Functional response of the hypopharyngeal glands to a social parasitism challenge in southern African honey bee subspecies

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Declarations.

Funding. This work was funded in parts by the University of Pretoria, the National Research Foundation of South Africa's incentive funding for rated researchers to C.W.W.P. and A.A.Y and National Research Foundations South African Research Chair in Mathematical Methods and Modelling in Biosciences and Bioengineering.

Conflict of interest/Competing interests. No competing interests declared.

Availability of data and material. All data are contained in the manuscript.

Code availability. Not applicable.

Authors' contributions. Christian W.W. Pirk, Abdullahi A. Yusuf and Zoë Langlands conceived and designed the study. Zoë Langlands performed the experiments. Zoë Langlands, Esther E. du Rand and Christian W. W. Pirk performed data analysis. Esther E. du Rand wrote the first draft, Christian W.W. Pirk and Abdullahi A. Yusuf contributed to revisions and secured funding. All authors gave final approval for publication.

Ethics approval. Not applicable.

Consents to participate. Not applicable.

Consent for publication. Not applicable.

Acknowledgements. Brendan Cooper for the use of his *A. m. capensis* colonies in Cape Town and the South African National Biodiversity Institute for the use of their research facilities. The beekeepers in Gauteng (South Africa) who donated Clone infested colonies for the purpose of this research.

Abstract

Hypopharyngeal gland (HPG) development in honey bee workers is primarily age-dependant and changes according to the tasks performed in the colony. HPG activity also depends on colony requirements and is flexible in relation to the need for feeding brood. Very little is known about HPG development in the honey bee subspecies found in Southern Africa. We examined HPG development in *Apis mellifera scutellata* and *A. m. capensis,* including *A. m. scutellata* colonies infested with an invasive parasitic clonal lineage of *A. m. capensis* known to manipulate food provisioning to the parasitic larvae by their *A .m*. scutellata hosts, under natural in-hive conditions in bees aged 0 to 14 days using light microscopy. We found marked differences in acini size (berry-like clusters of secretory cells) and the age at which maximum HPG development occurred between the subspecies and in the presence of the parasite. In *A. m. scutellata* workers, acini reached maximum size at six days. The acini of *A. m. capensis* workers were larger (up to double) than those of *A. m. scutellata* and reached maximum size at eight days. Whilst the HPG acini in *A. m. scutellata* workers infested with *A. m. capensis* Clones reached development sizes similar to those of *A. m. capensis* at day 10 and were 1.5 times larger than those of uninfested *A. m. scutellata*. This provides foundational insights into a functional response affecting the development of the HPG most likely associated with brood pheromone composition and how this is altered in the presence of a social parasite.

Keywords: brood-food glands / social parasitic Clone / brood pheromones / savannah honey bee / Cape honey bee

1. Introduction

Honey bee workers perform a series of tasks in the colony linked to their age. Age-polyethism sees workers in the early stages of their life perform tasks inside the nest such as cleaning, brood care, feeding of the queen, wax production, building comb, progressively taking over tasks at the periphery of the brood nest to the outside of the nest such as guarding and foraging (Rösch 1925, 1930; Lindauer 1952). This temporal polyethism is flexible and can adapt and respond to the development and growth of the colony, food availability, changing environmental conditions and season (Huang and Robinson 1996). Worker bees performing the different tasks in the nest can normally not be distinguished by external morphology, but these age-associated tasks are accompanied by various changes in anatomy, physiology and nutritional requirements (Huang et al. 1994; Huang and Robinson 1996; Naiem et al. 1999).

