared to the caste-related differences in *A. mellifera*.

5.1. Lifespan differences in reflection to telomerase and telomere length: advanced and primitive eusocial insects

My results support the hypothesis that there is a difference in the telomerase activity pattern between primitive and advanced eusocial species. Telomerase activity has been found elevated in most somatic tissues of honeybee queens (Korandová and Frydrychová, 2015) ▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮
▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮ Contrary to that, the telomerase activity in bumblebee queens is not commonly elevated in their somatic tissues. An upregulation of telomerase activity was only found in the fat body and only in the fat body of young pre-diapause queens. When the terminal restriction fragments were

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analysed in our laboratory using Southern hybridization, it was revealed that one-year old queens have shortened telomeres in comparison to workers and young queens (Koubová et al. 2019). This stands in opposition to the findings from honeybees, ███████ and ants (Korandová and Frydrychová, 2015; Jemielity et al., 2007, █████████████████████) showing no differences in telomere length between workers and queens of different ages. Thus, the telomerase activity evaluation, provided by the thesis, supports the hypothesis that extended lifespan of bumblebee queens is related to the long diapause period. It remains, however, unclear why differences in telomerase activity between castes in the advanced eusocial species do not lead to differences in telomere length.

5.2. Upregulation of telomerase activity in the young pre-diapause queens might be associated with diapause

The fat body serves as an important organ for energy storage, which the young queens need during diapause in order to survive. It has a strong metabolic activity and changes its structure and function throughout the lifespan of *B. terrestris* (Votavová et al., 2015). The development of this organ starts after the queen emerges from the pupa. Young queens only have a short time to build up energy reserves before they enter the diapause, which makes the build-up an urgent concern. Glycogen, the energy-storage molecule, is synthesized and stored in adipocytes and then used mostly during the diapause period (Alford, 2015).

We can speculate that the upregulated telomerase activity in the fat body of young pre-diapause queens is an important step for the intensification of metabolic activity in the fat body prior to diapause. This is supported by additional experiments performed in our laboratory, using 5-ethynyl-2´-deoxyuridine (EdU) staining to visualize DNA synthesis, and by the evaluation of amounts of nutrients in the fat body (Koubová et al., 2019). Experiments showed that DNA synthesis as well as the amount of nutrients, such as lipids and glycogen, and the fat body mass were strongly increased in the fat body of young pre-diapause queens (Koubová et al., 2019). This indicated that an increased telomerase activity correlates with increased DNA replication. The upregulated levels of DNA synthesis were observed in adipocytes and oenocytes. These cell types are polyploid and display DNA endoreduplication cycles, which is a phenomenon commonly found in tissues of high metabolic activity. Cells displaying DNA endoreduplication cycles reduce

the energy needed for cell proliferation through the absence of cell division, so the metabolic activity of those cells can be boosted instead (Larkins et al., 2001). Collectively, I can speculate that the upregulation of telomerase activity and DNA synthesis in the fat body of young pre-diapause queens both serve to build up a sufficient energy supply in time for the diapause. The results also strongly indicate that the lifetime differences between *B. terrestris* workers and queens could be related to the long period of queens spending in diapause.

I additionally tested telomerase activity during the larval development. The data is clearly consistent with the fact that telomerase activity is strongly related to cell proliferation. Moreover, it has been suggested that the stabilization of the telomeres by telomerase is not only enhancing the proliferation of epithelial cells, but that telomerase also has a positive effect on the expression of growth-promoting genes (Smith et al., 2003). We can speculate that the high activity during all the larval stage could therefore be linked to cell proliferation and the growing process of the larvae.

5.3. Is there a connection between telomerase regulation activity and endocrine control?

In our team it was evaluated if the differences in the telomerase activity in the fat body of old and young queens can be explained by changes in the cell signalling pathways with regard to the antagonistic pleiotropy theory of aging (Koubová et al., 2019). The antagonistic pleiotropy theory of aging suggests, that genes, that are likely to be selected during evolution, are favourable in the youth, however, lead to geroconversion during adulthood. Geroconversion means that inflammation, hyperfunction and malfunction, and therefore ultimately aging are promoted. Those favoured genes are commonly genes related to nutrient-sensing mechanisms. This includes the TOR pathway.

