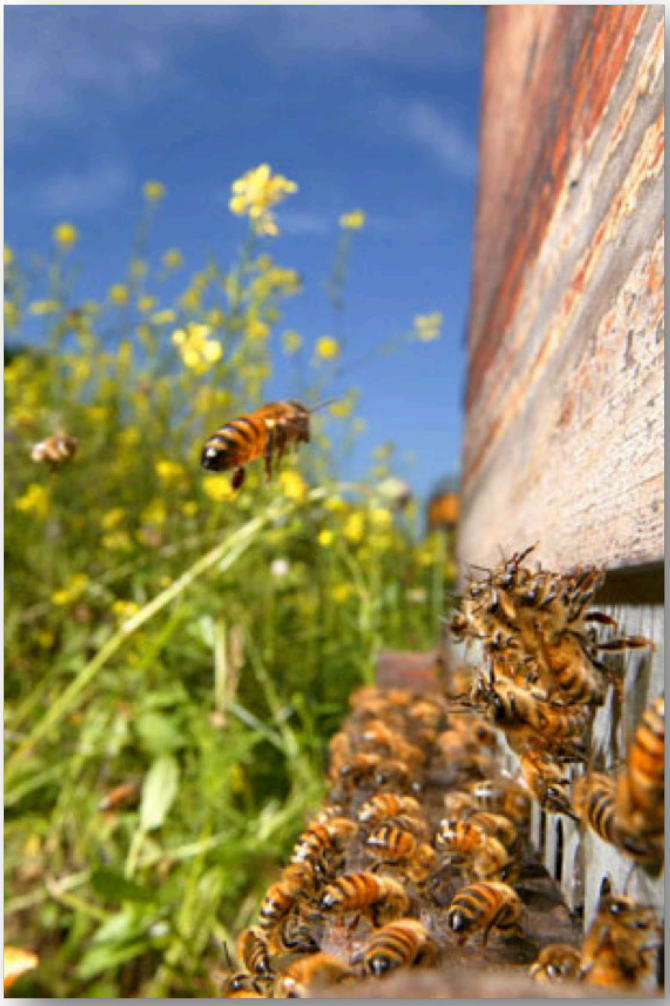
Physiological and behavioural stress responses in the social honeybee, *Apis mellifera*



Eric Tourneret ©

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Macquarie University,
Department of Biological Sciences.

This thesis is presented for the degree of Doctor of Philosophy

TABLE OF CONTENT

STATEMENT OF CANDIDATE

I certify that this thesis entitled "Physiological and behavioural stress responses in the social honeybee, *Apis mellifera*" has not previously been submitted for a degree nor has it been submitted as part of requirement for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Contributions of co-authors have been appropriately acknowledged, and so has any assistance that I have received in my research.

I certify that all information sources and literature used are indicated in the thesis.

As this research was on invertebrates, the research presented was not subject to approval by the university Ethics Review Committee.

Naïla Even (25/03/2014)

ACKNOWLEDGEMENTS

This journey started a long time ago when I was a teenager and discovered the joy, curiosity and peaceful state that the observation of bees made me dived in. I owe this passion to bees to my father, Daniel Even, who is still keeping bees, as a hobby in his yard in France. “Thanks Dad!”

My PhD adventure started with the set up of a completely new project for my supervisors and for me that we designed all together. I believe this great opportunity to think about and design a PhD project is not always possible when you are a PhD student. I am glad I had this opportunity. From this moment and until the end I loved my PhD, including the thinking, experimental design and set up, beekeeping sessions, data collection, lab experiments and data analysis but also even with the inevitable failures and numerous difficult moments to believe in myself and in the project. Finally the challenge of writing was still enjoyable despite events of my life (including the birth of my second child, car crash, legal troubles, job hunting and complex visa application). I owe this immense and enduring pleasure, to my most appreciated, devoted and talented supervisor, Andrew Barron, who did always understood the challenges of my life and will remain forever an academic inspiration for me. He always supported me to pass difficulties and especially to manage all together my family and my research. Every year I regretted missing the deadline for the nomination of the best supervisor, I am sure his future students will be better than me, “Thanks Andy!”

All along my PhD I had the chance to be guided and followed from far away by my second adjunct supervisor who visited us twice from France. Jean-Marc was extremely tolerant and supportive despite my long delays in giving feedback and advances in my work. I was always very lucky to receive, always very quickly, precious and very precise feedback on my work and writings: “Thanks Jean-Marc!

During my university career I had various opportunities to jump between different fields of biology, but the most inspiring for me was the study of animal behaviour and the proximate and ultimate mechanisms behind it. This lead me to do this wonderful PhD project in Brain Behaviour and Evolution (BBE) at Macquarie Universtity, a wonderful environment. I will miss BBEQs, the very scientifically animated lunches and parties. For that I have to say “thanks” to all my BBE friends and colleagues in a random order: Tina,

Miya, Vivian, Marianne, Eirik, Emilie, Rowan, Seppi, Antoine, Fernando, Veronica, Daniel, Dani and Dan, Dalila, Jenny, Marcus, Robby, Eloïse, Tim, Maria and Paul. “Thanks all!”

During this work I owe a lot of help from the volunteers and collaborators who helped me on various projects: Allan, Kritika, Flavie, Sonia, Stefan, Branden, the French Louise C. and the Aussie Louise C., but also for the unforgettable work on queenless colonies, Nick Naeger and Gene Robinson. Also I owe a lot of help for the English proof reading from Marianne and Rose. “Thanks to all of you!”

To face the last extreme stressful part of writing I could not have coped without the help of Maroubra beach environment. But more importantly, during this phase, the best behaved baby to ever exist on this planet happened to be mine! Aloïs never discovered the concept of crying, allowing me to sleep almost normally at nights and to write during the day. Also the “writing at home period” did overlap with the summer holidays of my 5 year old, Laëlie. This has been also wonderfully managed by the most extraordinary grand-mums (Mamie-Lo & Mamie-Fa) and grand-dad (Opa) who all flew from the other side of the planet to help out! This goes with special regards for Fabienne for doing it twice in 6 months! “Thanks mum!”

The final immense acknowledgements undeniably have to go toward my wonderful and lovely partner, Amaël, who always believes in me, understands me, tolerates my stress, and allows me to achieve and go beyond. With him I always succeed what I started, including building a happy family and completing this PhD project. His considerable immense support made him renounce the possibility of academic position opportunities in France to then follow me in Australia, this cost was even followed by facing difficulties of long periods of job hunting in a foreign language. For all this, I am extremely grateful. Without these efforts and the support from him I would not be here. “Thanks Amaël !”

SUMMARY

The honeybee *Apis mellifera* is the most important economic insect on the planet. It is also one the most important invertebrate models in neuroscience and social animal behaviour. A large part of honeybee research this past decade has been devoted to understanding the new sources of threats that bees are facing in the industrialised world in order to prevent the collapse of native and domesticated bee populations. More than ever we need to protect them and understand them better. This research project entitled "Physiological and behavioural stress responses in the social honeybee, *Apis mellifera*" explores specific responses to physical stressors but also considers stress responses in the social context of the bee hive that is vital for the individual honeybee to survive.

In **Chapter 1,** the thesis is introduced by a broad literature review to define the terms and concept of stress in honeybees. This first part develops the following questions: what is "stress"? what can we call a "stress response"? How could we measure a response to stress in the honeybee? What are the areas of stress research in bees that need focus in the following years? This review helps to define the key research questions addressed in the thesis, especially concerning the measures of behavioural and physiological responses to stressors but also the eventual roles of stress sensitivity in the organisation of honeybee society.

Chapter 2 reports the investigation of physiological responses (biogenic amines) and behavioural response (sting reflex) to physical stressors applied to bees for various durations. I found that dopamine and serotonin were increased after a long-term (three hours) stressor, but not after a short-term (up to twenty minutes) stressor. The nature of the stressor also affected differently the increase of octopamine and serotonin in the brain. These results were discussed in regard to the previously existing stress responses theories and mechanisms.

Chapter 3 explored the role that stress sensitivity could have in the organisation of division of labour. We found that the first bees to react to a threat, the guards, were more sensitive to a stressor than the bees later recruited to a threat, the soldiers. Stress responsiveness also varied with age, and the distinctions in stress responsiveness between soldiers and guards were less pronounced in young bees. Differences in brain biogenic amine levels were found between castes, but brain biogenic amine levels did not

correlate with stress responsiveness either between or within behavioural castes. This result supported the response threshold model of division of labour but is at odds with the defensive behavioural syndrome theory.

In **Chapter 4,** I tested the effect of systemic cocaine treatment, and the systemic injection of neuropeptides of interest (corazonin, allatostatin A and alatostatin CC) on stress responses. Allatostatin A and Corazonin were not found to affect behavioural stress response but Allatostatin CC partially reduced sting responsiveness. Cocaine treatment increased sting responsiveness. This chapter along with chapter 2 clarified the hypothetical model of physiological stress responses in honeybees presented in Chapter 1.

Chapter 5 focused on how a social stress (long term loss of the queen) affected colony organisation and some worker physiological traits. It is well known that the absence of the queen changes the distribution of reproductive functions as workers develop their ovaries to lay male-eggs. But this chapter uncovered that the distribution of other tasks were also disturbed, and workers tended to become generalists in performing multiple tasks at the same time like nursing, foraging and laying eggs. This study revealed the importance of studying stress at a social level, and how the homeostasis of the social organisation is fragile.

To conclude, **Chapter 6** discusses generally how this body of work addresses the initial research questions about stress in honeybees. Indeed this thesis yields some clarity about: how “stress” can be defined in honeybees, What are the physiological component of stress responses in honeybees, and finally how can stress affect the social organisation of honeybees both at the individual level and the colony level. Also some suggestions for future research possibilities are addressed.

PREFACE

The format of this PhD is based on published papers, papers in preparation and preliminary data for later publication. The thesis is an investigation of the stress responses of honeybees, and how they may contribute to the operation of honey bee society.

Chapter 5 is the result of a collaborative project in which I contributed to the conception, data collections, data analysis and writing. In all the other chapters I conceived, collected, analysed and reported most of the data. The specific contributions of every author for each part of each chapter is listed below.

All chapters are formatted for a single consistent thesis style, without modifying the content of published chapters. The style of the chapters in preparation will be later adjusted to fit the specific journal style for submission. The published versions of chapter 1 and 5 are presented in the appendix.

Published

Chapter 1: Naïla Even; Jean-Marc Devaud; Andrew B. Barron **2012**. "General Stress Responses in the Honey Bee." *Insects* 3(4) 1271-1298. Published version in appendix 1.

Contributions: NE conceived and wrote the manuscript figures and tables, JMD wrote the allatostatin paragraph and improve manuscript with comments. AB helped on the structure and writing of the whole document with constructive comments and corrections.

Chapter 5: Nicholas L. Naeger*, Marianne Peso*, Naïla Even*, Andrew B. Barron, Gene E. Robinson **2013** "Altruistic Behavior by Egg-Laying Worker Honeybees" *Current Biology* 23 (16) 1574-1578. Published version in appendix 2. *These authors contributed equally to this work.

Contribution: NLN, MP, NE, ABB, GER conceived the experiments. NLN, NE, MP collected the data. NLN, MP, NE, ABB performed the analysis. NLN and ABB wrote the manuscript. NLN, MP, NE, ABB, GER helped with the structure and corrections of the manuscript.

In preparation

Chapter 2: Naïla Even, Tina Peckmezian, Allan Taylor, Jean-Marc Devaud, Andrew Barron. "Brain biogenic amine levels and behavioural responses after physical stress in honeybees (*Apis mellifera*)" *Insect biochemistry and molecular biology*

Contribution: NE, JMD, ABB conceived the work. NE, AT, TP collected the data. NE performed the analysis. NE wrote the document. ABB and JMD helped with the structure and correction of the manuscript.

Chapter 3: Naïla Even, Jean-Marc Devaud, Andrew Barron. "Guard and soldier honeybees show distinct behavioural responses to aversive stimuli: implications for the organization of division of labour" *Behavioural Ecology and Sociobiology*

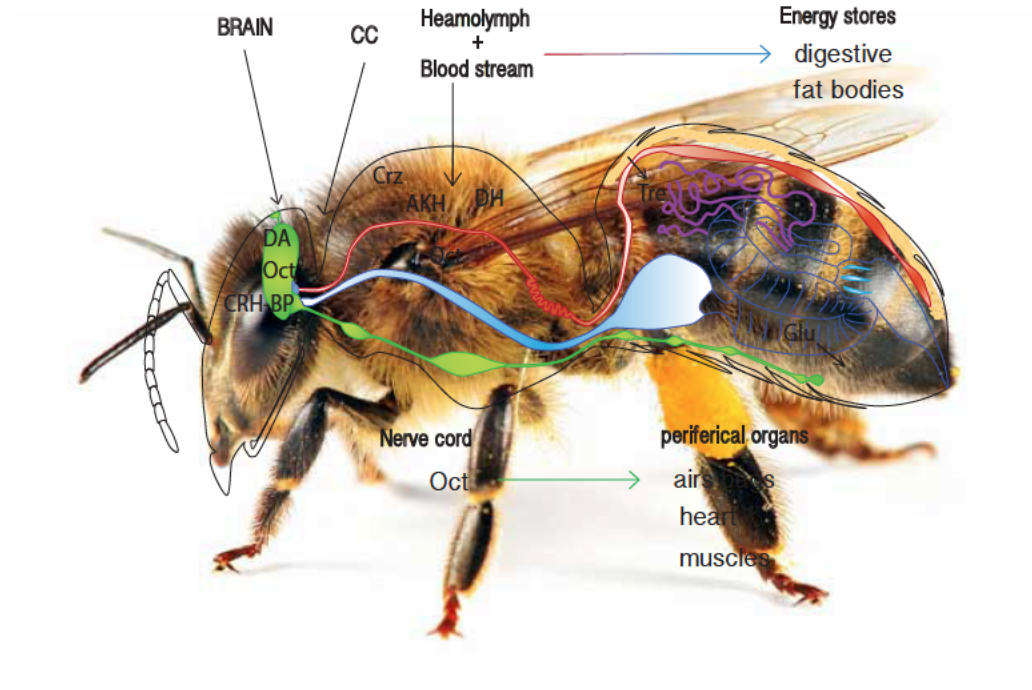
Contribution: Contribution: NE, JMD, ABB conceived the work. NE collected the data. NE performed the analysis. NE wrote the document. ABB and JMD helped with the structure and correction of the manuscript.

Chapter 4: Naïla Even, Louise Crepeau, Jean-Marc Devaud, Andrew Barron. "Honeybee sensitivity to electric shocks is affected by biogenic amines and neuropeptides" *Journal of insect physiology*

Contribution: Contribution: NE, JMD, ABB conceived the work. NE and LC collected the data. NE performed the analysis. NE wrote the document. ABB and JMD helped with the structure and correction of the manuscript.

Chapter 1

General stress responses in the honeybee



Abstract:

The biological concept of stress originated in mammals, where a "General Adaptation Syndrome" describes a set of common integrated physiological responses to diverse noxious agents. Physiological mechanisms of stress in mammals have been extensively investigated through diverse behavioral and physiological studies. One of the main elements of the stress response pathway is the endocrine hypothalamo-pituitary-adrenal (HPA) axis, which underlies the "fight-or-flight" response via a hormonal cascade of catecholamines and corticoid hormones. Physiological responses to stress have been studied more recently in insects: they involve biogenic amines (octopamine, dopamine), neuropeptides (allatostatin, corazonin) and metabolic hormones (adipokinetic hormone, diuretic hormone). Here, we review elements of the physiological stress response that are or may be specific to honeybees, given the economical and ecological impact of this species. This review proposes a hypothetical integrated honeybee stress pathway somewhat analogous to the mammalian HPA, involving the brain and, particularly, the neurohemal organ *corpora cardiaca* and peripheral targets, including energy storage organs (fat body and crop). We discuss how this system can organize rapid coordinated changes in metabolic activity and arousal, in response to adverse environmental stimuli. We highlight physiological elements of the general stress responses that are specific to honeybees, and the areas in which we lack information to stimulate more research into how this fascinating and vital insect responds to stress.

Introduction

Concept of stress

The term stress originated in physics to describe pressure and deformation in a system, but it has been adopted into a biological context through the work of Hans Selye (Chrousos, 1998; Chrousos, 2009; Selye, 1956). He recognized in mammals, as a "general adaptation syndrome," a similar suite of coordinated reactions to diverse noxious stimuli or "agents" (Selye, 1936). Selye's concept was at first criticized by physiologists as vague and immeasurable, but he subsequently clarified his concept defining several stress response elements, principally the hypothalamo-pituitary-adrenal (HPA) axis system. Stress is now recognized as a valid physiological concept, which allows organisms to respond to adverse environmental pressures (McEwen, 2009). Most studies use the word "stress" to describe negative treatments applied to organisms in an experiment, such as nutritional, heat or oxidative stress. Here, the triggering stimuli will be called "stressors" while "stress" will be considered as the response syndrome to any aversive or harmful treatment in a specific system. This understanding can be applied to different levels of organization: molecular, cellular, histological, physiological, even ecological or social, but this chapter will focus on physiological processes involved in an integrated response at the level of the organism. Also, the definition of stress should take into account the duration and intensity of the stressors involved, thereby the distinction between acute or chronic stress responses. Here, we review the putative elements participating in a physiological stress response, and propose an integrated model of the honeybee stress response. Although the model is based to a degree on the stress literature known from other insects (Boerjan et al., 2010a; Ivanovic, 1991; Johnson and White, 2009; Roeder, 2005), our intention is to build (as far as possible) a model that is honeybee specific. In doing so, we identify what is known about this particular species, and what may be assumed from our knowledge of other insects. Consistently, we wish to highlight the gaps in our existing knowledge to propose directions for future stress research.

Why study stress in honeybees?

The concept of stress is useful in understanding the physiological and behavioural responses of honeybees to harmful situations. This research is timely since, over the last years, beekeepers from different geographic areas have reported a marked increase in

honeybee colony failure rates and in the number of stressors affecting bees, including diseases, parasites, pesticides and poor nutrition (Neumann and Carreck, 2010; Ratnieks and Carreck, 2010; VanEngelsdorp et al., 2009). The syndrome termed "colony collapse disorder" seems to be the result of an accumulation of stressors chronically weakening honeybee colonies (Khoury et al., 2011; Oldroyd, 2007). As honeybees are the most important insects in agriculture for both pollination of diverse crops and honey production, the recent decline in their populations brings an urgent need to know more about the stress response systems of this ecologically and economically important insect.

In addition, the honeybee is an ideal insect model to understand the evolution of sociality. A key feature of honeybees is their high level of social organization and their well-developed system of division of labour among workers (Wilson, 1971). Honeybees exhibit age polyethism; young workers perform in-hive tasks (e.g., taking care of the brood), then become guards patrolling the entrance of the hive and later become foragers. Studying differences in stress responses across behavioral castes might help elucidate how a defined division of labour has evolved.

How does an organism react to stress?

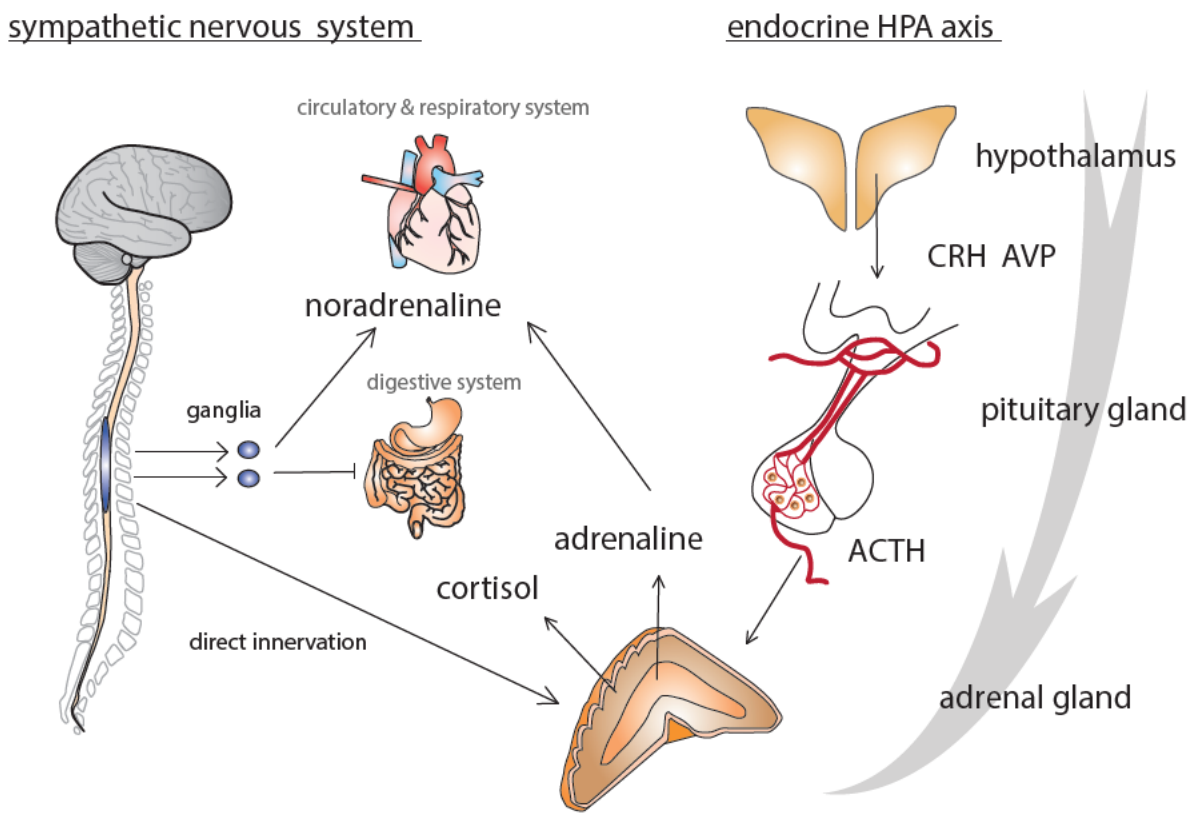
There are three stages to an organism's acute stress response: it first detects the stressor with sensory organs, then responds to it by defense or escape. Finally, if the stressor cannot be avoided and is sustained, the organism enters a state of exhaustion (Selye, 1956). Following detection of a stressor, mammalian physiological responses are coordinated by neural activity within the autonomic nervous system and the HPA axis (Figure 1). The first immediate response is an activation of sympathetic neurons, which stimulate the adrenal medulla to release adrenaline and noradrenalin into the blood. These two catecholamines increase heart rate and vasoconstriction. In parallel, the paraventricular nucleus (PVN) neurons in the hypothalamus release corticotrophin-releasing hormone (CRH) and arginine/vasopressin (AVP), which are conveyed to the nearby anterior pituitary gland via the blood stream. In response, the pituitary secretes adrenocorticotropic hormone (ACTH), which acts on the adrenal cortex to release glucocorticoids (such as cortisol) causing mobilization of energy reserves (*i.e.*, glucogenolysis in the liver)(Bamberger et al., 1996). Glucocorticoids also potentiate catecholamine release from the adrenal medulla. In parallel, adrenaline activates a release

of glucagon by the pancreas to further increase the catabolism of glycogen in the liver and raise glucose concentration in the blood. Other hormones such as cytokines or endogenous opioids may also be produced and/or released, depending on the nature, duration and intensity of the stressor, to act in diverse ways to limit the degree of tissue damage. Therefore, it is interesting to note that, additionally to the general stress response pathways described previously, certain stress responses can vary depending on the type and duration of the stressor.

Under chronic stress, the immune system, metabolic pathways and cognitive processes in the organism gradually weaken until exhaustion and failure are reached (McEwen, 2000; Selye, 1956). For example, repetitive HPA activation resulting in an excess of glucocorticoids in the blood can lead to metabolic diseases such as diabetes (Chrousos, 2009; Stratakis and Chrousos, 1995).

Cellular stress responses described in various models (bacteria, yeast, worms and flies) include the increased production or activation of antioxidant proteins and heat shock proteins (HSP) when facing high metabolic load or environmental stressors (Santoro, 2000; Takeda et al., 2008). Such proteins may be called “stress proteins” (Feder and Hofmann, 1999) and used as cellular stress biomarkers (Gibney et al., 2001; Nazir et al., 2003). These factors are induced by a variety of stressors such as extreme temperatures, elevated ion concentrations or toxic substances, all usually resulting in excessive amounts of denatured proteins (Stetler et al., 2010). Their actions are principally intracellular and hence we do not focus on them in this chapter that considers instead more integrated elements of a general stress response.

Figure 1. Human stress response pathways operating through the autonomic nervous system and the endocrine system. This diagram illustrates how neural and hormonal signals interact and complement each other through the regulatory action of the hypothalamo-pituitary-adrenal axis (HPA axis). ACTH: adreno-corticotropin hormone. AVP: arginin/vasopressin. CRH: cortico-releasing hormone. Adapted with permission from Macmillan Publishers Ltd.: Nature Reviews Neuroscience (Ulrich-Lai and Herman, 2009), copyright 2009.



Model of the honeybee stress response

How has stress been assessed in honeybees?

Multiple aspects of stress responses have been used to evaluate stress in honeybees, including behavioural, physiological or cellular stress responses. The many parameters used are listed in Table 1. Behavioural stress responses, usually immediate responses, characterized early on by Cannon (Cannon, 1915) as the "fight-of-flight" responses, are easy to observe in the honeybee. For example, extension of the sting (or stinging of a

target) has been used to evaluate sensitivity to stressors, and is widely considered as indicative of stress in honeybees, as well as an aggressive response. Physiological measures of stress responses in honeybees include hormonal titers and neurotransmitter levels, these parameters have been integrated into our model (see Tables 1 and 2, Sections 2.2 and 3 and Figure 2). Honeybee stress studies usually use acute stressors but the nature and duration of the stressors could sometimes be qualified as chronic (Table 1). Cellular stress responses have also been used in honeybees (Corona et al., 2005; Elekonich, 2009a; Gregorc and Bowen, 1999; Hranitz et al., 2010; Li et al., 2012; Severson et al., 1990), and Duell *et al.* (Duell et al., 2012) even suggest some cellular stress biomarkers as elements for a diagnostic of general stress in honeybees. Also, since exhaustion is the final stage of chronic stress response described by Selye (Selye, 1956), survival rate has been used to assess the degree of stress.

Based on the data available for honeybees (Table 1) and other insect species we have tried to synthetise a model of a general stress response pathway specific to the honeybee. It should be kept in mind that many proteins or genes of unknown function may be affected by stressors; we will only focus on a few of them, for which sequence homologies and/or functional data suggest a potential role in a physiological stress response.

Table 1. Stress response measures used in honeybees. This inventory illustrates the broad diversity of methods used to evaluate stress in honeybees. The table is divided into four parts, depending on the measure used: behavioural, physiological, cellular and survival. In the absence of an objective distinction between chronic and acute stressors, here we qualify stressors ingested or applied continuously during at least four hours as chronic (C); otherwise they are qualified as acute (A).

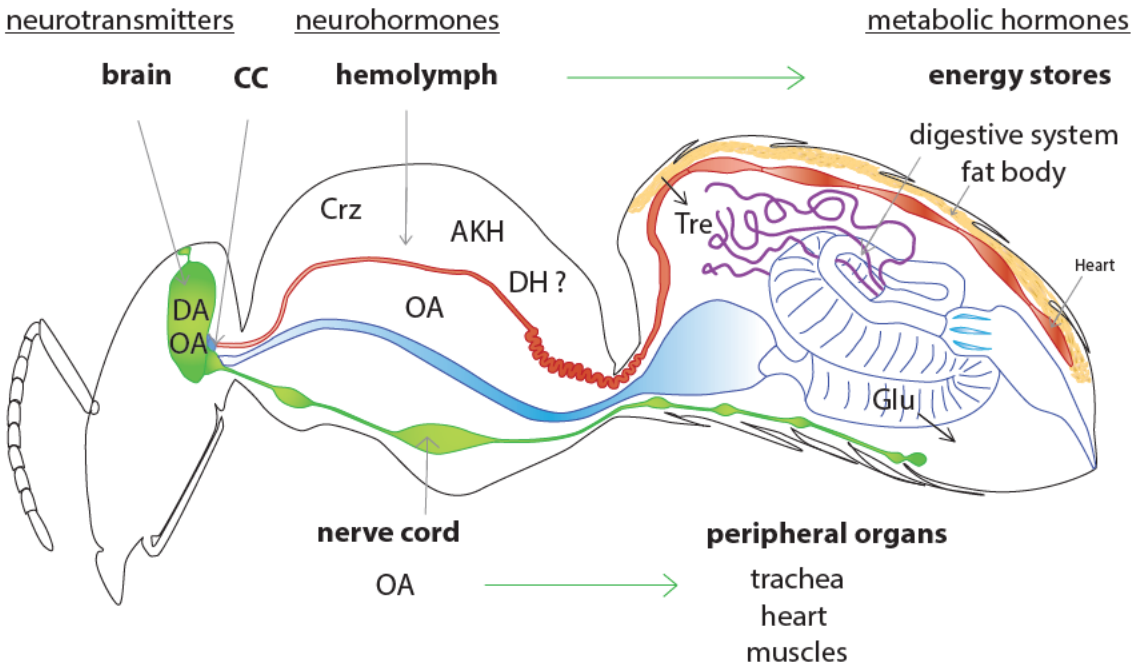
stress response measure	stressor	acute/chronic	variable	references
physiological responses				
juvenile hormone (RIA)	cold anesthesia, caging	A	task specialization, duration after treatment	(Lin et al., 2004)
brain biogenic amines (HPLC)	spinning, caging, chilling, CO_2	A	spinning speed, duration of stressor	(Chen et al., 2008)
brain biogenic amines (HPLC)	leg pinch	A	duration of stressor, age, season, patriline	(Harris and Woodring, 1992)
cellular stress responses				
HSP 70 (Elisa)	capture, transport, chilling, harnessing	A/C	ethanol concentration, duration of harness	(Hranitz et al., 2010b)
HSP70 (western) hsp70, hsc70 (q PCR)	heat	A	duration of stressor, age body part	(Elekonich, 2009b)
CRH-BP (qPCR)	cold, heat, UV light	A	intensity of stressor, caste, development stage, body part	(Liu et al., 2011)

stress response measure	stressor	acute/chronic	variable	references
behavioural response				
stinging response	electric shock	A	patriline	(Lenoir et al., 2006)
stinging response (delay)	electric shock	A	genotype, housing conditions, task specialization	(Uribe-Rubio et al., 2008)
sting extension	electric shock	A	genotype, exposure to alarm pheromone task specialization	(Balderrama et al., 1987; Balderrama et al., 2002; Roussel et al., 2009)
sting extension	electric shock	A	morphine and opioid peptides treatment	(Núñez et al., 1983; Núñez et al., 1997)
proboscis extension	soil-borne pollutants	C	treatment concentration	(Hladun et al., 2012)
survival				
survival	hyperoxia	C	learning performance	(Amdam et al., 2010)
survival	paraquat injection (oxidative stressor), hyperoxia	C	vitellogenin level, reproductive castes	(Corona et al., 2007; Seehuus et al., 2006)

Table 2. Main elements of the general stress response in the honeybee. The table gives the physiological role(s), known or hypothesized (as indicated by a question mark) for each element, following a classification based on chemical identity.

chemicals	abbreviation	nature	stress-related action	references
biogenic amines				
octopamine	OA	neurotransmitter neurohormone	enhances arousal, increases heart rate, modulates muscle activity	(Corbet, 1991; Farooqui, 2012; Papaefthimiou and Theophilidis, 2011; Pflüger et al., 2004)
dopamine	DA	neurotransmitter	modulates arousal	(Mustard et al., 2010)
peptides				
adipokinetic hormone	AKH	Hormone	mobilize energy in the fat body	(Kodrík, 2008)
cortico releasing hormone-binding protein	CRH-BP	chaperone?	potentiates or inhibits hormonal release ?	(Liu et al., 2011)
diuretic hormone-I	DH-I	hormone	stimulates diuresis induced by crop draining into hindgut after energy mobilization.	(Coast et al., 2002)
corazonin	Crz	neurohormone	activates metabolism?	(Veenstra, 2009a)
allatostatin-A	AST-A	neurohormone	activates gut contraction ? inhibits corazonin neurosecretion ?	(Veenstra, 2009a)
insulin-like peptide	ILP	?	regulates energy stores ?	(Corona et al., 2007)
proteins				
heat shock proteins	HSP70	chaperone	protects cells against oxidative stress and excess protein misfolding	(Elekonich, 2009; Hranitz et al., 2010)
ERK2	ERK2	?	protects cells against damage ?	(Li et al., 2012)
vitellogenin	Vg	antioxidant	protects cells against damage	(Seehuus et al., 2006)

Figure 2. Hypothesized model of the general stress system in the honeybee. The brain biogenic amines (OA) and dopamine (DA), acting as neurotransmitters or neuromodulators, would increase arousal, cognitive processes and sensitivity to various stimuli. Then, neurosecretory cells of the *corpora cardiaca* (CC), would release metabolically active hormones into the hemolymph. These may include corazonin (Crz), adipokinetic hormone (AKH), and possibly diuretic hormone-I (DH). This cocktail of hormones mobilize energy from the midgut and the fat body (see detail in Figure 3). Activation of the octopaminergic DUM (Dorsal Unpaired Median) neurons of segmental ganglia of the ventral nerve cord would stimulate activity of skeletal and visceral muscle. Metabolic hormones like allatostatin-A, tachykinin-related and insulin-related peptides can be released from peripheral neurosecretory cells, where they can modulate gut motility, and may also contribute to regulate the release of Crz, AKH and DH from the CC.

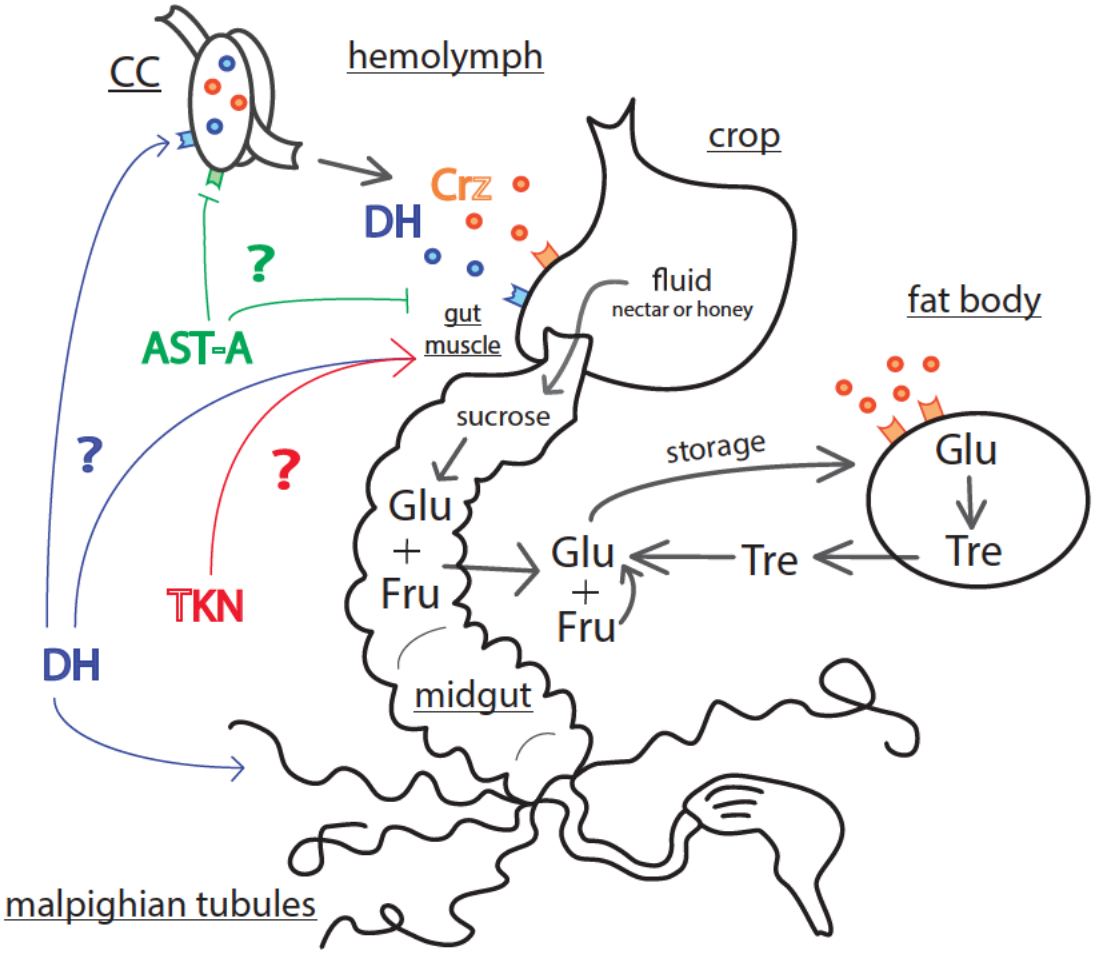


Model of the honeybee stress response

When faced with a stressor such as predation, robbing or adverse climatic conditions, a honeybee will need to increase her mobility and mobilize energy reserves to cope with the sudden increase of metabolic demand. We suggest here that this rapid change in physiology is achieved by a coordinated endocrine and neuroendocrine response (Figure 2).

As soon as the stressor is detected via appropriate receptors (e.g., olfactory, mechanosensory or visual), our model proposes that there will be release of octopamine and dopamine within the brain, thus increasing arousal (Corbet, 1991) (see Section 3.1). Other signals like cortico-releasing hormone-binding protein (CRH-BP) might also participate in the brain stress response (Liu et al., 2011). Octopamine is also released into the hemolymph (Davenport and Evans, 1984) from neurohemal cells (Kreissl et al., 1994) to act on many organs and coordinate a physiological response to the stressor (see Section 3.2.1). Peripheral octopamine increases heart rate, and may modulate ventilation and stimulate mobilization from muscles (Papaefthimiou and Theophilidis, 2011; Verlinden et al., 2010). The activation of the neurosecretory cells of the *corpora cardiaca* (CC), the major brain neurosecretory organ, stimulates the release of several neurohormones: adipokinetic hormones (AKH) and possibly corazonin, into the hemolymph (Boerjan et al., 2010a; Kodrík, 2008; Veenstra, 2009a) to mobilize energy from body stores (see Section 3.2). Finally, we suggest here (from honeybee physiology studies) that hormonal factors including AKH and candidates like allatostatin-A (AST-A), diuretic hormones (DHs) and tachykinins (Veenstra, 2009a), could reinforce the liberation of trehalose and glucose from the fat body, but also from the main energy store of the honeybee, the crop (Blatt and Roces, 2001; Crailsheim, 1988; Veenstra, 2009a) (see Section 3.3 and Figure 3).

Figure 3. Hypothetical model of energy mobilization in honeybee. Glucose (Glu) and trehalose (Tre) are the main sources of energy in the hemolymph. Trehalose is stored in the fat body and, when necessary, is released into the hemolymph to be metabolized into glucose. Another source of hemolymph glucose is sucrose from nectar contained in the crop. If hemal carbohydrate levels drop, an influx of nectar is passed from the crop to the midgut via muscle contractions. In the midgut, sucrose is metabolized into fructose (Fru) and glucose, which are then transported to the hemolymph. The passage of nutrients from the crop to the midgut is allowed by contraction of the gut muscle, also named the proventriculus. During normal metabolic demands this influx from the crop depends on the carbohydrate concentration in the hemolymph (Blatt and Roces, 2001; Blatt and Roces, 2002). In energy-demanding situations, this process might be boosted by tachykinin-related peptides (TRPs), diuretic hormone-I (DH-I), corazonin (Crz) and adipokinetic hormone (AKH) while an inhibitory effect from allatostatin A (AST-A) secreted from the midgut would be relieved. DH-I may also exert feedback on corazonin-secreting cells of the *corpora cardiaca* (CC).