Congruently, the morphological and physiological status of the hypopharyngeal glands (HPG) or brood-food glands changes with age or according to the tasks performed by the workers in the colony. These paired glands are located in the bee's head and produce the proteinaceous secretions (jelly) that are fed to the queen, larvae, drones and nest mates (Crailsheim 1992). Each gland consist of small oval bodies (acini) that are linked to axial or terminal secretory ducts (Kratky 1931; Snodgrass 1956). The size of the HPG are often used to describe the physiological status of worker bees (associated with the age-related task performed), as the size is closely related to the rate of protein synthesis in the glands (Brouwers et al. 1987; Knecht and Kaatz 1990; Deseyn and Billen 2005). The HPG are generally well developed in nurses of European honey bee subspecies at the age of 6 to14-days old with large functional secreting acini (Crailsheim and Stolberg 1989; Lass and Crailsheim 1996). The primary source of protein for the synthesis of the protein-rich jelly is pollen, which is ingested in large quantities by the nurse bees (Crailsheim et al. 1992). At the onset of foraging, the HPG degenerate which results in decreased protein synthesis and the expression of digestive enzymes that are involved in converting sucrose into simple sugars, such as α-glucosidase, amylase, glucose oxidase, etc.

(Rösch 1930; Ohashi et al. 1999). Apart from age, HPG development is also influenced by the consumption of different types of pollen with varying levels of protein content (Wright et al. 2018), with the development being positively correlated with protein consumption (Altaye et al. 2010).

The secretory activity of the HPG also largely depends on the requirements of the colony and is flexible in relation to the brood status of the colony or the need for feeding brood (Hrassnigg and Crailsheim 1998a). The developing larvae release a complex of pheromones to signal their protein requirements and need for care to the worker bees (Winston 1987; Le Conte et al. 1995; Pankiw 2004). These pheromones stimulate HPG development, protein synthesis (Huang et al. 1989; Huang and Otis 1989; Mohammedi et al. 1996) and increases the amount of extractable proteins in the HPG (Mohammedi et al. 1996; Pankiw 2004). The pheromone complex vary with larval age and enables the nurse bees to differentiate between larvae of different ages and regulate the type and amount of food provisioned based on the age of the developing larvae (Leimar et al. 2012; Wang et al. 2014) . Larvae can signal for food when hungry (Le Conte et al. 1990; He et al. 2016), but the extent to which larvae can manipulate the pheromones released to manipulate the type and amount of food provisioned by the nurse bees is still largely an unanswered question. Intriguingly, it has been shown that the brood of a parasitic lineage of *Apis mellifera capensis* (one of the honeybee subspecies native to Southern Africa) manipulates nurse bees from other subspecies to provision them with more food than they would their own brood (Allsopp et al. 2003; Boot et al. 2006).

Very little is known about HPG development in the two honey bee subspecies found in Southern Africa, *Apis mellifera scutellata* Lepeletier and *Apis mellifera capensis* Escholtz. *Apis mellifera scutellata* is found in the northern regions of South Africa and extends northwards across Botswana, Namibia and into East Africa (Hepburn and Radloff 1998). While *A. m. capensis* is native to the western and eastern Cape, confined largely to the fynbos in the

southern-western corner of South Africa (Hepburn and Radloff 1998). One of the main differences between these two subspecies is the ability of *A. m. capensis* workers to produce diploid female offspring through the process of thelytokous parthenogenesis (Onions 1912; Verma and Ruttner 1983), rapidly activate their ovaries and synthesise a queen-like pheromone bouquet (Hepburn 1992; Dietemann et al. 2007; Lattorff et al. 2007; Zheng et al. 2010), which allows these workers to develop into false queens. A specific invasive lineage of these *A. m. capensis* laying workers (referred to as Clones) has evolved through a short-term evolutionary process into a facultative reproductive social parasite of *A. m. scutellata* and *A. m. capensis* (Härtel et al. 2006; Moritz et al. 2008). This social parasite infiltrates susceptible host colonies (Neumann and Hepburn 2002) and once inside, it beginsproducing a queen-like pheromonal bouquet (Sole et al. 2002; Dietemann et al. 2007; Zheng et al. 2010; Okosun et al. 2017) and rapidly activates its ovaries (Hepburn 1992; Neumann and Hepburn 2002) becoming false queens in the host colony. The Clone workers (the false queens) become reproductively active after claiming pheromonal dominance in the host colony, taking over the reproductive role in the colony leading to the eventual collapse of the colony (Neumann and Hepburn 2002).