TOR proteins are evolutionary highly conserved kinases, which play an important role in the protein biosynthesis by importing and recognizing amino acids. The TOR proteins can be found at the core of the two complexes TORC1 and TORC2 (Schonbrun et al., 2009). TOR pathway is activated by growth factors, nutrients, and hormones (e.g. insulin), and it is responsible for cell division, body growth, metabolic regulations and also involved in caste differentiation of honeybees (Blagosklonny, 2014, 2010; Corona et al., 2016; Mutti et al., 2011). Forkhead box (FOXO) is a transcription factor that suppresses the TOR pathway and geroconversion. FOXO is involved in cell proliferation, metabolism,

DNA repair and oxidative stress responses and consequently extends lifespan (Blagosklonny, 2010; Tia et al., 2018). Lifespan extension in vertebrates and invertebrates is linked to the suppression of TOR. In the fission yeast, where two TOR complexes exist, TORC1 and TORC1, it was found that TORC1 is required for telomere length maintenance and TORC2 is required in the DNA damage repair mechanism (Schonbrun et al., 2009). When the relation between telomerase activity and the activity of TOR and FOXO was tested in bumblebees a negative correlation was found between the telomerase activity and TOR, while a positive correlation was observed between telomerase activity and FOXO (Koubová et al., 2019). ████████████████████████████
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Another endocrine signalling molecule that could have an effect on the regulation of telomerase activity is vitellogenin. Vitellogenin (Vg) is a lipoprotein and a yolk precursor and therefore important for reproduction. Vg is synthesized in the fat body and released into the haemolymph. Its synthesis is triggered by juvenile hormone (JH). Juvenile hormone is an important insect hormone regulating growth, development and lifespan. It has a gonadotropin function and a positive correlation with Vg in various insect species. This is contrasted by honeybees, where JH lost its gonadotropin function and shows no positive correlation to Vg. Although the haemolymph of young, virgin queens shows both high levels of JH and Vg, later in their life the JH level decreases, while the Vg level stays high. In comparison to queens, honeybee workers show low levels of Vg and high levels of JH. However, Vg and JH levels of workers change during their lifespan. During the first few weeks of the adult life of a honeybee worker, when workers perform tasks inside the hive, the JH titre is low and the Vg level is high. After the workers switch to being foragers, the JH titre rises and the Vg synthesis is downregulated (Corona et al., 2007). Although in honeybees the gonadotropin function of JH is lost, JH and therefore also Vg are linked to caste differentiation and the division of labour (Amsalem et al., 2014). Beside this, Vg is believed to be involved in anti-oxidant and anti-stress defence as well as in immune reactions of honeybees (Blacher et al., 2017; Rueppell et al., 2007; Rueppell and Schrempf, 2017).

In bumblebees it was found that JH is needed for oocyte development and egg-laying and shows a positive correlation with Vg. This means that, in contrast to honeybees, JH in bumblebees kept its gonadotropin function. Levels of JH and Vg are influenced by the social context of the colony and it is speculated that JH could have therefore played an

important role in the evolution of eusociality in Hymenopteran species (Shpigler et al., 2014).

The role of JH and Vg in caste differentiation has been found also in termites. However, termites show a positive correlation between JH and Vg as well as a gonadotropic function of JH. This makes termites more similar to the closely related, but solitary cockroaches, rather than to the eusocial, distantly related honeybees (Korb and Hartfelder, 2008).

5.4. Is telomerase activity affected by mating?

Furthermore, the data of the thesis indicates that the reproduction process affects the activity of telomerase. Queens of the same age differed in the levels of telomerase activity, depending if they mated or not. This finding is consistent with the cost of reproduction phenomenon arguing that longevity is decreased upon reproduction. It also supports our hypothesis that the lifespan regulation in bumblebee queens differs from that in queens or kings of advanced eusocial species. ████████████████████

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████████ Similarly, in association with higher fecundity rate in ant queens, an upregulation of some longevity-related genes was found. These include superoxide dismutase (*SOD*) and catalase (*CAT*), which are key players in antioxidant defence, or Neural Lazarillo (*NLaz*), which is an insect homologue of Apolipoprotein D, showing common ancestral functions like longevity, stress resistance and homeostasis of carbohydrates and triglycerides. It is assumed that the differential gene expression and signaling pathways lead to a change in metabolism and stress tolerance in mated queens (Negroni et al., 2019; Page and Amdam, 2007; Von Wyschetzki et al., 2015).