Molecular signals of the honeybee stress response

Stress indicators within the brain

Biogenic amines

The role of catecholamines as hormones and neuromodulators in the acute stress response is extremely well conserved and well documented in vertebrates (Kvetnansky et al., 2009). In insects, the biogenic amines octopamine and dopamine are also involved in responses to stressors (Bicker and Menzel, 1989; Roeder, 1999; Roeder, 2005). Their respective receptors are phylogenetically related to adrenergic and dopaminergic receptors (Evans and Maqueira, 2005; Farooqui, 2012; Scheiner et al., 2006), showing a strong conservation of both structure and function through more than 500 million years of evolution. These amines regulate many aspects of insect physiology and behaviour (Farooqui, 2012; Scheiner et al., 2006), but principally have been shown to increase arousal state and motor activity in several insect species (Corbet, 1991; Sombati and Hoyle, 1984). In *Drosophila*, dopamine modulates sleep and locomotion, thus paralleling the functions of dopamine in mammalian circadian rhythms and arousal state (Andretic et al., 2005; Van Swinderen and Andretic, 2011). Similarly, octopaminergic neurons from the *pars intercerebralis* regulate the sleep:wake cycle (Crocker et al., 2010) ("endogenous arousal") and octopamine signaling has been implicated in arousal increase in response to environmental stressors ("exogenous arousal") (Corbet, 1991). Both forms of arousal are inversely regulated by dopamine, which exerts an inhibitory control on stressor-induced locomotor hyperactivity (Lebestky et al., 2009). In the honeybee, there is evidence that dopamine and octopamine modulate motor activity (Fussnecker et al., 2006; Mustard et al., 2010). In many insects, including honeybees, octopamine treatments have been shown to increase sensitivity to sensory inputs (Barron et al., 2007; McQuillan et al.; Menzel et al., 1999; Pribbenow and Erber, 1996; Sombati and Hoyle, 1984). Moreover, two studies have shown that exposure to physical stressors modifies brain levels of octopamine and dopamine in honeybees (Chen et al., 2008; Harris and Woodring, 1992).

Both octopamine and dopamine also modulate learning of a stressful event, particularly dopamine (Agarwal et al., 2011; Heisenberg, 2003; Vergoz et al., 2007). In this regard, the functions of these biogenic amines parallel those of catecholamines (adrenaline and noradrenalin) in mammals, which modulate not only the initial

neurohormonal cascade of the stress response, but also the learning of a stressful event (Roozendaal and McGaugh, 2011). Therefore, both in mammals and invertebrates, signaling through biogenic amines mediates both the initial stress response and the capacity to learn about the stressors triggering the response, thus potentially modulating behavioral and physiological reactions upon further exposure to these stressors.

Cortico-releasing hormone-binding protein (CRH-BP) and its putative diuretic hormone ligand (DH-I)

Cortico-releasing hormone (CRH), also called cortico-releasing factor (CRF), is a crucial signaling element within the vertebrate HPA axis (Huising et al., 2004). Its action is negatively regulated by the CRH-binding protein (CRH-BP) (Westphal and Seasholtz, 2006) The CRH receptor and CRH-BP are strikingly conserved both structurally and functionally throughout vertebrates as hormonal regulatory elements of the stress response (Chang and Hsu, 2004; Huising and Flik, 2005; Lovejoy and Balment, 1999). CRH-BP even shows a degree of conservation in honeybees (Huising and Flik, 2005). The predicted *Apis mellifera* CRH-BP shares only 25%–29% identity with the vertebrate CRH-BP, but the sequence comparison reveals that amino acids potentially crucial for the 3D structure (cysteines forming bisulfure bridges) (Huising and Flik, 2005) and for ligand binding are conserved. Interestingly, its homolog in the Asian honeybee, *Apis cerana* (*Acc*CRH-BP), is expressed as the transcriptional level in the brain (Liu et al., 2011), and upregulated following application of various acute stressors such as UV light, heat or cold (Liu et al., 2011). This increase by diverse stressors strongly suggests a signaling role in general stress pathways, even though the role of CRH-BP in insects (chaperone protein or link with the hormonal cascade) needs to be explored (Westphal and Seasholtz, 2006).

Despite this apparent conservation of CRH-BP in insects, no obvious homolog of CRH has been found yet, but precursor peptides of the vertebrate CRH family display similarities with the insect diuretic hormone-I (DH-I, also named DH31 in *Drosophila*) (Chang and Hsu, 2004; Huising and Flik, 2005; Zandawala, 2012) which has been suggested to be a good candidate ligand for CRH-BP (Huising and Flik, 2005). Still, a clear link between DH-I and the stress response is lacking, but we note that the regulation of water balance via DH-I action on the excretory system (Coast et al., 2002) could be essential to mobilize energy sources from the honeybee crop. Since DH-I is detected in the

CC (Boerjan et al., 2010b), it might well be part of a coordinated neuroendocrine cascade preparing the honeybee for rapid energy mobilization in energy-demanding situations (see Section 3.3 and Figure 3). Therefore, we think that DH-I and CHR-BP are good candidates as putative elements of the stress response whose action would be worth considering in the future.

Coordinated peripheral stress responses

In the periphery, the immediate physiological stress response might be coordinated by nerve signals allowing a very fast reaction, but (as in vertebrates) neuroendocrine systems seem to also play a major role in honeybees and other insects. Important components are neurosecretory cells of the CC, which integrate neuronal signals and may trigger broad effects in a variety of target cells through endocrine signals in the hemolymph (Scharrer, 1967). Like the vertebrate pituitary gland, the insect CC houses many neuroendocrine cells that play a central role in the regulation of diverse metabolic functions (De Loof et al., 2012).

Octopamine

Additionally to its role as a neurotransmitter and a neuromodulator in the brain, octopamine also acts in periphery, mainly as an endocrine signal. Increases of octopamine level in the hemolymph have been measured in energetically demanding, “fight-or-flight” situations (Davenport and Evans, 1984; Farooqui, 2012; Roeder, 2005; Scheiner et al., 2006; Verlinden et al., 2010). A large literature from locusts, cockroaches, flies and moths demonstrates that many insect organs are sensitive to octopamine, including flight and visceral muscles (Luffy and Dorn, 1992; Malamud et al., 1988; Orchard and Lange, 1985), reproductive organs (Avila et al., 2012; Lange and Orchard, 1986; Monastirioti, 2003; Orchard and Lange, 1987; Stevenson et al., 1994), heart (Collins and Miller, 1977; Hertel and Penzlin, 1992; Johnson et al., 1997; Prier et al., 1994), air bags (Zeng et al., 1996), sense organs (Farooqui, 2007, 2), metabolic tissues such as the fat body (Arrese and Soulages, 2010; Downer, 1979; Gole and Downer, 1979; Meyer-Fernandes et al., 2000; Orchard et al., 1982) and malpighian tubules (Goosey and Candy, 1982; Martin et al., 1989). These two latter organs have key roles in energy mobilization in honeybees (see Section 3.3 and Figure 3). Hence, octopamine is in the position to trigger broad and coordinated physiological changes such as the ones expected in a general stress response

(David and Lafon-Cazal, 1979; Lam et al.). Several authors have proposed it to be the major stress hormone in insects, including honeybees (Adamo and Baker, 2011; Farooqui, 2012; Roeder, 2005; Scheiner et al., 2006; Verlinden et al., 2010), but its specific action on each cell population remains to be clarified in detail (Farooqui, 2012; Scheiner et al., 2006).

In response to threats, octopamine primes flight and leg muscles in locusts (Duch and Pflüger, 1999; Orchard et al., 1993; Pflüger et al., 2004). A similar action in honeybees has not been demonstrated yet, but would be consistent with its positive action on locomotor (specifically flight) activity (Fussnecker et al., 2006). In parallel, octopamine seems to also be a cardiostimulant and an activator of the respiratory system to increase oxygen supply to muscles. Modulation of the respiratory system by octopamine is not well understood in insects, but octopamine can stimulate respiratory activity through increasing the hemolymph circulation in the Dobson fly, *Corydalus cornutus*, and the locust *Schistocerca americana* (Bellah et al., 1984; Sombati and Hoyle, 1984). Octopamine was also shown to stimulate respiratory neurons in *Locusta migratoria* (Ramirez and Pearson, 1991). Evidence of similar respiratory effects of octopamine in honeybees is still lacking, and thus more research is needed in this area. Recently, Papaefthimiou and Theophilidis (Papaefthimiou and Theophilidis, 2011) have shown *in vitro* a biphasic effect of octopamine on heart activity in honeybees. A high concentration of octopamine increases the contraction frequency of the heart, but a low concentration has the opposite effect. The authors argue that this double action may indicate the presence of different types of octopamine receptors on the heart, but another explanation may be due to the participation of diverse signaling pathways depending on OA concentration, since receptor activation can trigger different intracellular signals for different OA concentrations, at least *in vitro* (Huang et al., 2012).

To perform all these diverse functions, octopamine very likely acts both as a neurotransmitter and as a neurohormone (Roeder, 1999). While the distribution of octopaminergic neurons has been described in detail in the honeybee brain and subesophageal ganglion (Bicker, 1999; Kreissl et al., 1994; Schröter et al., 2007; Sinakevitch et al., 2005) very little is known about its distribution in the nerve cord and motor nerves. This state of knowledge contrasts heavily with the well-characterized network of extensive efferent unpaired octopaminergic neurons in locusts and

cockroaches (Bräunig, 1997; Bräunig and Pflüger, 2001; Field et al., 2008; Stevenson and Sporhase-Eichmann, 1995). Thus, data from these species suggest that such neurons might act similarly to the sympathetic vertebrate system by releasing octopamine from varicosities directly near the organs (glands, peripheral flying or leg muscles) (Bräunig, 1995; Bräunig et al., 1994). In the honeybee, octopamine-like immunoreactivity in varicose structures of CC suggests a possible (neuro)endocrine source of octopamine (Kreissl et al., 1994). Additionally, our model assumes that a network of peripheral octopaminergic neurons exists in honeybees as in locusts, but information on this is currently lacking. It will be important to confirm the presence of octopaminergic neurohemal structures on the surface of peripheral nerves similar to those described in locusts, which are only inferred in honeybees for now, based on comparison with various insects (Farooqui, 2012; Stevenson and Sporhase-Eichmann, 1995).

Octopamine is released in energy demanding "fight-or-flight" situations to increase the honeybees' state of general arousal and we can therefore consider it as a stress hormone in insects (Adamo and Baker, 2011; Farooqui, 2012; Roeder, 2005). Interestingly, octopaminergic neurosecretory cells innervate the honeybee CC (Bicker, 1999; Kreissl et al., 1994), thus suggesting that octopamine could also regulate the release of several neuropeptides from this structure (including the stress candidates discussed hereafter).

Corazonin

The cardioacceleratory function of this 11-aminoacid neuropeptide was first described in 1989 by Veenstra in *Periplaneta americana* (Veenstra, 1989). Now we know that this effect is probably restricted to cockroaches only, while corazonin has been shown to have diverse effects in other insects such as silkworms, locusts, flies, and moths. In locusts (both *Locusta migratoria* and *Schistocerca gregaria*), corazonin is involved in the induction of the gregarious phase (Tawfik et al., 1999), and in ecdysis in the moth *Manduca sexta* (Kim et al., 2004). Also, a metabolic function of corazonin as a nutritional stress hormonal signal has been recently suggested by Veenstra (Veenstra, 2009a), based on the localization of the peptide precursor and its receptor in *Drosophila*. Corazonin is produced by neurosecretory cells projecting into the CC in many insects, including *Drosophila*, locusts and honeybees (Roller et al., 2006; Verleyen et al., 2006). In *Drosophila*, corazonin receptors have been found in the heart, fat body, salivary glands

and gut (Chintapalli et al., 2007). Additionally, in *Drosophila*, corazonin neurons express diuretic hormone receptors and an AST-A receptor (Johnson et al., 2005). This has led Veenstra to suggest a model in which corazonin is released in response to peripheral feedback from the gut in a hunger state, and acts to mobilize energy (see Section 3.3 and Figure 3). Phylogenetic analyses support an ancestral hormonal role for corazonin in regulating metabolic functions, more specifically because corazonin receptors belong to the ancient GnRH-AKH receptor family (Hansen et al., 2010; Park et al., 2002; Veenstra, 2009a). Based on these recent findings on the role of corazonin in insects we propose, following others (Boerjan et al., 2010a), that it may have an important hormonal role in the honeybee acute and chronic stress response, although this hypothesis has yet to be directly tested.

Allatostatins

Allatostatins (ASTs) are insect neuropeptidic hormones first identified as regulators of growth during development, on the basis of their ability to reduce juvenile hormone (JH) release by the Corpora allata (Bendena et al., 1999). However, there is growing evidence that not all members of the AST family play this biological function (Audsley and Weaver, 2009; Bendena et al., 1999; Stay and Tobe, 2007). Among the three major AST types (A, B and C), only A and C are present in honeybees (Audsley and Weaver, 2009; Stay and Tobe, 2007). In addition, honeybees (as along with some other insect species) have two closely related C-type peptides, AST-C and AST-CC (Veenstra, 2009b).

ASTs are important regulators of food intake and/or digestive functions in several insect species (Meyering-Vos and Woodring, 2008; Robertson et al., 2012; Veenstra et al., 2008; Wang et al., 2012; Wilson and Christie, 2010), but this might be part of a much broader spectrum of inhibitory functions (Veenstra, 2009b). They are present in the midgut of several species as well as in the CC (Audsley et al., 2008; Mayoral et al., 2010; Veenstra et al., 2008), and are known to release neuroendocrine signals regulating energy supply from the digestive tract (see below and Figure 3). Hemal AST-A has been suggested to modulate CC function (Veenstra, 2009a): low food content in the gut reduces circulating AST-A released by midgut secretory cells; this in turn relieves inhibition of CC neuroendocine cells containing corazonin and diuretic hormones. This postulated role in response to nutritional stress has been recently challenged by recent work in *Drosophila*,

showing that genetic manipulations of AST-A alter feeding behaviour without apparent consequences on energy reserves or metabolism (Hergarden et al., 2012). Thus, whether an AST-mediated nutritional feedback loop exists remains an open question. It is worth mentioning that at least the AST-A type may be expressed in the honeybee CC (Kreissl et al. but see Boerjan et al., 2010b), which places it in a position of possibly participating in energy mobilization control, in particular under conditions of chronic and acute nutritional stress.

Adipokinetic hormone (AKH)

AKH is perhaps the most important metabolic regulator described in insects (Arrese and Soulages, 2010). This octapeptide is synthesized in the CC and released into the hemolymph to increase catabolism in the fat body, ultimately leading to increased circulating trehalose levels (Gade and Auerswald, 2003; Ivanovic, 1991) (see detail in the following section), similarly to the action of glucagon in vertebrates (Bharucha et al., 2008; Isabel et al., 2005; Lee and Park, 2004). In cockroaches, AKH stimulate spiking from peripheral octopaminergic neurons and locomotion (Wicher, 2007). Interestingly, the demonstration that octopamine mediates AKH release into the hemolymph in the locust CC (Pannabecker and Orchard, 1986; Pannabecker and Orchard, 1987; Passier et al., 1995) provides further evidence of a precise interplay between arousal and hunger. In addition, recent papers support a role for AKH in a general stress response in various insects (Candy, 2002; Kodrík, 2008; Kodrík and Socha, 2005). Insecticide treatment inducing oxidative stress leads to increased hemolymph titers in the locust *Schistocerca gregaria* (Candy, 2002) and in the firebug *Pyrrhocoris apterus* (Kodrík and Socha, 2005). Indeed, AKH has the capacity to trigger antioxidant processes (Večeřa et al., 2012; Velki et al., 2011). In the latter species, mechanical stressors had a similar effect (Kodrík and Socha, 2005), thus strongly arguing for AKH-mediated actions as pivotal element of a widespread stress response. However, honeybees CC contain a lower amount of AKH than other insects (half that of *Gryllus bimaculatus* and *Acheta domesticus*), and AKH has a minor hypertrehalosemic effect in honeybees (Lorenz et al., 1999), Thus, the role of AKH in stress responses in honeybees may be less prominent than in other insects. This can possibly be explained by the specific mechanisms honeybees use to mobilize energy (Section 3.3).

Mechanisms of energy mobilization in honeybees

Increased energy mobilization triggered by hormonal signals plays a very important role in stress responses. In insects, carbohydrates (especially trehalose) are the most important energy source (Thompson, 2003). However, available trehalose is rapidly depleted, and in several insects, a sustained effort such as a long flight requires the release of trehalose from the main energy store, the fat body (Arrese and Soulages, 2010; Van der Horst, 2003). Consequently, mobilization of energy from energy stores by hormonal factors is an essential part of the stress response. By contrast, honeybees show specific mechanisms for energy mobilization which appear related to their social organization. Strikingly, forager honeybees have fat bodies almost entirely depleted and seem to use the sugar energy reserve carried in their crops to sustain the energetic demands of flight (Blatt and Roces, 2001; Crailsheim, 1988). This observation would also explain why foragers express almost no AKH in their CC, and have lower abdominal glycogen stores (Panzenbock and Crailsheim, 1997; Woodring et al., 1994).

Honeybees appear to have a quite specific mechanism for regulating hemolymph sugar levels (Figure 3), according to a model proposed by Blatt and Roces (Blatt and Roces, 2001). In an energy-demanding situation, trehalose synthesis by the fat body is not fast enough to match rates of trehalose consumption, so circulating trehalose levels decline. This stimulates the passage of nectar from the impermeable crop storage organ to the midgut by the contraction of the proventriculus (gut muscle between the crop and midgut) (Blatt and Roces, 2002). From the midgut, sugars are digested, absorbed and more glucose and fructose are transported into the hemolymph. Therefore, upon high metabolic demand, while the trehalose level decreases in the hemolymph, those of fructose and glucose increase, maintaining a stable sugar concentration (Figure 3). Moreover, the honeybee genome sequence suggests the loss of two important insect enzymes converting gluconeogenic substrates to trehalose and glycogen (Kunieda et al., 2006), which are both stored in fat body and considered as the primary energy storage molecules in insects. This implies that in the honeybee, regulation of sugar transport from the gut probably plays a more important role in energy balance than the regulation of trehalose release from the fat body. Interestingly, in honeybees injection of CC extracts into the hemolymph has a hypertrehalosemic effect (Woodring et al., 1994), thus candidates for this function are expected to be found in this gland. As discussed above,

hormonal candidates performing this role in honeybees might include corazonin, DH-I and possibly AKH (Figure 3).

Hormones activating the mobilization of glucose from the crop by stimulating the proventriculus and midgut remain to be identified. In *Drosophila* (Veenstra, 2009a), it has been suggested that the (neuro)hormones: AST-A and tachykinin, released from secretory cells of the midgut or the nerve cord, might play this role (Veenstra, 2009a) (Figure 3). Tachykinin and tachykinin-related peptides (TRPs) are known in insects to be myostimulatory of the insect midgut muscle (Nassel, 1999), and therefore would be good candidates for modulating release of nectar from the honeybee crop. One "tachykinin-like receptor" has been identified in honeybees from sequence homology with *Drosophila* (Hauser et al., 2006), but its function remains unclear, especially as many TRPs have been described and seem to have diverse functions in insects (Nassel, 1999). Nine different TRPs have been localized all along the nervous system of the honeybee (Boerjan et al., 2010b), but none of them was detected in CC neurosecretory cells. If TRPs act to upregulate metabolism in stress, they would need to be released from the efferent peripheral nerves directly to the proventriculus.

It should be noted that insulin and vitellogenin pathways have also been linked to energy store mobilization and oxidative stress in insects (Broughton et al., 2005; Seehuus et al., 2006). Insulin/insulin-like growth factor signaling (IIS) pathways appears to regulate fat stores in *Drosophila* (Broughton et al., 2005) and confer oxidative stress resistance in *Drosophila* and honeybees (Corona et al., 2007). Vitellogenin expression seems to be triggered by IIS pathways (Corona et al., 2007). In reproductive females, vitellogenin is a glycolipoprotein stored in the fat body and usually released into the hemolymph before being stored within oocytes. In the sterile honeybee worker, vitellogenin expression is inhibited by JH (Pinto et al., 2000), thus giving foragers a lower amount of vitellogenin (perhaps reinforced by their decrease of fat body mass). However, vitellogenin seems to be protective against oxidative stress, perhaps via certain antioxidant properties, and may account for longevity in reproductive insects, e.g., queen bees (Corona et al., 2007; Seehuus et al., 2006). Presently, the metabolic role of these molecules needs to be clarified in honeybees in order to be integrated within our model of endocrine regulation of energy sources.

Gaps in knowledge and urgent questions

The sections above help build a model of a general stress response in the adult honeybee, as presented in Figure 2. However, as mentioned, in some instances specific data from this particular species are lacking.

What is the role of JH in the stress response?

JH acts on development and sexual maturation in insects; it is produced in the CC and released under the neural control of brain peptidergic innervation. JH is typically described as the master larval developmental hormone, boosting growth of insects, inhibiting metamorphosis and initiating reproductive traits in adults (Riddiford, 2012). Still, JH has been described more recently as a stress hormone in *Drosophila*, as JH levels drop after exposure to various stressors (Gruntenko et al., 2012). As JH tends to have long-lasting effects, this hormone may be more likely to be involved in chronic than acute stress response. Whether JH acts as a stress hormone in honeybees is less clear, particularly since, in the adult worker bee, it has a species-specific function: that of a regulator of division of labor. JH titers are low in young nurse bees but higher in foragers. Indeed, pharmacological elevation of JH levels or injection of JH analogs accelerates the onset of foraging of young bees (Robinson, 1987; Schulz et al., 2002a). Perhaps because of this, studies of the possible role of JH in stress have thus far given confusing results. Lin *et al.* (Lin et al., 2004) could not find a consistent change in JH levels after application of various stressors in honeybees, and found a response only if JH levels were initially low (in which case JH levels increased after caging or cold anesthesia). If JH levels were already high, stress seemed to decrease JH levels. These differences probably result from the dynamics of mechanisms for metabolism and recycling of JH when JH levels are very low or very high. As a consequence of this additional complexity, the precise roles of JH in the honeybee stress response are presently unclear, but given the importance of this hormone system, this is certainly an area demanding further study (Lin et al., 2004).

Can dopamine be considered as a stress hormone?

A potential role of hemal dopamine in stress is suggested by work on *Drosophila* as dopamine increased heart rate, while mutations impairing dopamine synthesis had the opposite effect (Johnson et al., 1997). In cockroaches, one study also found dopamine in

the CC (Shimizu et al., 1991), thus suggesting a neurohormonal role. In the hemolymph dopamine levels have been rarely quantified in honeybees, but Bateson *et al.* (Bateson et al., 2011), found a decrease in dopamine levels in the head hemolymph of honeybees 30 minutes after a strong vibration stressor. Clearly more work is needed here to explore the possible role of dopamine in endocrine acute stress response.

Neuropeptides in the CC?

Neuropeptides are an emerging area of research, and many members of this large family have never been studied. The neurosecretory cells of the CC contain numerous unstudied peptides (Scharrer, 1967), which would be good candidates as stress neurohormones. Corazonin, DH, AKH, tachykinins and AST-A have been mentioned previously, but many other peptides might play metabolic roles in the honeybee (Woodring et al., 1994). In addition, looking at the location of neurohormone receptors is important to understand how the stress system operates. More details on the distribution of neuropeptides and their receptors might highlight their targets, the responses they elicit, as well as the feedback loops regulating the system.

Stress responses and immune system

As in vertebrates, the immune response can be affected negatively by stressors. This may be a direct effect of stress hormones (biogenic amines and AKH), as shown in some insect species (Adamo, 2012; Adamo and Parsons 2006). Depending on the context and the stressor characteristics, the immune response has been shown to be boosted by stressful events or stress hormones (Baines et al., 1992; Mowlds et al., 2008). This can be understood as a way of maintaining immune equilibrium in a harmful environment (Adamo, 2012; Adamo et al., 2008). In honeybees, stress and immune responses do not seem to have been considered together yet, but recently several detrimental synergistic effects of various combinations of stressors suggest a link between them (Alaux et al., 2010; Aufauvre et al., 2012; Köhler et al., 2012 ; Vidau et al., 2011). As proposed by some of the authors of those studies, such a link may be highly relevant to understand the recent decrease of honeybee populations (Köhler et al., 2012; Vidau et al., 2011).

Task specialization and sensitivity to stress

The high level of sociality and the complex system of division of labor are essential characteristics of honeybees (Wilson, 1971). Here, we propose that at least some elements of the stress response may have been adapted in specific ways to contribute to the evolution of division of labor. During its lifetime, an individual honeybee progresses through a succession of specialized behavioral states whose sequence follows an internal developmental program modulated by various social signals (pheromones) emitted from the colony (Robinson, 1992; Slessor et al., 2005). Honeybees typically begin their adult life undertaking in-hive activities such as brood nursing, cleaning and food storing, then guarding the hive entrance against intruders and predators and finally foraging for food sources (mostly pollen and nectar). The transition to foraging is a major event is a honeybee's life, and corresponds to multiple changes in hormonal activity, brain circuits, and physiology (Robinson, 1992; Winston, 1987).

There has been a lot of research on the mechanisms underlying and organizing this division of labor, which have been shown to involve octopamine, JH and vitellogenin (Fahrbach and Robinson, 1996; Robinson, 1987; Robinson, 1992; Wagener-Hulme et al., 1999). We suggest here that modulation of stress reactivity may be linked to the evolution of task specialization in honeybees. The fact that octopamine is two to four times more abundant in brains of foragers than those of nurse bees (Harris and Woodring, 1992) may predispose foragers to attain more easily or rapidly a state of higher energy mobilization. Indeed foragers appear to be the colony members most exposed to stressors: foraging is energetically demanding, and exposes honeybees to more adverse environments (e.g., predators, insecticides) than working within the hive (Williams et al., 2008). Elevated brain octopamine levels, as a potential result of chronic stressor exposure (Adamo and Baker, 2011), may prepare honeybees to cope with the higher stress levels caused by foraging, and the hormonal state of a forager bee may resemble that of nurses bees under chronic stress.

Further, chronic stressors applied at the colony level and experimental elevation of brain octopamine levels both accelerate the onset of foraging in the honeybee (Higes et al., 2008; Khoury et al., 2011; Schulz et al., 2002b). A high brain level of octopamine may also make foragers more sensitive to hunger, which could motivate them to gather food.

The forager's state and number appear to be as a response to colony stress, and foragers are also the behavioral caste exposed to the greatest stress. Therefore, knowing the molecular pathways and physiological mechanisms that regulate chronic and acute stress responses at the individual level are of great interest for developing strategies to improve the health and longevity of honeybee colonies.

Conclusions

The model developed here describes a general stress response in honeybees. It provides a framework to facilitate our understanding of how honeybees can respond to stressors, and is also aimed at stimulating research to improve our knowledge of the physiological pathways involved.

Our comparison of vertebrate and honeybee stress response pathways suggests a parallel organizational structure in the two groups, including regulation of arousal and cognitive functions in the brain by catecholamines, coupled with neurohormonal signals stimulating energy mobilization in the periphery. Yet, the extent to which the stress response pathways are evolutionarily conserved remains unclear. Some elements, like CRH-BP, may offer examples of conservation of function, but others, particularly neuropeptide hormones, are likely to be specific to insects or invertebrates. In this regard, it is worth noting that several key neuropeptides cited here (AKH, tachykinin, DH) are among the most highly conserved neuropeptides among insect species, perhaps indicating the operation of strong stabilizing selection (Hauser et al., 2010).

In this review, we also highlighted the aspects of the stress response that appear to be specific to honeybees as a result of their peculiar social organization. Specifically, we have summarized particular mechanisms enabling an increase of glucose from the crop.

Finally, pursuing studies on stress in honeybees is essential for developing standard methods to assess stress in this insect of major economical importance. Relevant and robust criteria to evaluate stress symptoms would be useful as basic indicators of health in honeybees, and the development of standardized assays would improve risk assessment for pesticide and other agricultural practices on honeybee populations. Thus, knowing more about stress in honeybees is now crucial to design strategies for the protection of this fragile, but ecologically and economically important, insect.

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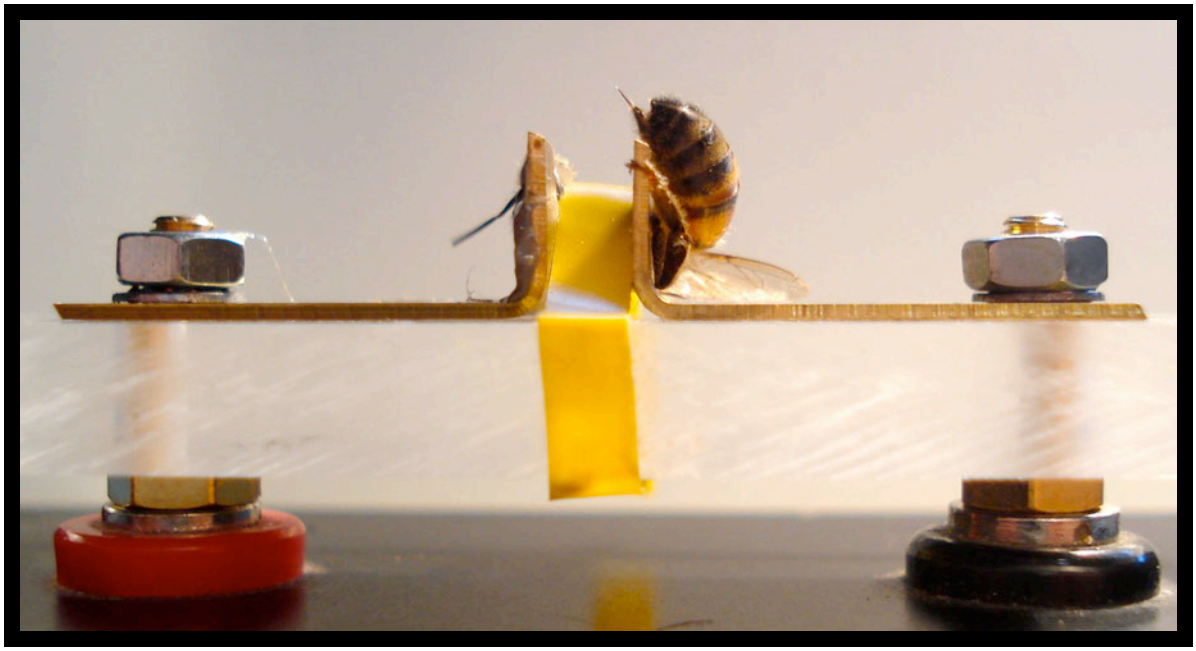
Chapter 2

Brain biogenic amine levels and behavioural responses after physical stress in honeybees (*Apis mellifera*)



Abstract

Behavioural and physiological responses to stress in insects can be understood using the concept of stress created and developed with mammal models. Brain biogenic amines have previously been studied as possible mediators of responses to physical stress in insects, including in honeybees (*Apis mellifera*). Yet, in this latter species the available results are not always consistent and no clear picture emerges, possibly because of the use of different protocols. Here, we examine the relationship between physical stress and brain levels of biogenic amines, and in particular the possible influence of the type and duration of stress. To achieve this we performed two experiments using different physical stressors that were applied for either short or longer times. In both experiments we recorded sting responses and brain biogenic amine levels (octopamine, dopamine, serotonin and tyramine) to measure individual behavioural and physiological stress responses. Short-term physical stress did not increase brain biogenic amine levels, however longer-term physical stress did increase brain levels of octopamine and serotonin. We discuss our results in terms of understanding stress mechanisms in the honeybee.

Introduction:

The concept of stress has been developed in rodents and is typically illustrated and measured by behavioural responses categorized as fight or flight, and by neuro-hormonal physiological responses (Chrousos, 2009; Selye, 1936). The general adaptation syndrome (GAS) developed by Hans Seyle describes three important consecutive phases of an organism's stress responses: alarm, resistance and exhaustion after prolonged exposure to a stressor (Selye, 1956). These phases can be applied to behavioural and physiological stress responses and provide a framework for understanding how an organism responds to a threat. The "alarm phase" corresponds to an individual's perception of the stressor, then the body enters a "resistance phase" and suppresses secondary functions like immunity, reproduction and digestion to mobilize energy toward emergency functions such as blood circulation, respiration and motility. If the threat is maintained, it leads to a fatigue of the system or "exhaustion". The physiological mediators of these responses (amines and neuropeptides) are at the interface between the brain and peripheral organs to coordinate appropriate response to the environmental aversive stimuli.

Similarly to vertebrates, individual behavioural stress responses also include aggression and escape responses in insects (Ivanovic, 1991; Johnson and White, 2009). In honeybees, the sting response is an obvious aggressive response to a direct or indirect threat to their body, therefore it is currently used to measure a behavioural stress response in this species (Lenoir et al. 2006; Roussel et al. 2009; Uribe-Rubio et al. 2008).

In insects biogenic amines (BA) including octopamine (OA), dopamine (DA), serotonin (or 5-hydroxytryptamine 5-HT) and tyramine (TYR) are good candidates for physiological stress signals (Ivanovic, 1991; Johnson and White, 2009). Depending on the location and the pharmacology of the receptors they act on, as well as on their mode of release, BAs can operate as neurotransmitters, neuromodulators or neurohormones (Blenau and Baumann 2001). There is some evidence for changes in brain BA levels in response to physically challenging situations in various insect models (Chentsova et al., 2002; Davenport and Evans, 1984; Hirashima and Eto, 1993; Ivanovic, 1991; Verlinden et al., 2010) including honeybees (Chen et al., 2008; Harris and Woodring, 1992). OA and DA, in particular have been related to stress functions in the American cockroach and in fruit flies (Chentsova et al., 2002; Gruntenko and Rauschenbach, 2008; Neckameyer and

Weinstein, 2005; Roeder, 2005). A series of experiments in fruit flies using transitory *corpora allata* ablation supports the hypothesis that DA and OA levels are indicators of thermal and nutritional stress. These studies reveal an age-dependent mediator role of DA (either inhibitor or stimulator) in the reciprocal regulation and balance of juvenile hormone and ecdysone levels (Chentsova et al., 2002; Gruntenko and Rauschenbach, 2008; Gruntenko et al., 2005; Gruntenko et al., 2012). These hormones are released from endocrine glands (*corpora cardiaca* and *corpora allata*) that receive innervation from the suboesophageal ganglion (SOG) of the brain, now called gnathal ganglia (GNG) according to a new nomenclature (Ito et al., 2014). In honeybees, the expression of OA and DA receptors in the GNG suggests that these amines may play a role in the modulation of stress-induced hormonal responses (Even et al., 2012; Kreissl et al., 1994; Schäfer and Rehder, 1989). Interestingly, Kreissl and colleagues (1994) were able to detect OA in very fine fibers around the *corpora cardiaca* only in starved animals, suggesting that this network of neurons might be involved in neurosecretatory hormone release during nutritional stress. Additionally, 5-HT is an interesting candidate for physiological responses in insects as it is also present in GNG (Rehder et al., 1987) and has been linked to aggression and circadian rhythms in flies and bees (Blenau and Thamm, 2011).

Previously, two studies in honeybees have examined brain BA level changes after physical stress. Harris and Woodring (1992) applied mechanical stress (pinching the legs for various time periods). They reported significant increases of brain levels of OA and 5-HT (but not DA) after 10 minutes of leg pinching. On the other hand, another study revealed a drastic decrease in brain OA and DA levels after catching a bee, keeping it in a tube for 30 minutes and spinning it in a centrifuge (Chen et al., 2008). These studies suggest that brain BA levels changes after physical stress can vary depending on the duration and nature of the stressor.

In this study we tested how brain BA levels are changed by various types and durations of physical stress. In experiment one, we describe short-term stress responses after various durations of thorax pinching. We used sting extension measures as a behavioural measure of stress sensitivity, and whole and regional brain BA levels. In experiment two, we tested if brain BA levels vary according to the nature and duration of physical stress. We measured whole brain BA levels in bees which were either flying freely, caged, harnessed, or harnessed and shocked (equivalent to three hours of physical

stress). We measured shock sensitivity and brain BA levels in the same bees to examine possible correlations between BA levels and stress sensitivity.

Methods:

Experiment one: Short-term stress experiment using thorax pinch:

Bees used for this experiment were foragers from a hive kept in a flight-cage during the Australian summer season. Bees were directly caught at a sucrose feeder with calibrated forceps (Fig. 1) to ensure that a consistent pressure was applied (no deformation of the cuticle was observed), then held for varying lengths of time (3 min, 5min, 10min, 20 min) by their thorax, based on the protocol from Harris and Woodring (1992). As soon as they were caught, the duration and frequency of the sting extension was immediately recorded in the computer with J-watcher software (version 1.0; Blumstein, 2006) while holding the bee. To collect bee brains for BA analysis, bees were flash-frozen immediately in liquid nitrogen at the end of the specific interval of time for which they were held in forceps. They were stored in Eppendorf tubes in dry ice and then at -80°C until dissection and analysis.

Experiment two: Long term stress experiment using various stressors:

Bees from this experiment were collected from three different colonies kept at Macquarie University. For each treatment 60 Foragers (20 per colony) were collected at the hive entrance on their return from their foraging trip (this total number was slightly reduced because of losses during the experiment and brain dissection). Foragers caught at the hive, and flash-frozen in liquid nitrogen without forceps handling (bees were simply pushed in liquid nitrogen) were used as controls submitted to minimal physical stress. The three experimental groups consisted of foragers that were first collected individually in 15mL plastic tubes and then cold-anesthesised on ice for maximum 10 minutes. Some of these bees were introduced individually into well-ventilated transparent plexiglas boxes (12 x 7 x 10 cm) and were left on the bench (3hours) next to the two other groups of bees (*caged* group). The other bees were harnessed to a horizontal holder made of two copper plates connected to a current generator, that allowed administering a mild electric shock to the restrained bees (Vergoz et al., 2007). Non-salt conductive gel (Spectragel, Parker) was applied between the bee and the plates to improve electric conductivity (Fig.

2). Afterwards, the bees were either left on the bench 3hours (*harnessed* group) or left on the bench (2hours) and then received a series of twelve shocks (one hour process) with increasing voltage (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8 and 10 V) separated by 5-minute intervals (*shocked* group). This assay determined an individual shock sensitivity score to test the correlation of behaviour sensitivity with the individual BA levels. The score of each shocked bee correspond to the number of sting extensions throughout the sequence of 12 shocks. A honeybee displaying a high number of sting extensions was therefore considered to be more sensitive to electric shocks. All bees from the three experimental groups were flash-frozen into liquid nitrogen at the end of the shocks and stored at -80°C.

Figure 1 Illustration of the pinch experiment. Bees were caught at a sucrose feeder between calibrated forceps to apply consistent pressure on the thorax. The sting extension was immediately recorded in a laptop with J-wacher software to measure frequency and cumulative duration of sting extension in stressed bees.