The Clone workers do not contribute to the worker tasks in the colony and rely on the host workers for food and brood care (Neumann and Hepburn 2002). Clone larvae receive more food from *A. m. scutellata* workers in comparison to the amount host workers feed their own larvae (Allsopp et al. 2003; Boot et al. 2006). Similarly, non-parasitic *A. m. capensis* larvae also receive more food when reared by another subspecies such as *A. m. scutellata* (Allsopp et al. 2003) or European honey bees (Beekman et al. 2000). In contrast, *A. m. scutellata* larvae reared in *A. m. capensis* colonies receive only half the food they are usually provided with from their own nurse bees (Allsopp et al. 2003). This suggest that the Clone brood's demand or signalling for food (hunger signal) is stronger than both *A. m. capensis* and *A. m. scutellata* brood. This stronger demand for food presumably affects the development and size of the HPG of the nurse bees provisioning food for the Clone brood.

Focussing on the two subspecies of honey bees native to South Africa, the objective of this study was to investigate whether HPG development in bees aged 0 to 14 days is a functional response by answering the following questions. Do workers of *A. m. capensis* develop their HPG faster compared to workers of *A. m. scutellata* due to the stronger brood signal? Secondly, what is the effect of the presence of Clones (parasitic *A .m.* capensis) on the development of the HPG of the host colony workers?

2. Materials and Methods

2.1 Sampling worker bees of known age.

Healthy, queenright colonies of *Apis mellifera scutellata* were maintained in an apiary located at the University of Pretoria experimental farm (25°44'50.8"S 28°15'31.9"E, Pretoria, South Africa). Colonies of *A. m. scutellata* infested with parasitic *Apis mellifera capensis* Clones (hereafter referred to as Clones) were donated by local beekeepers from the Gauteng province of South Africa and maintained in quarantined areas at the University of Pretoria experimental farm apart from the apiary that houses the healthy *A. m. scutellata* colonies to avoid spill over infestation to healthy non-infested *A. m. scutellata* colonies. Healthy, queenright colonies of *Apis mellifera capensis* were maintained in an apiary in their native range in the Table Mountain Nature Reserve, Western Cape (33°58'09.7"S 18°27'04.3"E, Cape Town, South Africa). In total, six colonies (two healthy colonies of *A. m. scutellata,* two healthy colonies *A. m. capensis* and two *A. m. scutellata* colonies infested with Clones) were used in this study. To obtain bees of a known age, a brood frame was collected from each of two healthy *A. m. scutellata* and two healthy *A. m. capensis* colonies and incubated at 35 °C and 50% RH in darkness to simulate in hive conditions. Three hundred newly emerged bees (≤ 24 h old) were collected from each frame and marked on the thorax using a paint marker (Schneider, Germany). The marked newly emerged bees were returned back to their respective source hives. In addition, a brood frame was collected from two other healthy *A. m. scutellata* colonies, incubated and 300 newly emerged bees (≤ 24 h old) marked on the thorax and placed

in two queenright *A. m. scutellata* colonies infested with Clones kept in the quarantine site on the experimental farm of the University of Pretoria. For the next consecutive 14 days a sample of ~20 marked bees were collected daily from each of the six colonies used in this study using an aspirator. The age of the bees corresponded to the day on which they were collected, i.e. aged 0 to 14 days old, with day 0 being newly emerged bees (20 bees) collected immediately after emergence from each brood frame, prior to being returned to the colonies to obtain a base line for measurements.