Collectively, it shows that the longevity of the reproductives of eusocial insects is enhanced by the reproduction process, whereas in solitary or primitive social species the lifespan is shortened by reproduction. Findings on telomerase activity, the role of the reproduction process and the endocrine signaling pathways in terms of lifespan and the caste determination process in advanced and primitive eusocial insects, in comparison to solitary insects, are summarized in Table 1.

Table 1. Summary of the differences found between the advanced eusocial, primitive eusocial and solitary organisms.

		ADVANCED EUSOCIAL INSECTS		PRIMITIVE EUSOCIAL INSECTS Bumblebees	SOLITARY INSECTS
		Honeybees	Termites		
Lifespan		• extended lifespan in reproductive females (Rueppell and Schrempf, 2017)	• extended lifespan in reproductive females and males (Thorne et al., 2002)	• extended lifespan in reproductive females (Lopez-Vaamonde et al., 2009)	• shortened lifespan in reproductive individuals (Aurori et al., 2014)
Telomerase in somatic tissues of adults in terms of :	Activity	• high activity in somatic tissues of reproductive females (Korandová and Frydrychová, 2015)	████	• in somatic tissues high activity only in the fat body of young pre-diapause queens (Koubová et al., 2019)	• low activity in most somatic tissues (Korandová et al., 2014)
	Reproduction	• not tested	• positive correlation (Jemielity et al., 2007)	• negative correlation (Koubová et al., 2019)	• not tested
	DNA synthesis	████	████	• positive correlation (Koubová et al., 2019)	████
Endocrine regulators	Juvenile hormone and vitellogenin	• JH lost its gonadotropin function in queens (Amsalem et al., 2014)	• JH acts as gonadotropin with positive regulation of queen fertility (Korb and Belles, 2017)	• JH acts as gonadotropin with positive regulation of queen fertility (Amsalem et al., 2014)	• JH acts as gonadotropin with positive regulation of female fertility (Korb and Belles, 2017)
		• Vg and JH are associated with caste and social context (Amsalem et al., 2014; Eyer et al., 2017)	• Vg and JH are associated with caste and social context (Korb and Belles, 2017)	• Vg and JH are associated with caste and social context (Shpigler et al., 2014)	N/A

		• negative correlation of JH with Vg (Rueppell et al., 2016)	• positive correlation of JH and Vg (Korb and Belles, 2017)	• positive correlation of JH with Vg (Jedlicka et al., 2016; Shpigler et al., 2014)	• a positive correlation of JH with Vg (Korb and Belles, 2017)
	TOR	• gero-promoting factor (Blagosklonny, 2010) • negative correlation with telomerase activity (our unpublished data) • involved in caste determination (Simpson et al., 2011)	N/A	• negative correlation with telomerase activity (Koubová et al., 2019)	• reduced activity in response to starvation (stress) (Teleman et al., 2005)
	FOXO	• gerorepressor (Blagosklonny, 2010) • ▮▮▮▮▮	N/A	• positive correlation with telomerase activity (Koubová et al., 2019)	• activation in response to starvation (stress) (Teleman et al., 2005)

6. Conclusion & future directions

It can be concluded that the telomerase activity pattern in advanced (honeybees, termites) and primitive (bumblebees) eusocial species differ significantly. It was confirmed that the somatic tissues of *B. terrestris*, with exception of the fat body, have low levels of telomerase activity. The elevated telomerase activity in the fat body of young *B. terrestris* queens together with the high level of DNA synthesis might be connected to the impending diapause. The main hypothesis, which is supported by the data, is therefore, that the elevation of both telomerase activity and DNA synthesis serves to accelerate the build-up of an adequate energy storage in the fat body prior to the long diapause. There might also be a connection to the mating process or regulated cell signalling pathways such as the TOR pathway. However, these conclusion needs to be more deeply investigated by future studies.

In the future, the link between telomerase regulation and the nutrient-sensing pathways could be investigated by using RNA interference (RNAi) assays. Silencing the telomerase subcomplex TERT in bumblebees with the help of RNAi can give important information if the regulation of the endocrine pathways plays a role in the telomerase activity and telomere length regulation. Furthermore, differential expression analysis using RNA sequencing could be employed for mated and unmated queens to determine differences in the level of gene expression.

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