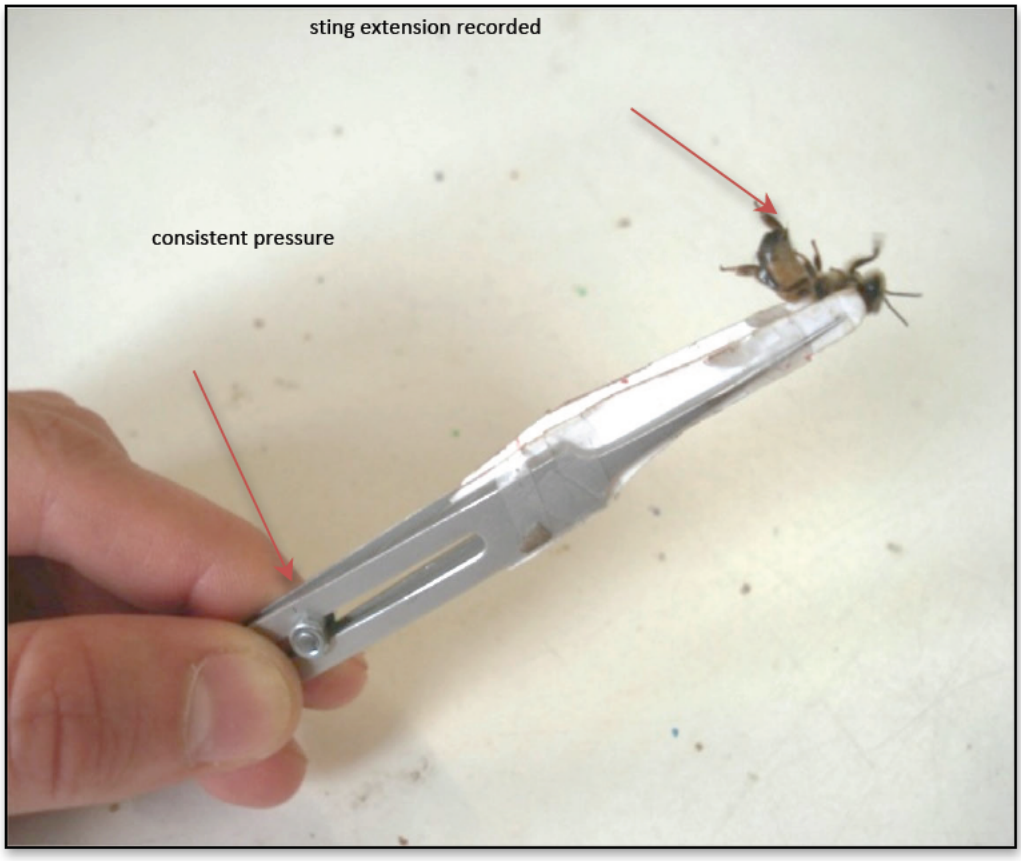
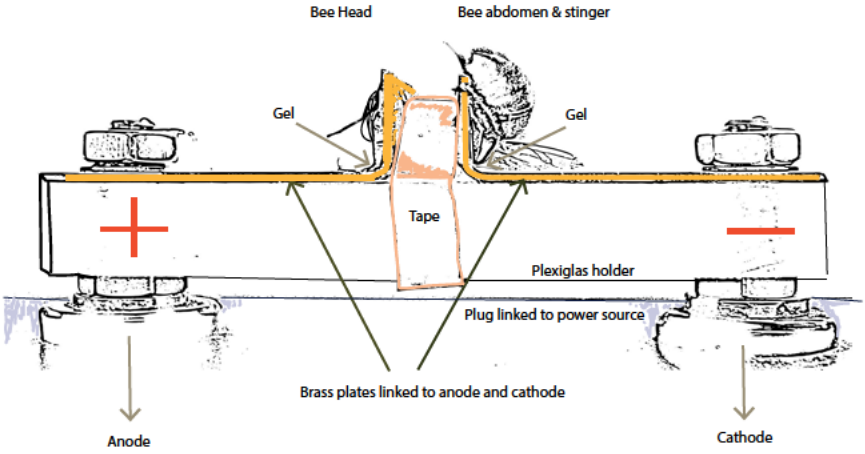


Figure 2 Drawing of the harnessing holder to deliver electric shocks. The bee is placed on top of 2 brass plates linking a positive pole (head) and a negative (abdomen).



Brain dissection

Frozen brains, were freeze-dried for 50 min at 290 mTorr (VirTis Benchtop™), and dissected under a dissecting microscope, on dry ice in order to avoid thawing and thus to preserve the BA content. Each brain was dissected from the head capsule (experiment 1 and 2) and then separated into four different regions of interest (experiment 1). Optic lobes (OLs) were first separated, then the GNG and finally the central brain was separated in two equal parts, one containing the antennal lobes (ALs) the other containing the mushroom bodies (MBs) (see Fig3).

BA quantification

Brain BA levels were measured with high-pressure liquid chromatography (HPLC) following the protocol used by Søvik et al. (Søvik et al., 2013) using an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clare, CA, USA), coupled to an electrochemical detector (ESA coulechem III) connected to a dual electrode analytical cell (ESA, Chelmsford, MA, USA).

Whole brains or dissected brain regions were centrifuged at 13,500 rpm for 5 minutes at 4°C before being homogenised by ultrasonication in 60µL (25µL for brain regions) of HPLC solvent [0.2M perchloric acid containing 10pg/µL dihydrobenzylamine (DHBA, internal standard)]. Homogenates were incubated on ice at 0°C for 20 minutes in the dark, then centrifuged at 13,500 rpm for 15 min at 4°C. The supernatant was collected and transferred into a dark sample vial, for loading into the HPLC autosampler maintained at 4°C. For a whole brain, 10µL per sample were injected into the HPLC, and 13µL for brain regions.

BAs contained in the samples were separated through a 100 mm column (Hypersil 5µm octadecylsilane package column, Thermo Fisher Scientific, Waltham, MA, USA) prior to quantification across a 5011 dual channel microelectrode analytical cell connected to a Coulochem III electrochemical detector (ESA). BA amounts in samples were quantified relative to a standard curve obtained by series of 7 dilutions of BA (OA, DA, 5HT, TYR) in perchloric acid. The dilutions ranged from 10 pg/µL to 2.5pg/µL for DA and 5-HT, 5 pg/µL to 1.25pg/µL for OA and TYR. These 7 standards were injected before and after each of the 24 randomised sample injections allowing a quantification of the samples relative to

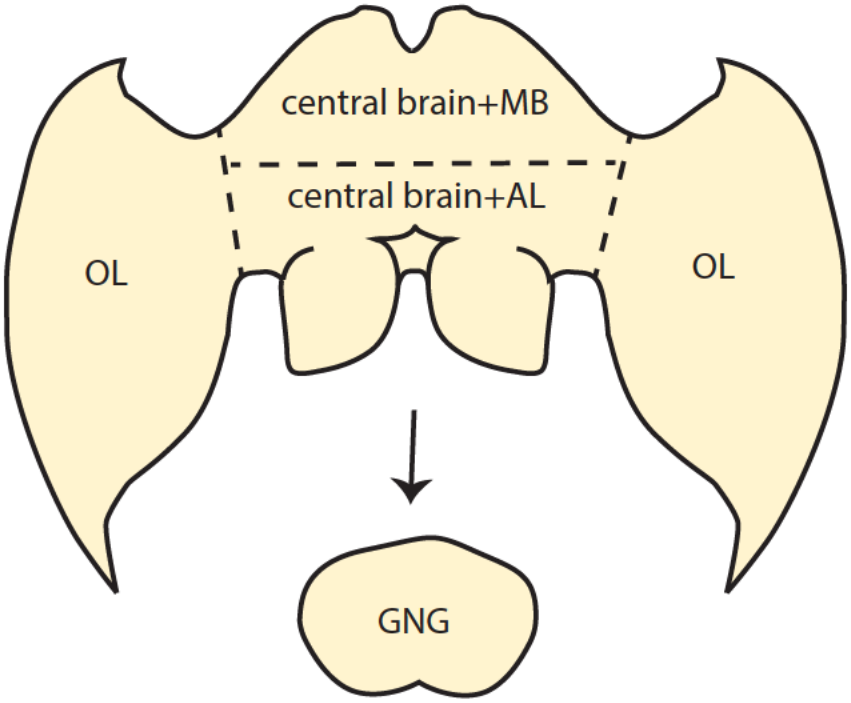
the average of the two linear standard curves obtained. All standards and samples contained a known amount of DHBA (10pg/µL), against which BA amounts were quantified. For each BA, a linear regression of the peak area relative to the area of the DHBA peak was fitted against the calculated standard curve. The integration of the signal was performed with the software Chemstation (Agilent). All compounds (Sigma-Aldrich, St Louis, MO, USA) were stored frozen in highly-concentrated aliquots used to make fresh standard dilutions for each day of an HPLC run.

OA, DA, 5-HT and TYR were quantified in the whole brain in experiment one. However TYR was not present in sufficient quantities in brain regions to be detected by our system (experiment 2).

Statistical analysis

All the analyses were performed using GraphPad Prism (version 5.0b) except the MANOVA (Fig 7) which was performed with R version 3.0.0 (R Core Team, 2013). For experiment one, the sting frequency and duration were analysed with an analyses of variance (ANOVA). For experiment one and two the HPLC data were tested to fit a Gaussian distribution with D'Agostino and Pearson omnibus normality test before being analysed independently with ANOVA or Kruskal-Wallis tests. Post-hoc tests (Tukey's test or Dunn's multiple comparison test) were used to compare the differences between HPLC runs conducted on different days. In experiment two, the BA dataset was analysed additionally with MANOVA to test interrelatedness between amines. For the correlations between behavioural and physiological measures (Expt. 2), the Pearson's r coefficient was used unless the data set did not follow a Gaussian distribution, in which case the Spearman r coefficient was calculated.

Figure 3: Dissection of the brain regions in four parts. OL: optic lobes, GNG: Gnathal ganglia, MB: central brain including mushroom bodies, AL: central brain including antennal lobes.



Results:

Experiment one: Behavioural phases of a short-term physical stress response

Sting frequency (Figure 4A) and duration (Figure 4B) varied between four different groups of bees pinched during either 3 minutes, 5 minutes, 10 minutes or 20 minutes (Sting frequency: ANOVA, $F_{3,62}$ = 5.280, p= 0.0027; Sting duration: ANOVA, $F_{3,62}$= 3.542, p = 0.0199). During the first 3 min, the bee extends its sting briefly (Fig 4B) but frequently (Fig 4A). During intermediate timings, 5 and 10 minutes, we observed a drastic decrease in the number of sting extensions (Fig4 A & C), but these individual sting extensions had an increased duration, also reflected in the total sting duration (Fig4 B). Finally, between 10 and 20 minutes the bees stopped extending their stings (Fig4 A, B, C).

To illustrate further the tendency of the behavioural response over time, durations of sting per 30 sec windows were calculated for the group of bees that were thorax pinched for 20 min (Fig4-C). The corresponding change in sting response over time, fits an inverse exponential curve following the equation $Y=Y_{max}\, e^{k}$. In this equation, Y_{max} is equal to 17.7 seconds and correspond to the maximum sting duration recorded, the parameter K is a rate constant express in inverse seconds is equal to -0.3632.

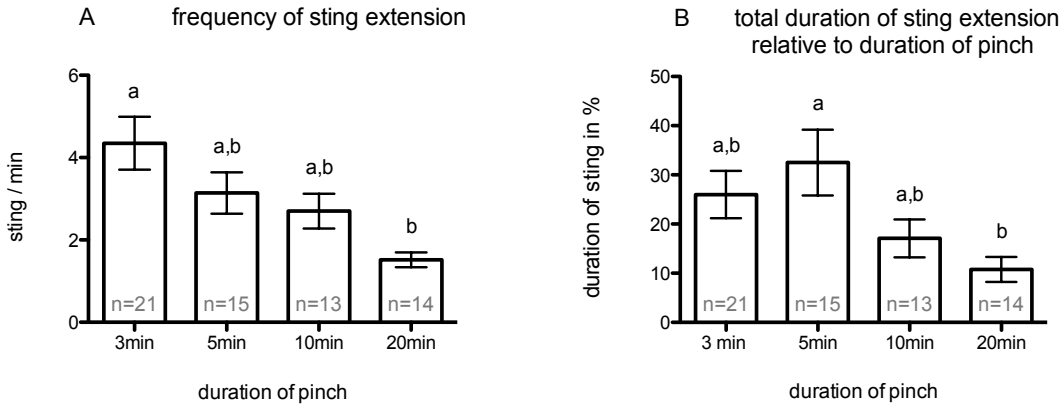
Experiment one: Brain BA levels after short-term physical stressor.

When we analysed the brains of pinched *vs.* control bees we found no significant differences in brain BA levels (Fig 5). In addition, there was no correlation in response to pinching between an individual's level of either amine and its sting frequency (Table 1).

Considering analyses of BA at the brain region level (Fig 6 & 7) we found different amount of each BA between regions (Fig 6). As the four brain regions studied here are variable in size (OL bigger than AL & MB which are themselves bigger than GNG) proportion of each BA were calculated relative to the whole brain (Fig 6). DA level is higher in mushroom bodies than the two other BA, and less dopamine occurs in OL compare to the OA and 5-HT (Fig 6).

We found no significant differences in BA content in any brain region following different durations of thorax pinching in AL, MB and GNG. Mean results and the corresponding statistics are summarised in fig 7 and Table 2. 5-HT levels in the OL increased significantly after 5 min of thorax pinching (Kruskal-Wallis, $H_{4,114} = 10.86$, $p= 0.0282$; Dunn's multiple comparison test, $p < 0.05$).

Figure 4: Behavioural stress responses illustrated by the sting extension frequency and duration while pinched during 20 minutes. (A) Frequency of sting extension during four stress durations: 3, 5, 10, 20 minutes. Each bar represents the mean (±SEM) frequency of sting extensions for each group. Data are analyzed with an ANOVA ($p = 0.0027$, $F_{3,59} = 5.280$). (B) Total duration of sting extension for four durations: 3, 5, 10, 20 minutes. Each bar represents the mean (±SEM) duration of sting extensions for each group. Data are analyzed with ANOVA ($p = 0.0199$, $F_{3,59} = 3.542$). A post hoc Tukey's Test was used to compare the differences between columns, different letters are significantly different with $p < 0.05$. (C) Duration of sting extension per 30 seconds windows during 20 minutes of pinch. The curve fits an inverse exponential function. The equation of the curve was determined with a one-phase decay curve fit in GraphPad Prism where the plateau was constrained to be equal to zero.



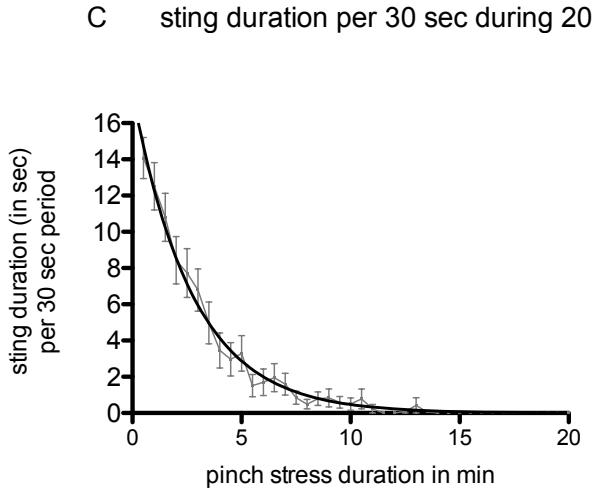


Figure 5: Brain biogenic amines levels after various durations of pinch: 3, 5, 10 and 20 minutes (on graph respectively noted 3,5,10,20) in forager bees. Each bar represents the average (± SEM) amount of the corresponding biogenic amine in whole brains. The numbers inside the columns correspond to sample sizes. Data were analysed with a Kruskal-Wallis test. No significant variations were recorded for any of the biogenic amines: octopamine ($H_{4,123}$ = 6.922, p =0.1401); dopamine ($H_{4,123}$ = 5.941, p =0.2036); serotonin ($H_{4,124}$ = 2.8, p= 0.5919); tyramine ($H_{4,84}$ =5.226, p= 0.2649).

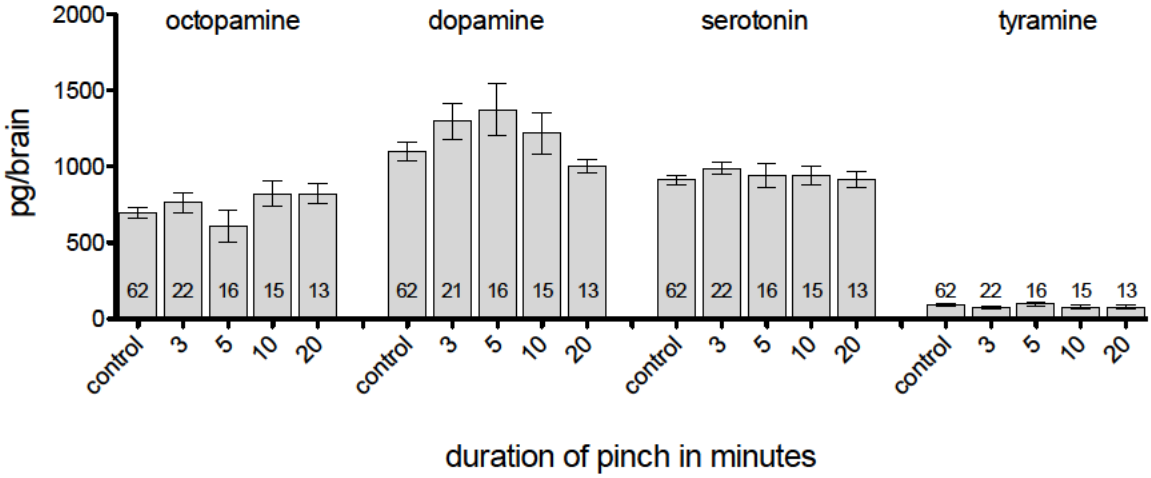
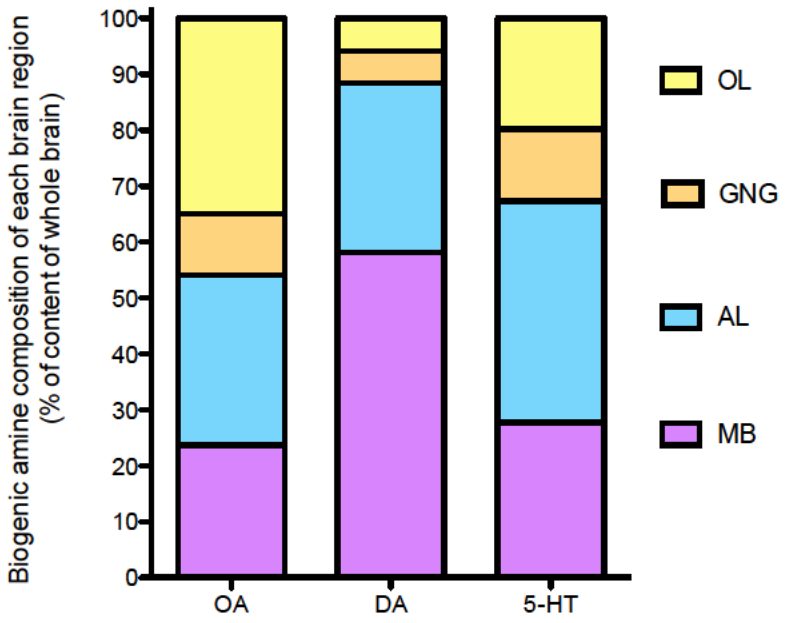


Figure 6: Proportion of biogenic amines per brain region in percentages. The average amount of amines from all of the groups (experiment one) was calculated for each brain region to compare the proportions of biogenic amines in each brain region AL: Antennal lobes; DA: dopamine; GNG: gnathal ganglia; MB: Mushroom bodies; OL: optique lobe; OA: octopamine; 5-HT: serotonin.



Experiment two: Brain BA levels after various and longer physical stressors

BA level differences were interrelated to each other in this experiment (MANOVA, $F_{3,194}$ = 2.673, p = 0.00477; Fig7). There was no significant difference in DA (ANOVA; $F_{3,194}$= 1.008, p = 0.390) levels between groups (Fig 8), however differences between treatment groups were observed for brain levels of OA (ANOVA; $F_{3,194}$= 5.250, p=0.0016) and 5HT (ANOVA; $F_{3,198}$= 4.075, p=0.0078). Whole brain amounts of OA and 5-HT significantly increased in the brains of bees harnessed on their back compared to control bees (Tukey's multiple comparison test, OA: $p < 0.001$; 5-HT: $0.001 < p < 0.01$). When bees were caught and caged or harnessed and shocked, they showed intermediate increases of OA and 5-HT which were not significantly different from those of free-flying controls (Tukey's multiple comparison test, $p>0.05$). The shocked group allowed us to measure a score of behavioural sensitivity as determined by counting the total number of stings (see Materials and Methods): this parameter did not correlate with any of the brain BA levels at the end of the shocks (Fig 9).

Figure 7: Variation of biogenic amines in four different brain regions after various pinch duration. Each bar represents the mean (± SEM) amount of whole brain biogenic amines. The numbers inside the columns correspond to the sample size. Data were analysed with Kruskal-Wallis test (Statistical analysis results are presented in table 2). Dunn multiple comparison test was used for across-group comparisons for each BA (* $p<0.05$). No significant variations were recorded for any of the biogenic amines levels in Antennal lobes (AL), Mushroom bodies (MB) and gnathal ganglia (GNG). Bees pinched bees for 5 mins had higher levels of serotonin than controls (Dunn multiple comparison test, $p < 0.05$).

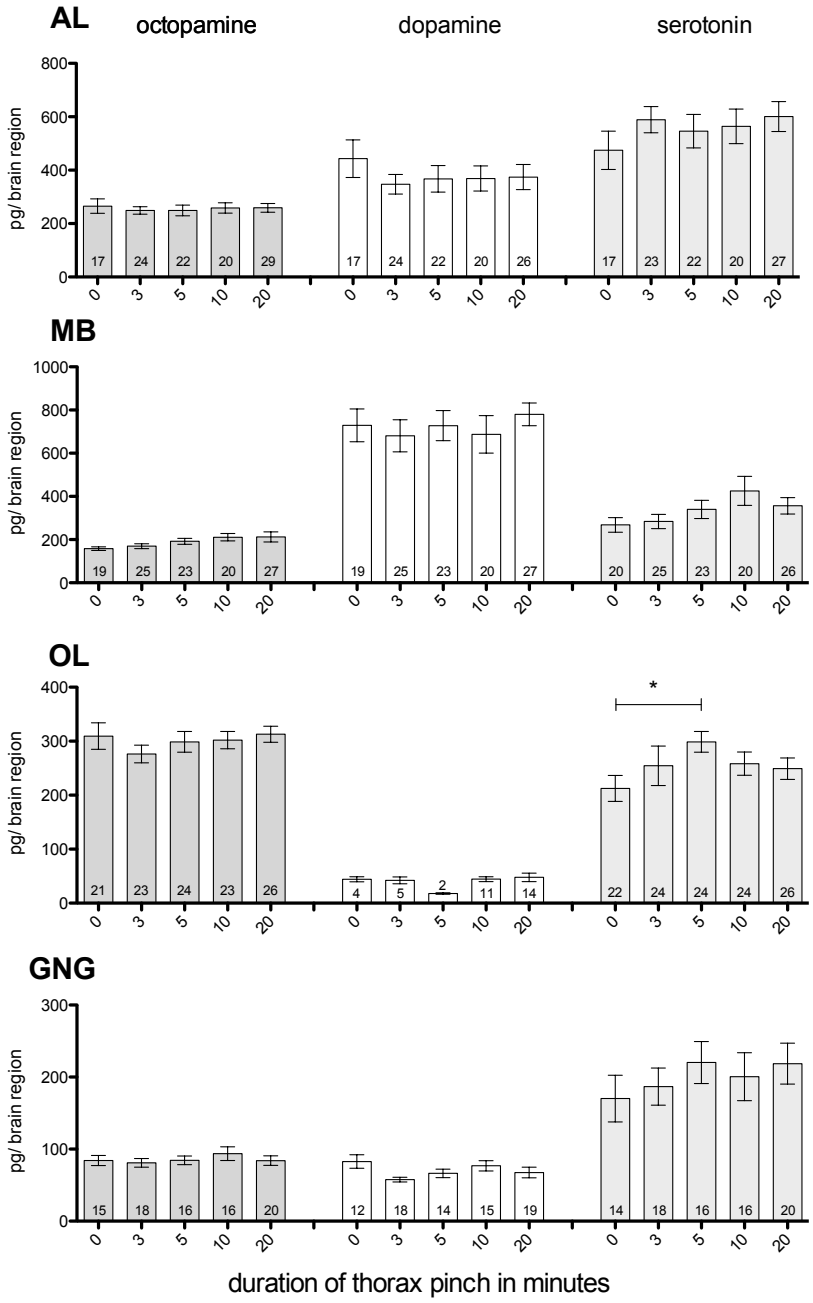


Figure 8: Whole brain biogenic amine levels after long stress applications. Each bar represents the mean amount (± SEM) of the corresponding brain amine in whole brains of bees from groups submitted to increased levels of stress (free-flying, caged, harnessed, and shocked). The numbers inside the columns correspond to sample sizes. A post hoc Tukey's test was used for across-group comparisons for each BA. Significant increases were found in octopamine and serotonin brain levels when bees were harnessed compared to the control free flying bees [octopamine ($p = 0.0016$, $F_{3,195} = 5.280$), serotonin ($p = 0.0078$, $F_{3,195} = 4.075$)]. No differences were found in dopamine levels [dopamine ($p = 0.3903$, $F_{3,194} = 1.008$)]. An additional multivariate analysis of variance did show that the amount of each amine depend on each others for each treatment (MANOVA, $F_{3,194} = 2.673$, $p = 0.00477$). **: $0.005 < p < 0.001$; ***: $p < 0.001$)

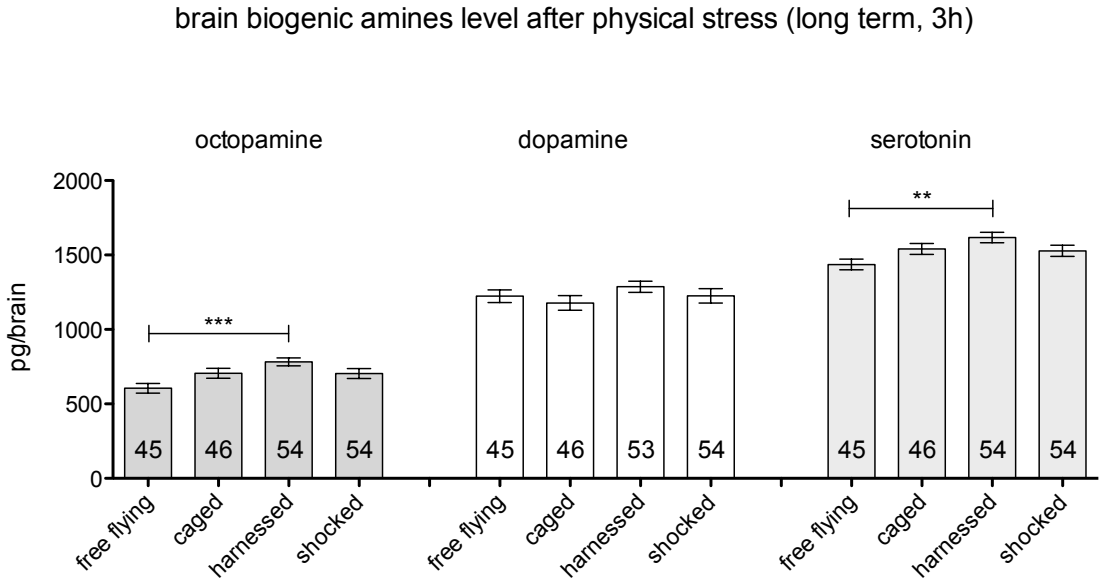
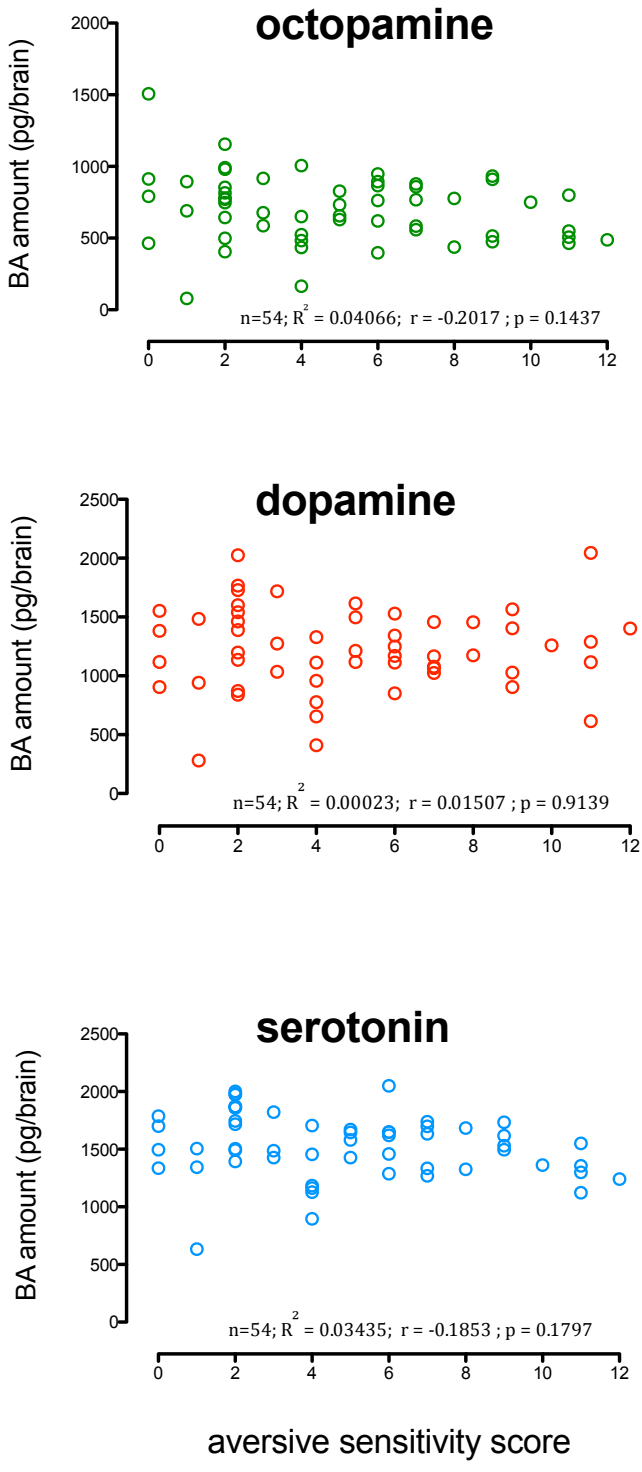


Figure 9: Correlation between the whole brain amine levels and the behavioral stress sensitivity score of shocked bees. Brain biogenic amine levels plotted against the sensitivity score of individual bees from the shocked group. None of the brain biogenic amines are correlated with the individual honeybee sensitivity to the stressor. Each pair of variables were tested with a normality test (D'Agostino and Pearson omnibus). ns = non significant $p < 0.05$.



Discussion:

These two experiments investigated behavioural and physiological stress responses in honeybees. After thorax pinching during 5-20 min (considered here as a short stress), none of the four measured BA levels varied in the whole brain, and only 5-HT increased locally in OL. Applying another restraint stress (harnessing) for a longer time, OA and 5-HT levels increased significantly overall in the brain.

Behavioural stress responses were described for the first time by Walter Cannon as the now famous "fight or flight" syndrome (Cannon, 1915). Later Hans Selye (1936) detailed these responses by describing three main phases in the general stress response, applicable to any biological response: the alarm, resistance and exhaustion phases (Chrousos, 1998; Selye, 1956) using physiological stressors as examples. Similarly here, the intensity of the behavioural response to stress (duration and frequency of sting extensions), varies during continuous stress application, It clearly decreased from a strong response in the first seconds (which would correspond to the "alarm" phase) to a complete absence of response in the last minutes (thus corresponding to the "exhaustion" phase). This analogy is presented in Fig 10 A and B. In our observation, (fig 10 B) the nociceptive stimulus induced a reflex response therefore the biological response is immediate and intense. Indeed, sting extension results from a reflex response with no need to build up and increase in intensity, which is the case for physiological responses to toxin, infections as initially describe by Selye (Selye, 1936). Also, it should be noted that the boundaries of the intermediate "resistance" phase cannot be determined precisely as the response intensity fits with a continuous exponential decrease.

No changes were detected in whole brain BA levels as compared to controls, irrespective of the duration of thorax pinching on a small timescale (3-20 minutes). Since this might be a result of local variations in specific brain regions compensating for each other, we examined specifically BA levels in brain regions. In particular, we were interested in possible changes in the GNG whose neurons innervate the endocrine glands which produce and release the putative stress hormones (Eichmüller et al., 1991; Kreissl et al., 1994; Kreissl et al., 2010). No changes in BA levels were detected in this neuropil after various durations of thorax pinching with our quantification method over this timescale. However, a significant increase in 5-HT amounts was recorded in the OLs after

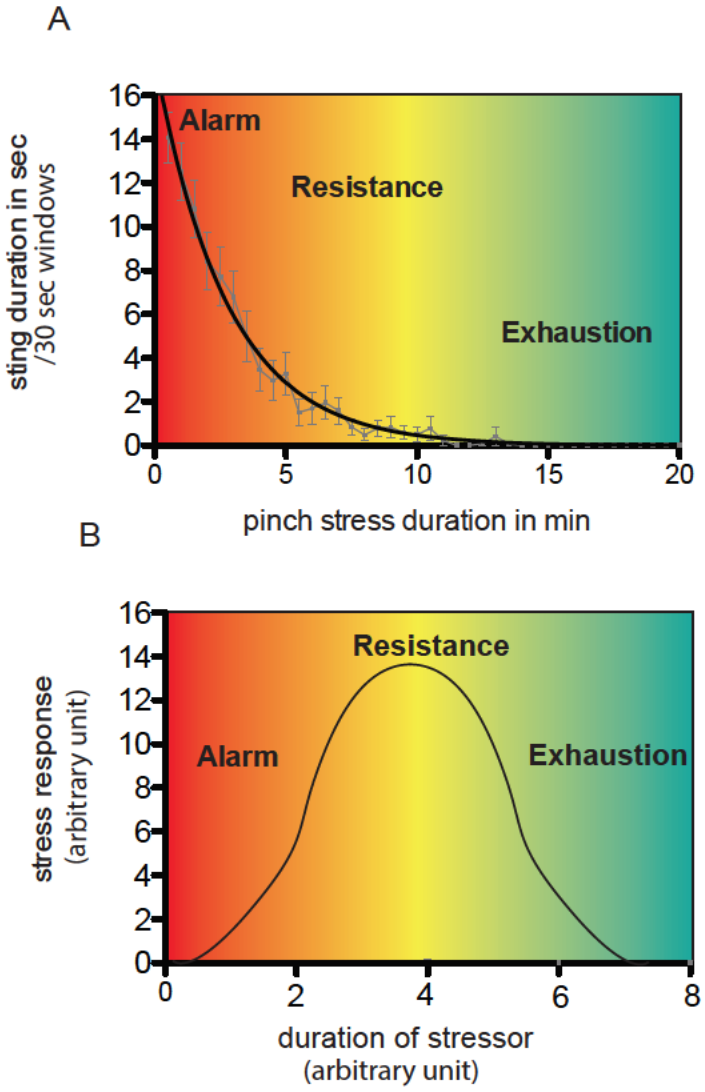
5 minutes of pinching but not after longer stress applications. This neurotransmitter has already been shown to modulate visual reflexes (measured with antennal responses) when injected in different visual neuropils in honeybees (Erber and Kloppenburg, 1995). We can hypothesized that short-term physical stress might therefore induce a fine tune visual sensorimotor signals by serotonin to help to focus on important stimuli.

This first series of results on short-term responses, differ from those of Harris and Woodring (1992), who found increases of OA and 5-HT whole-brain levels after 10 minutes of leg pinching. This discrepancy might arise from a variation in dissection protocols: while we dissected frozen brains, Harris and Woodring (1992) dissected the bee brains in cold saline solution. This latter technique does not impede a possible contamination of brain samples by BAs present in the hemolymph. Indeed, studies using physical short-term stress have shown increases in BA levels in the hemolymph or body periphery (Adamo et al., 1995; Davenport and Evans, 1984; Even et al., 2012; Johnson and White, 2009; Stevenson et al., 2000; Verlinden et al., 2010). It can also be noted that Harris and Woodring (1992) were pinching the leg and not the thorax which could induce a stronger stimulus and therefore be reflected in these different BA level responses.

In experiment two, when the physical stressor was applied for longer, the levels of brain OA and 5-HT increased in harnessed bees as compared to control (free-flying) bees, while intermediate increase levels were found in caged and shocked bees. This result suggests that the nature or intensity of stressor may influence the BA brain profile. Yet, it seems surprising that the greater variations were recorded in a group submitted to an intuitively intermediate level of stress. However, this may be explained by possible local variations remaining undetected here, because we did not quantify BA amounts in separate brain regions in this experiment. For example, a hypothetical increase in some BA levels in the GNG with increasing stress intensity might be masked by other variations (e.g. in the OLs at intermediate stress levels). Harnessing is of common use in honeybee studies, especially when studying responsiveness or learning (Felsenberg et al., 2011; Vergoz et al., 2007). Since changes in brain BA levels have been linked to the behavioural performances related to these processes (Perry and Barron, 2013; Scheiner et al., 2006), our results reveal a possible confounding effect of stress when looking for brain correlates of such performances, and underline the importance of timing and controls when addressing these questions.

All our brain BA level measures need to be understood with respect to BA neurotransmission mechanisms and the protocols used here. Brain BA neurotransmitters are recycled after release in the synapse either by re-uptake in terminal vesicles (by transporters) or enzymatic inhibition (e.g. by N-acetylation) inside the vesicles, or in the synaptic gap (Scavone et al., 1994; Wierenga and Hollingworth, 1990). Besides, the homogenization by sonication of brain tissue before HPLC analysis, as used here, does not allow the separation of extra- and intra-cellular BA levels. For both of these reasons, in the whole

Fig 10. Temporal dynamic of stress response. Analogy of the pattern found in a honeybee stress response (Experiment1) with the general adaptation syndrome (B) (Selye, 1950). In the "Alarm" phase the stressor is detected before the body react in a "Resistance" phase with increase of biological responses, if the stressor is maintained the body enter in an "Exhaustion" phase. These consecutive stages were described initially following physiological stressors. Here an intense physical stressor, source of nociceptive stimuli, induces an intense sting reflex response immediately, which merge "Alarm" phase to the beginning of "Resistance" phase.



brain, an elevation in BA level cannot be a record of a synaptic release alone, but can only be detected if there is an influx from the periphery to the brain or a net synthesis. In this study, when the bees are harnessed for three hours the duration of the stressor might allow such an influx or synthesis of BA to accumulate in the brain. Similarly a decrease in BA level could only be detected if there was a net degradation post-release without new synthesis of neurotransmitters. As a result, balancing of increased synthesis and degradation cannot be detected with our method, especially in the whole brain where the size of the network would dilute any detectable effect. Such a net degradation might explain the drop in the levels of OA and 5-HT when the bees are shocked in the long-term stress experiment compared to other stressed groups (harnessed and caged). That is the increased level of OA and 5-HT induced by the harnessing might be reduced by a net degradation of released BA from the additive effect of electric shock.

While BA release alone could not be detected in the whole brain, it might be detected at the brain region level by a massive BA flow between different brain regions. In this study 10 minutes of thorax pinch induced an increased 5-HT in OLs, possibly resulting from a significant synaptic serotoninergic release in this visual region of the brain.

Interestingly, BA levels seem to be interdependent as OA and 5-HT increase in a similar pattern through the long-term stress treatments (MANOVA analysis in experiment two). This result may indicate that BAs share some of their metabolic pathways but also their pharmacological properties. Tyrosine is the direct precursor of TYR, itself a precursor of OA as well as a degradation product of DA. DA is also an indirect product of Tyrosine *via* dihydroxyphenylalanine, known as DOPA (Farooqui, 2012). Biosynthesis of one BA may therefore affect the level of others.

More studies on pharmacological and metabolic mechanisms would complement our data to clarify mechanisms of BA turnover and the correlated increase in OA and 5-HT under various physical stress conditions. Our HPLC system was inappropriate to detect BA metabolites such as N-acetyldopamine or norepinephrine (Sasaki and Nagao, 2001).

It was also surprising that no change in DA levels after stress were found when DA has been linked to stress in insects in the literature. Stress has been shown to increase DA precursor levels in fruit flies (Gruntenko and Rauschenbach, 2008; Neckameyer and

Weinstein, 2005). In honeybees, however, brain DA levels decrease after physical stress when bees are caught in small tubes or spun in a centrifuge (Chen et al., 2008). This last result is not repeated in our study as we only found stress to affect OA and 5-HT levels, these differences of results might be a further evidence for different stressors affecting different BA systems.

To summarise, BA levels don't seem to be indicative of short-term physical stress in honeybees since 20 minutes of thorax pinching did not cause any detectable changes in brain BA levels. This interpretation is reinforced by the absence of any correlation between sting frequency and brain BA levels during applied pinch stress. On the contrary, our results demonstrated a potential role for OA and 5-HT in the response to longer stress exposures, which might justify their use as stress indicators in similar context.