2.2 Measurements of Hypopharyngeal Gland development.

Collected individual bees were weighed before the head was removed. The head was weighed and place in bee saline solution and stored (< 48 h) at 4 ˚C before being dissected. The heads were dissected under a stereoscopic microscope (Olympus SZ51, Tokyo, Japan) by cutting from the ocelli to the mandibles and removing the HPG (Altaye et al. 2010). Each gland was mounted in a drop of distilled water on a glass slide and sealed with clear nail varnish before being photographed using a transmission light microscope (Vickers Instrument, York, England) equipped with a Moticam (Motic®, Moticam 5.0 MP, China). Using the photographs, the area (μm^2) of 15 randomly selected acini per bee were measured by tracing the circumference using ImageJ image processing and analysis software (version 1.48, US National Institute of Mental Health, Bethesda, Maryland, USA). Diameters of the acini were measured at the same time (the maximum width across the acinus parallel to the axial duct of the gland (see Démares et al. 2017). The volume of the acini were estimated by calculating the volume of a sphere using the measured diameters. In total the HPG of 90 workers were assessed for each age group (0 to14-days old), i.e. 30 workers each (15 workers per colony per day) of *A. m. capensis*, *A. m. scutellata*, and Clone infested *A. m. scutellata*.

2.3 Assessing colony condition.

The condition of each of the colonies used for the experiment was assessed at the end of the trial period using the Liebefeld method (Delaplane et al. 2013). The individual frames in each hive were inspected and the percentage nectar, capped honey, pollen, open brood, sealed brood, empty comb and drone brood present were noted. The presence or absence of the queen was determined prior to selecting a specific colony for the experimental trial by assessing the brood and egg laying patterns and searching for fresh, single cell laid eggs.

2.4 Statistical analyses.

All data were evaluated for normality using the Shapiro–Wilks and Lilliefors tests and homoscedasticity using Levene's test prior to analysis. Generalised linear models were used to evaluate the differences in HPG acini area, diameter and volume of *A. m. capensis, A. m. scutellata* and Clone infested *A. m. scutellata* workers (acini volume, diameter and area set as the dependent variables, subspecies and age as a categorical factor and head mass as a continuous factor). Correlation analyses (nonparametric, Spearman Rank test) were performed to evaluate the relationship between the fresh head mass and body mass, as well as fresh head mass, area, diameter and volume of HPG acini. The alpha level was set to 0.05 for all analyses. All analyses were performed using STATISTICA version 13.2 (TIBCO software Inc., USA).

3. Results

3.1 Hypopharyngeal gland development.

Apis mellifera capensis and *A. m. scutellata* showed similar patterns of change in HPG acini area, diameter and volume with respect to age (Fig 1), gradually increasing up to a maximum before decreasing slowly. The HPG acini area, diameter and volume in *A. m. capensis* workers aged from 0 to 14 days, ranged from 3 702 to 105 666 μm^2, 29 to 281 µm and 184 846 to 12 289 569 μm^3, respectively and reached peak values at day 8 (mean ± SE: 58 540 ± 3 315 μm^2, 208 ± 6 µm, 5 639 938 ± 474 150 μm^3) (GLM $F_{(42, 1217)} = 5.1413$, $P < 0.05$). In comparison, the

HPG acini area, diameter and volume of the same aged *A. m. scutellata* workers ranged from 3 843 to 59 617 µm^2, 54 to 211 µm and 89 087 to 5 376 276 µm^3, respectively and reached peak values earlier at days 5 and 6 (mean ± SE: 33 626 ± 10 863 µm^2, 160 ± 5 µm, 2 558 702 ± 317 010 µm^3) (GLM $F_{(42, 1181.4)} = 4.532$, $P < 0.05$). *Apis mellifera capensis* HPG acini were invariably larger than that of *A. m. scutellata*, apart from day 0 (bees less than 24 h old) when *A. m. capensis* HPG acini were significantly smaller than that of *A. m. scutellata*. From days 7 to 14, the differences in size increased and became significant (GLM $F_{(42, 3240.2)} = 5{,}6492$, $P < 0.05$) (Fig 1). On Day 14, the HPG acini of *A. m. capensis* were still more than twice the size of that of *A. m. scutellata* despite the gradual shrinking of HPG acini observed in both subspecies after peak values were reached (Fig 1).

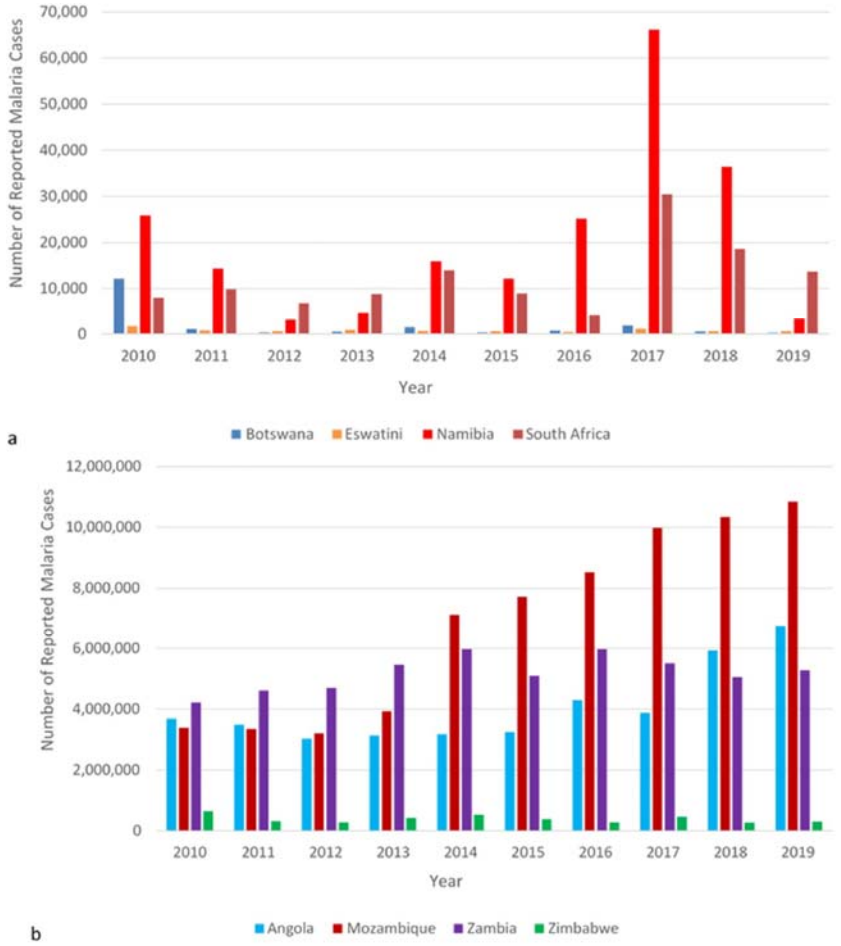

Figure 1. Development of HPG in *A. m. capensis*, *A. m. scutellata*, and clone infected *A. m. scutellata* workers aged 0 to 14 days. The HPG acini **a** area, **b** diameter, and **c** volume displayed the typical convex development curve. Mean ± SE; $N = 30$ workers per age

The HPG acini area, diameter and volume in Clone infested *A. m. scutellata* workers aged from 0 to 14 days, ranged from 4 877 to 58 790 µm^2, 65 to 218 µm, 103 355 to 5 714 809 µm^3, respectively and only reached peak values at day 10 (mean ± SE: 42 559 ± 2 497 µm^2, 184 ± 6 µm, 3 721 425 ± 35 0340 µm^3) (GLM $F_{(42, 742.39)} = 1.8108$, $P< 0.05$). The development of HPG acini in the Clone infested *A. m. scutellata* closely followed the development curve of uninfected *A. m. scutellata* and were similar in size during the first 6 days. From day 7, the HPG acini in uninfested *A. m. scutellata* worker slowly started to shrink, while those of Clone infested *A. m. scutellata* workers continued to increase in size until reaching maximum values at day 10 (Fig 1). From day 9 to day 14, the size of HPG acini of Clone infested *A. m. scutellata* workers more closely resembled that of *A. m. capensis* and were significantly larger (GLM $F_{(42, 3240.2)} = 5,6492$, $P< 0.05$) (Fig 1) than those of uninfested *A. m. scutellata* workers.