Supplements

Table 1: Correlation tests between the total brain amine levels and the sting extension frequency for all the different durations (3, 5, 10, or 20 min). Each pair of variables was tested with a normality test (D'Agostino and Pearson omnibus). According to the result the Pearson (parametric) or Spearman (non-parametric) test was applied. ns = non significant (P> 0.05).

	number of XY pairs	Pearson r * spearman r	P value	P value summary	R^2	95% CI
OA x 3 min sting frequency	18	0.4605	0.0544	ns	0.2121	-0.008201 to 0.7633
OA x 5 min sting frequency	14	-0.03883	0.8952	ns	0.001507	-0.5580 to 0.5022
OA x 10 min sting frequency	11	0.3389	0.308	ns	0.1148	-0 3277 to 0.7802
OA x 20 min sting frequency	13	0.3466*	0.2459	ns		-0 2698 to 0.7615
DA x 3 min sting frequency	18	-0.08436	0.7393	ns	0.007117	-0.5304 to 0.3983
DA x 5 min sting frequency	14	0.1389	0.6357	ns	0.0193	-0.4229 to 0.6236
DA x 10 min sting frequency	11	-0.004783	0.9889	ns	0.00002288	-0 6030 to 0.5969
DA x 20 min sting frequency	13	0.2071	0.4973	ns	0.04287	-0 3883 to 0.6805
5HT x 3 min sting frequency	18	0.304*	0.22	ns		-0 2043 to 0.6832
5HT x 5 min sting frequency	14	0.1389	0.6357	ns	0.0193	-0.4229 to 0.6236
5HT x 10 min sting frequency	11	0.1563	0.6463	ns	0.02442	-0.4896 to 0.6914
5HT x 20 min sting frequency	13	-0.04375	0.8872	ns	0.001914	-0.5808 to 0.5199
TYR x 3 min sting frequency	14	0.272	0.3469	ns	0.07397	-0 3023 to 0.7014
TYR x 5 min sting frequency	8	0.05784	0.8918	ns	0.003345	-0 6744 to 0.7327
TYR x 10 min sting frequency	10	0.5046*	0.144	ns		
TYR x 20 min sting frequency	9	0.4745	0.1968	ns	0.2252	-0 2770 to 0.8658

Table 2. Statistical results details of regional brain biogenic amines (BA) levels variation after short-term stress (experiement one). Data presented on fig 7. Data were analysed with Kruskal-Wallis test. AL: Antennal lobes; MB: Mushroom bodies; GNG: gnathal ganglia; OL: Optique lobe. ns = non significant; * p< 0.05.

	BA	n	Df	H	P value summary	p value
AL	octopamine	111	4	0.6589	ns	0.9563
	dopamine	103	4	1.021	ns	0.9066
	serotonin	103	4	2.527	ns	0.6398
MB	octopamine	108	4	8.922	ns	0.0631
	dopamine	108	4	2.935	ns	0.5688
	serotonin	108	4	4.972	ns	0.2902
GNG	octopamine	79	4	0.8259	ns	0.9349
	dopamine	72	4	6.665	ns	0.1547
	serotonin	78	4	1.616	ns	0.8060
OL	octopamine	114	4	10.86	ns	0.6080
	dopamine	111	4	2.707	ns	0.1996
	serotonin	30	4	5.994	*	**0.0282**

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Chapter 3

Guard and soldier honeybees show distinct behavioural responses to aversive stimuli: implications for the organisation of division of labour

Abstract

The honeybee is one of the best examples of a complex animal society in which worker bees show a clear division of labour. The behavioural role of each individual is influenced by their age, brain biogenic amine profile, and also variation in responsiveness to key task related stimuli, like sucrose or pheromones. Here we explore if sting responsiveness to aversive stimuli varies between behavioural castes. We examined two defensive subcastes (guards and soldiers) who are more likely to respond to aversive stimuli in their tasks. We found that guards were very sensitive to an aversive electric shock when compared to soldiers and foragers, but the degree of difference was dependent on age. Nurses, unexpectedly, were highly sensitive to aversive shocks independent of the age of the nurses. Quantification of brain biogenic amines levels showed clear differences between the behavioural castes, but did not correlate with shock sensitivity. These results emphasized a distinction between the two defensive subcastes: guards and soldiers, and are discussed in regard to current division of labour theories.

Introduction

In honeybees, there is a clear division of reproductive functions with morphologically distinct reproductive queen and sterile worker castes (Winston, 1987). The sterile female workers perform all of the colony's work. All workers are morphologically identical to each other, however different individuals specialize on different behavioural roles in their colony such as foraging, nursing or defensive functions (Seeley, 1995). Division of labour is currently explained by various complementary models, including: social inhibition, temporal polyethism (Robinson, 1992), and response threshold models (Beshers & Fewell, 2001). These behavioural castes are organised principally by worker age (Seeley, 1982). Young bees perform in-hive tasks like taking care of the queen, feeding the brood, building the combs, and storing food. At around two weeks of age they start to go out of the hive and perform orientation flights, before progressing to outside tasks like pollen and nectar foraging. At around two weeks of age, some of those bees, but not all, will also perform guarding tasks (Moore et al., 1987).

Defensive roles are important for the survival of the honeybee colony as they protect against intraspecific robbing or predation (Breed et al., 1992; Breed et al., 2004). Guard bees patrol the entrance, and are recognisable by their distinct posture with their antennae typically directed forwards and their forelegs up (Breed et al., 1990; Butler and Free, 1951; Moore et al., 1987). They inspect the entrance to prevent entry by bee pests or non-nestmates to prevent robbing or intraspecific parasitism (Breed et al., 1992). When a significant disturbance occurs at the hive entrance guards react in releasing alarm pheromones (Moore et al., 1987). This alarm triggers a second wave of defensive bees to fly out from inside the hive. These are the "soldiers", which pursue and attack larger predators by stinging them (Butler and Free, 1951; Free, 1961). "Guards" and "soldiers" bees are two distinct subcastes described in the literature. Soldiers are a similar age to foragers, but with less flight experience (evidenced by less wing wear, (Breed et al., 1990; Cunard and Breed, 1998). They also show physiological traits that distinguish them from foragers and guards, such as hypopharyngeal gland development, and brain biogenic amine levels differences (Huang et al., 1994; Wagener-Hulme et al., 1999). Moreover, genotypic differences have been shown between guards and soldiers in several studies (Alaux et al., 2009; Arechavaleta-Velasco et al., 2003; Guzman-Novoa et al., 2004; Hunt et al., 2007; Uribe-Rubio et al., 2013).

To defend the colony, guards and soldiers act sequentially. Guards patrol the entrance all the time (Moore et al., 1987) and are the first to react to a threat by releasing alarm pheromones. This alarm triggers the second stage of defence by recruiting the soldiers from inside the hive to attack (Butler and Free, 1951; Free, 1961). If the threat to the colony is great, the recruitment of workers by alarm pheromone will be high, and in these cases it is not uncommon to find guarding and foraging bees mixed in the attacking pool of soldiers (Breed et al., 2004). This plasticity in division of labour illustrates that these categories of guard and soldier are not completely distinct, and can have overlapping behaviours especially attacking and attraction to alarm pheromones. For this reason some studies do not draw a distinction between guards and soldiers, with the term soldiers not yet universally accepted in the social insect literature (Breed et al., 2004). Those studies that do draw this distinction, define guards as bees from the entrance, and soldiers after recruitment following an effective disturbance and at a longer distance from the entrance (Breed et al., 1990) to differentiate the first- and second-wave of bees reacting to the threat.

Division of labour, and the plasticity of behavioural roles can be understood through the response threshold model theory (Beshers and Fewell, 2001). In this theory, individual task specialisations are explained by differences in the responsiveness to key task-related stimuli. It is expected that bees more sensitive to a specific stimulus (e.g. nectar or sugars) will perform specific tasks related to this stimulus (e.g. foraging). Stimuli can include communicative signals like pheromones (from the brood, the queen, or to alarm…), environmental cues that signal the needs of the colony (pollen and nectar stores, infected brood cells…) or a combination of both (Fewell and Winston, 1992; Pankiw and Page, 2001). Many honeybee tasks have been linked to specific variation in threshold responses towards task-related stimuli: nursing and brood pheromones (Pankiw and Page, 2001), queen care (Pham-Delegue et al., 1991), hygienic behaviour (Masterman et al., 2001), undertaker tasks (Trumbo et al., 1997), fanning (Cook and Breed, 2013), foraging (Scheiner and Erber, 2009). In this study we address the hypothesis that guards which are the first to react to threat will have a lower threshold response to a stressor compared to other types of bees who react only in a second stages when recruited.

Individual differences in thresholds have been largely linked to hereditable factors (Robinson and Page, 1988; reviewed in Zayed and Robinson, 2012), changes in stimulus

responsiveness with age (Pankiw and Page, 1999; Paxton et al., 1994), and some neuromodulation of gustatory and visual stimuli responses (Erber et al., 1993). The influence of genetic background on defence behaviour is particularly well illustrated by numerous studies in Africanised versus European bees (Guzman-Novoa et al., 2004; Uribe-Rubio et al., 2008). These two lines of bees differ in their defence behaviour and sting response threshold to shocks. Africanised bees, more aggressive are more sensitive to shocks than European bees (Uribe-Rubio et al., 2008).

Response thresholds understood via "behavioural syndromes"

Response threshold models have proven an extremely useful conceptual tool for exploring division of labour, but the models do have some limitations and their application to social insect systems is not without some problems. For application to biological systems, response threshold models of task specialisation must consider variation in responsiveness to multiple environmental stimuli (Barron and Robinson, 2008; Beshers and Fewell, 2001). A documented challenge to the response threshold model of division of labour, however, has been observed among different types of forager honeybee (Page et al., 1998). Sucrose sensitivity is higher in nectar foragers than in pollen and water foragers (Pankiw and Page, 1999; Scheiner et al., 2001; Scheiner et al., 2003). A prediction from the response threshold model would be that foragers with the highest sensitivity to sucrose should collect nectar (rich in sucrose) rather than pollen or water. It seems that the response threshold model cannot be simply applied to the food preferences of forager bees. Why this is so is not well understood. One possible explanation is that reinforcing social feedback that foragers receive from the colony on the resources they return (Tezze and Farina, 1999) inhibits high-sucrose-sensitivity foragers from returning to the hive with low sugar concentration nectars. The social feedback from the colony instead focuses these high-sucrose sensitivity foragers on pollen which provides the most energy gain for the colony (Scheiner and Erber, 2009; Scheiner et al., 2004), as previously shown in field experiments (Frisch, 1967; Seeley, 2009). This example illustrates how the response threshold model does not simply apply to all aspects of bee foraging.

Analyses of defensive behaviour have also presented some challenges to interpretation by the response threshold model (Roussel 2009). Roussel et al. (2009)

assessed guarding bees as being less sensitive to a stressful stimulus (electric shock) than foragers. This result is counterintuitive regarding the response threshold model as guard bees should be more sensitive to threats than foragers because they are to be the first to react. This result is also surprising as bees of 14-20 days (the age of guards) have been shown to be more sensitive to electric shock than older bees (forager age) and callow bees (Kolmes and Fergusson-Kolmes, 1989; Paxton et al., 1994). The authors argued that a high sensitivity would be costly for the colony (i.e. not adaptive), because bees die after stinging, therefore it is not in the colony's interest to expend guards on low-level threats.

Both of these challenges to the response threshold model have been explained by the proposition of behavioural syndromes mostly based on the dilemma of nectar foragers response threshold. The variability of responsiveness for sucrose has been correlated to variation in other traits and responsiveness to stimuli, like phototaxis, odour responses, learning (Scheiner et al., 2001; Scheiner et al., 2003; Scheiner et al., 2004) and locomotion, leading to the proposition of a "forager behaviour syndrome" (Pankiw, 2005). In this explanation, behavioural specialization should be considered as the outcome of a complete syndrome rather than responsiveness to any single stimulus. Similarly a "defensive behaviour syndrome" was suggested as low aversive learning performances were found to correlate with low shock sensitivity in guards compared to forager bees (Roussel et al., 2009). These two parameters would thus reflect the correlation of broader defensive behaviours such as reactions to intruders, attraction to alarm pheromones or to moving objects (Tedjakumala and Giurfa, 2013). Yet, within this theoretical frame the distinction between guards and foragers remains unclear.

In this study we measured the response threshold to electric shock in guard, soldier, nurse and forager bees to examine whether the division of labour between these groups could be best explained by a response threshold or a behavioural syndrome model. Guards are expected to have a lower threshold reponse than foragers and soldiers in a response threshold model in contrast to the behavioural syndrome model which expect that defensive bees (soldiers and guards) have a higher threshold than foragers (Roussel et al., 2009).

Age and in division of labour

Age, is one of the most important factors to consider in the influence of individual task choice (Robinson, 1992; Winston, 1987). The effect of age and behavioural caste on response thresholds can be studied separately using an artificially constructed colony composed of bees from the same age (Barron et al., 2002; Schulz and Robinson, 1999; Wagener-Hulme et al., 1999). In these colonies a division of labour occurs via environmental clues and individual genotypic background that is independent of age. Consequently in this study we made use of single age cohort colonies to test how age and behavioural role, affected sting responsiveness to electric shock.

Brain biogenic amines in division of labour

In honeybees, variation in brain biogenic amine levels has been also related both to stimulus responsiveness, behavioural specialization and stress responses (see chapter 2). Also brain levels of the biogenic amines: octopamine (OA), dopamine (DA), and serotonin (5-HT) have been correlated to age, and shown to increase in various tasks in bees as they age (Wagener-Hulme et al., 1999). Callow bees have very low levels of the three biogenic amines and forager bees have the highest level. Intermediate age bees have intermediate levels of brain biogenic amines (Wagener-Hulme et al., 1999).

Experiments

Our objective in this study was to carefully examine how variation in responsiveness to stressful shock stimuli might correlate with task specialization, considering particularly the guard and soldier subcastes. In experiment 1 and 2 we evaluated the sensitivity to electric shock in various types of bees including guards and soldiers using a sting threshold measure in a harnessed set-up. Then we tested in experiment 3 how age affects shock sensitivity using single age cohort colonies when bees artificially divide the labour independently from their age. Finally in experiment 4 using the bees sampled in experiment 1 and 2 we investigated how brain levels of biogenic amines vary with behavioural castes and stress.

Methods

Experiment 1

The aim of the first experiment was to compare sensitivity to electric shock for callow bees, foragers and guards (defined as the first reacting bees at the entrance). This first experiment tested whether the sensitivity of guards versus foragers to electric shock corresponded to the response threshold model.

Collection of bees: To collect guards, black feathers were shaken close to the hive entrance (between 5cm to 10cm away) for 5 to 10 seconds to attract only the first reacting bees. The first bees to attack the feathers were trapped in a cubic Perspex container. In this situation no obvious recruitment of soldiers (second wave of attacking bees) was noticed. Bees were collected gently in 15mL tubes, which were immediately immersed in wet ice.

Foragers bees were collected at the entrance of the hive on their return to their foraging trip directly into 15mL tubes. Pollen foragers were distinguished by the pollen load on their legs, and nectar foragers by their extended abdomen. Callow bees were collected directly from brood frames, and were distinguished from the other bees by their white furry hairs on the thorax. As each collection was separated by 15 minutes, the waiting time before shock was not negligible. The order of each caste collection was counterbalanced between experimental days. Collection of a specific subcaste was collected first on one day, second another day then third *etc*...

Shock sensitivity assessment: The same procedure was used as in chapter 1. In brief, bees were anaesthetized by chilling on ice (between 5-10 min) directly after collection. After attachment to the shocking base with connecting gel, bees were left for 45 minutes, and then received a series of shocks of increasing voltage (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 volts). This assay determined an individual shock sensitivity score for each shocked bee corresponding to the number of sting extensions throughout the sequence of 12 shocks. Sting extensions were recorded to calculate the proportion of bees demonstrating a sting response at each voltage in each group. If the proportion of bees stinging at a low voltage is high, this group of bees will be qualified as very sensitive to shocks. At the end

of the shock test, bees were then frozen in liquid nitrogen for the analysis of brain biogenic amine levels in experiment 4.

Experiment 2:

The aim in this experiment was to explore more precisely the shock sensitivity of nurses and foragers and to compare them to the two types of defensive bees: guards and soldiers. To further emphasise the separation between guards and soldiers, in experiment 2 guards were sampled at the nest entrance and soldiers as the second attacking pool after recruitment.

Collection of bees in experiment 2: Foragers were collected similarly to that described for experiment 1. Nurse bees were collected directly on a brood frame by sampling bees inserting their head into a cell containing larvae of 3 days old or less (aged by instar). To guarantee a separation between guards and soldiers, in experiment 2, we collected guards following methods used in previous studies defining them as bees patrolling the entrance in advance of a threat (Moore et al., 1987). Hence, guards were detected on the board of the entrance of the hive, anntenating with returning foragers and displaying typical guard behaviour (pursuing, front legs up, antennae pointing forward [see Breed et al., 2004]). For the collection of soldiers, the hive was first threatened by shaking a stick at the entrance, then black feathers were shaken at one meter from the entrance of the hive to collect the bees that flew to a threat as the second wave of bees reacting to a disturbance. In this collection, there was an obvious recruitment of soldiers from inside, all leaving the hive together following the disturbance: a dozen to a hundred depending on the size of the colony itself. Once sampled, these bees were used to assay responsiveness to electric shock as in experiment 1, and then frozen in liquid nitrogen for brain biogenic amine analysis. In this experiment bees of each subcaste were also collected and immediately frozen in liquid nitrogen without exposure to shock as a control group as testing the effect of stress on brain biogenic amine levels between subcastes (experiment 4).

Experiment 3:

The goal of experiment 3 was to test the influence of age on sting responsiveness using single age cohort colonies, and to test if differences in the sensitivity of the

behavioural castes were influenced either by age or behaviour. Collection of bees and shock responsiveness of bees were performed with the methods described for experiment 2.

Foundation of single age cohort colonies: Three single-age cohort colonies based on previous published methods (Schulz and Robinson, 1999; Wagener-Hulme et al., 1999) were built during the Australian summer in February, October and December 2012 respectively. For each colony approximately ten brood frames were collected from 10 to 15 hives located on two different apiaries in a Sydney suburban area. The frames were incubated at 32 degrees Celsius and 65% humidity. For the following two days bees that emerged from brood frames overnight were painted on the thorax with enamel paint, using a different colour for each day to allow recognition of bee age. The colony contained bees born on consecutive days. Between 2000 and 3000 bees were painted each day, resulting in three colonies of 4500, 5500 and 6000 bees. As soon as the bees were painted they were inserted in a 4-frame nucleus hive. Two frames contained honey and pollen and two empty frames to encourage foraging and provide room for brood. A young fertilized queen was introduced to each colony. The hives were kept in the incubator for the two first days with a plastic mesh on top to allow air circulation. On day three the hive was placed outside and bees were free to fly outside. Forager bees and eggs were observed at day 8 in each hive indicating a successful onset of foraging and brood rearing behaviour. Bees of the different behavioural castes were collected and tested at two different time points. First, middle age 13-14 days old bees were used, corresponding to the typical age of defensive bees in a normal demography colony (Moore et al., 1987). In the second collection we used older bees of 28-29 days old, which corresponds to the age of summer foragers, and which is an old age for nurses (Robinson, 1992).

Experiment 4:

This experiment tested if differences in sensitivity of the behavioural castes could be related to any variation in brain biogenic amine levels. For this, we used brains extracted from bees sampled in experiments 1 and experiment 2.

Biogenic amines level quantification: Brain biogenic amine levels were measured with high-pressure liquid chromatography (HPLC) following the protocol used by Søvik et al. (Søvik et al., 2013) using an Agilent 1200 Series HPLC system (Agilent Technologies,

Santa Clare, CA, USA) coupled to an electrochemical detector (ESA coulechem III) and connected to a dual electrode analytical cell (ESA, Chelmsford, MA, USA). The full description of the protocol is detailed in chapter 2.

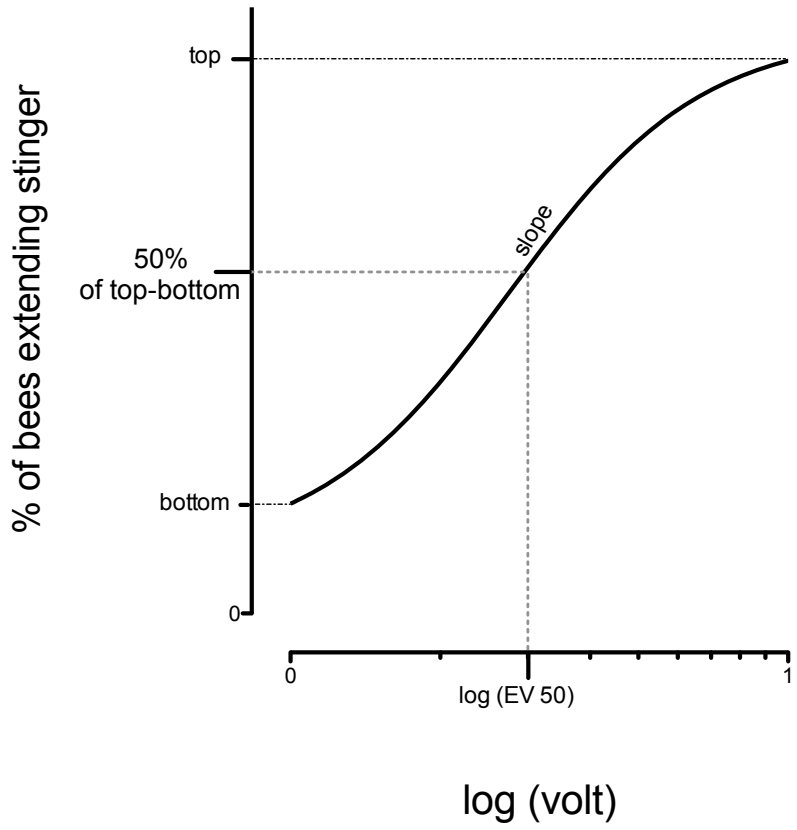
Statistical analysis

All statistical analyses were performed using Graph Pad Prism (version 5.0b). In experiment 1, 2, and 3 the sensitivity to electric shock effect was detected not only at one voltage but across an increasing voltage scale, therefore a dose-response analysis was performed.

First the voltage scale was transformed in a logarithmic scale to apply a nonlinear fit (proportion of sting response fit against the log of the voltage). The equation (Y=Bottom + (Y=Bottom + (Top-Bottom)/(1+10^((LogEV50-X)*Slope)))) was used to fit the data (Fig 1) and tested with Akaike information criterion test was apply to determine if the equation of best fit. Among the parameters the Log of half the maximum effective voltage (EV50) for each fit was calculated. Log EV50 gives the log of the voltage corresponding to the response half way between minimum and maximum of the response curve (Fig 1). Then an extra-sum-of-square (F test) was used to test if the fits were significantly different. This test compares whether the parameter log EV50 is significantly shared or not by each fit. The value EV50 is used in this paper as a comparative measure describing at which voltage each behavioural caste is more likely to start to extend the sting on the scale (used here as the response threshold).

In experiment 4, biogenic amine levels presented in fig 4a were analysed with a Kruskal-Wallis Test, followed by a post-hoc test (Dunn multiple comparison test) to compare the differences between columns. The differences in biogenic amines levels before and after stress in various subcastes, were analysed with a Mann Whitney test, and are reported directly on the graph. The effect of caste or stress or the interaction of both variables on sting responsiveness were analysed with a two way ANOVA (Table 4). The differences of brain biogenic amine levels between castes were analysed with ANOVA then with Tukey post-hoc test (Table 5).

Figure 1: Graphic representation illustrating the parameters used for the equation fit. Log of half the maximum effective voltage (EV50) for each fit was calculated. Log EV50 gives the log of the voltage corresponding to the response half way between minimum (bottom) and maximum (top) of the response curve. The equation (Y=Bottom + (Y=Bottom + (Top-Bottom)/(1+10^((LogEV50-X)*Slope)))) was used to fit the data. If the slope is equal to 1 the equation contains three parameters, if the slope is different from 1 the equation contain 4 parameters. Akaike information criterion was used to select the equation that is most likely to have generated the data.



Results

In experiment 1 (Fig 2), the sting response threshold to electric shock of callows, guards and foragers was examined. The three response curves are significantly different ($F_{(4,24)}$ = 107.5; $p < 0.0001$). Callow bees showed a very high response threshold (EV50 = 7.1 volt) meaning a greater proportion of callow started to sting at higher voltages compared to guards and foragers. Guard bees were the most sensitive in this assay, with a very low response threshold to shocks (EV50 = 2.2 volts) and foragers showed an intermediate response threshold compared to callows and guards (EV50 = 3.6 volts).

Experiment 2 (Fig 3) examined the shock sensitivity of nurses, guards, soldiers and foragers. Guards were significantly more responsive than foragers and soldiers (Table 1), and were more likely to start to extend their sting at lower voltage (EV50 = 1.3 Volts). Nurses appear also to be very sensitive to low-voltage shocks with a low EV50 (2.6 volts) compared to foragers (EV50 = 6.8 volts) and soldiers (EV50 = 5.8 volts), however the response curve of nurses were not significantly different from forager and soldiers with borderline statistical values (respectively p= 0.0522 and p= 0.053).

Experiment 3 (Fig 4) tested the shock sensitivity of nurses, guards and soldiers sampled from a single age cohort colony (SCC) at two ages. At 13-14 days old (Fig 4A), the nurses were the most responsive to electric shock with a response threshold of 1.9 volt. Foragers, guards, and soldiers were less sensitive and had respectively a sting response threshold of 3.1 volts, 3.2 volts and 3.8 volts. Nurses' response curves differed significantly from guards and soldiers, but not from foragers (Table 2). At 28-29 days old (Fig 4B), nurses were again the most sensitive to electric shock with a response threshold of 1.9 volts. Foragers, guards, and soldiers were less sensitive and have respectively a sting threshold of 6.4 volts, 4.6 volts and 4.0 volts. Nurses' response curve differed significantly from guards and foragers but not from soldiers (Table 3).

In experiment 3, hypopharyngeal gland sizes were significantly larger in nurses than in other subcastes at both age 13-14 days old (ANOVA, $F_{3,286}$= 23.60, p<0.0001) and 28-29 days old (ANOVA, $F_{3,282}$= 24.65, p<0.0001) when data were pooled from all colony replicates (Fig 5).

Figure 2: Shock sensitivity of callow bees, guards and foragers (experiment 1). Proportion of bees displaying a sting extension in response to a shock (grey lines) was fitted against the log of the voltage applied at each shock. All the fitted curves (colour) are significantly different ($F_{(4,24)}$, 107.5; $p < 0.0001$). The guard bees are the most sensitive to electric shock and tend to sting at 2.2 volt. Foragers tend to sting at 3.6 volt; New born bees are the least sensitive to electric shock as they tend to sting at 7.1 volt.

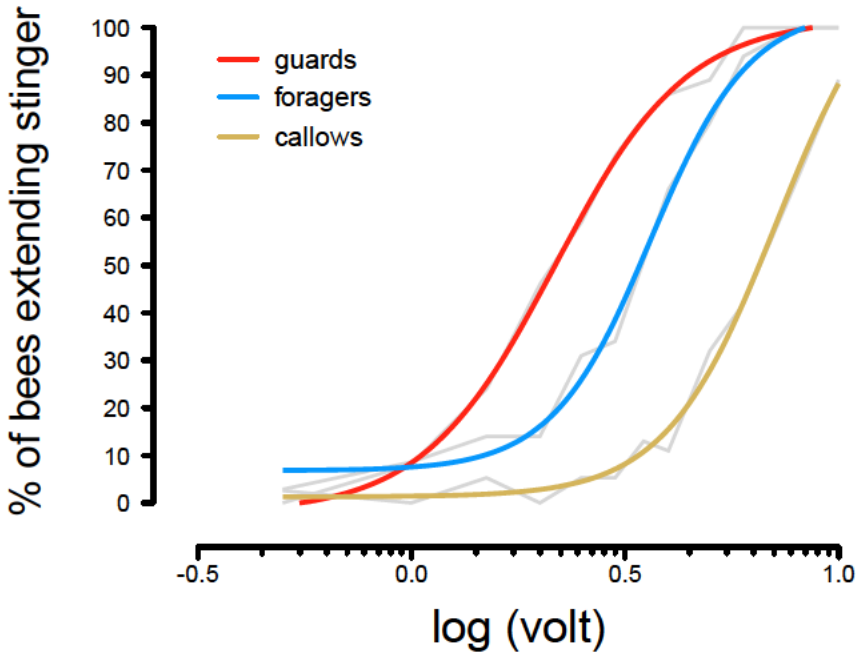


Figure 3: Shock sensitivity of nurses, guards, soldiers and foragers (experiment 2). Proportion of bees displaying sting extensions fit against the log of the voltage applied at each shock. The nurses and the guard bees are the most sensitive to electric shock and tend to sting respectively at 2.6 volt and 1.3 volt. Foragers and soldiers are the least sensitive to electric shock as they tend to sting at 6.8 volts and 5.8 volts. respectively. The response curve for nurses is significantly different from that of soldiers ($F_{(1,18)}$4.322; p = 0.0522) and foragers ($F_{(1,18)}$ 4.276; p = 0.0533). The response curve for guards is significantly different than that of soldiers ($F_{(1,18)}$ 11.76; p = 0.003) and foragers ($F_{(1,18)}$ 10.30; p = 0.0049).

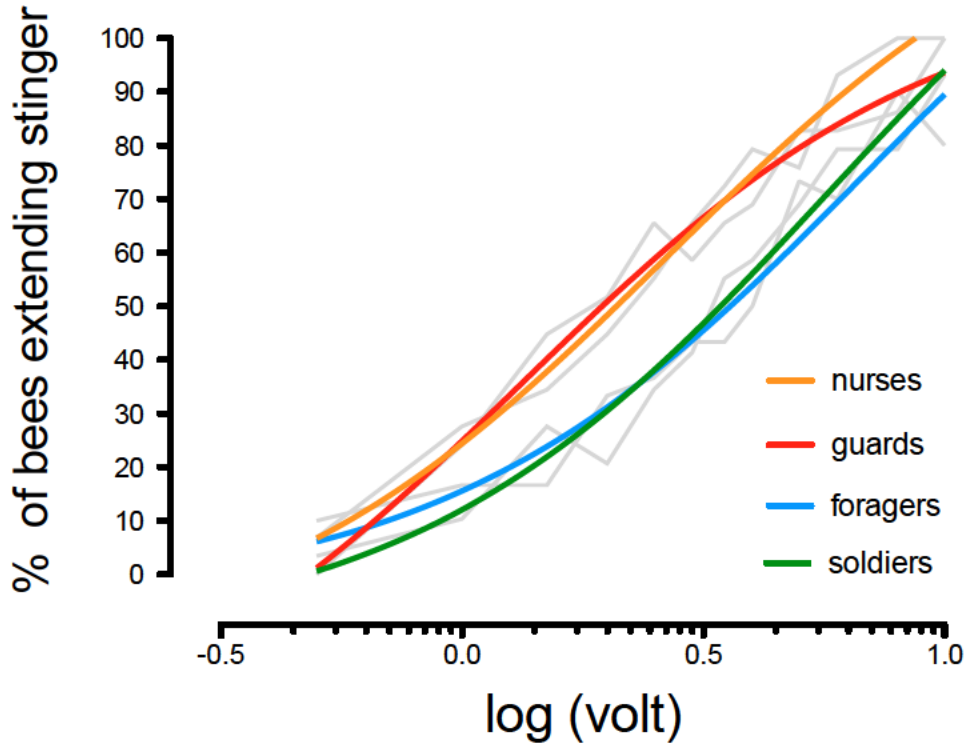
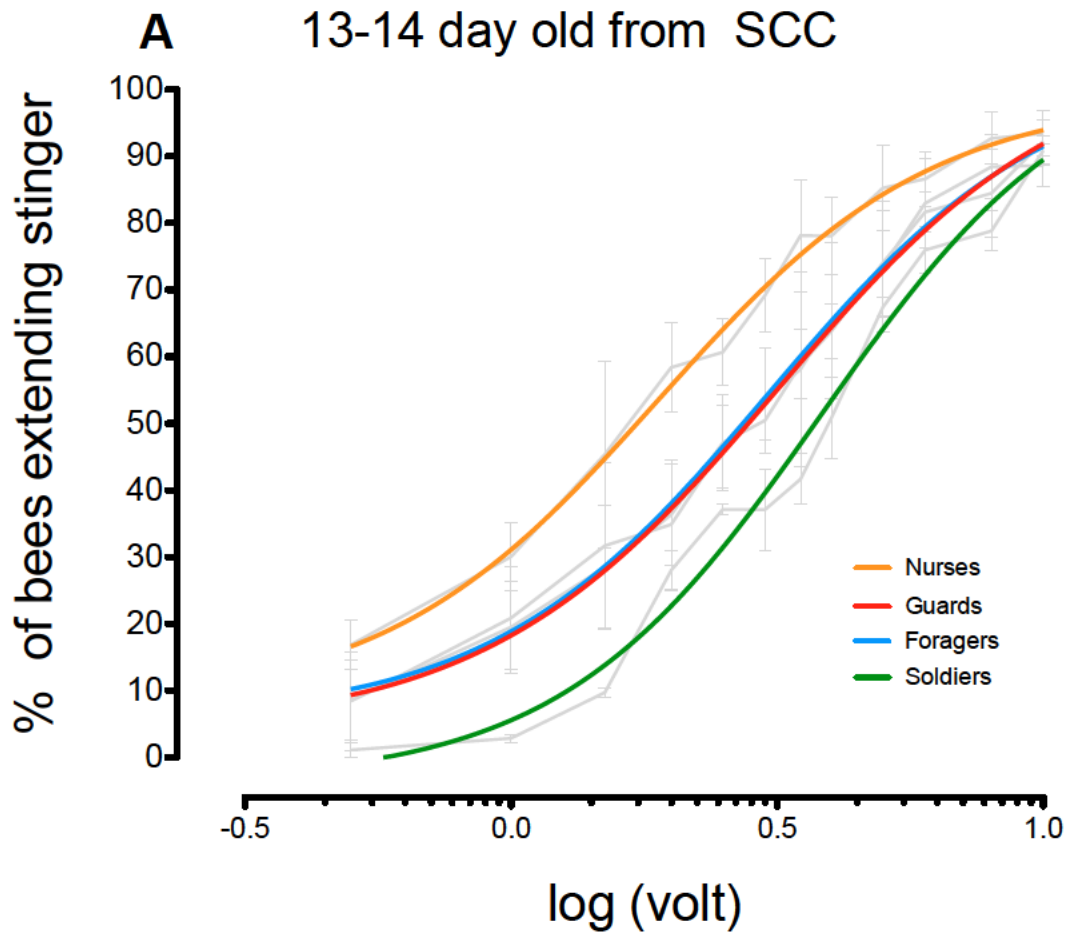


Fig 4: Shock sensitivity of various types of bees of similar age issued from a single age cohort colony (SCC). Proportion of bees displaying sting extensions was fit against the log of the voltage applied at each shock. (A) bees of 13-14 days old. The nurses are the most sensitive to electric shock and tend to sting at 1.9 volts. Foragers, guards, and soldiers are less sensitive and have sting thresholds of 3.1 volts, 3.2 volts and 3.8 volts respectively. The response curve for nurse significantly from guards ($F_{(1,66)}$ 3.998; p = 0.0497) and soldiers ($F_{(1,54)}$ 10.15; p = 0.0024). (B) bees of 28-29 days old. The nurses were the most sensitive to electric shock and tend to sting at 1.950 volts. Foragers, guards, and soldiers were less sensitive and have sting thresholds of 6.436 volts, 4.631 volts and 4.038 volts respectively. Nurses significantly from guards ($F_{(1,64)}$ 5.241; p = 0.0254) and foragers ($F_{(1,64)}$ 7.554; p = 0.0078).



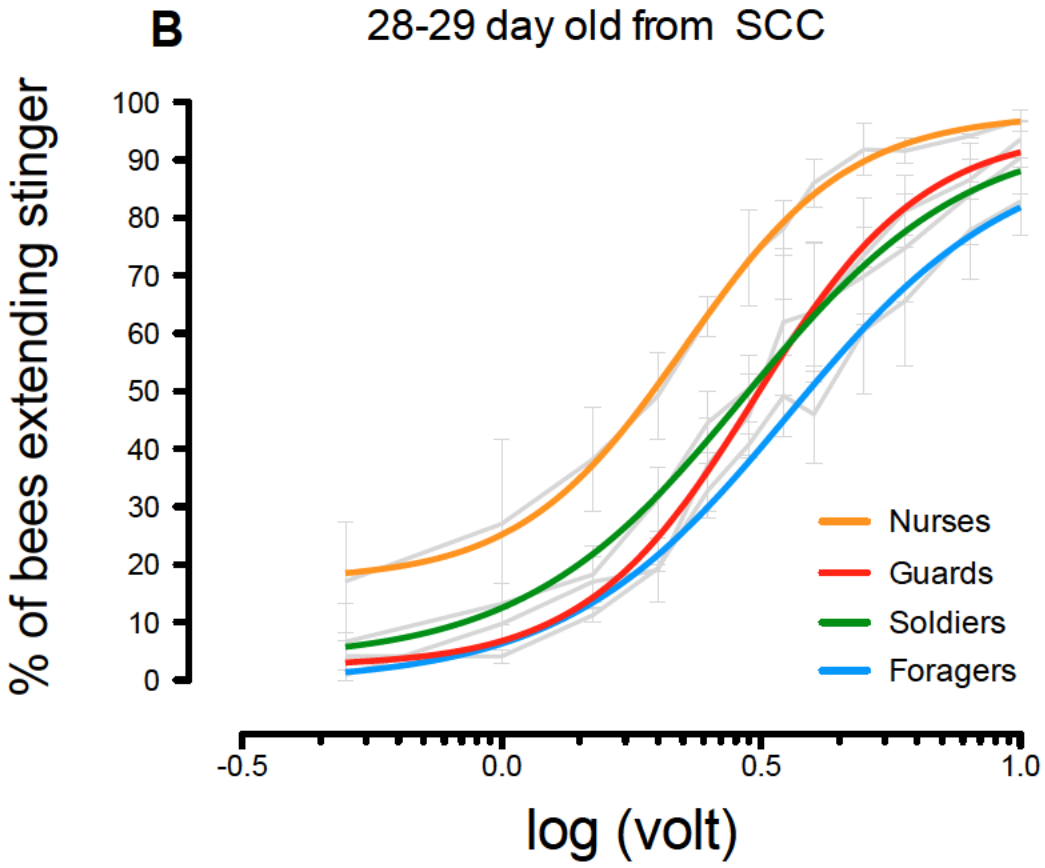

B
28-29 day old from SCC
% of bees extending stinger
100
90
80
70
60
50
40
30
20
10
0
-0.5
0.0
0.5
1.0
log (volt)
Nurses
Guards
Soldiers
Foragers

Table 1: Results of pairwise comparison stress response curves between subcastes in normal demography colonies, using F test. Results presented in Fig 2

(group1 *vs* group 2)	N	$F_{(DFd,DFn)}$	*p* value	EV 50 (group1- group2)
nurses *vs* guards	58	$3.709_{(1,18)}$	0. 0701	2.6 – 1.3
nurses *vs* soldiers	58	$4.322_{(1,18)}$	0. 0522	2.6 – 5.8
Nurses *vs* foragers	59	$4.276_{(1,18)}$	0.0533	2.6 – 6.8
guards *vs* foragers	59	$10.30_{(1,18)}$	**0.0049**	**1.3 – 6.8**
guards *vs* soldiers	58	$11.76_{(1,18)}$	**0.003**	**1.3 – 5.8**
forager *vs* soldiers	59	$0.0942_{(1,18)}$	0.7624	6.8- 5.8

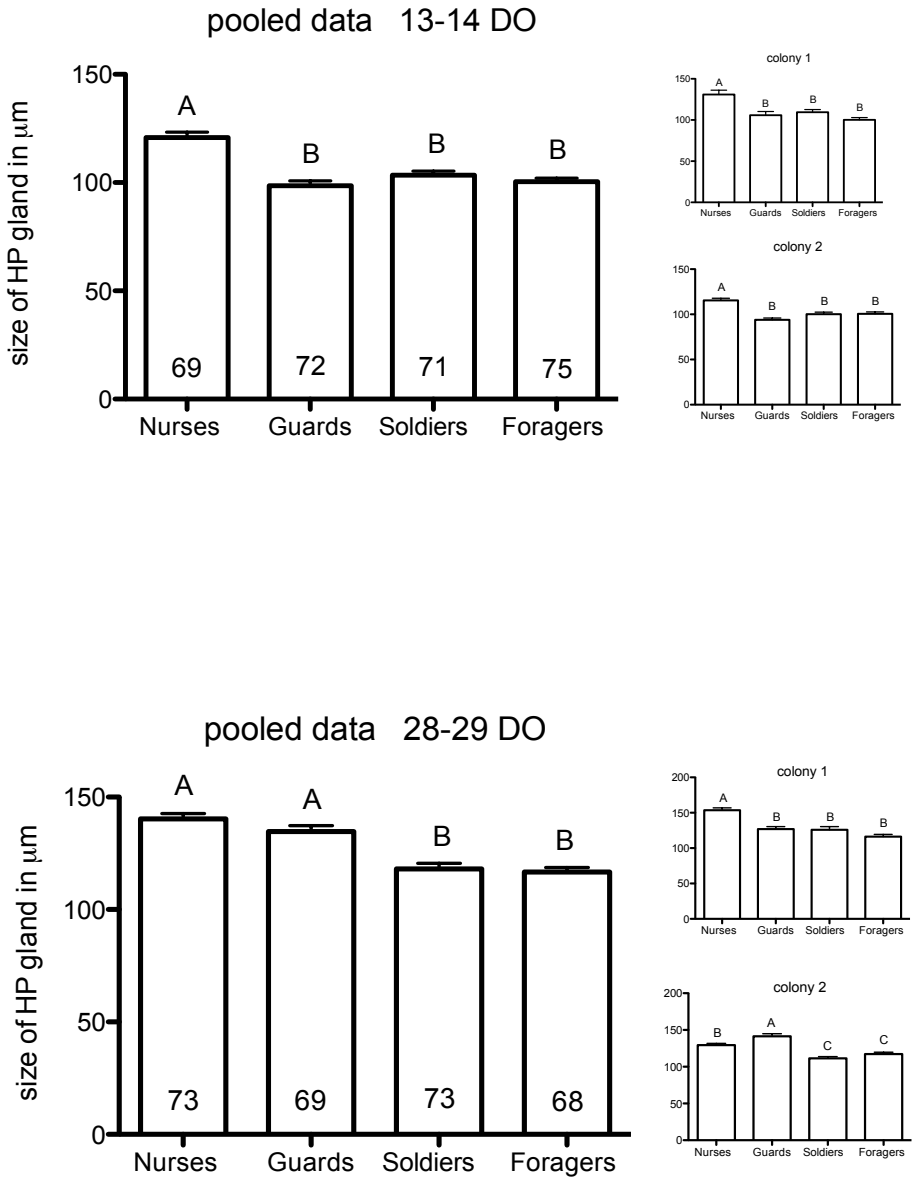
Table 2: Results of pairwise comparisons of stress sensitivity response curves between subcastes in 13-14 day old bees from single age cohort colonies, using F test. Results presented in Fig 3a.