3.2 Fresh head mass.

As expected, the changes in fresh head mass of *A. m. capensis* and *A. m. scutellata* and Clone infested *A. m. scutellata* workers with respect to age showed similar patterns to the age-dependant HPG development or changes in HPG size (Hrassnigg and Crailsheim 1998a), see Fig 2. There was a significant correlation between fresh head mass and HPG acini size (area, diameter and volume) (Spearman rank order correlation: $rs =0.52$; $P<0.05$), as well as fresh head mass and fresh body mass (Spearman rank order correlation: $rs=0.71$; $P< 0.05$). From day 2, the fresh head mass of *A. m. capensis* workers increased dramatically, at least 2 mg on average higher than that of *A. m. scutellata* (Fig 2), confirming the higher rate of protein incorporation into the HPG as suggested in the sizes of the acini in the *A. m. capensis* workers. The correlation between fresh head and body mass were lowest for *A. m. capensis* workers, confirming their heads are heavier in relation to their body or the rate of protein intake is higher when compared to *A. m. scutellata*.

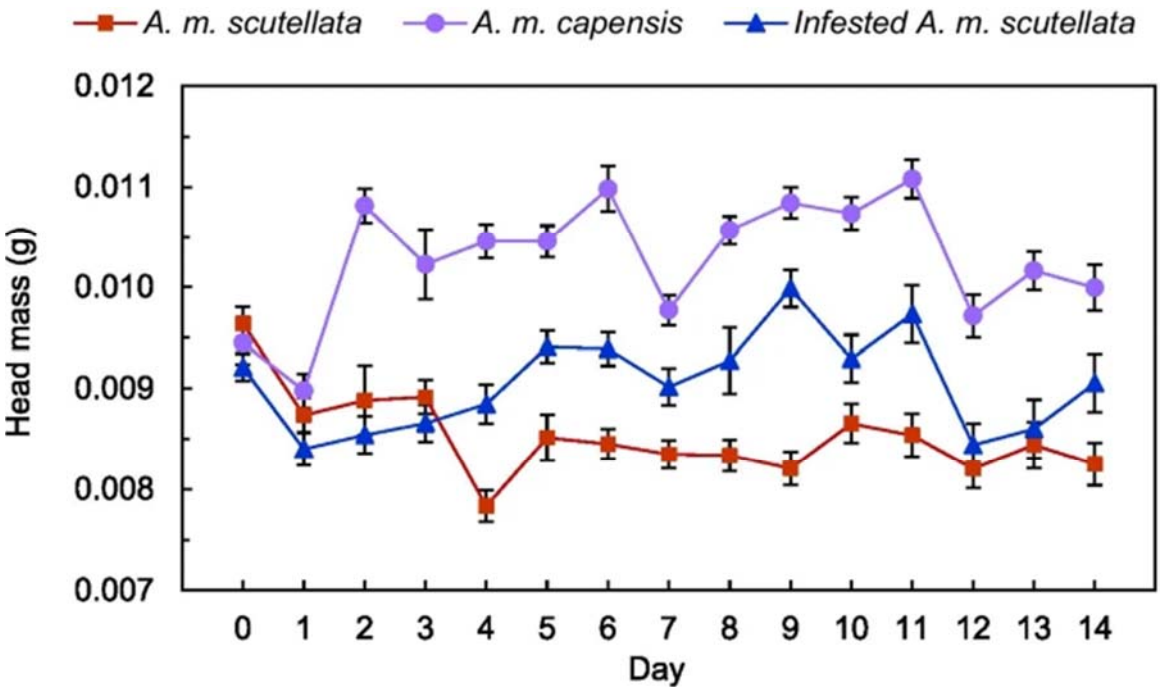

Figure 2. The fresh head mass of *A. m. capensis*, *A. m. scutellata*, and clone infected *A. m. scutellata* workers aged 0 to 14 days. Mean ± SE; $N = 30$ workers per age