(group1 *vs* group2)	N	$F_{(DFd,DFn)}$	*p* value	EV 50 (group1- group2)
nurses *vs* guards	237	**$3.998_{(1,66)}$**	**0.0497**	**1.9 – 3.2**
nurses *vs* soldiers	191	**$10.15_{(1,54)}$**	**0.0024**	**1.9 – 3.8**
nurses *vs* foragers	234	$2.229_{(1,66)}$	0.1402	1.9 – 3.1
guards *vs* foragers	235	$0.019_{(1,66)}$	0.8929	3.2 – 3.1
guards *vs* soldiers	192	$2,277_{(1,54)}$	0.2634	3.2 – 3.8
forager *vs* soldiers	189	$0.915_{(1,54)}$	0.343	3.1 – 3.8

Table 3: Results of pairwise comparisons of stress sensitivity response curves between subcastes in 28-29 day old bees from single age cohort colonies, using F test. Results presented in Fig3b.

(group1 *vs* group2)	N	$F_{(DFd,DFn)}$	*p* value	EV 50 (group1- group2)
nurses *vs* guards	175	**5.241 $_{(1,64)}$**	**0.0254**	**1.9 – 4.6**
nurses *vs* soldiers	176	2.258 $_{(1,64)}$	0.1131	1.9 – 4.0
nurses *vs* foragers	170	**7.554 $_{(1,64)}$**	**0.0078**	**1.9 – 6.4**
guards *vs* foragers	169	0.652 $_{(1,64)}$	0.4221	4.6 – 6.4
guards *vs* soldiers	175	0.055 $_{(1,64)}$	0.8155	4.6 – 4.0
forager *vs* soldiers	170	0.709 $_{(1,64)}$	0.4027	6.4 – 4.0

Figure 5. Hypopharyngeal gland measures in single age cohort bees. Each bar represents the mean (± SEM) of hypopharyngeal (HP) gland acini diameters from bees performing different tasks of known age (13-14 Days old and 28-29 days old). Glands were measured from the same bees used for stress response threshold tests. Data are pooled from two single age cohort colonies (detailed in the inset). Numbers inside columns represent sample size. Data were analysed with ANOVA and Tukey post-hoc tests, significantly different acini sizes are indicated with different letters above the columns.



In experiment 4, brain biogenic amines levels varied between subcastes in both experiment 1 and 2 (Fig 6 A-B). In bees from experiment 1, when brain biogenic amines was measured after aversive shocks in callows, foragers and guards we found that callows had significantly lower brain levels of OA, DA, 5-HT and TYR than foragers and guards (see Fig 6 for statistical details).

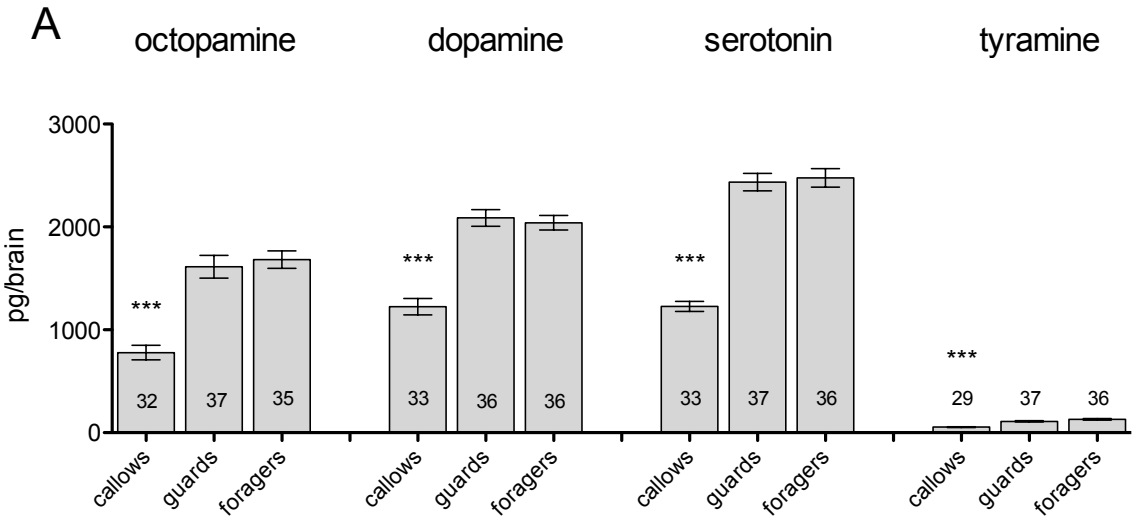
With bees from experiment 2, we measured brain biogenic amine levels in nurses, foragers, guards, and soldiers subcastes (Fig 6B) sampled with or without the application of the shock stress. When considering both variables (castes and stress), no significant interactions between variables were found (Table 1). Subcaste affected levels of OA, DA and 5-HT which were higher in the brains of guards, soldiers and foragers when compared to nurses (see detail of post-hoc results in Table 4). Stress did not affect OA, DA, 5-HT and TYR when subcaste was not considered as a variable (Table 5). However a pairwise comparison showed a slight increase of OA in the brain of soldiers (Mann Whitney; $U= 117$; $p= 0.049$) and a decrease of DA in the brain of nurses (Mann Whitney; $U= 181$; $p= 0.045$) after being shocked, compared to the control (Fig 6B). Finally a post-Hoc analysis showed that nurses have significantly lower amounts of OA, DA and 5-HT (see Table 5) and foragers have higher levels of OA than guards (Tukey's test; $p= 0.032$) independently of stress.

Using the results of experiments 1,2 and 4, we correlated biogenic amine levels with individual stress responsiveness scores. In experiment 1 and 4 no correlation between sensitivity score and levels of each brain biogenic amine were found in the three groups tested: callow, guards, foragers (Fig 7A). In experiment 2 and 4 a similar analysis found a negative correlation between DA levels and sensitivity score in soldiers (Fig 7B) but not in the other subcastes.

Fig 6- Brain biogenic amines levels in various castes. Each bar represents the mean (± SEM) amount of whole brain biogenic amines.

A- callow bees, foragers and guards after aversive shocks. Data were analysed with Kruskal-Wallis tests. Dunn multiple comparison tests were used to compare the differences between columns. The numbers inside the columns correspond to the sample sizes. Callow bees had significantly lower amounts of biogenic amine. [octopamine ($H_{2,105}$ = 52.03, $p < 0.0001$); dopamine ($H_{2,106}$ = 45.65, $p < 0.0001$); serotonin ($H_{2,105}$ = 58.95, $p < 0.0001$); tyramine ($H_{2,101}$ = 48.78, $p < 0.0001$)].

B- Brain biogenic amine levels in nurses, guards, soldiers and foragers in stressed or unstressed group. Effects of stress [control (cont.) versus stressed] were analysed with Mann-Whitney tests and *p*-values are written on top of compared columns. Interaction and individual effects of independent variables (caste and treatments) on brain biogenic amines are summarized in table 4 and 5.



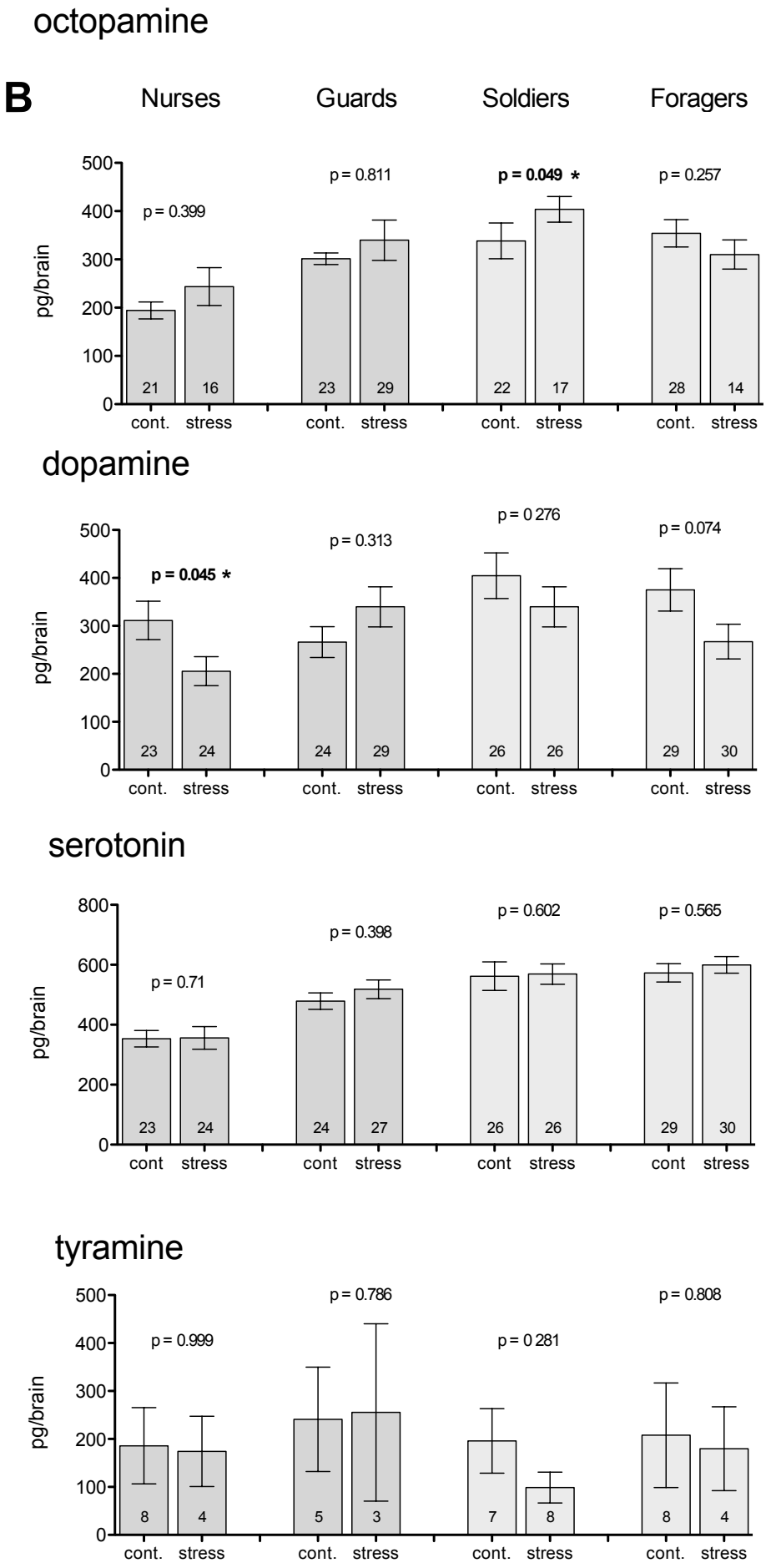
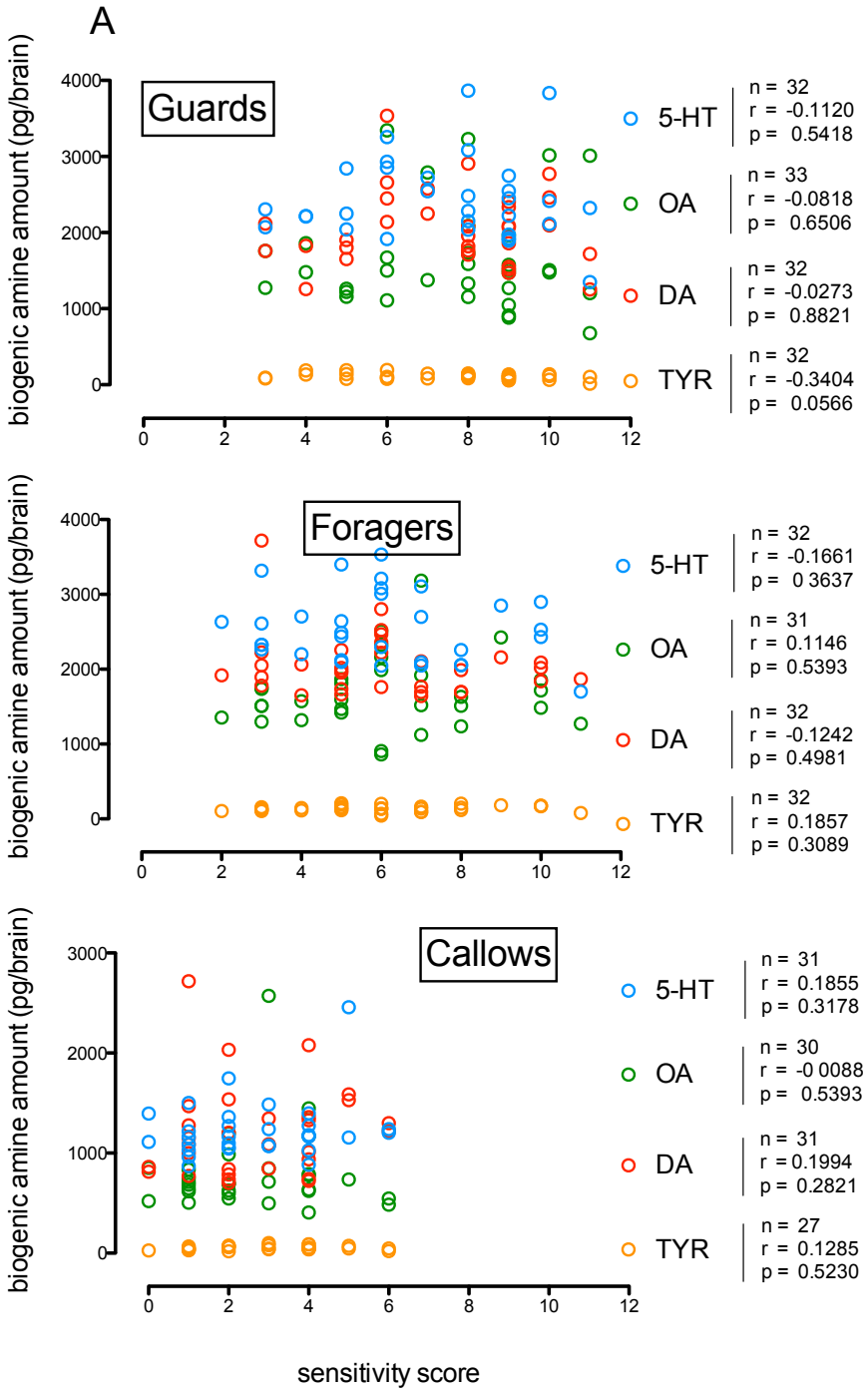
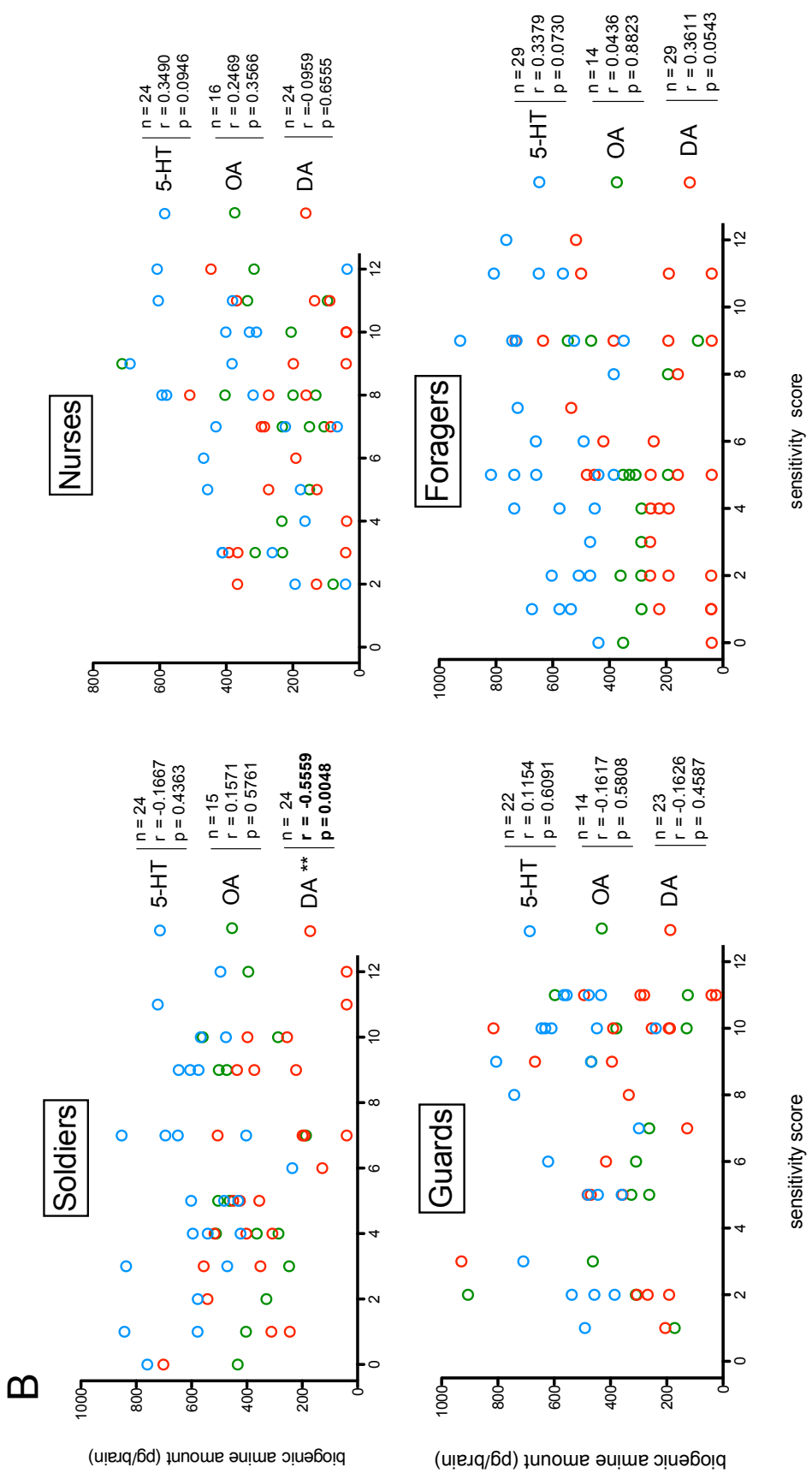

octopamine
B
Nurses
Guards
Soldiers
Foragers
pg/brain
p = 0.399
p = 0.811
p = 0.049 *
p = 0.257
21
16
23
29
22
17
28
14
cont.
stress
dopamine
p = 0.045 *
p = 0.313
p = 0.276
p = 0.074
23
24
24
29
26
26
29
30
serotonin
p = 0.71
p = 0.398
p = 0.602
p = 0.565
23
24
24
27
26
26
29
30
cont
stress
tyramine
p = 0.999
p = 0.786
p = 0.281
p = 0.808
8
4
5
3
7
8
8
4

Figure 7. Correlation between sensitivity score and brain biogenic amine levels in each subcaste. OA: octopamine; DA: dopamine; 5-HT: serotonin; TYR: tyramine. A- Data from experiment 1. B- Data from experiment 2. Tyramine level sample size were too low to be included in this analysis. Dopamine levels were negatively correlated to sensitivity to shock in soldier subcaste. ** p<0.01




B
Soldiers
Nurses
Guards
Foragers
5-HT
OA
DA
n = 24
r = -0.1667
p = 0.4363
n = 15
r = 0.1571
p = 0 5761
n = 24
r = -0.5559
p = 0.0048
DA **
n = 24
r = 0.3490
p = 0.0946
n = 16
r = 0.2469
p = 0.3566
n = 24
r =-0 0959
p =0.6555
n = 22
r = 0.1154
p = 0.6091
n = 14
r = -0.1617
p = 0.5808
n = 23
r = -0.1626
p = 0.4587
n = 29
r = 0.3379
p = 0.0730
n = 14
r = 0.0436
p = 0.8823
n = 29
r = 0.3611
p = 0.0543
biogenic amine amount (pg/brain)
sensitivity score

Discussion

Here we have shown that the responsiveness to a stressor (an electric shock) of bees is influenced by both behavioural caste and age. This influence was expected because these different roles are taken by bees of different ages. Also defensive reactions of bees to a threat towards the colony can be described in sequential stages, where guards are the first to react, then soldiers and finally foragers and maybe nurses. Yet, the influence of both factors on sensitivity differs depending on the model considered for division of labour. On one hand, the response threshold model would predict the stress sensitivity to be the highest in guards and lowest in foragers. On the other hand, if we apply the behavioural syndrome theory we expect guards and soldiers to respond similarly with both showing a lower sensitivity to stress than foragers (Roussel et al., 2009; Tedjakumala and Giurfa, 2013).

In this study we found exactly what the response threshold model would predict for defensive subcastes in a normal demography colony at the hive entrance. Guards (i.e.: bees that are waiting for threat at the hive entrance or the first to react to a threat very close to the entrance during the first seconds) were more likely to sting at lower voltages compare to the forager and soldier group. This result shows guards are more responsive to shocks compared to foragers and soldiers. This result is also consistent with another very recent study, testing the stinging reactions of guards and soldiers on an electric grid, where guards were also more reactive to sting than soldiers and foragers (Uribe-Rubio et al., 2013). As guards are more sensitive to an aversive stimulus than soldiers and foragers, they can be considered to act as a "threat sensor" that would trigger a more intense response from recruited soldiers if necessary.

Our result is at odds with the proposed "defence behaviour syndrome" (Roussel et al., 2009) which found a low sensitivity to shock in a pool of recruited attacking bees compare to foragers. Yet, this discrepancy may be attributed to different methodologies for collecting bees. Here, we tried to separate the bees participating in the two successive waves of attack, unlike Roussel and colleagues (2009). Thus, in their case it is probable that the attacking pool included both guards and soldiers, thus making difficult a clear distinction in the response threshold measure.

Another possible explanation is the possible effect of age as a confounding factor. Using single-age cohort colonies, which avoids this effect, we found that the sensitivities of guards, soldiers, and foragers were no longer distinct from each other (although guards tended to be less sensitive than soldiers when aged 13-14 days). Thus, age and behaviour interact together to affect stress sensitivity, so that any variation in the age composition in the tested samples across studies may result in discrepancies.

Callow bees had a very low responsiveness to shock, as expected, because of their immature nervous system (Burrell and Smith, 1994). By contrast, unexpectedly nurses were very responsive to electric shocks, and this effect persisted regardless of age, although they would be the last ones expected to react to a threat to the colony. This illustrates a possible limitation of the application of the response threshold model to all aspects of honeybee division of labour. Clearly nurses are not the first to react to a threat despite high sting sensitivity suggesting more than a single factor determines which bees participate in defensive roles. The sucrose sensitivities of nectar and pollen foragers also diverge from the simple predictions of the response threshold model. In that regard, the foraging behavioural syndrome was useful to clarify the distribution of foraging tasks as correlated with response threshold to sucrose sensitivity and other stimuli like odour, light, water, and pollen (Erber et al., 2006; Pankiw, 2005; Scheiner and Erber, 2009; Scheiner et al., 2004).

This high sensitivity of nurses, as compared to defending and foraging bees, might reflect important physiological differences between in-hive bees and those spending a large part of their time outside. Specifically, nurses have a lower titer of juvenile hormone and hyper-developed hypopharyngeal glands compared to older bees performing outside tasks (Huang et al., 1994; Jassim et al., 2000). Consistently, here we found that hypopharyngeal gland development was not affected by age but rather confirms that the development of these glands was related to behaviour as nurses had more developed glands than guards, soldiers and foragers independently of age. This result also demonstrated that we had a clear physiological trait related to division of labour in our single age cohort colonies.

Complementary to this analysis, our measurements of brain levels of biogenic amines reinforce previous variations of such levels according to behavioural tasks (Schulz

and Robinson, 1999; Schulz et al., 2002; Wagener-Hulme et al., 1999). In particular, we found that both nurses and callow bees had lower levels of brain biogenic amines compared to older tasks bees (guards, soldiers, and foragers), especially OA, which is consistent with previous studies (Schulz and Robinson, 1999; Schulz et al., 2002; Wagener-Hulme et al., 1999). We also noticed differences in 5-HT between guards and foragers reinforcing the concept of distinct behavioural and physiological castes. This result also confirms previous results (Wagener-Hulme et al., 1999).

Given the additional interplay between brain biogenic amines and response to stress (see chapter 2), we also looked for possible variation in brain amine levels that might explain stress sensitivity differences. However, in this study neither variations nor interactions were found within or between groups in overall comparisons. In pairwise comparisons, only two subcastes showed significant differences (although borderline p values) in brain biogenic amines levels when comparing stressed and unstressed animals: OA was higher only in the group of stressed soldiers compared to unstressed, and DA lower in the group of stressed nurses compared to unstressed (Fig 5). Moreover the individual aversive sensitivity score did not correlate with any brain biogenic amine levels in most of the subcastes (Fig 6). Only one correlation showed that within soldiers the low responding bees had higher brain DA levels after being shocked (Fig 6-B). These results may indicate a different modulation of stress by OA and DA in soldiers and DA in nurses, as compared to other subcastes. Still, the limited statistical significance of the two first results and the absence of clear patterns of variation in biogenic amines levels related to stress responsiveness does not allow us to explain stress responsiveness differences between subcastes. As discussed in the previous chapter, the method used here does not differentiate intra- and extra-cellular biogenic amine amounts, neither do they detect any bio-enzyme related to biogenic amine turnover. As a result, net increases (synthesis) or decreases (degradation) following release can be detected, but not synthesis and degradation compensating each other. It is therefore possible that the actions of these biogenic amines are restricted to local neuropils, so that whole brain measures do not allow us to detect clear differences between subcastes when under stress. This measure would be interesting considering that Schulz and colleagues (1999) found specific brain regional increase of OA and 5-HT in antennal lobes independently of age, supporting their role in division of labour.

Conclusion

In this study we have shown that stress sensitivity can be influenced by age and by task specialisation. Our results indicate that the observed behavioural differences between two defensive subcastes, guards and soldiers, can be explained by response threshold model, but that this simple model fails to explain the nurses' high sensitivity to shock. More experiments comparing the respective responses of nurses and foragers to stressors would be necessary to find an appropriate explanation of this result. Despite a known variation of brain biogenic amine levels with age, behavioural task and stress exposure, whole brain biogenic amine measures cannot explain shock responsiveness differences in our study. Nevertheless, these results confirmed previous data showing increase of brain biogenic amines in guards, soldiers and foragers compared to callow and nurse honeybees. More brain regional studies would be necessary to understand physiological mechanisms responsible for the different sting response threshold between subcastes.

Supplements

Table 4: Results of two-way ANOVA for brain levels of biogenic amines as a function of subcaste (nurse, guards, soldiers, or forager) and stress (stressed or control) or both. Results represented in figure 5b.

Variables	df	F	p -value	Significance
octopamine				
subcaste	3	6.918	**0.0002**	***
Stress	1	0.8304	0.3635	ns
*Subcaste * Stress*	3	0.9903	0.3989	ns
dopamine				
subcaste	3	3.532	**0.0493**	***
Stress	1	2.660	0.0616	ns
*Subcaste * Stress*	3	2.365	0.0722	ns
serotonin				
Subcaste	3	18.74	**<0.0001**	***
Stress	1	0.6331	0.4272	ns
*Subcaste * Stress*	3	0.1244	0.9456	ns
tyramine				
Subcaste	3	0.5767	0.6335	ns
Stress	1	0.6911	0.4104	ns
*Subcaste * Stress*	3	0.1783	0.9105	ns

Table 5: Result of post-Hoc Tukey's test after multivariate analysis for brain levels of biogenic amines as a function of subcaste only including: nurses, guards, soldiers, and foragers. Result represented in Fig 6 B.

variables	*df*	p value	significance
octopamine			
nurses vs guards	3	0.020	*
nurses vs soldiers	3	<0.0001	***
nurses vs foragers	3	0.002	**
guards vs soldiers	3	0.272	ns
guards vs foragers	3	0.772	ns
soldiers vs foragers	3	0.841	ns
dopamine	3		
nurses vs guards	3	0.650	ns
nurses vs soldiers	3	0.032	*
nurses vs foragers	3	0.381	ns
guards vs soldiers	3	0.366	ns
guards vs foragers	3	0.977	ns
Soldiers vs foragers	3	0.585	ns
serotonin	3		
nurses vs guards	3	<0.0001	***
nurses vs soldiers	3	<0.0001	***
nurses vs foragers	3	<0.0001	***
guards vs soldiers	3	0.197	ns
guards vs foragers	3	0.04	*
vs oldiers vs foragers	3	0.918	ns
tyramine	3		
nurses vs guards	3	0.908	ns
nurses vs soldiers	3	0.966	ns
nurses vs foragers	3	0.997	ns
guards vs soldiers	3	0.685	ns
guards vs foragers	3	0.977	ns
Soldiers vs foragers	3	0.909	ns

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Chapter 4

Honeybee sensitivity to electric shocks is affected by biogenic amines and neuropeptides

Abstract

It is unclear whether insects possess endogenous mechanisms to reduce sensitivity to acute physical stress. To explore this issue we tested here if several neuropeptides and an antagonist of a biogenic amine transporter could modulate responsiveness to electric shock in honeybees. Here we found that the neuropeptide allatostatin CC decreased responsiveness to electric shock when injected into the hemolymph at the neck by the *corpora allata*. The neuropeptides allatostatin A and corazonin also have been suggested to be involved in physiological stress responses in bees, but haemolymph injection of these neuropeptides had no effect on honeybee sting response to shocks. Finally, increasing extracellular amounts of biogenic amines by treatment with cocaine increased the sensitivity to electric shock. This confirms the importance of biogenic amines, particularly dopamine and octopamine in stress responses in honeybees. Our results support the role of allatostatin CC as a possible endogenous analgesic in honeybees, however more work is needed to precisely define the roles of allatostatin CC and the biogenic amines in the modulation of sensitivity to acute physical stress.

Introduction

Worldwide, honeybee populations are declining due to a plethora of increasing stressors in their environment (Oldroyd, 2007). For this reason, understanding the physiological and behavioural stress response pathways in honeybees is fundamental. In insects, neuroendocrine pathways seem to be involved in general stress responses (see Chapter 1; Ivanovic, 1991; Johnson and White, 2009). Specific neuropeptides and biogenic amines (Johnson and White, 2009) have been identified as signalling agents in the stress response systems of diverse insect species like flies, cockroaches and locusts (Johnson and White, 2009). However the specific neuromodulatory elements involved in the stress response in bees remain unclear (Chapter 1). Here we examined the effect of injection of neuropeptides related to the general physiological stress response (two allatostatins and corazonin) and treatment with volatilized cocaine (an antagonist of biogenic amine transporters leading to increased extracellular biogenic amine concentrations) on the sting response of bees to electric shock, in order to identify factors that might affect responsiveness to acute physical stress in bees. Below we discuss each of the neuromodulators we have chosen to focus on, and why they might be involved in stress responsiveness or possible analgesia in bees.

Allatostatins

The "allatostatins" (AST) are a group of structurally different peptides, which historically have all been identified as inhibitors of release of the insect growth hormone (juvenile hormone) from the *corpora allata*. This allatostatic function has been identified in various insect groups, which has resulted in three distinct subtypes of allatostatin. The A subtype was first identified in cockroaches, followed by the cricket subtype (B), and then the subtype C in the moth (Bendena et al., 1999). Recently a paralog gene *Ast-CC* was found additionally to *Ast-C* to encode for an ASTC-like peptide in arthropods (including honeybees); the peptide was called ASTCC (Veenstra, 2009a). Only subtypes A, C and CC are present in the honeybee (Boerjan et al., 2010a; Veenstra et al., 2008). ASTs are now recognized to have extremely diverse inhibitory roles (Audsley and Weaver, 2009; Stay and Tobe, 2007).

In honeybees, ASTC and ASTCC peptides (ASTCs), have a similar affinity for the ASTC receptor, of which there is only one in honeybees (Urlacher, 2011). When injected in

the brain through the median ocellus, both peptides altered appetitive learning and sting responses to electric shocks in a strongly dose-dependent manner (some doses reducing and others increasing sting responsiveness to shock). Treatment with opioid antagonists blocked the effect of ASTs on sting responsiveness to shock (Urlacher, 2011). These neuropeptides have therefore been suggested as candidates to perform a possible analgesic function in the honeybee (Urlacher, 2011) perhaps similarly to the endogenous opioid peptides of vertebrates.

Sequence homologies lend some support to a possible functional conservation of ASTCs as endogenous analgesics in the honeybee. Opioid receptors or peptides have never been reported in honeybee. Indeed, it has been suggested that opioid receptors diversified from somatostatin and the neuropeptide B/W family in early vertebrates (Dreborg et al., 2008) and are therefore specific to vertebrates. However, interestingly the ASTC receptors of *Apis mellifera* and *Drosophila melanogaster* share a strong sequence similarity with the vertebrate somatostatin receptor (Kreienkamp et al., 2002; Urlacher, 2011; Veenstra, 2009a). Also, the ASTC receptor has the closest sequence similarity to the vertebrate opioid family of receptors, sharing 60% aminoacid identity with the intercellular loop of vertebrate μ opioid receptors (Urlacher, 2011). These observations suggest, along with the functional results, that the ASTC signalling pathway of the honeybee may have an ancient evolutionary relationship with the derived vertebrate opioid peptides (Urlacher, 2011). Urlacher reported injections of ASTCC into the bee brain via the median ocellar tract reduced responsiveness to electric shock. ASTCC is present in *corpora allata* (Boerjan et al., 2010a), which is a neurosecretory organ. Here we tested if this analgesic effect was also seen when ASTCC was injected directly into the hemolymph (near the *corpora allata*) to act peripherally and mimic a neurohormonal release.

Allatostatin A (ASTA) peptides are also likely to play a role in physiological stress responses in the honeybee. Localisation of the ASTA peptides and their receptors along with functional studies demonstrate their inhibitory roles on gut contractions and feeding behaviour in insects (Audsley and Weaver, 2009; Chintapalli et al., 2007; Veenstra, 2009a; Veenstra, 2009b; Veenstra et al., 2008). In honeybees we hypothesized that AST-A plays an inhibitory role on energy mobilization as part of the stress response (Chapter 1). It remains to be tested if the analgesic function proposed for the ASTCs are specific to this group, or if it generalises to other allatostatins also. Here we examined if ASTA also had an

analgesic function by testing whether the sting response to electric shock was modified by the injection of ASTA into the hemolymph.

Corazonin

The neuropeptide corazonin has also been identified as part of the physiological stress response of honeybees (Chapter 1). Corazonin was initially described as a cardio-accelerator in cockroaches (Veenstra, 1989), but additional endocrine roles for corazonin, including gregarisation in locusts and ecdysis in moth have been reported (see chapter 1 for details; Boerjan et al., 2010b; Veenstra, 2009b). Corazonin's endocrinal role in metabolism is suggested by both the histology and phylogeny of the peptide. Specifically, corazonin-expressing neurons innervate the endocrine cells of the *corpora cardiaca* in honeybees (Roller et al., 2006; Verleyen et al., 2006).

The corazonin receptor has been localised in the heart, fat body, salivary gland and, to a lesser extent, in the brain of *Drosophila* (Chintapalli et al., 2007). It is related to the ancient Gonadotropic-Realising Hormone-Adipokinetic-Hormone (GnRH-AKH) receptor family which include important receptors for peptides signalling stress responses in vertebrates (Hansen et al., 2010; Park et al., 2002).

Furthermore, in insects AKH is the neuropeptide which seems to play a key role in the general stress responses in insects. AKH stimulates various physiological mechanisms necessary to cope with various kinds of stressors like increased heart beat (cockroaches), locomotion (in firebug), muscle tonus (cockroaches) and diverse metabolic actions (reviewed in Kodrík, 2008). However AKH is generally weakly expressed in bees (Lorenz et al., 1999), but the phylogenetic link between corazonin and AKH peptides (Hansen et al., 2010), and the pleiotropic endocrine roles of corazonin make it a good candidate to act in energy mobilisation in honeybees as part of the stress response. Moreover several studies (Boerjan et al., 2010b; Veenstra, 2009b, see also Chapter 1) have suggested the possibility that corazonin might be a hormone activating physiological stress response pathways in insects as well as honeybees.

Biogenic amines and cocaine

Cocaine is a plant-derived defence compound that blocks biogenic amine re-uptake transporters across many animal phyla (Barron et al., 2010). Evidence indicates that brain levels of biogenic amines increase after exposure to long-term stress (see chapter 2). Also, octopamine has been related to an increase in the sensitivity to sensory inputs. For example, in honeybees, octopamine treatment enhances response to specific olfactory stimuli (Barron and Robinson, 2005; Barron et al., 2002; Barron et al., 2007; Menzel et al., 1999), gustatory stimuli (Pribbenow and Erber, 1996), visual stimuli (Pribbenow and Erber, 1996) and aversive stimuli (Burrell and Smith, 1995). Burrell and Smith (1995), in particular, showed that octopamine enhanced sting responses and electromyogram activity in the sting apparatus to high vibration stimuli in isolated abdomens, suggesting various peripheral targets for octopamine (muscular and/or motoneural receptors) controlling this reaction.

Cocaine has been shown to sustain elevated extracellular dopamine levels after exogenous dopamine application (Søvik, 2013). This result suggests that cocaine affects dopamine reuptake, increasing extracellular dopamine signals. The dopamine transporter described in the honeybee (*Am*DAT) share a high proportion of amino-acids with dopamine (74%) and octopamine (51%) transporters of the moth *Trichoplusia ni* [NCBI Basic Local Alignment Selection Tool; (Altschul et al., 1990)]. The pharmacology of *Am*DAT has not been described yet, but it is not impossible that *Am*DAT could transport octopamine as well as dopamine as it has already been shown in *Trichoplusia ni* (Gallant et al., 2003). This suggests the possibility that cocaine might also increase extracellular octopamine concentrations. By treating bees with cocaine prior to stress, we examined if increasing extracellular biogenic amine levels in the brain and periphery would increase honeybees' sensitivity to electric shock.

Materials and methods

Solution preparation

Corazonin (p-QTFTYSHGWTN-amide), AST-A (GRQPYSFGL-amide), and Allatostatin-CC (GQAKGRVYWRCYFNAVTCF) were all synthetized by GL Biochem (Shanghai). AST-CC was cyclised (between the two cysteine residues). The three peptides were dissolved in pure

ethanol and then diluted in PBS to reach a concentration of 10^{-2}M. Due to this dilution in ethanol, the minimum ethanol concentration present in these initial solutions was 33%. For this reason, we made all further dilutions of peptides (10^{-4} M, 10^{-7}M, 10^{-9}M) in a solution of 33% ethanol in PBS. This required two neuropeptide control groups: injection of PBS alone and injection of PBS + ethanol (33%) to detect any possible effects of ethanol. Phenylmethane-sulfonylfluoride (PMSF), a protease inhibitor, has been shown to be effective against neuropeptide degradation in the honeybee hemolymph when injected prior to each peptide injection (Urlacher, 2011). Thus, PMSF was first diluted in ethanol 30mM and then in PBS to obtain a 1mM solution, which was injected prior to each peptide and control injection (method described in Urlacher 2011).

Honeybee collections

Honeybee colonies were kept in Macquarie University Fauna Park. Experiments were performed during the Australian summer (from September to April). Pollen foragers were collected at the entrance of the hive as they returned from their foraging trip. Each bee was collected individually in a 15 mL plastic tube and immediately placed in an ice bath to be anaesthetized.

Injection method

Anaesthetized bees were harnessed on their back individually to the plate used for delivering the shock. The head of each bee was then extended dorsally, using blue tack to attach the mandibles, allowing the stretched ventral membrane of the neck to be exposed for internal injection. The bee was placed under a dissecting microscope prior to injection. 200nL of solution was injected in the membrane of the honeybee neck (see Figure 1) with a Hamilton syringe (Nanofil, equipped with a NF33BV2 needle, WPI). Every peptide injection was preceded by an injection of 200nL of PMSF 1mM immediately before.

Cocaine treatment

Using the same method as for the injection experiment, anaesthetized bees were harnessed to the plate used for delivering the shocks. Then the bees were treated with volatilised freebase cocaine (Sigma-Aldrich; St Louis, MO, USA) using the methods of Søvik *et al.* (2013) that were inspired by (McClung and Hirsh, 1998). Briefly, cocaine was dissolved in 100% ethanol then 2µL was pipetted carefully onto a nichrome wire filament.

This was allowed to thoroughly air dry leaving a deposit of cocaine coating the filament and eliminating all ethanol. Treatment of bees (one at the time) was done in a 50cm^3 airtight container encapsulating the filament. The filament was heated for ten seconds to allow it to reach 200°C within five seconds without surpassing 350ºC (Martin et al., 1989). Bees remained in the container for 50 additional seconds after the current was turned off (one minute in total). For controls, pure ethanol was pipetted onto the filament, which was allowed to air dry before treatment.

Shock sensitivity test

The same shock procedure was used as in chapter 2 and 3. In brief, after attachment of the bee to the shock platform with non-salt conductive gel, bees were left for 45 minutes, and then received a series of shocks of increasing voltage (1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 volts). The number of sting extensions was recorded to calculate the proportion of bees stinging at each voltage in each group. 60 bees (5 per treatment group) were injected and tested on a given experimental day. As the injection of 60 bees took 60 minutes, the delay between injection and shock tests was randomized between groups.

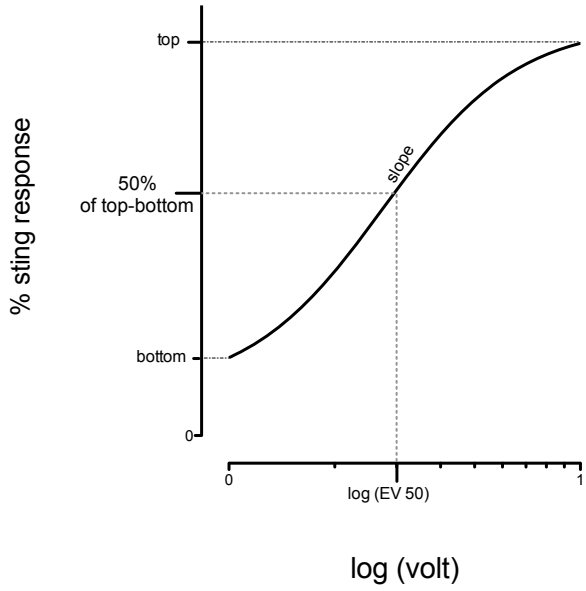
Statistical analysis

Sensitivity to electric shock was assessed through responses to an increasing series of voltages. A dose-response analysis was conducted using Prism, with the dose corresponding to the shock voltage, and with the response as the proportion of bees extending the sting at each voltage. Separate fits were created for each dose of each treatment.

The voltage scale was log-transformed in order to apply a nonlinear fit (proportion of bees demonstrating the sting response fit against the log of the voltage) for each treatment group. Akaike information criterion was used to select the model that is most likely to have generated the data. As a result, the equation (Y=Bottom + (Y=Bottom + (Top-Bottom)/(1+10^((LogEV50-X)*Slope)))) was used to fit the data (Fig 1). These equations calculate the Log of half the maximum effective voltage (EV50) for each fit. Log EV50 gives the log of the voltage corresponding to the response half way between minimum and maximum of the response curve. Then extra-sum-of-square test (F test)

was used to test if the fits were significantly different. This test compared whether the parameter log EV50 was significantly different between treatment groups.

Figure 1: Graphic representation illustrating the parameters used for the equation fit. Log of half the maximum effective voltage (EV50) for each fit was calculated. Log EV50 gives the log of the voltage corresponding to the response half way between minimum (bottom) and maximum (top) of the response curve. The equation (Y=Bottom + (Y=Bottom + (Top-Bottom)/(1+10^((LogEV50-X)*Slope)))) was used to fit the data. If the slope is equal to 1 the equation contains three parameters, if the slope is different from 1 the equation contain 4 parameters. Akaike information criterion was used to select the equation that is most likely to have generated the data.



Results

In three different pharmacological experiments, ethanol did not affect sensitivity to electric shocks as the sensitivity curves of the groups: PBS and PBS + ethanol did not differ significantly in any of the three experiment (Fig 2, 3, 4, Table 1). The presence of 33 % of ethanol in the injected solution did not modify the sensitivity to electric shock.

Table 1: Summary of pairwise comparison (F test) indicating no differences in EV50 between the two control groups in the three experiments testing effect of peptide: allatostatin CC (ASTCC), allatostatin A (ASTA) and corazonin.

	N	DFn, DFd	F	P value	EV 50 (PBS–PBS+Ethanol)
ASTCC PBSvs PBS+Ethanol	170	1,8	0.2054	0.6624	1.137 - 2.195
ASTA PBS vs PBS+Ethanol	120	1,8	1.227	0.3002	1.823 – 2.203
Corazonin PBS vs PBS+Ethanol	98	1,8	1.966	0.1985	1.099 – 2.588

The four doses of corazonin and ASTA injected into the honeybee hemolymph did not modify the sensitivity to electric shocks (Fig 2 & 3) compared to respective controls PBS + ethanol (corazonin: F test; $F_{5,24}$= 1.170; p= 0.3525; ASTA: F test; $F_{5,24}$= 1.263; p= 0.3119).

Figure 2. Sting Extension Responses (SER) after injecting various doses of corazonin in the honeybee neck. Proportion of sting extension fit against the log of the voltage applied at each shock. (F test; $F_{5,24}$=1.170; p= 0.3525).

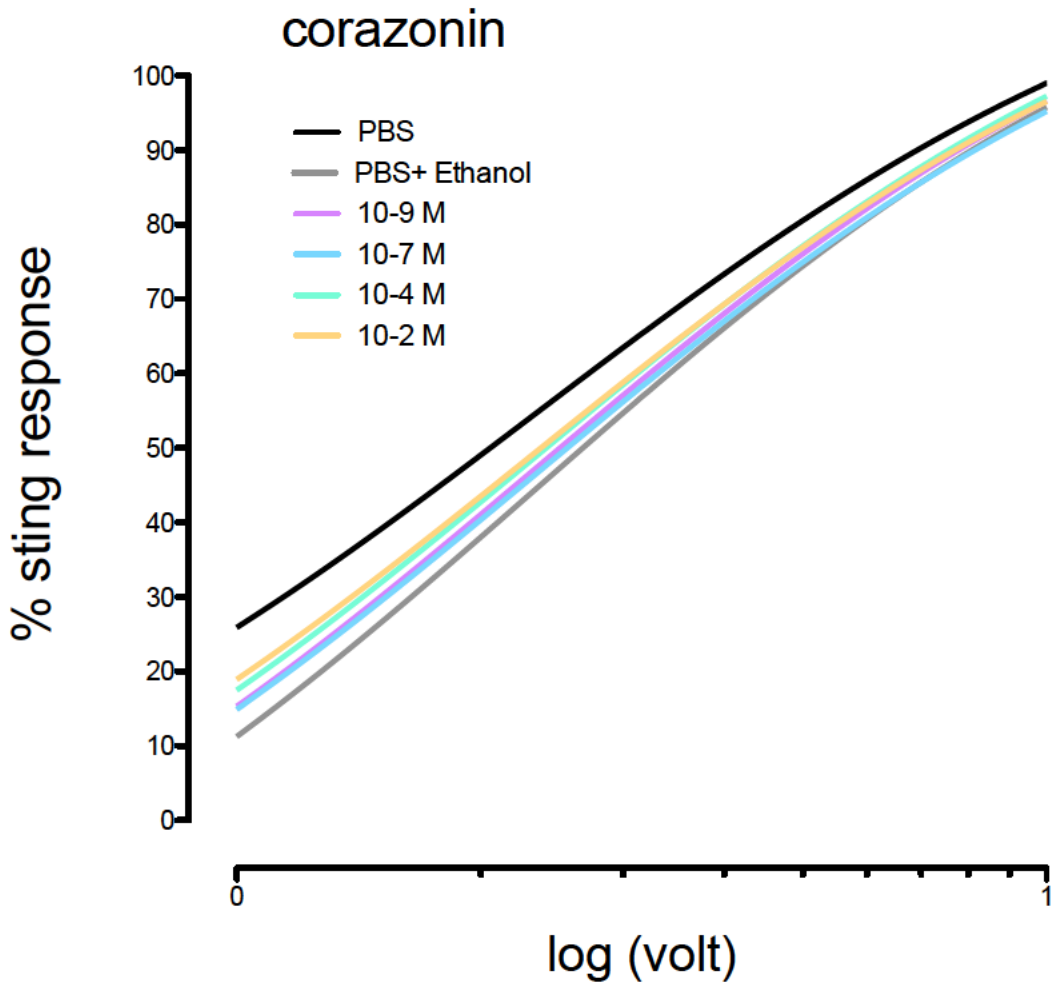
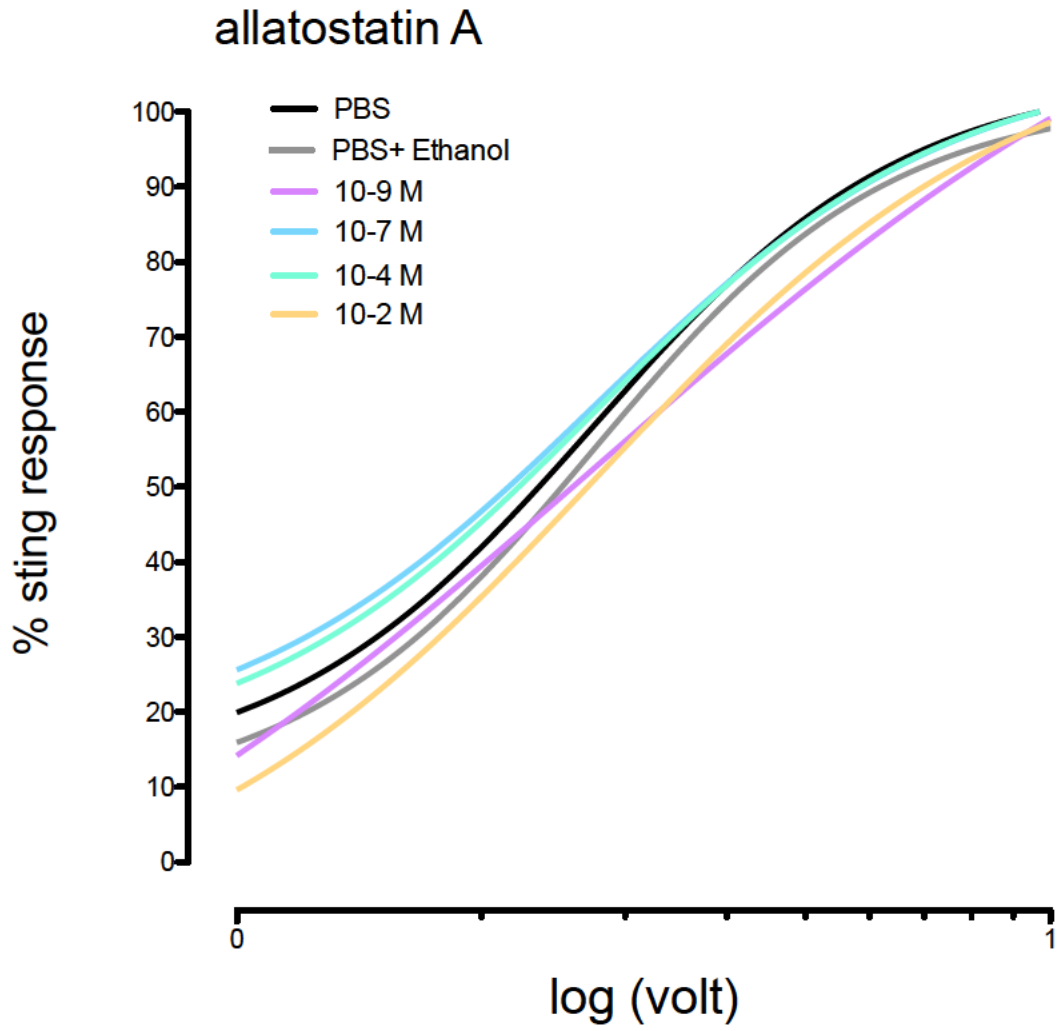


Figure 3. Sting Extension Responses after injecting various doses of ASTA in the honeybee neck. Proportion of sting extension fit against the log of the voltage applied at each shock. (F test; $F_{5,18}$=1.724; p= 0.1802).



In the experiment ASTCC injections changed responsiveness to shock (F test; $F_{5,18}$= 3.895; p= 0.0144). Two different doses (10^{-2} M and 10^{-7} M) of ASTCC injection decreased the sensitivity to electric shock but no significant differences were recorded when injecting 10^{-4} M, 10^{-9} M concentrations compared to control (Fig 4, Table 2). A further small study did not find any effect of 10^{-3}M injection compared to controls (F test; $F_{1,6}$=1.579; p= 0.2556) (Fig4 inset).

Figure 4. Sting Extension Responses after injecting various doses of ASTCC in the honeybee neck. Proportion of sting extension fit against the log of the voltage applied at each shock. (F test; $F_{5,18}$=3.108; p= 0.0340). Comparisons between groups are summarised in table 1. **Inset:** Additional separate experiment testing the effect of a concentration of ASTCC 10^{-3} M compared to a group control (PBS+ ethanol).

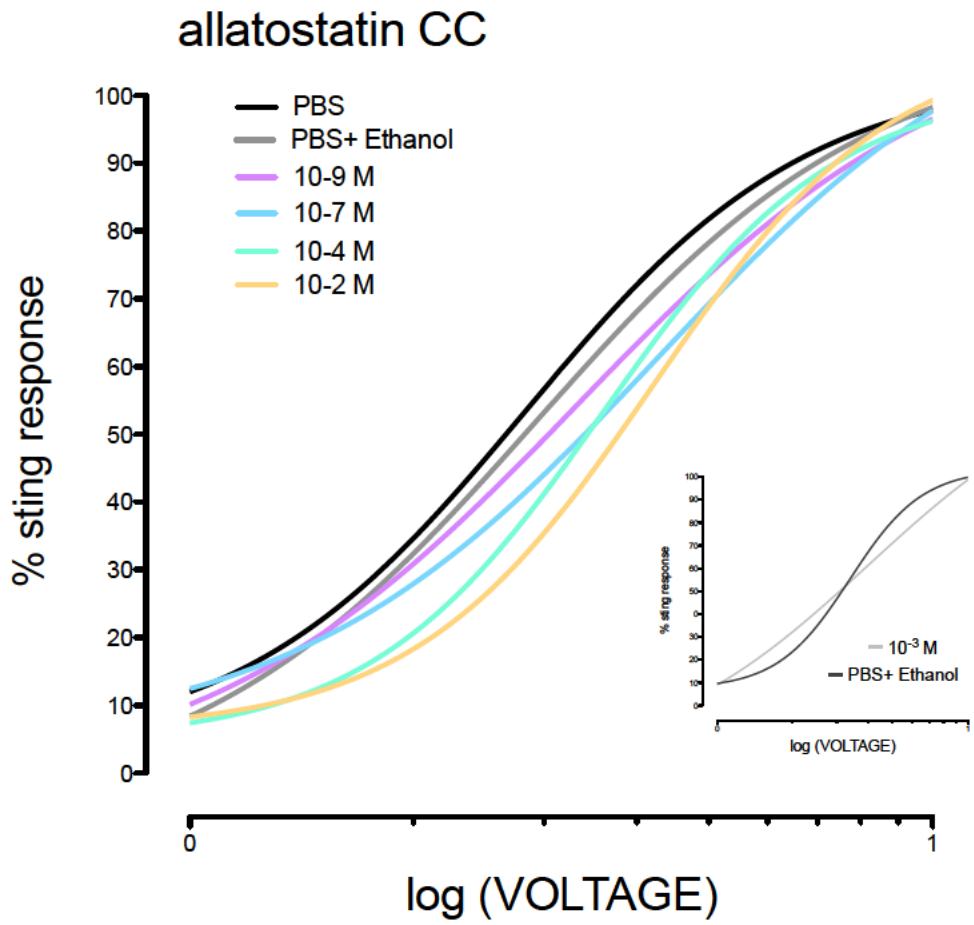


Table 2: Summary of pairwise comparisons (F test) indicating the ASTCC doses reducing sensitivity to electric shock.

	N	DFn, DFd	F	P value	EV 50 (PBS– PBS+Ethanol)
PBS+Ethanol vs 10-2M	170	1,6	9.254	0.0227*	2.203 - 4.922
PBS+Ethanol vs 10-4M	170	1,6	3.837	0.0979	2.203 - 3.134
PBS+Ethanol vs 10-7M	170	1,6	6.975	0.0385*	2.203 - 5.032
PBS+Ethanol vs10-9 M	170	1,6	1.129	0.3288	2.203 - 2.953

Treatment with cocaine increased the sensitivity to electric shock (Fig 5) in a dose-dependent manner (F test; $F_{4,45} = 3.741$; $p = 0.0101$). Specifically, the low concentration 5µM did not affect sting responses compared to the control group but the bees treated with 10µM and 20µM responded significantly more to electric shocks than the control group. Finally the 50µM cocaine treatment induced a highly significant increase of sting sensitivity to electric shocks compared to the control group (Table 3).

Figure 5: Dose-dependant pharmacological modulation of sting sensitivity to shock following treatment with different doses of cocaïne. Proportion of sting extension fit against the log of the voltage applied at each shock.

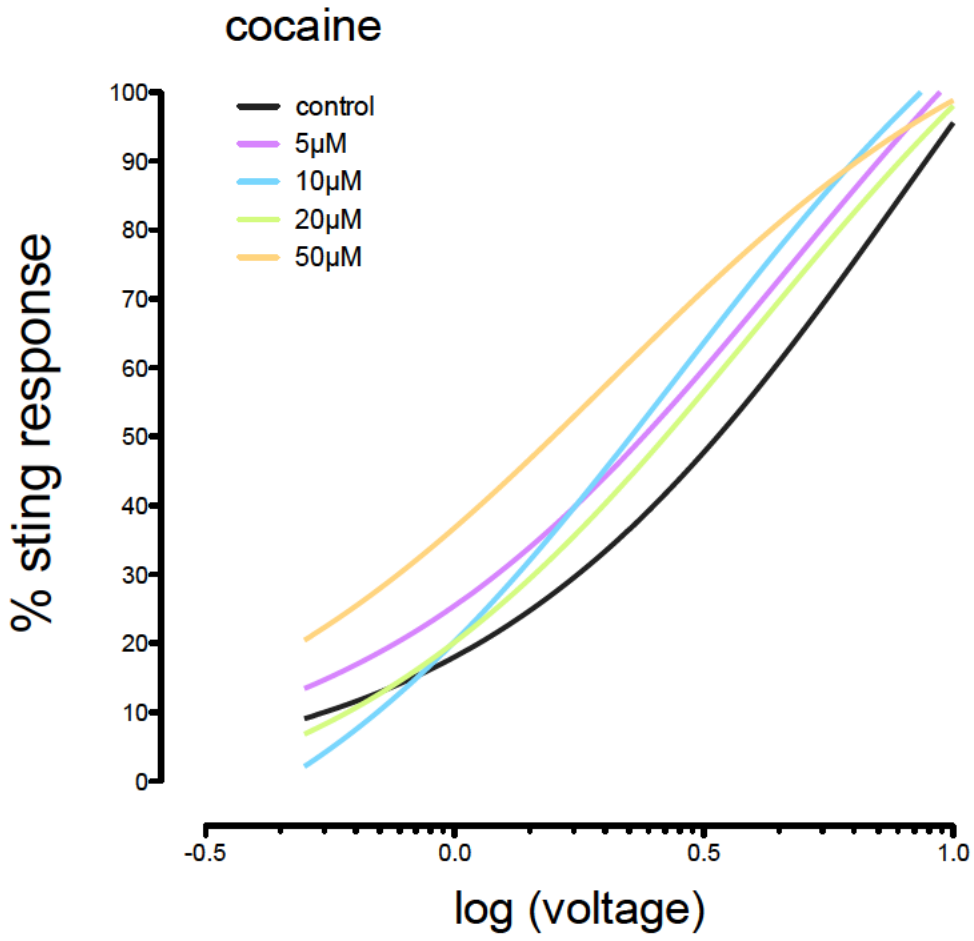


Table 3: Summary of pairwise comparison (F test) indicating the cocaine doses increasing sensitivity to electric shock.

	N	DFn, DFd	F	P value	EV 50 (PBS+Ethanol - Dose)
Control vs. 5µM	80	1,18	2.124	0.1623	8.330 - 4.716
Control *vs.* 10µM	80	1,18	7.240	**0.0149***	8.330 - 2.789
Control *vs.* 20µM	80	1,18	5.510	**0.0305***	8.330 - 4.062
Control *vs.* 50µM	80	1,18	18.95	**0.0004*****	8.330 - 2.045

Discussion

Injections with corazonin and ASTA into the hemolymph had no effect on responsiveness to electric shocks, however two doses of ASTCC reduced responsiveness to shocks, while volatilized cocaine treatment increased responsiveness in a dose dependent manner.

Do bees have an endogenous "pain-killer"?

Urlacher (2011) proposed both functional and phylogenetic arguments supporting the view that ASTCC is related to the ancestor of the vertebrate opioid peptide family; therefore ASTCC, was hypothesised to have a physiological analgesic function in honeybees. Similarly to Urlacher (2011), we found that ASTCC can reduce the responsiveness to electric shock when the peptide is injected into the circulatory hemolymph. Haemal injection of two different doses (10^{-2} M, 10^{-7} M) reduced sting responsiveness while the intermediate doses (10^{-3} M, 10^{-4} M) and the lower dose (10^{-9} M) seems to have no effect. This unusual dose-response relationship is hard to explain. Urlacher (2011) also reported complex dose-response relationships between ASTCC and sting responsiveness to shock. When ASTCC was injected into the brain via the median ocellus, high concentrations (10^{-2}M and 10^{-3} M) induced analgesia, intermediate concentrations (10^{-4} M and 10^{-7} M) did not have any effect, while a low concentration (10^{-9} M) induced hyperalgesia (increase of sensitivity to electric shocks) (Urlacher 2011). ASTCC had an analgesic effect here for 2 doses only, but it is possible that the intermediate doses might have had too weak an effect to be detected statistically with our method (as for example the dose 10^{-4} M has a p value relatively low 0.0979 and EV50 of 3.597 volts as the control EV50 is 2.917 volts). The effective concentration will depend on the location of the injection either into the brain or in the circulatory hemolymph. Our method differed

from Urchaler (2011) in that we injected into the haemolymph close to the brain, not the brain itself since we wanted to explore possible peripheral and neurosecretory functions of ASTCC. The mammalian opioid system also acts both centrally and at the periphery, opioid receptors are located on central and peripheral inhibitory (GABAergic) neurons to disinhibit descending pathways such as noradrenergic neurons to produce analgesia (Kapitzke et al., 2005; Pan et al., 2004; Vaughan et al., 1997). In insect, *Drosophila* ASTC receptors (Drosophila has 2 ASTC receptors) have been localised centrally and in periphery. Both *Drosophila* ASTC receptors have similar distribution patterns: more pronounced in the brain, but also slightly present in many peripheral tissues most importantly in thoracico-ganglion, crop, midgut, salivary gland and in male accessory organs (Chintapalli et al., 2007). The localization of ASTC receptors in the honeybee would be a first interesting step to help understand the actions of ASTCs both centrally and in the periphery, and how ASTC might have this analgesic effect.

Both ASTA and corazonin do not appear from our results to affect sting responsiveness to shock. ASTA and corazonin peptides might modulate stress responses in other ways, such as affecting energy mobilisation (Veenstra, 2009b) similar to glucagon in vertebrates (Bednářová et al., 2013). Such specific activation (by corazonin) or inhibition (by ASTA) of energy mobilisation as a response to stress could be tested, for example by measuring sugar titers in hemolymph after various doses of both peptides.

Because peptide neurohormones are quite small molecules with a simple amino acid sequence, they appear to have changed quite rapidly during evolution, since even a single amino acid change is proportionally a large change in the peptide sequence. However, similar functions are maintained over evolutionary time despite diverging peptide-receptor couples. An important example is found in the intermediate ACP

(AKH/corazonin peptide) found in mosquitoes but not in the honeybee (Hansen et al., 2010) which is suggested to be a stress signalling peptide. Another example is the glucagon-insulin pathway regulating homeostasis as part of the stress response in mammals, which mirrors the coupled AKH-insulin-like peptide pathways in insects (Bednářová et al., 2013). Our result for ASTCC in addition to that of Urlacher (2011) might be another example illustrating conservation of function (ie: analgesic effect to protect the homeostasis of the body) without conservation of structure (no opioid system described in the honeybee but evidence of common ancestor).

The general stress response model of honeybee (Chapter 1) suggests a crucial role of circulating biogenic amines to signal stress responses (Papaefthimiou et al. 2011; Farooqui 2012). In this study the level of biogenic amine release in the brain and in the periphery was increased with cocaine, and the behavioural responses to an acute stressor were tested. The results show a clear dose-dependent effect of cocaine on sting responsiveness to electric shock (Fig 5). In vertebrates, cocaine affects specifically dopamine signalling by increasing its extracellular concentration as it blocks its reuptake transporter. In honeybees, cocaine treatment increases expression of the honeybee transporter (*Am*DAT) and delays a dopamine external treatment to return to its basal level, thus suggesting a similar effect as in vertebrates (Søvik, 2013). Here, higher doses of cocaine increased the proportion of bees that stung at lower voltages, meaning that cocaine increase the sensitivity to the stressor. The delivery by volatilised treatment does not allow us to differentiate whether the main effect of cocaine resulted from actions at the periphery, or in the brain, or both. However as cocaine's primary action of cocaine is to alter extracellular levels of biogenic amines our findings are consistent with the fact that increased extracellular biogenic amines increases the response to an electric stressor. Overall this result is consistent with the hypothetical model proposed in chapter 1. In that

chapter, we proposed that octopamine and possibly dopamine are released broadly to act on various target organs to coordinate simultaneous responses to cope with stress-inducing stimuli. These responses include elevated heart beat, locomotion, energy mobilisation in fat bodies or crop, inhibition of reproductive function and increased sensitivity to various senses.

In Summary, the haemal ASTCC peptide seems to modulate stress response by acting as a mild analgesic in response to a noxious stimulus, while ASTA and corazonin may modulate stress response following exposure to other stressors as they do not seem to affect a general behavioural stress response here. Their actions in energy mobilisation in stress response remain to be tested. Finally, using cocaine as a pharmacological blocker of biogenic amine transporter affecting dopamine and maybe octopamine levels, we proposed a relationship between extracellular biogenic amine levels and responsiveness to a physical stressor.

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Chapter 5

Altruistic Behaviour by Egg-Laying Worker Honeybees

Abstract:

If a honeybee (*Apis mellifera*) colony loses its queen, worker bees develop their ovaries and produce male offspring (Seeley, 1985). Kin selection theory predicts that the degree of altruism in queenless colonies should be reduced because the relatedness of workers to a hivemate's offspring is less in queenless colonies than it is to the daughters of the queen in queenright colonies (Ratnieks, 1998; Ratnieks and Wenseleers, 2008; Ratnieks et al., 2006). To explore this hypothesis, we examined the behaviour and physiology of queenless egglaying workers. Queenless bees engaged in both personal reproduction and the social foraging and defense tasks that benefited their colony. Laying workers also had larger brood-food-producing and wax glands, showing metabolic investments in both colony maintenance and personal reproduction. Whereas in queenright colonies there is a very clear age-based pattern of division of labour between workers, in queenless colonies the degree of individual specialization was much reduced. Queenless colonies functioned as a collective of reproductive and behaviourally generalist bees that cooperatively maintained and defended their nest. This social structure is similar to that observed in a number of primitively social bee species (Michener, 1974). Laying workers therefore show a mix of selfish personal reproduction and altruistic cooperative behaviour, and the queenless state reveals previously unrecognized plasticity in honeybee social organization.

Results and Discussion

Honeybees (*Apis mellifera*) form one of the most complex animal societies. Within a colony, the single queen is typically the sole reproductive, supported by thousands of her daughters, who form a highly specialized and sterile worker caste. Kin selection theory has provided a framework for understanding the evolution of these pronounced social and altruistic traits (Foster et al., 2006; Hamilton, 1964; West-Eberhard, 1975). The theory proposes that altruistic traits would be selected for and spread if they increase the reproductive success of the altruists' relatives (Foster et al., 2006; West-Eberhard, 1975). The unusual kin structure of queenright haplodiploid hymenopteran honeybee colonies provides conditions that promote both the evolution of worker altruism and mutual enforcement of worker sterility by policing (Queller and Strassmann, 1998; Ratnieks, 1988). Indeed, both evidence and theory suggest that the level of altruism seen in an animal society (considered in terms of investment in colony maintenance and raising relatives' offspring rather than personal reproduction) is a function of the relatedness structure of the colony (Wenseleers and Ratnieks, 2006a; Wenseleers and Ratnieks, 2006b; Wenseleers et al., 2004).

If workers are unable to raise a replacement queen, the colony becomes hopelessly queenless. In this phase, the only reproductive options available to workers are to produce their own male offspring (workers cannot mate, and their haploid eggs develop into males) or assist other workers in reproducing and thereby raise their nephews (Page and Erickson, 1988; Page and Metcalf, 1984; Seeley, 1985). The relatedness structure of a queenless honeybee colony is radically different from a queenright colony (Wenseleers et al., 2004), and under such conditions, the level of altruism displayed by workers is expected to decrease and the degree of reproductive conflict to increase (Wenseleers and Ratnieks, 2006a; Wenseleers et al., 2004). It is well known that many queenless workers develop their ovaries and lay eggs (Figure 1A). Under those circumstances, it is commonly assumed that reproductive workers selfishly prioritize their own reproduction over colony tasks; this raising of sons offers a direct fitness benefit, as compared to assisting with raising less-related nephews or brothers (Cardoen et al., 2011; Hillesheim et al., 1989; Woyciechowski and Kuszewska, 2012), and should cause workers to stop performing the demanding and risky foraging and defensive tasks that benefit the colony (Cardoen et al., 2011; Mattila et al., 2012). However, the behaviour of workers in

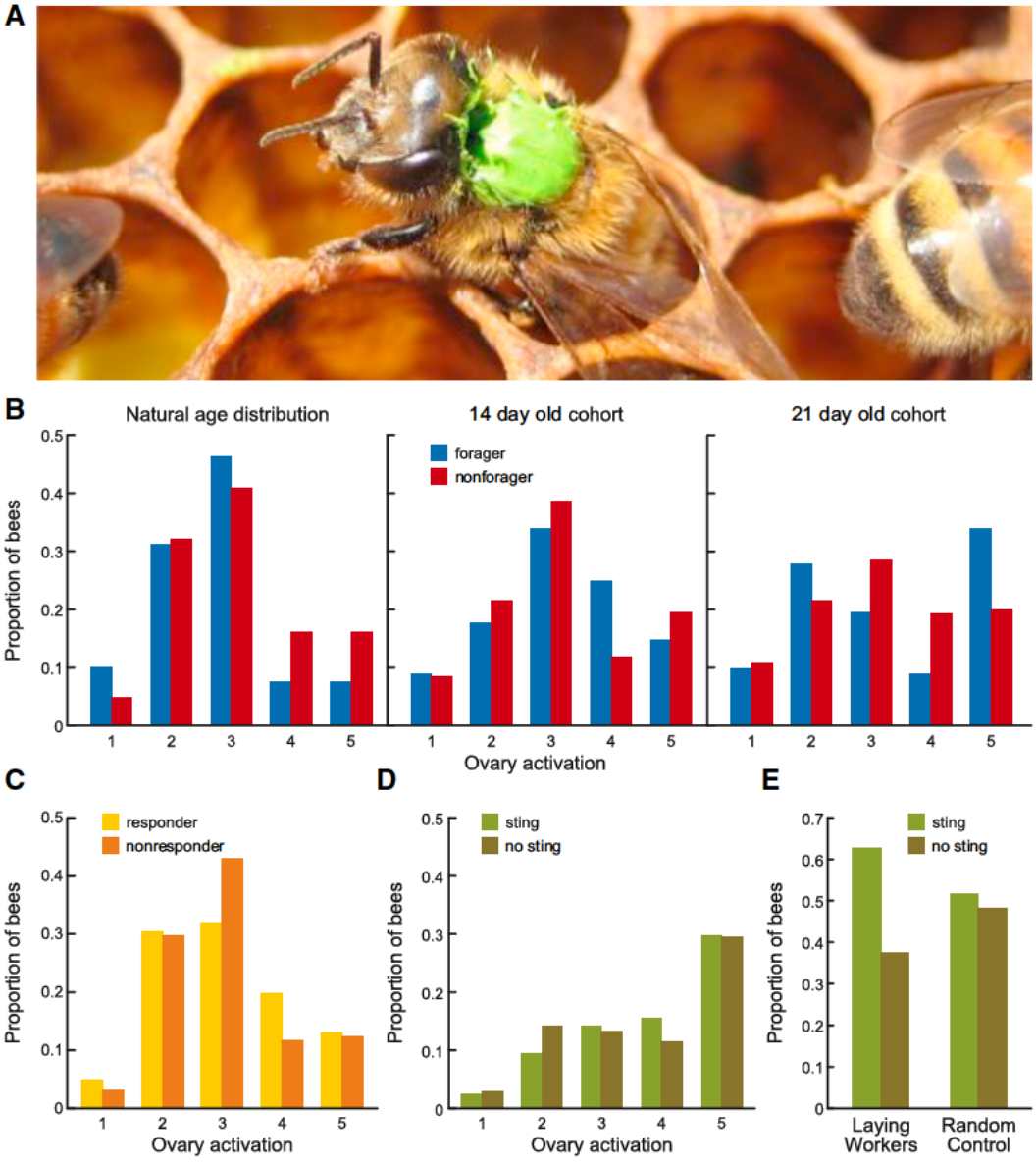
queenless honeybee colonies has been little studied. Here, we examined the behaviour and physiology of workers in hopelessly queenless colonies to determine whether altruism persists, and to examine the nature of social organization in the queenless condition.

Foraging benefits the colony but is both metabolically costly (Williams et al., 2008) and risky (Woyciechowski and Moroń, 2009) for the individual bee. To determine whether laying worker bees engaged in personal reproduction continued altruistic behaviours, we sampled forager and nonforager bees from queenless colonies and dissected them to assess their level of ovary activation (Hess, 1942). We found no difference in the degree of ovary activation between forager and nonforager bees (Figure 1B). Furthermore, in comparisons of age-matched samples taken from three independent queenless colonies, at 14 days of age there was no difference in the level of ovary activation between foragers and nonforagers, but at 21 days of age the overall degree of ovary activation was higher in foragers, and foragers were more likely to have fully developed ovaries (containing at least one developed egg) than nonforagers (Wald χ^2 = 9.216, n = 73, df = 1, p = 0.002). In addition, bees that were marked in the act of laying were as likely to be later observed foraging as bees that did not lay (Wald χ^2 = 0.300, n=30, df=1, p=0.5839; see Figure S1). For these analyses, ovary development was scored on a five-point scale following Hess (1942). Collapsing these data to a binary scale considering levels 1 and 2 as inactive and levels 3+ as active (a common convention for these data), we found that ovary development was significantly influenced by the presence or absence of the queen (generalized mixed model assuming binomial error: analysis of deviance: $p < 0.001$) and varied between colonies in our study ($p = 0.038$), but there was still no significant difference in levels of ovary activation between foragers and non-foragers ($p = 0.426$). A similar mode of analysis confirmed no difference in ovary development between foragers and nurses ($p = 0.785$), source colony ($p = 0.226$), or age ($p = 0.724$) in the age-matched samples collected at 14 and 21 days old. In summary, several experiments conducted with seven different colonies showed that reproductive workers in queenless colonies are as likely to forage as bees with less-developed ovaries.

Foraging is individually costly, but participating in colony defense is suicidal because the act of stinging causes the death of the individual worker. To test whether laying workers altruistically engage in colony defense, we disturbed a queenless colony by removing the

hive cover and shook a black lure over the exposed honeycombs to then sample bees that attacked the lure and bees that did not. There was no difference in the level of ovary activation between attackers and nonattackers (Figure 1C). We also tested the likelihood to sting in response to an electric shock in a laboratory assay for aggressiveness (Uribe-Rubio et al., 2008). Level of ovary activation had no effect on the likelihood of stinging in response to the shock stimulus (Figure 1D). Furthermore, there was no difference in the likelihood to sting for bees with fully developed ovaries compared to those without (Fisher's exact $p = 1.0$), or those observed to have laid an egg compared to randomly sampled control bees (Fisher's exact $p = 0.279$; Figure 1E). Taking these results together, multiple experiments conducted with five colonies indicated that reproductive workers in queenless colonies are as likely to engage in colony defense as bees with less developed ovaries.