Table 1 Strength and nutritional status of the experimental colonies according to the Liebefeld method. All colonies where housed in standard 10 framed Longstroth hives.

Colony	Number of full honey Supers Present	Colony component composition (%)						
		Pollen	Open Brood	Closed Brood	Nectar	Honey	Empty Comb	Drone Brood
A. m. capensis 1	2	18.5	12.5	12.5	24.5	0	30	2
A. m. capensis 2	2	7	27	24	26.5	0	15.5	0
A. m. scutellata 1	0	12	11	32	14	0	29	2
A. m. scutellata 2	0	4	6.5	22	41.5	17.5	4.5	4
Clone infested *A. m. scutellata* 1	0	*	*	*	*	*	*	*
Clone infested *A. m. scutellata* 2	0	18	7	15	28.5	9	22.5	0

1. *The hive used for Clone treatment 1 absconded after 8 days and colony condition assessment was not possible.

3.3 Colony strength and nutritional status.

Based on assessment using the Liebefeld Method, strength of the colonies used in this study were similar, indicating that the colonies have similar nutritional status (Delaplane et al. 2013), and therefore differences observed in HPG development between tests groups are highly unlikely due to differences in the nutritional status of colonies. (see Table 1).

2. Discussion

The size of the HPG acini clearly changed with age and followed the typical convex development curve (Crailsheim and Stolberg 1989) (see Fig 1). However, there were marked differences in HPG development between the two honey bee subspecies in acini size and the age at which maximum development were reached. In *A. m. scutellata* workers, HPG acini increased until 6 days of age and from then slowly decreased. At 6 days-old, acini volume were on average two thirds bigger than those of 0 day-old workers, but by 14 days of age the acini had shrunk again, resembling that of newly emerged or 0 day-old workers. The observed HPG acini sizes were much larger than the sizes previously recorded for caged *A. m. scutellata* workers maintained under laboratory conditions without brood (Altaye et al. 2010; Démares et al. 2017). But were consistent with HPG acini sizes observed in *A. m. carnica* workers (its European cousins) kept in similar natural or in-hive conditions in the presence of brood (Crailsheim and Stolberg 1989; Crailsheim 1992). In comparison, the HPG acini of *A. m. capensis* workers were generally larger than those of *A. m. scutellata* and took longer to reach peak size, reaching maximal values in 8 day-old workers. At this age, the acini volume was seven times larger than that of newly emerged workers and double the peak acini volume of *A. m. scutellata* workers (see Fig 1). The HPG acini also seem to remain inflated for a longer period of time after reaching peak values compared to that of *A. m. scutellata.* In 14 day-old *A. m. capensis* workers, acini were still four times bigger than that of newly emerged or 0 day-old workers.