Figure 1. Reproductive worker honeybees do not avoid risky behaviors. (A) A worker honeybee, previously marked as a forager, laying an egg. (B) Distribution of mixed-age foragers and non- foragers with different levels of ovary activation (n = 119 foragers and 125 nonforagers; ordered logit, pseudo R^2 = 0.0305, p = 0.287) and age-matched cohorts of 14 days (n = 68 foragers and 153 nonforagers; ordered logit, pseudo R^2 = 0.0027, p = 0.590) and 21 days (n = 133 foragers and 140 nonforagers; ordered logit, pseudo R^2 = 0.0269, p = 0.042). (C) No differences were observed in ovary activa- tion between bees that defended the hive against a simulated vertebrate predator (n = 207) and those that did not (n = 195) (ordered logit, pseudo R^2 = 0.0065, p = 0.084). (D) No differences were observed in ovary activa- tion between bees that responded to a 9V electric shock by stinging (n = 75) and those that did not (n = 60) (ordered logit, pseudo R^2 = 0.0065, p = 0.159); bees observed laying eggs were purpose- fully oversampled. (E) No differences were observed in likelihood of stinging in response to 9V electric shock between bees observed laying eggs (n = 48) and randomly sampled hivemates (n = 87) (Fisher's exact test, p = 0.279).



In addition to engaging in personally risky behaviours that benefit the colony, reproductive workers in queenless colonies also metabolically invested in brood food and wax production for the good of their colony. Queenright honeybees show precise task-related physiological specializations, with a negative association between ovary development and development of the brood-food-producing hypopharyngeal glands (HPGs) (Woyciechowski and Kuszewska, 2012), demonstrating a physiological trade-off between personal reproduction and investment in colony maintenance. By contrast, we observed a significant positive correlation between ovary development and HPG development in bees from queenless colonies (Figure 2A). There was also a significant positive correlation between ovary development and the number of fully formed wax flakes produced by the abdominal wax glands (Figure 2B).

The observed coactivation of HPGs and wax glands in queenless bees deviates markedly from the precise task related physiological specializations typically seen in workers from queenright colonies, which have a predictable age-based system of division of labour (Robinson, 1992; Seeley, 1985). To explore this further, we performed direct comparisons of bees in queenright and queenless colonies of similar population sizes. After marking all foragers over the course of at least two days, we collected them along with samples of nonforaging hivemates and measured development of the ovaries, HPGs, and wax glands. Foragers in queenless colonies (N = 4) had greater HPG (ordered logit, n = 194, pseudo R^2 = 0.1772, $p < 0.0001$; Figure 2C) and wax gland development (ordered logit, n = 195, pseudo R^2 = 0.1445, p = 0.001; Figure 2D) than those in queenright colonies (N = 3). Similar results were obtained for a sample of queenless foragers that were 8 weeks old and were known to have been foraging since 3 weeks of age (Figure S2).

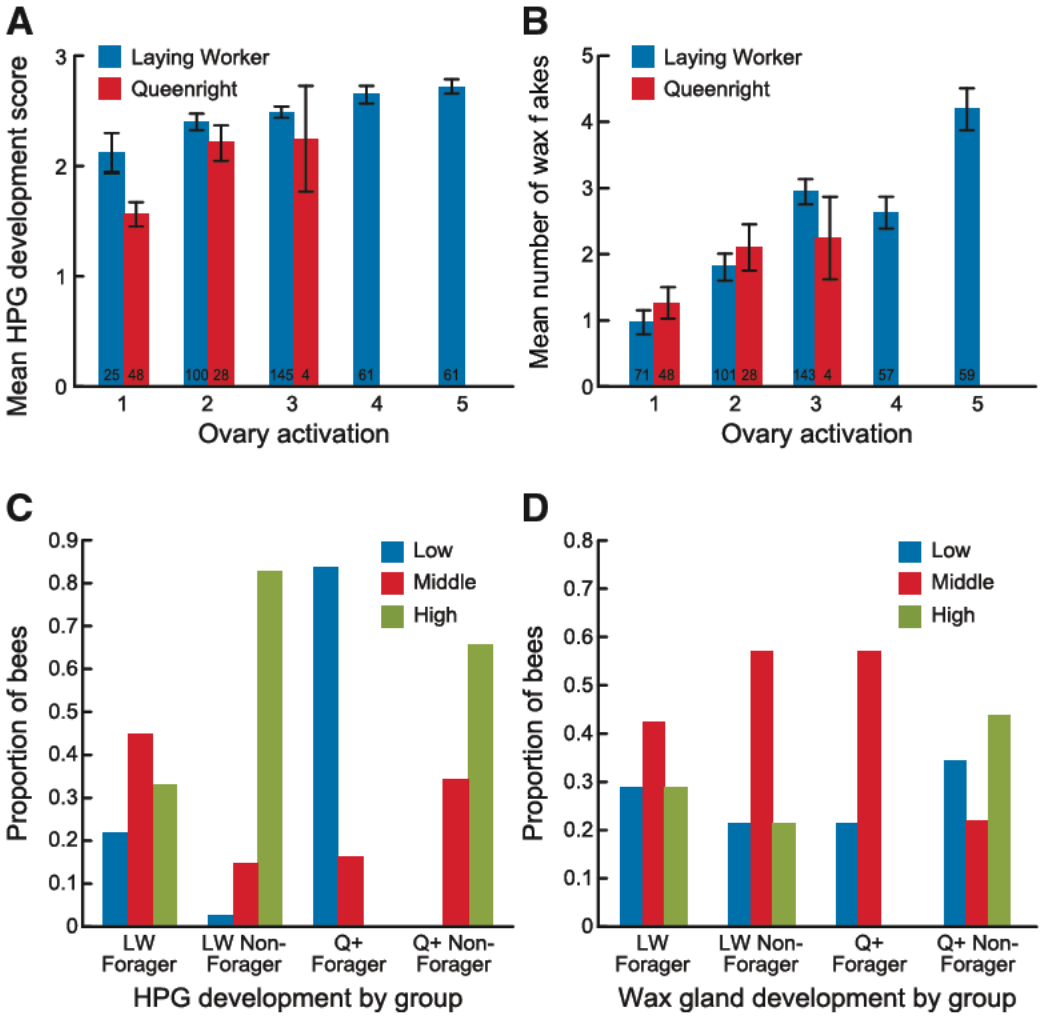
Principal component analysis based on ovary, HPG, and wax gland development measurements (N = 6 colonies, n = 165 individuals) revealed that whereas queenright workers differentiated into separate forager and nonforager clusters, queenless workers did not (Figure 3). These results indicate that task specialization has broken down in queenless colonies, with forager bees maintaining the capacity to engage in brood care and colony maintenance tasks as well as personal reproduction.

Previous reports have shown a negative association between level of ovary development and level of foraging activity (Dampney et al., 2002; Hillesheim et al., 1989;

Mattila et al., 2012) and HPG development (Woyciechowski and Kuszewska, 2012) in queenright colonies, where full ovary activation and worker reproduction are very rare. Although in this study we did not directly compare worker activity levels in queenright and hopelessly queenless colonies, we have conclusively shown that queenless workers split investment between both their own personal reproduction and the altruistic behaviours of foraging, colony defense, and maintenance, and that engaging in personal reproduction does not reduce the likelihood of bees engaging in colony defense and foraging roles.

In a queenright colony, worker task specialization is organized by temporal polyethism, with bees beginning life engaged in in-hive tasks and delaying high-risk colony defense and foraging tasks until later in life (Jeanne, 1986; Tofilski, 2002; Tofilski, 2009). Elements of this pattern were preserved in queenless colonies, in that most queenless workers commenced foraging when >2 weeks old (comparable to behavioural development in queenright colonies; Figure S3). Beginning high-risk foraging tasks later in life is a common pattern across social insects and appears to be an evolved strategy to maximize lifespan, lifetime colony investment, and personal reproduction (Jeanne, 1986; Tofilski, 2006). This basic pattern was preserved in queenless colonies, but unlike in queenright colonies, bees did not then exclusively specialize on foraging.

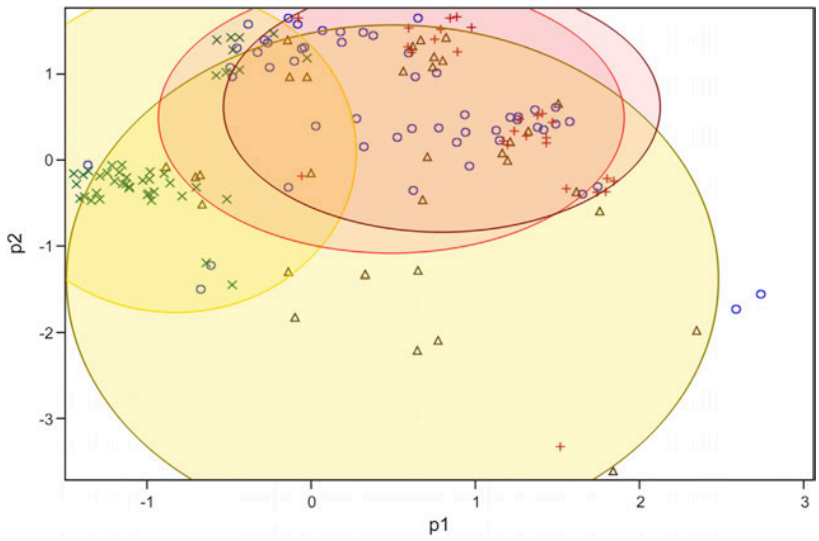
Figure 2. Reproductive worker honeybees maintain physiology for hive Tasks (A and B) The level of hypopharyngeal gland (HPG) development (A) and wax production (B) is positively correlated with the level of ovary activation in queenless colonies (least-squares regression, n = 392, R^2 = 0.049, p < 0.0001 for HPG; least-squares regression, n = 431, R^2 = 0.163, p < 0.0001 for wax). Workers in queenright colonies rarely have highly activated ovaries. Sample sizes are shown at the base of the bars; error bars represent SEM. (C and D) Workers in colonies with laying workers were more likely to maintain HPGs (C; ordered logit, n = 194, pseudo R^2 = 0.1772, p < 0.0001) and wax glands (D; ordered logit, n = 195, pseudo R^2 = 0.1445, p = 0.001) while foraging than workers in queenright colonies.



The generalist behaviour of reproductive workers in queenless colonies that forage and defend the hive while maintaining the ability to care for brood, build comb, and lay eggs is similar to solitary or primitively social bees. Queenless honeybee colonies resemble a communal form of social organization called ‘‘quasisociality,’’ defined as individuals of a single generation that share a nest and exhibit cooperative brood care (Michener, 1974). This type of sociality is exhibited by many euglossine orchid bees (Michener, 1974), the most closely related extant taxon to the honeybees (Cardinal and Danforth, 2011). The queenless state thus exposes heretofore unrealized plasticity in honeybee social organization, with queenless bees manifesting an atavistic social structure typical of many primitively social species.

Our data support the predictions from kin selection models that reproductive conflict is increased in queenless colonies (Wenseleers et al., 2004), but altruism is far from eliminated, and individual bees split investment between selfish and altruistic behaviour. For a hopelessly queenless colony, there may be a strong selective advantage for reproductive workers to prolong the life of their failing

Figure 3. Honeybee Colonies with Laying Workers Lose Division of Labour. Principal component analysis of HPG, wax gland, and ovary development revealed that foragers (green, 3) and nonforagers (brown, 6) from queenright colonies formed distinct clus- ters, whereas foragers (blue, B) and nonforagers (red, +) from colonies with laying workers did not.



Methods

Honeybee Colonies

All colonies were mixed races of *Apis mellifera*, mostly ligustica. Colonies 1– 4 were each established on January 13, 2011 at Macquarie University, North Ryde campus, New South Wales, Australia. Each colony was started from a 1 kg ‘‘package’’ of bees from Australian Queen Bee Exporters. Packages are artificial swarms created by collecting young worker bees en masse from the brood nests of many different colonies and represent a mix of genotypes. They were installed into hives with five honeycomb frames: two frames of honey; one frame with a cell diameter appropriate for male larvae; and two frames that contained a mix of empty cells, pollen, honey, and worker brood. The hives were monitored for replacement queen cells, which were removed in colonies 1, 2, and 4. Colony 3 was allowed to rear a replacement queen to serve as a queenright control. After worker-laid brood appeared, frames of honeycomb containing brood were taken from queenright colonies and placed in an incubator at 34˚C. One-day-old adult workers that came from these frames were marked with a paint dot on the thorax and introduced as cohorts of 1,000 individuals into each of the colonies. Colonies 5–10 were created by moving five frames of honeycomb (as above) and several thousand workers from a large colony into a new hive. Colonies 5, 6, 7, and 9 were created queenless, whereas colonies 8 and 10 had the queen moved along with her workers to the new hive. The four queenless colonies were monitored, and the rearing of replacement queens was prevented to force the colonies to become hopelessly queenless. Colonies 5 and 6 were established in Sydney, Australia, and colonies 7–10 were established at the University of Illinois Bee Research Facility, Urbana, Illinois, USA. Colonies were transported to a new location to prevent the bees from flying back to the original hive. Experiments were not started until the first worker-laid brood appeared.

Foraging Assays

To compare ovary activation between foragers and nonforagers of known age, we monitored the paint-marked cohorts in colonies 1, 2, and 4 for at least four periods of 15 min per day before midday and another four periods after midday. Foragers were identified by either a visible pollen load on the corbicula or a distended abdomen and were painted on the abdomen with a unique color for each day. This continued from day 8

to day 21 of age. During this interval, frames were occasionally removed from the hive, and bees observed in the act of laying eggs were marked with a paint dot, as were random control bees nearby. The foraging behaviour of these bees was recorded. On days 14 and 21, the hive was opened, and bees marked as foragers and bees from the cohort without a foraging mark were collected. For bees of natural age demographics, all of the foragers from colonies 3 and 5–10 were marked over the course of 2–4 days. Foragers and nonforagers were then collected as they returned to the hive and from inside the hive, respectively. Additionally, 8-week-old bees were collected from colonies 1 and 2 to test whether the maintenance of developed glands into the foraging phase was a result of a younger age at first forage.

Defensive Assays

Defensive behaviour was measured in colonies 1, 2, 4, 5, and 6 by removing a honeycomb frame from the colony and waving a black lure over it. The lure consisted of small leather patch with three stings on it from bees from another colony, surrounded by a ball of black feathers. Bees that flew to and attacked the lure were collected, as well as those that did not respond (considered controls). For the electric shock assays, bees from these colonies that were directly observed laying eggs as well as random nearby control bees were collected individually into vials. Bees were then transferred to a 12 x 3 x 12 cm arena with a floor composed of parallel stainless steel wires 2 mm in diameter. A BK Precision 1696 power supply was used to apply a constant 9V stimulus; this voltage was shown in pilot experiments as well as previous studies (Uribe-Rubio et al., 2008) to be a good discriminating voltage between bees that will versus those that will not sting. Two experimenters, blind to the behavioural group of the bee, observed whether or not the bee stung at the device. Bees were then collected into ethanol for ovary dissections.

Dissections and Gland Scoring

All dissections were performed under dissecting microscopes with the experimenter blind to the behavioural group of the bee. The level of ovary activation was scored on a 1–5 scale in accordance with Hess (1942). HPGs were scored on a 1–3 scale, with a score of 1 representing completely underdeveloped or atrophied glands and a score of 3 representing fully developed glands that filled the internal space between the brain and anterior cuticle. Wax gland development was scored by counting the number of

fully formed wax flakes on the abdominal sternites. Zero or one flakes were considered ‘‘low,’’ two or three flakes ‘‘middle,’’ and four or more flakes ‘‘high’’ in terms of gland development.

Statistical Analysis

Wald χ^2, Fisher’s exact tests, and least-squares regressions were performed using MYSTAT 12 (Cranes Software International). Ordered logits were performed using STATA version 9.2 (StataCorp). For comparing ovary activation between foragers and nonforagers or defensive and nondefensive bees, level of ovary activation was analyzed with an ordered logit model with behavioural classification, colony number, and the interaction as explanatory variables. For comparing HPG activation, the level of activation was also analyzed with an ordered logit model with colony type (queenless or queenright) and colony number as explanatory variables. Principal component analysis was performed in SAS 9.2 (SAS Institute). HPG, wax, and ovary data were transformed using PROC PRINQUAL, and principal components were generated using PROC FACTOR, with jitter applied to allow multiple points occupying the same two-dimensional space to be visible.

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Suplemental figures

Fig S1: **Bees directly observed laying eggs were as likely as random bees to later be observed foraging**.

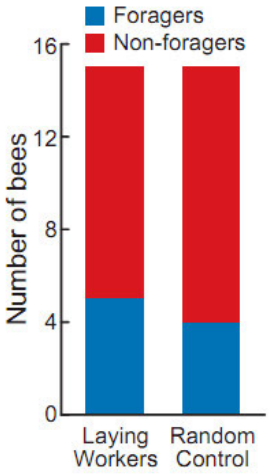


Fig S2: **Eight-week-old bees maintained developed HPGs, wax glands, and ovaries**. This includes bees that had been foraging from before their third week of age, suggesting that the gland development found in other experiments is a result of a loss of division of labor and not a lack of time for the glands to atrophy. Error bars represent SEM.

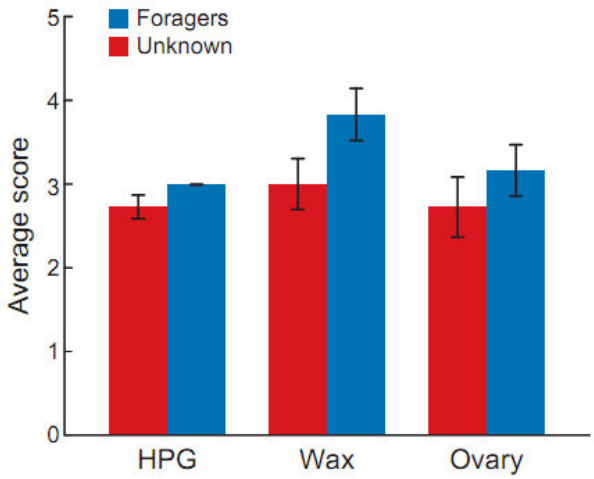
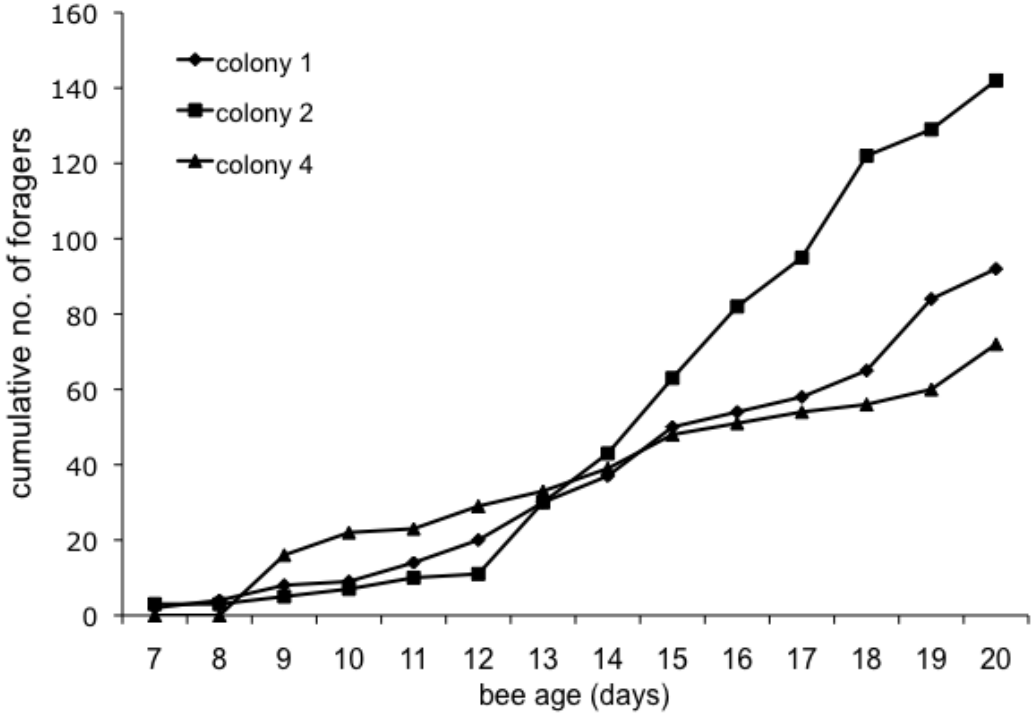


Figure S3 **Assessment of Rates of Behavioral Development in Queenless Colonies**. Cumulative number of bees beginning foraging between 7 and 20 days of age in three independent queenless colonies. In each colony bees were from an age matched and marked cohort of 1,000 bees added to each colony. Rates of onset of foraging in queenless colonies were comparable to those documented from queenright colonies.



Chapter 6

General discussion

As the largest animal biomass on land, insects play vital roles in terrestrial ecosystems, like turning soil, recycling biological matter, primary herbivores in many food chains and pollination (Resh and Cardé, 2009). This latter role is indispensable for the fertilisation of many plants of ecological and economical importance. During the last decade, a decrease of pollinators was recorded worldwide, including the drastic decline of domesticated honeybee populations in the United States (Oldroyd, 2007; VanEngelsdorp et al., 2009). In recent years, researchers have identified multiples potential sources of stress to be responsible for those declines (Ratnieks and Carreck, 2010). Honeybees are threatened by the use of new pesticides (Henry et al., 2012), the worldwide dispersion of parasites, diseases and predators of honeybees (Higes et al., 2008; Johnson et al., 2009), the intensification of beekeeping practices and a reduction in their food diversity (Naug, 2009). To protect honeybees, it is therefore necessary to understand better how they respond to stress physiologically, behaviourally and socially. This thesis furthers the understanding of stress responses in honeybees by addressing several questions: 1 - How can we define "stress" in honeybees? 2 - What are the physiological stress response systems specific to honeybees? 3 - How can stress affect the social organisation of honeybees?

Here I have presented new discoveries regarding these three questions. First, the definition of stress in the honeybee is explained, developed and supported by results, in chapters 1 and 2. Second, some of the physiological factors hypothesised in chapter 1 are confirmed to be part, or not, of the honeybee stress response in chapters 2 and 4. Third, stress is shown to interact with the division of labour within colonies, in chapters 3 and 5, where differences in individual stress sensitivity may explain the organisation of colony tasks, while long-term social stress radically changes such organisation.

1. Definition of stress

Stress is recognized to be a biological concept describing the impact of adverse conditions which disturb the optimal or equilibrium state of a system. Organisms cope with these adverse environmental pressures (stressors) by coordinated physiological and behavioural responses (stress responses) (McEwen, 2009). Different stressors (e.g. physical, toxic, immune, nutritional, thermic, predation…) induce similar responses, (e.g. mobilisation of energy, analgesia, alertness…) to try to avoid or fight its deleterious

effects. Long-term exposure to a stressor can induce three phases of response described by Hans Selye: the alarm, resistance and exhaustion phases (Selye, 1956). This definition of stress is well illustrated in chapter 2, which shows that honeybees stop displaying the sting response after strong and sustained trials in which they are pinched over a prolonged time.

The concept of stress can be applied to all levels of analysis in biology: cells, organs, individuals, societies and ecosystems (Kassahn et al., 2009). In the remaining chapters of this thesis I explored stress responses at two levels. In chapters 2, 3, and 4, I examined stress responses at the individual level measuring physiological and behavioural responses in single honeybees. In chapter 5, I evaluated the impact of a stressor at the level of the colony, which is sometimes qualified as a super-organism (Hölldobler et al., 2009) as it reproduces itself as a unit, and survival of one individual is impossible without a stable colony organisation. I considered behavioural responses and the collective organisation of the whole colony.

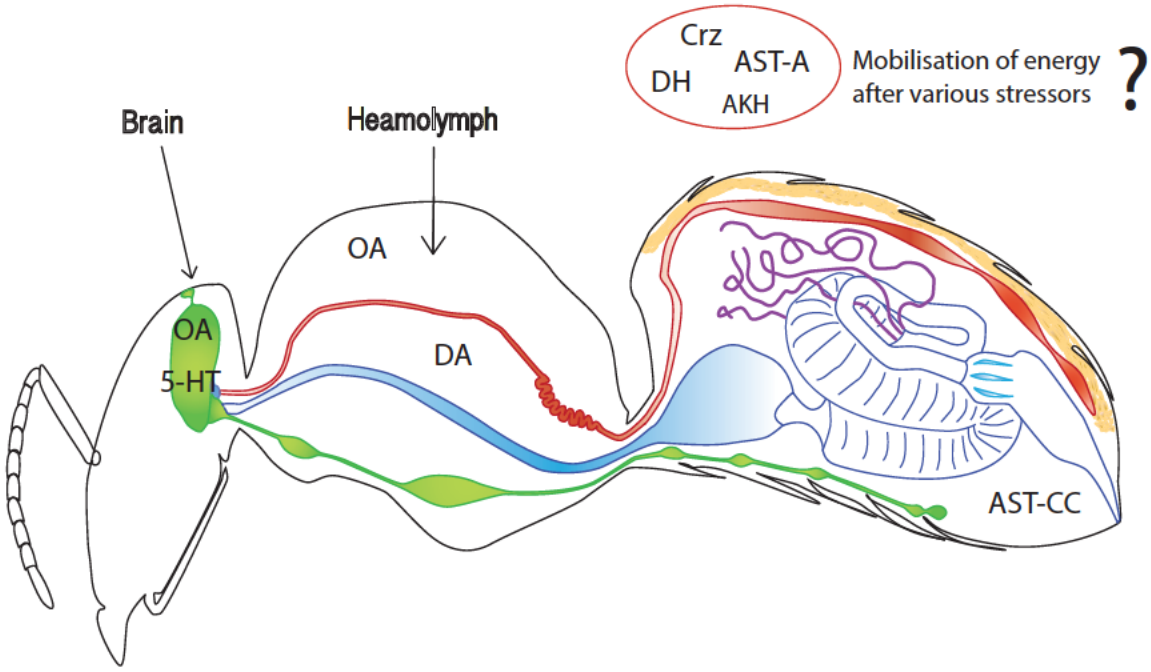
2. Honeybee general stress responses

Chapter 1 hypothesised that stressors induce coordinated physiological responses to stress in honeybees. Based on the wider literature on insect and honeybee stress (see chapter 1), octopamine and dopamine were suggested to increase in the brain, affecting arousal, cognitive abilities and sensitivity to stimuli, as means of coping with the stressor. In chapter 2 we found that one type of physical stressor (harnessing) did increase octopamine and serotonin levels in the brain, but not those of dopamine, after sufficient exposure (three hours). Additionally to an effect on biogenic amine levels in the brain, the initial model strongly suggested that stress would increase circulating amounts of biogenic amines in the hemolymph, certainly octopamine and maybe dopamine. This was strongly supported by the results of chapter 4. In that study volatile treatment of cocaine, which increases systematic biogenic amine stocks, also increased here in a dose-dependant manner the sting response to aversive stimuli. To be able to differentiate which biogenic amines are targeted, it would be interesting to perform similar tests in the future with direct injections of each biogenic amine in the hemolymph or in various regions of the brain. Although it was stated in chapter 2 that whole brain biogenic amine levels are not always the best measure to evaluate their roles, measuring hemolymph

titers would be relevant. Despite efforts to perform such measures in this project, biogenic amine measures from hemolymph failed for different reasons, which included a very high variability of hemolymph total volumes between individuals, but also high pressure liquid chromatography (HPLC) detection ability since confounding products in hemolymph overlay the biogenic amines signals making any quantification of biogenic amine amounts impossible. Another approach, voltammetry, could be very useful for exploring further roles of biogenic amines in stress responses. This technique allows measuring direct release of biogenic amines (due to their specific oxidation proprieties) around synapses (Vickrey et al., 2009). For example this tool could be used to link the application of a stressor (e.g. an electric shock) to an increase of biogenic amine release in various locations in the body (near any organ or brain region).

Additionally, chapter 1 suggested the importance of neuropeptides in the general signalling of stress in the honeybee. Among interesting candidates, I chose to test corazonin and two types of allatostatins (A and CC) present in the honeybee. I tested their impact on behavioural stress responses by measuring the sting response to electric shock. Only allatostatin CC reduced sting responsiveness to the stressor. This result was consistent with previous studies suggesting a role of allatostatin CC in endogenous analgesia (Urlacher, 2011). However, the fact neither allatostatin A nor corazonin affect behavioural stress responses invites further studies exploring their specific metabolic roles. Such studies would complement the two models suggested in chapter 1. For example, it would be interesting to look at corazonin and allatostatin A in addition to diuretic hormones and adipokinetic hormone peptides in energy mobilisation following various stress treatments. I have summarised in Figure 1 the components that were linked to stress responses as a result of work in this thesis.

Figure 1: Summary of the physiological signals studied in this thesis that are linked to stress responses and their potential location of action (brain or hemolymph). AKH: adipokinetic hormone, AST-A: allatostatin A, AST-CC: allatostain CC, Crz: corazonin, DA: dopamine, 5-HT: serotonin, OA: octopamine.



3. The role of stress in the division of labour

The fitness of an insect colony is dependant on the reproductive success of the colony as a unit. Therefore if the colony is under threat by any stressor, the colony reacts as a unit. This is well illustrated in chapters 3 and 5 of this thesis.

In chapter 3, the organisation of defensive tasks was shown to be related to the individual stress sensitivity of the two defensive subcastes: guards and soldiers, thus supporting the "response threshold model". Guards, which are more sensitive to aversive stimuli, act like a sensor for the colony. If the colony is threatened, for example by predators or robber bees, guards are first to respond and will trigger an aggressive response by another subcaste, the soldiers, which are less sensitive to aversive stimuli but do react to alarm signals. This organisation allows the colony to respond accurately to the intensity of the disturbance created by intruders or robber bees. If this defence system

fails, then the threat to the colony is sustained and it will result in the death of the colony, corresponding to the "exhaustion" phase described at the individual level.

Chapter 5 shows that the effect of sustained social stress by the prolonged absence of the queen affects the division of labour within the colony. This situation, which is not uncommon in nature, destabilises the social organisation for colony tasks and reproductive functions. Some workers develop ovaries and begin to lay male-destined eggs, and here we show that the clear distinction in the distribution of colony tasks, paired with physiological traits, disappears. Bees from queenless colonies simultaneously activate hypopharyngeal glands, wax production and ovaries, all while foraging. These traits do not co-occur in queenright colonies. Queenless bees, in becoming task generalists, have to maintain the huge metabolic load of having active ovaries and diverse gland development, but in parallel they also forage, which is known to be extremely metabolically stressful (Williams et al., 2008). In this study we showed that disrupting the colony disrupted individual physiology and probably lifespan (Tofilski, 2002; Tofilski, 2006).

Chapter 5 reveals also how social organisation depends on the homeostasis of the biological organism, here the colony. Social stress triggered by the absence of queen pheromone, also provoked indirectly a series of other social stresses such as the absence of new brood, population decrease, ageing of the whole population. These symptoms can be common to several threatening situations affecting mortality rates of young bees or brood. We could even qualify these symptoms as "general responses to colony threat", in a similar way as they apply to the individual level. To explore this, further studies should focus on colony dynamics and mortality when colonies are facing multiple stressors. Colony dynamics studies have already shown that in theory, when the death rate of foragers is affected, say from stressors like nutrition or disease (Higes et al., 2008; Khoury et al., 2013), the colony population can collapse in just a few days, as observed in "colony collapse disorder" (Khoury et al., 2011). Clearly there is a need to further understand stress responses at the level of the colony, so as to be able in the future to prevent sudden dramatic colony losses.

In conclusion, this thesis emphasizes the need for future integrative studies of stress mechanisms to determine the precise roles and targets of octopamine, dopamine,

serotonin, allatostatins. Also, it highlights that the fragile homeostasis of a honeybee colony can be threatened by diverse stressors, and that the social mechanisms used by colonies to cope with multiple stressors are important to understand. Knowing the mechanisms and the elements of stress responses in honeybees might help in the future, to evaluate the level of stress that an individual or a colony is facing. Measuring and understanding stress is therefore vital to protect this important insect.

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Appendix 1

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Review

General Stress Responses in the Honey Bee

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Abstract: The biological concept of stress originated in mammals, where a "General Adaptation Syndrome" describes a set of common integrated physiological responses to diverse noxious agents. Physiological mechanisms of stress in mammals have been extensively investigated through diverse behavioral and physiological studies. One of the main elements of the stress response pathway is the endocrine hypothalamo-pituitary-adrenal (HPA) axis, which underlies the "fight-or-flight" response via a hormonal cascade of catecholamines and corticoid hormones. Physiological responses to stress have been studied more recently in insects: they involve biogenic amines (octopamine, dopamine), neuropeptides (allatostatin, corazonin) and metabolic hormones (adipokinetic hormone, diuretic hormone). Here, we review elements of the physiological stress response that are or may be specific to honey bees, given the economical and ecological impact of this species. This review proposes a hypothetical integrated honey bee stress pathway somewhat analogous to the mammalian HPA, involving the brain and, particularly, the neurohemal organ *corpora cardiaca* and peripheral targets, including energy storage organs (fat body and crop). We discuss how this system can organize rapid coordinated changes in metabolic activity and arousal, in response to adverse environmental stimuli. We highlight physiological elements of the general stress responses that are specific to honey bees, and the areas in which we lack information to stimulate more research into how this fascinating and vital insect responds to stress.

Keywords: honey bee; *Apis mellifera*; stress; *corpora cardiaca*; dopamine; octopamine; allatostatin; corazonin; adipokinetic hormone; diuretic hormone

1. Introduction

1.1. Concept of Stress

The term stress originated in physics to describe pressure and deformation in a system, but it has been adopted into a biological context through the work of Hans Selye [1 3]. He recognized in mammals, as a "general adaptation syndrome," a similar suite of coordinated reactions to diverse noxious stimuli or "agents" [4]. Selye's concept was at first criticized by physiologists as vague and immeasurable, but he subsequently clarified his concept defining several stress response elements, principally the hypothalamo-pituitary-adrenal (HPA) axis system. Stress is now recognized as a valid physiological concept, which allows organisms to respond to adverse environmental pressures [5]. Most studies use the word "stress" to describe negative treatments applied to organisms in an experiment, such as nutritional, heat or oxidative stress. Here, the triggering stimuli will be called "stressors" while "stress" will be considered as the response syndrome to any aversive or harmful treatment in a specific system. This understanding can be applied to different levels of organization: molecular, cellular, histological, physiological, even ecological or social, but this review will focus on physiological processes involved in an integrated response at the level of the organism. Also, the definition of stress should take into account the duration and intensity of the stressors involved, thereby the distinction between acute or chronic stress responses. Here, we review the putative elements participating in a physiological stress response, and propose an integrated model of the honey bee stress response. Although the model is based to a degree on the stress literature known from other insects [6 9], our intention is to build (as far as possible) a model that is honey bee specific. In doing so, we identify what is known about this particular species, and what may be assumed from our knowledge of other insects. Consistently, we wish to highlight the gaps in our existing knowledge to propose directions for future stress research.

1.2. Why Study Stress in Honey Bees?

The concept of stress is useful in understanding the physiological and behavioral responses of honey bees to harmful situations. This research is timely since, over the last years, beekeepers from different geographic areas have reported a marked increase in honey bee colony failure rates and in the number of stressors affecting bees, including diseases, parasites, pesticides and poor nutrition [10 12]. The syndrome termed "colony collapse disorder" seems to be the result of an accumulation of stressors chronically weakening honey bee colonies [13,14]. As honey bees are the most important insects in agriculture for both pollination of diverse crops and honey production, the recent decline in their populations brings an urgent need to know more about the stress response systems of this ecologically and economically important insect.

In addition, the honey bee is an ideal insect model to understand the evolution of sociality. A key feature of honey bees is their high level of social organization and their well-developed system of division of labor among workers [15]. Honey bees exhibit age polyethism; young workers perform in-hive tasks (e.g., taking care of the brood), then become guards patrolling the entrance of the hive and later become foragers. Studying differences in stress responses across behavioral castes might help elucidate how a defined division of labor has evolved.

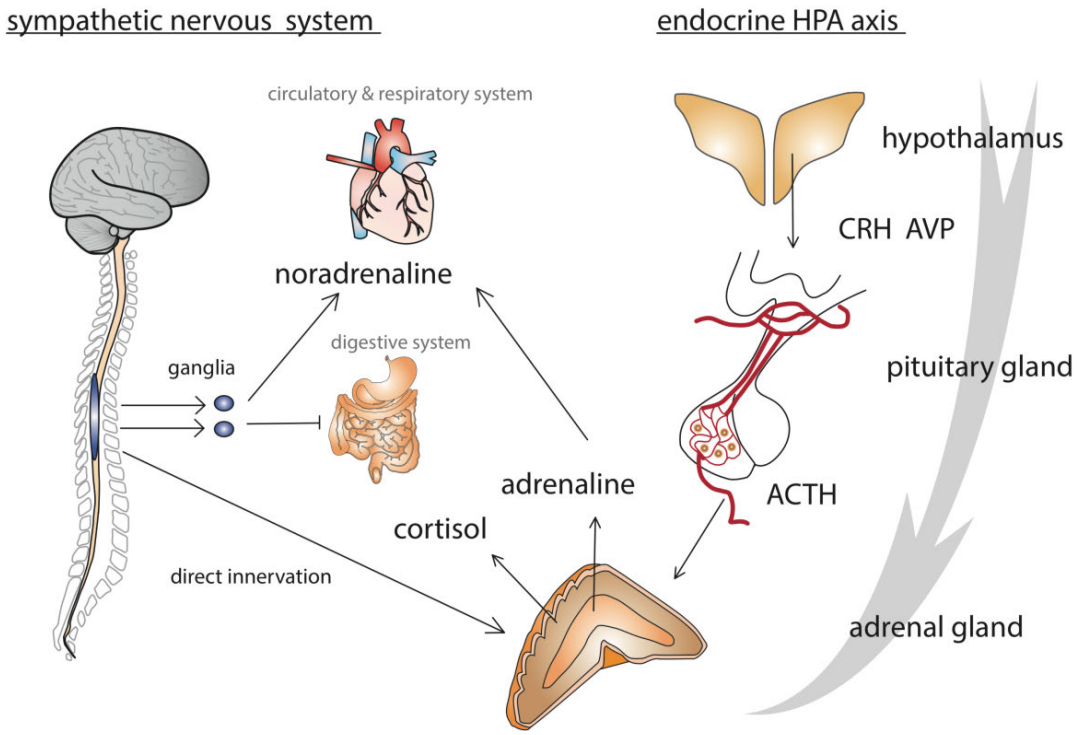
1.3. How Does an Organism React to Stress?

There are three stages to an organism's acute stress response: it first detects the stressor with sensory organs, then responds to it by defense or escape. Finally, if the stressor cannot be avoided and is sustained, the organism enters a state of exhaustion [1]. Following detection of a stressor, mammalian physiological responses are coordinated by neural activity within the autonomic nervous system and the HPA axis (Figure 1). The first immediate response is an activation of sympathetic neurons, which stimulate the adrenal medulla to release adrenaline and noradrenalin into the blood. These two catecholamines increase heart rate and vasoconstriction. In parallel, the paraventricular nucleus (PVN) neurons in the hypothalamus release corticotrophin-releasing hormone (CRH) and arginine/vasopressin (AVP), which are conveyed to the nearby anterior pituitary gland via the blood stream. In response, the pituitary secretes adrenocorticotropic hormone (ACTH), which acts on the adrenal cortex to release glucocorticoids (such as cortisol) causing mobilization of energy reserves (*i.e.*, glucogenolysis in the liver) [16]. Glucocorticoids also potentiate catecholamine release from the adrenal medulla. In parallel, adrenaline activates a release of glucagon by the pancreas to further increase the catabolism of glycogen in the liver and raise glucose concentration in the blood. Other hormones such as cytokines or endogenous opioids may also be produced and/or released, depending on the nature, duration and intensity of the stressor, to act in diverse ways to limit the degree of tissue damage. Therefore, it is interesting to note that, additionally to the general stress response pathways described previously, certain stress responses can vary depending on the type and duration of the stressor.