Several factors could be responsible for the large variation observed in HPG development between the two subspecies, including physiological differences, differences in the food provisioned to worker larvae (amount and quality), variation in the blend, amount or period of release of brood pheromone, colony condition (brood status, pollen stores, etc), the type of pollen consumed and environmental conditions (Huang and Otis 1989; Crailsheim et al. 1992; Le Conte et al. 1995; Mohammedi et al. 1996; Hrassnigg and Crailsheim 1998a; Allsopp et al. 2003; Di Pasquale et al. 2016). The effect of colony condition (amount of brood, pollen stores, etc) was controled for by means of selecting experimental colonies in comparable conditions and is negalible (see Table 1). It is also unlikely that the amout of food provisioned by nurse bees plays a major role since *A. m. capensis* and *A. m. scutellata* larvae are provisioned with the same amount of food by their sisters (Allsopp et al. 2003). However, the quality (amount of protein) likely differ to some extent as *A. m. capensis* workers raised in their own colonies are more queen-like compared to *A. m. scutellata* workers raised in their own colonies (Hepburn 1992; Allsopp et al. 2003; Boot et al. 2006). Caste in honey bees is determined by the differential feeding of female larvae with larvae destined to become queens receiving more protein (Beetsma 1979). It has been shown that HPG activity (protein synthesis) and acini size is positively correlated (Deseyn and Billen 2005).

Comparing the HPG development of *A. m. capensis* and *A. m. scutellata* with Clone infested *A. m. scutellata* workers provided some unique additional insights into the large variations observed between *A. m. capensis* and *A. m. scutellata* HPG development. The HPG acini of Clone infested *A. m. scutellata* workers reached development sizes similar to that of *A. m. capensis* workers (up to 1.5 times larger than *A. m. scutellata* workers from healthy colonies) and remained inflated for a longer period of time like the HPG of *A. m. capensis* workers (see Fig 1), demonstrating that the Clone's higher demand for food (Allsopp et al. 2003; Boot et al. 2006) indeed affects the development and size of the HPG of the host nurse bees provisioning the food for the Clone brood. It strongly suggests that the observed variation between the subspecies (*A. m. capensis* and *A. m. scutellata*) in gland size and development is not due to the

physiological differences, but rather due to a functional response to external stimuli such as the brood signal that are involved in regulating gland size. Furthermore, *A. m. scutellata* and Clone infested *A. m. scutellata* colonies were exposed to the same environmental conditions and had access to the same type of pollen resources. Taken together, this suggests that variation in the blend, amount or period of release of brood pheromones are likey the major contributors to the observed variations in HPG size and driver of the observed functional response.

The HPG will develop in the presence of brood and after the worker has started to consume stored pollen (Hrassnigg and Crailsheim 1998b), whereas protein synthesis in the glands are stimulated by the complex of brood pheromones released by the larvae. Metz et al. (2010) found marked differences in the fatty acid ester components of brood pheromone produced by *A. m. scutellata* and European honey bee subspecies, including the proportions and concentrations of two components (methyl palmitate and ethyl oleate) known to increase the activity in the HPG of nurse bees (Mohammedi et al. 1996). It is conceivable that a similar difference would exist between the two South African subspecies as well. Moreover, brood pheromones also regulate the type and amount of food provisioned by the nurse bees. The 'hunger signal' released by *A. m. capensis* is stronger than that released by *A. m. scutellata* brood, yet *A. m. capensis* and *A. m. scutellata* worker larvae receive the same amount of food when raised in their own colonies (Allsopp et al. 2003), but the quality or protein content most likely differ as mentioned earlier (Allsopp et al. 2003; Boot et al. 2006), supporting the possibility of differential brood pheromone blends being released by the two South African subspecies. Additionally, a variation in the period of brood pheromone release would be consistent with the observed variation in the period the HPG remained inflated.

In conclusion, this study described and compared HPG development in two subspecies of South African honey bees under natural in-hive conditions in bees aged 0 to 14 days old. There were marked differences in HPG development between the two honey bee subspecies in acini

size and the age at which maximum development were reached. These differences are most likely caused by difference in the brood pheromones released by the developing larvae of the subspecies. However, the complex and quantities of brood pheromones produced *A. m. scutellata* and *A. m. capensis* for each larval stage is still unknown and requires further investigating. In addition, the study demonstrated how the presence of a facultative reproductive parasite (parasitic *A. m. capensis* or the Clone) impacts the HPG development of the host colony's workers, providing functional insights into this unique social parasite.

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