Under chronic stress, the immune system, metabolic pathways and cognitive processes in the organism gradually weaken until exhaustion and failure are reached [1,17]. For example, repetitive HPA activation resulting in an excess of glucocorticoids in the blood can lead to metabolic diseases such as diabetes [2,18].

Cellular stress responses described in various models (bacteria, yeast, worms and flies) include the increased production or activation of antioxidant proteins and heat shock proteins (HSP) when facing high metabolic load or environmental stressors [19,20]. Such proteins may be called "stress proteins" [21] and used as cellular stress biomarkers [22,23]. These factors are induced by a variety of stressors such as extreme temperatures, elevated ion concentrations or toxic substances, all usually resulting in excessive amounts of denatured proteins [24]. Their actions are principally intracellular and hence we do not focus on them in this review that considers instead more integrated elements of a general stress response.

Figure 1. Human stress response pathways operating through the autonomic nervous system and the endocrine system. This diagram illustrates how neural and hormonal signals interact and complement each other through the regulatory action of the hypothalamo-pituitary-adrenal axis (HPA axis). ACTH: adreno-corticotropin hormone. AVP: arginin/vasopressin. CRH: cortico-releasing hormone. Adapted with permission from Macmillan Publishers Ltd.: Nature Reviews Neuroscience [25], copyright 2009.



2. Model of the Honey Bee Stress Response

2.1. How Has Stress Been Assessed in Honey Bees?

Multiple aspects of stress responses have been used to evaluate stress in honey bees, including behavioral, physiological or cellular stress responses. The many parameters used are listed in Table 1. Behavioral stress responses, usually immediate responses, characterized early on by Cannon [26] as the "fight-of-flight" responses, are easy to observe in the honey bee. For example, extension of the sting (or stinging of a target) has been used to evaluate sensitivity to stressors, and is widely considered as indicative of stress in honey bees, as well as an aggressive response. Physiological measures of stress responses in honey bees include hormonal titers and neurotransmitter levels these parameters have been integrated into our model (see Tables 1 and 2, Sections 2.2 and 3 and Figure 2). Honeybee stress studies usually use acute stressors but the nature and duration of the stressors could sometimes be qualified as chronic (Table 1). Cellular stress responses have also been used in honey bees [27 32], and Duell *et al.* [33] even suggest some cellular stress biomarkers as elements for a diagnostic of general stress in honey bees. Also, since exhaustion is the final stage of chronic stress response described by Selye [1], survival rate has been used to assess the degree of stress.

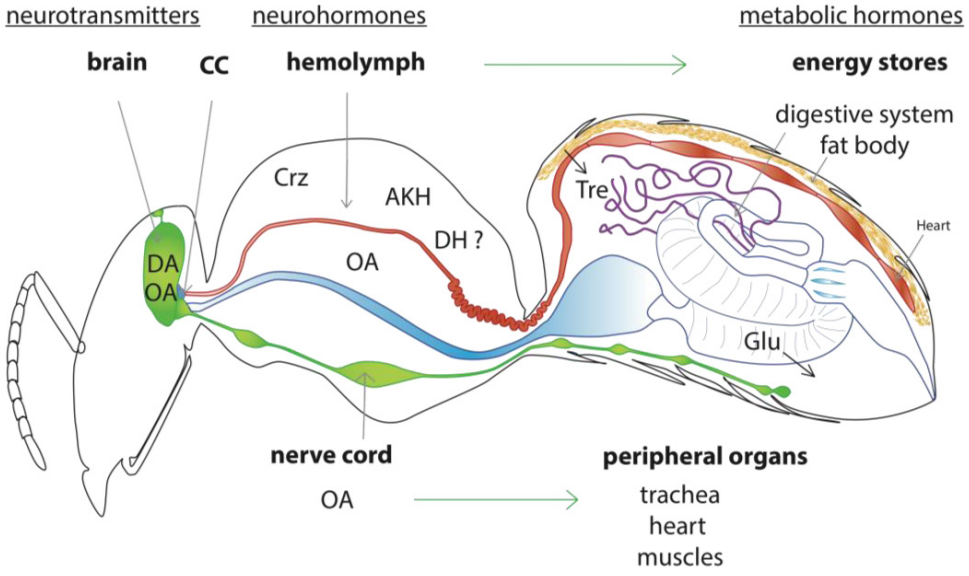
Table 1. Stress response measures used in honey bees. This inventory illustrates the broad diversity of methods used to evaluate stress in honey bees. The table is divided into four parts, depending on the measure used: behavioral, physiological, cellular and survival. In the absence of an objective distinction between chronic and acute stressors, here we qualify stressors ingested or applied continuously during at least four hours as chronic (C); otherwise they are qualified as acute (A).

stress response measure	stressor	acute/ chronic	variable	references
physiological responses				
juvenile hormone (RIA)	cold anesthesia, caging	A	task specialization, duration after treatment	Lin *et al.*, 2004 [34]
brain biogenic amines (HPLC)	spinning, caging, chilling, CO_2	A	spinning speed, duration of stressor	Chen *et al.*, 2008 [35]
brain biogenic amines (HPLC)	leg pinch	A	duration of stressor, age, season, patriline	Harris and Woodring, 1992 [36]
cellular stress responses				
HSP 70 (Elisa)	capture, transport, chilling, harnessing	A/C	ethanol concentration, duration of harness	Hranitz *et al.*, 2010 [31]
HSP70 (western) hsp70, hsc70 (q PCR)	heat	A	duration of stressor, age body part	Elekonich, 2009 [28]
CRH-BP (qPCR)	cold, heat, UV light	A	intensity of stressor, caste, development stage, body part	Liu *et al.*, 2011 [37]
behavioral response				
stinging response	electric shock	A	patriline	Lenoir *et al.*, 2006 [38]
stinging response (delay)	electric shock	A	genotype, housing conditions, task specialization	Uribe-Rubio *et al.*, 2008 [39]
sting extension	electric shock	A	genotype, exposure to alarm pheromone task specialization	Balderrama *et al.*, 1987, 2002 Roussel *et al.*, 2009 [40 42]
sting extension	electric shock	A	morphine and opioid peptides treatment	Núñez *et al.*, 1983, 1997 [43,44]
proboscis extension	soil-borne pollutants	C	treatment concentration	Hladun *et al.*, 2012 [45]
survival				
survival	hyperoxia	C	learning performance	Amdam *et al.*, 2010 [46]
survival	paraquat injection (oxidative stressor), hyperoxia	C	vitellogenin level, reproductive castes	Seehuus *et al.*, 2006; Corona *et al.*, 2007 [47,48]

Table 2. Main elements of the general stress response in the honey bee. The table gives the physiological role(s), known or hypothesized (as indicated by a question mark) for each element, following a classification based on chemical identity.

chemicals	abbreviation	nature	stress-related action	references
biogenic amines				
octopamine	OA	neurotransmitter neurohormone	enhances arousal, increases heart rate, modulates muscle activity	Corbet, 1991; Farooqui, 2012, Papaefthimiou and Theophilidis, 2011, Pflüger *et al.*, 2004 [49–52]
dopamine	DA	neurotransmitter	modulates arousal	Mustard *et al.*, 2010 [53]
peptides				
adipokinetic hormone	AKH	hormone	mobilize energy in the fat body	Kodrik, 2008 [54]
cortico releasing hormone-binding protein	CRH-BP	chaperone?	potentiates or inhibits hormonal release ?	Liu *et al.*, 2011 [37]
diuretic hormone-I	DH-I	hormone	stimulates diuresis induced by crop draining into hindgut after energy mobilization.	Coast *et al.*, 2002 [55]
corazonin	Crz	neurohormone	activates metabolism?	Veenstra, 2009 [56]
allatostatin-A	AST-A	neurohormone	activates gut contraction ? inhibits corazonin neurosecretion ?	Veenstra, 2009 [56]
insulin-like peptide	ILP	?	regulates energy stores ?	Corona *et al.*, 2007 [48]
proteins				
heat shock proteins	HSP70	chaperone	protects cells against oxidative stress and excess protein misfolding	Hranitz *et al.*, 2010, Elekonich, 2009 [28,31]
ERK2	ERK2	?	protects cells against damage ?	Li *et al.*, 2012 [30]
vitellogenin	Vg	antioxidant	protects cells against damage	Seehuus *et al.*, 2006 [47]

Figure 2. Hypothesized model of the general stress system in the honey bee. The brain biogenic amines (OA) and dopamine (DA), acting as neurotransmitters or neuromodulators, would increase arousal, cognitive processes and sensitivity to various stimuli. Then, neurosecretory cells of the *corpora cardiaca* (CC), would release metabolically active hormones into the hemolymph. These may include corazonin (Crz), adipokinetic hormone (AKH), and possibly diuretic hormone-I (DH). This cocktail of hormones mobilize energy from the midgut and the fat body (see detail in Figure 3). Activation of the octopaminergic DUM (Dorsal Unpaired Median) neurons of segmental ganglia of the ventral nerve cord would stimulate activity of skeletal and visceral muscle. Metabolic hormones like allatostatinA, tachykinin-related and insulin-related peptides can be released from peripheral neurosecretory cells, where they can modulate gut motility, and may also contribute to regulate the release of Crz, AKH and DH from the CC.



Based on the data available for honey bees (Table 1) and other insect species we have tried to synthesize a model of a general stress response pathway specific to the honey bee. It should be kept in mind that many proteins or genes of unknown function may be affected by stressors; we will only focus on a few of them, for which sequence homologies and/or functional data suggest a potential role in a physiological stress response.

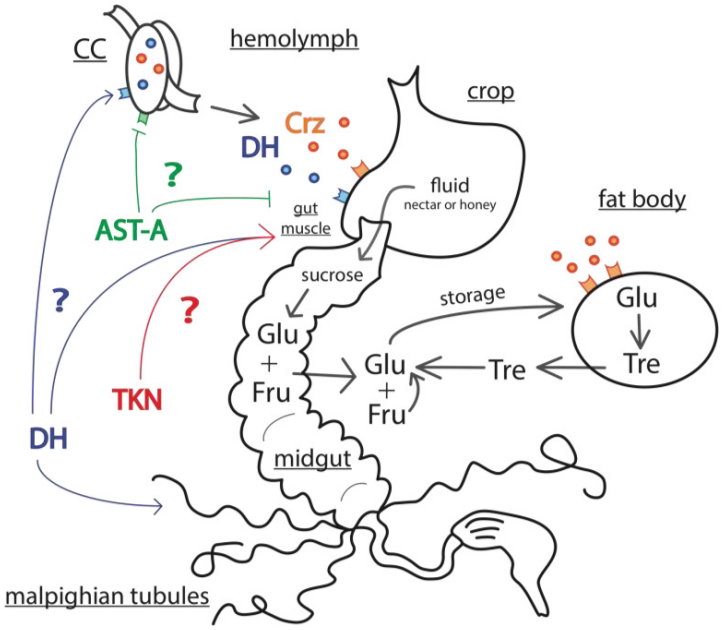
2.2. Model of the Honey Bee Stress Response

When faced with a stressor such as predation, robbing or adverse climatic conditions, a honey bee will need to increase her mobility and mobilize energy reserves to cope with the sudden increase of metabolic demand. We suggest here that this rapid change in physiology is achieved by a coordinated endocrine and neuroendocrine response (Figure 2).

As soon as the stressor is detected via appropriate receptors (e.g., olfactory, mechanosensory or visual), our model proposes that there will be release of octopamine and dopamine within the brain, thus increasing arousal [49] (see Section 3.1). Other signals like cortico-releasing hormone-binding

protein (CRH-BP) might also participate in the brain stress response [37]. Octopamine is also released into the hemolymph [57] from neurohemal cells [58] to act on many organs and coordinate a physiological response to the stressor (see Section 3.2.1). Peripheral octopamine increases heart rate, and may modulate ventilation and stimulate mobilization from muscles [51,59]. The activation of the neurosecretory cells of the *corpora cardiaca* (CC), the major brain neurosecretory organ, stimulates the release of several neurohormones: adipokinetic hormones (AKH) and possibly corazonin, into the hemolymph [6,54,56] to mobilize energy from body stores (see Section 3.2). Finally, we suggest here (from honey bee physiology studies) that hormonal factors including AKH and candidates like allatostatin-A (AST-A), diuretic hormones (DHs) and tachykinins [56], could reinforce the liberation of trehalose and glucose from the fat body, but also from the main energy store of the honey bee the crop [56,60,61] (see Section 3.3 and Figure 3).

Figure 3. Hypothetical model of energy mobilization in honey bee**.** Glucose (Glu) and trehalose (Tre) are the main sources of energy in the hemolymph. Trehalose is stored in the fat body and, when necessary, is released into the hemolymph to be metabolized into glucose. Another source of hemolymph glucose is sucrose from nectar contained in the crop. If hemal carbohydrate levels drop, an influx of nectar is passed from the crop to the midgut via muscle contractions. In the midgut, sucrose is metabolized into fructose (Fru) and glucose, which are then transported to the hemolymph. The passage of nutrients from the crop to the midgut is allowed by contraction of the gut muscle, also named the proventriculus. During normal metabolic demands this influx from the crop depends on the carbohydrate concentration in the hemolymph [61,62]. In energy-demanding situations, this process might be boosted by tachykinin-related peptides (TRPs), diuretic hormone-I (DH-I), corazonin (Crz) and adipokinetic hormone (AKH) while an inhibitory effect from allatostatin A (AST-A) secreted from the midgut would be relieved. DH-I may also exert feedback on corazonin-secreting cells of the *corpora cardiaca* (CC).



3. Molecular Signals of the Honey Bee Stress Response

3.1. Stress Indicators within the Brain

3.1.1. Biogenic Amines

The role of catecholamines as hormones and neuromodulators in the acute stress response is extremely well conserved and well documented in vertebrates [63]. In insects, the biogenic amines octopamine and dopamine are also involved in responses to stressors [8,64,65]. Their respective receptors are phylogenetically related to adrenergic and dopaminergic receptors [50,66,67], showing a strong conservation of both structure and function through more than 500 million years of evolution. These amines regulate many aspects of insect physiology and behavior [50,67], but principally have been shown to increase arousal state and motor activity in several insect species [49,68]. In *Drosophila*, dopamine modulates sleep and locomotion, thus paralleling the functions of dopamine in mammalian circadian rhythms and arousal state [69,70]. Similarly, octopaminergic neurons from the *pars intercerebralis* regulate the sleep:wake cycle [71] ("endogenous arousal") and octopamine signaling has been implicated in arousal increase in response to environmental stressors ("exogenous arousal") [49]. Both forms of arousal are inversely regulated by dopamine, which exerts an inhibitory control on stressor-induced locomotor hyperactivity [72]. In the honey bee, there is evidence that dopamine and octopamine modulate motor activity [53,73]. In many insects, including honey bees, octopamine treatments have been shown to increase sensitivity to sensory inputs [68,74 77]. Moreover, two studies have shown that exposure to physical stressors modifies brain levels of octopamine and dopamine in honey bees [35,36].

Both octopamine and dopamine also modulate learning of a stressful event, particularly dopamine [78 80]. In this regard, the functions of these biogenic amines parallel those of catecholamines (adrenaline and noradrenalin) in mammals, which modulate not only the initial neurohormonal cascade of the stress response, but also the learning of a stressful event [81]. Therefore, both in mammals and invertebrates, signaling through biogenic amines mediates both the initial stress response and the capacity to learn about the stressors triggering the response, thus potentially modulating behavioral and physiological reactions upon further exposure to these stressors.

3.1.2. Cortico-Releasing Hormone-Binding Protein (CRH-BP) and Its Putative Diuretic Hormone Ligand (DH-I)

Cortico-releasing hormone (CRH), also called cortico-releasing factor (CRF), is a crucial signaling element within the vertebrate HPA axis [82]. Its action is negatively regulated by the CRH-binding protein (CRH-BP) [83] The CRH receptor and CRH-BP are strikingly conserved both structurally and functionally throughout vertebrates as hormonal regulatory elements of the stress response [84 86]. CRH-BP even shows a degree of conservation in honey bees [85]. The predicted *Apis mellifera* CRH-BP shares only 25% 29% identity with the vertebrate CRH-BP, but the sequence comparison reveals that amino acids potentially crucial for the 3D structure (cysteines forming bisulfure bridges) [85] and for ligand binding are conserved. Interestingly, its homolog in the Asian honey bee, *Apis cerana* (*Acc*CRH-BP), is expressed as the transcriptional level in the brain [37], and upregulated following

application of various acute stressors such as UV light, heat or cold [37]. This increase by diverse stressors strongly suggests a signaling role in general stress pathways, even though the role of CRH-BP in insects (chaperone protein or link with the hormonal cascade) needs to be explored [83].

Despite this apparent conservation of CRH-BP in insects, no obvious homolog of CRH has been found yet, but precursor peptides of the vertebrate CRH family display similarities with the insect diuretic hormone-I (DH-I, also named DH31 in *Drosophila*) [84,85,87] which has been suggested to be a good candidate ligand for CRH-BP [85]. Still, a clear link between DH-I and the stress response is lacking, but we note that the regulation of water balance via DH-I action on the excretory system [55] could be essential to mobilize energy sources from the honey bee crop. Since DH-I is detected in the CC [88], it might well be part of a coordinated neuroendocrine cascade preparing the honey bee for rapid energy mobilization in energy-demanding situations (see Section 3.3 and Figure 3). Therefore, we think that DH-I and CHR-BP are good candidates as putative elements of the stress response whose action would be worth considering in the future.

3.2. Coordinated Peripheral Stress Responses

In the periphery, the immediate physiological stress response might be coordinated by nerve signals allowing a very fast reaction, but (as in vertebrates) neuroendocrine systems seem to also play a major role in honey bees and other insects. Important components are neurosecretory cells of the CC, which integrate neuronal signals and may trigger broad effects in a variety of target cells through endocrine signals in the hemolymph [89]. Like the vertebrate pituitary gland, the insect CC houses many neuroendocrine cells that play a central role in the regulation of diverse metabolic functions [90].

3.2.1. Octopamine

Additionally to its role as a neurotransmitter and a neuromodulator in the brain, octopamine also acts in periphery, mainly as an endocrine signal. Increases of octopamine level in the hemolymph have been measured in energetically demanding, “fight-or-flight” situations [8,50,57,59,67]. A large literature from locusts, cockroaches, flies and moths demonstrates that many insect organs are sensitive to octopamine, including flight and visceral muscles [91 93], reproductive organs [94 98], heart [99 102], air bags [103], sense organs [104], metabolic tissues such as the fat body [105 109] and malpighian tubules [110,111]. These two latter organs have key roles in energy mobilization in honey bees (see Section 3.3 and Figure 3). Hence, octopamine is in the position to trigger broad and coordinated physiological changes such as the ones expected in a general stress response [112,113]. Several authors have proposed it to be the major stress hormone in insects, including honey bees [8,50,59,67,114], but its specific action on each cell population remains to be clarified in detail [50,67].

In response to threats, octopamine primes flight and leg muscles in locusts [52,115,116]. A similar action in honey bees has not been demonstrated yet, but would be consistent with its positive action on locomotor (specifically flight) activity [73]. In parallel, octopamine seems to also be a cardiostimulant and an activator of the respiratory system to increase oxygen supply to muscles. Modulation of the respiratory system by octopamine is not well understood in insects, but octopamine can stimulate respiratory activity through increasing the hemolymph circulation in the Dobson fly, *Corydalus cornutus*, and the locust *Schistocerca americana* [68,117]. Octopamine was also shown to stimulate

respiratory neurons in *Locusta migratoria* [118]. Evidence of similar respiratory effects of octopamine in honey bees is still lacking, and thus more research is needed in this area. Recently, Papaefthimiou and Theophilidis [51] have shown *in vitro* a biphasic effect of octopamine on heart activity in honey bees. A high concentration of octopamine increases the contraction frequency of the heart, but a low concentration has the opposite effect. The authors argue that this double action may indicate the presence of different types of octopamine receptors on the heart, but another explanation may be due to the participation of diverse signaling pathways depending on OA concentration, since receptor activation can trigger different intracellular signals for different OA concentrations, at least *in vitro* [119].

To perform all these diverse functions, octopamine very likely acts both as a neurotransmitter and as a neurohormone [64]. While the distribution of octopaminergic neurons has been described in detail in the honey bee brain and subesophageal ganglion [58,120 122] very little is known about its distribution in the nerve cord and motor nerves. This state of knowledge contrasts heavily with the well-characterized network of extensive efferent unpaired octopaminergic neurons in locusts and cockroaches [123 126]. Thus, data from these species suggest that such neurons might act similarly to the sympathetic vertebrate system by releasing octopamine from varicosities directly near the organs (glands, peripheral flying or leg muscles) [127,128]. In the honey bee, octopamine-like immunoreactivity in varicose structures of CC suggests a possible (neuro)endocrine source of octopamine [58]. Additionally, our model assumes that a network of peripheral octopaminergic neurons exists in honey bees as in locusts, but information on this is currently lacking. It will be important to confirm the presence of octopaminergic neurohemal structures on the surface of peripheral nerves similar to those described in locusts, which are only inferred in honey bees for now, based on comparison with various insects [50,123].

Octopamine is released in energy demanding "fight-or-flight" situations to increase the honey bees' state of general arousal and we can therefore consider it as a stress hormone in insects [8,50,114]. Interestingly, octopaminergic neurosecretory cells innervate the honey bee CC [58,120], thus suggesting that octopamine could also regulate the release of several neuropeptides from this structure (including the stress candidates discussed hereafter).

3.2.2. Corazonin

The cardioacceleratory function of this 11-aminoacid neuropeptide was first described in 1989 by Veenstra in *Periplaneta americana* [129]. Now we know that this effect is probably restricted to cockroaches only, while corazonin has been shown to have diverse effects in other insects such as silkworms, locusts, flies, and moths. In locusts (both *Locusta migratoria* and *Schistocerca gregaria*), corazonin is involved in the induction of the gregarious phase [130], and in ecdysis in the moth *Manduca sexta* [131]. Also, a metabolic function of corazonin as a nutritional stress hormonal signal has been recently suggested by Veenstra [56], based on the localization of the peptide precursor and its receptor in *Drosophila*. Corazonin is produced by neurosecretory cells projecting into the CC in many insects, including *Drosophila*, locusts and honey bees [132,133]. In *Drosophila*, corazonin receptors have been found in the heart, fat body, salivary glands and gut [134]. Additionally, in *Drosophila*, corazonin neurons express diuretic hormone receptors and an AST-A receptor [135]. This has led Veenstra to suggest a model in which corazonin is released in response to peripheral feedback from the

gut in a hunger state, and acts to mobilize energy (see Section 3.3 and Figure 3). Phylogenetic analyses support an ancestral hormonal role for corazonin in regulating metabolic functions, more specifically because corazonin receptors belong to the ancient GnRH-AKH receptor family [56,136,137]. Based on these recent findings on the role of corazonin in insects we propose, following others [6], that it may have an important hormonal role in the honey bee acute and chronic stress response, although this hypothesis has yet to be directly tested.

3.2.3. Allatostatins

Allatostatins (ASTs) are insect neuropeptidic hormones first identified as regulators of growth during development, on the basis of their ability to reduce juvenile hormone (JH) release by the CC [138]. However, there is growing evidence that not all members of the AST family play this biological function [138 140] Among the three major AST types (A, B and C), only A and C are present in honey bees [139,140]. In addition, honey bees (as along with some other insect species) have two closely related C-type peptides, AST-C and AST-CC [141].

ASTs are important regulators of food intake and/or digestive functions in several insect species [142 146], but this might be part of a much broader spectrum of inhibitory functions [141]. They are present in the midgut of several species as well as in the CC [143,147,148], and are known to release neuroendocrine signals regulating energy supply from the digestive tract (see below and Figure 3). Hemal AST-A has been suggested to modulate CC function [56]: low food content in the gut reduces circulating AST-A released by midgut secretory cells; this in turn relieves inhibition of CC neuroendocine cells containing corazonin and diuretic hormones. This postulated role in response to nutritional stress has been recently challenged by recent work in *Drosophila*, showing that genetic manipulations of AST-A alter feeding behavior without apparent consequences on energy reserves or metabolism [149]. Thus, whether an AST-mediated nutritional feedback loop exists remains an open question. It is worth mentioning that at least the AST-A type may be expressed in the honey bee CC [150] (but see [88]), which places it in a position of possibly participating in energy mobilization control, in particular under conditions of chronic and acute nutritional stress.

3.2.4. Adipokinetic Hormone (AKH)

AKH is perhaps the most important metabolic regulator described in insects [109]. This octapeptide is synthesized in the CC and released into the hemolymph to increase catabolism in the fat body, ultimately leading to increased circulating trehalose levels [7,151] (see detail in the following section), similarly to the action of glucagon in vertebrates [152 154]. In cockroaches, AKH stimulate spiking from peripheral octopaminergic neurons and locomotion [155]. Interestingly, the demonstration that octopamine mediates AKH release into the hemolymph in the locust CC [156 158] provides further evidence of a precise interplay between arousal and hunger. In addition, recent papers support a role for AKH in a general stress response in various insects [54,159,160]. Insecticide treatment inducing oxidative stress leads to increased hemolymph titers in the locust *Schistocerca gregaria* [159] and in the firebug *Pyrrhocoris apterus* [160]. Indeed, AKH has the capacity to trigger antioxidant processes [161,162]. In the latter species, mechanical stressors had a similar effect [160], thus strongly arguing for AKH-mediated actions as pivotal element of a widespread stress response. However,

honey bees CC contain a lower amount of AKH than other insects (half that of *Gryllus bimaculatus* and *Acheta domesticus*), and AKH has a minor hypertrehalosemic effect in honey bees [163], Thus, the role of AKH in stress responses in honey bees may be less prominent than in other insects. This can possibly be explained by the specific mechanisms honey bees use to mobilize energy (Section 3.3).

3.3. Mechanisms of Energy Mobilization in Honey Bees

Increased energy mobilization triggered by hormonal signals plays a very important role in stress responses. In insects, carbohydrates (especially trehalose) are the most important energy source [164]. However, available trehalose is rapidly depleted, and in several insects, a sustained effort such as a long flight requires the release of trehalose from the main energy store the fat body [109,165]. Consequently, mobilization of energy from energy stores by hormonal factors is an essential part of the stress response. By contrast, honey bees show specific mechanisms for energy mobilization which appear related to their social organization. Strikingly, forager honey bees have fat bodies almost entirely depleted and seem to use the sugar energy reserve carried in their crops to sustain the energetic demands of flight [60,61]. This observation would also explain why foragers express almost no AKH in their CC, and have lower abdominal glycogen stores [166,167].

Honey bees appear to have a quite specific mechanism for regulating hemolymph sugar levels (Figure 3), according to a model proposed by Blatt and Roces [61]. In an energy-demanding situation, trehalose synthesis by the fat body is not fast enough to match rates of trehalose consumption, so circulating trehalose levels decline. This stimulates the passage of nectar from the impermeable crop storage organ to the midgut by the contraction of the proventriculus (gut muscle between the crop and midgut) [62]. From the midgut, sugars are digested, absorbed and more glucose and fructose are transported into the hemolymph. Therefore, upon high metabolic demand, while the trehalose level decreases in the hemolymph, those of fructose and glucose increase, maintaining a stable sugar concentration (Figure 3). Moreover, the honey bee genome sequence suggests the loss of two important insect enzymes converting gluconeogenic substrates to trehalose and glycogen [168], which are both stored in fat body and considered as the primary energy storage molecules in insects. This implies that in the honey bee, regulation of sugar transport from the gut probably plays a more important role in energy balance than the regulation of trehalose release from the fat body. Interestingly, in honey bees injection of CC extracts into the hemolymph has a hypertrehalosemic effect [167], thus candidates for this function are expected to be found in this gland. As discussed above, hormonal candidates performing this role in honey bees might include corazonin, DH-I and possibly AKH (Figure 3).

Hormones activating the mobilization of glucose from the crop by stimulating the proventriculus and midgut remain to be identified. In *Drosophila* [56], it has been suggested that the (neuro)hormones: AST-A and tachykinin, released from secretory cells of the midgut or the nerve cord, might play this role [56] (Figure 3). Tachykinin and tachykinin-related peptides (TRPs) are known in insects to be myostimulatory of the insect midgut muscle [169], and therefore would be good candidates for modulating release of nectar from the honey bee crop. One “tachykinin-like receptor” has been identified in honey bees from sequence homology with *Drosophila* [170], but its function remains unclear, especially as many TRPs have been described and seem to have diverse functions in insects [169].

Nine different TRPs have been localized all along the nervous system of the honey bee [88], but none of them was detected in CC neurosecretory cells. If TRPs act to upregulate metabolism in stress, they would need to be released from the efferent peripheral nerves directly to the proventriculus.

It should be noted that insulin and vitellogenin pathways have also been linked to energy store mobilization and oxidative stress in insects [47,171]. Insulin/insulin-like growth factor signaling (IIS) pathways appears to regulate fat stores in *Drosophila* [171] and confer oxidative stress resistance in *Drosophila* and honey bees [48]. Vitellogenin expression seems to be triggered by IIS pathways [48]. In reproductive females, vitellogenin is a glycolipoprotein stored in the fat body and usually released into the hemolymph before being stored within oocytes. In the sterile honey bee worker, vitellogenin expression is inhibited by JH [172], thus giving foragers a lower amount of vitellogenin (perhaps reinforced by their decrease of fat body mass). However, vitellogenin seems to be protective against oxidative stress, perhaps via certain antioxidant properties, and may account for longevity in reproductive insects, e.g., queen bees [47,48]. Presently, the metabolic role of these molecules needs to be clarified in honey bees in order to be integrated within our model of endocrine regulation of energy sources.

4. Gaps in Knowledge and Urgent Questions

The sections above help build a model of a general stress response in the adult honey bee, as presented in Figure 2. However, as mentioned, in some instances specific data from this particular species are lacking.

4.1. What Is the Role of JH in the Stress Response?

JH acts on development and sexual maturation in insects; it is produced in the CC and released under the neural control of brain peptidergic innervation. JH is typically described as the master larval developmental hormone, boosting growth of insects, inhibiting metamorphosis and initiating reproductive traits in adults [173]. Still, JH has been described more recently as a stress hormone in *Drosophila*, as JH levels drop after exposure to various stressors [174]. As JH tends to have long-lasting effects, this hormone may be more likely to be involved in chronic than acute stress response. Whether JH acts as a stress hormone in honey bees is less clear, particularly since, in the adult worker bee, it has a species-specific function: that of a regulator of division of labor. JH titers are low in young nurse bees but higher in foragers. Indeed, pharmacological elevation of JH levels or injection of JH analogs accelerates the onset of foraging of young bees [175,176]. Perhaps because of this, studies of the possible role of JH in stress have thus far given confusing results. Lin *et al.* [34] could not find a consistent change in JH levels after application of various stressors in honey bees, and found a response only if JH levels were initially low (in which case JH levels increased after caging or cold anesthesia). If JH levels were already high, stress seemed to decrease JH levels. These differences probably result from the dynamics of mechanisms for metabolism and recycling of JH when JH levels are very low or very high. As a consequence of this additional complexity, the precise roles of JH in the honey bee stress response are presently unclear, but given the importance of this hormone system, this is certainly an area demanding further study [34].

4.2. Can Dopamine Be Considered As a Stress Hormone?

A potential role of hemal dopamine in stress is suggested by work on *Drosophila* as dopamine increased heart rate, while mutations impairing dopamine synthesis had the opposite effect [102]. In cockroaches, one study also found dopamine in the CC [177], thus suggesting a neurohormonal role. In the hemolymph dopamine levels have been rarely quantified in honey bees, but Bateson *et al.* [178], found a decrease in dopamine levels in the head hemolymph of honey bees 30 minutes after a strong vibration stressor. Clearly more work is needed here to explore the possible role of dopamine in endocrine acute stress response.

4.3. Neuropeptides in the CC?

Neuropeptides are an emerging area of research, and many members of this large family have never been studied. The neurosecretory cells of the CC contain numerous unstudied peptides [89], which would be good candidates as stress neurohormones. Corazonin, DH, AKH, tachykinins and AST-A have been mentioned previously, but many other peptides might play metabolic roles in the honey bee [167]. In addition, looking at the location of neurohormone receptors is important to understand how the stress system operates. More details on the distribution of neuropeptides and their receptors might highlight their targets, the responses they elicit, as well as the feedback loops regulating the system.

4.4. Stress Responses and Immune System

As in vertebrates, the immune response can be affected negatively by stressors. This may be a direct effect of stress hormones (biogenic amines and AKH), as shown in some insect species [179,180]. Depending on the context and the stressor characteristics, the immune response has been shown to be boosted by stressful events or stress hormones [181,182]. This can be understood as a way of maintaining immune equilibrium in a harmful environment [179,183]. In honey bees, stress and immune responses do not seem to have been considered together yet, but recently several detrimental synergistic effects of various combinations of stressors suggest a link between them [184 187]. As proposed by some of the authors of those studies, such a link may be highly relevant to understand the recent decrease of honey bee populations [186,187].

4.5. Task Specialization and Sensitivity to Stress

The high level of sociality and the complex system of division of labor are essential characteristics of honey bees [15]. Here, we propose that at least some elements of the stress response may have been adapted in specific ways to contribute to the evolution of division of labor. During its lifetime, an individual honey bee progresses through a succession of specialized behavioral states whose sequence follows an internal developmental program modulated by various social signals (pheromones) emitted from the colony [188,189]. Honey bees typically begin their adult life undertaking in-hive activities such as brood nursing, cleaning and food storing, then guarding the hive entrance against intruders and predators and finally foraging for food sources (mostly pollen and nectar). The transition to foraging is

a major event is a honey bee's life, and corresponds to multiple changes in hormonal activity, brain circuits, and physiology [188,190].

There has been a lot of research on the mechanisms underlying and organizing this division of labor, which have been shown to involve octopamine, JH and vitellogenin [175,188,191,192]. We suggest here that modulation of stress reactivity may be linked to the evolution of task specialization in honey bees. The fact that octopamine is two to four times more abundant in brains of foragers than those of nurse bees [36] may predispose foragers to attain more easily or rapidly a state of higher energy mobilization. Indeed foragers appear to be the colony members most exposed to stressors: foraging is energetically demanding, and exposes honey bees to more adverse environments (e.g., predators, insecticides) than working within the hive [193]. Elevated brain octopamine levels, as a potential result of chronic stressor exposure [114], may prepare honey bees to cope with the higher stress levels caused by foraging, and the hormonal state of a forager bee may resemble that of nurses bees under chronic stress.

Further, chronic stressors applied at the colony level and experimental elevation of brain octopamine levels both accelerate the onset of foraging in the honey bee [14,194,195]. A high brain level of octopamine may also make foragers more sensitive to hunger, which could motivate them to gather food.

The forager's state and number appear to be as a response to colony stress, and foragers are also the behavioral caste exposed to the greatest stress. Therefore, knowing the molecular pathways and physiological mechanisms that regulate chronic and acute stress responses at the individual level are of great interest for developing strategies to improve the health and longevity of honey bee colonies.

5. Conclusions

The model developed here describes a general stress response in honey bees. It provides a framework to facilitate our understanding of how honey bees can respond to stressors, and is also aimed at stimulating research to improve our knowledge of the physiological pathways involved.

Our comparison of vertebrate and honey bee stress response pathways suggests a parallel organizational structure in the two groups, including regulation of arousal and cognitive functions in the brain by catecholamines, coupled with neurohormonal signals stimulating energy mobilization in the periphery. Yet, the extent to which the stress response pathways are evolutionarily conserved remains unclear. Some elements, like CRH-BP, may offer examples of conservation of function, but others, particularly neuropeptide hormones, are likely to be specific to insects or invertebrates. In this regard, it is worth noting that several key neuropeptides cited here (AKH, tachykinin, DH) are among the most highly conserved neuropeptides among insect species, perhaps indicating the operation of strong stabilizing selection [196].

In this review, we also highlighted the aspects of the stress response that appear to be specific to honey bees as a result of their peculiar social organization. Specifically, we have summarized particular mechanisms enabling an increase of glucose from the crop.

Finally, pursuing studies on stress in honey bees is essential for developing standard methods to assess stress in this insect of major economical importance. Relevant and robust criteria to evaluate stress symptoms would be useful as basic indicators of health in honey bees, and the development of

standardized assays would improve risk assessment for pesticide and other agricultural practices on honey bee populations. Thus, knowing more about stress in honey bees is now crucial to design strategies for the protection of this fragile, but ecologically and economically important, insect.

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Appendix 2

Published version of chapter 5

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Report

Altruistic Behavior by Egg-Laying Worker Honeybees

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Summary

If a honeybee (*Apis mellifera*) colony loses its queen, worker bees develop their ovaries and produce male offspring [1]. Kin selection theory predicts that the degree of altruism in queenless colonies should be reduced because the relatedness of workers to a hivemate's offspring is less in queenless colonies than it is to the daughters of the queen in queenright colonies [2–4]. To explore this hypothesis, we examined the behavior and physiology of queenless egg-laying workers. Queenless bees engaged in both personal reproduction and the social foraging and defense tasks that benefited their colony. Laying workers also had larger brood-food-producing and wax glands, showing metabolic investments in both colony maintenance and personal reproduction. Whereas in queenright colonies there is a very clear age-based pattern of division of labor between workers, in queenless colonies the degree of individual specialization was much reduced. Queenless colonies functioned as a collective of reproductive and behaviorally generalist bees that cooperatively maintained and defended their nest. This social structure is similar to that observed in a number of primitively social bee species [5]. Laying workers therefore show a mix of selfish personal reproduction and altruistic cooperative behavior, and the queenless state reveals previously unrecognized plasticity in honeybee social organization.

Results and Discussion

Honeybees (*Apis mellifera*) form one of the most complex animal societies. Within a colony, the single queen is typically the sole reproductive, supported by thousands of her daughters, who form a highly specialized and sterile worker caste. Kin selection theory has provided a framework for understanding the evolution of these pronounced social and altruistic traits [6–8]. The theory proposes that altruistic traits would be selected for and spread if they increase the reproductive success of the altruists' relatives [7, 8]. The unusual kin structure of queenright haplodiploid hymenopteran honeybee colonies provides conditions that promote both the evolution of worker altruism and mutual enforcement of worker sterility by policing [9, 10]. Indeed, both evidence and theory suggest that the level of altruism seen in an animal society (considered in terms of investment in colony maintenance and raising relatives' offspring rather than personal reproduction) is a function of the relatedness structure of the colony [11–13].

If workers are unable to raise a replacement queen, the colony becomes hopelessly queenless. In this phase, the only reproductive options available to workers are to produce their own male offspring (workers cannot mate, and their haploid eggs develop into males) or assist other workers in reproducing and thereby raise their nephews [1, 14, 15]. The relatedness structure of a queenless honeybee colony is radically different from a queenright colony [11], and under such conditions, the level of altruism displayed by workers is expected to decrease and the degree of reproductive conflict to increase [11, 12]. It is well known that many queenless workers develop their ovaries and lay eggs (Figure 1A). Under those circumstances, it is commonly assumed that reproductive workers selfishly prioritize their own reproduction over colony tasks; this raising of sons offers a direct fitness benefit, as compared to assisting with raising less-related nephews or brothers [16–18], and should cause workers to stop performing the demanding and risky foraging and defensive tasks that benefit the colony [18, 19]. However, the behavior of workers in queenless honeybee colonies has been little studied. Here, we examined the behavior and physiology of workers in hopelessly queenless colonies to determine whether altruism persists, and to examine the nature of social organization in the queenless condition.

Foraging benefits the colony but is both metabolically costly [20] and risky [21] for the individual bee. To determine whether laying worker bees engaged in personal reproduction continue altruistic behaviors, we sampled forager and nonforager bees from queenless colonies and dissected them to assess their level of ovary activation [22]. We found no difference in the degree of ovary activation between forager and nonforager bees (Figure 1B). Furthermore, in comparisons of age-matched samples taken from three independent queenless colonies, at 14 days of age there was no difference in the level of ovary activation between foragers and nonforagers, but at 21 days of age the overall degree of ovary activation was higher in foragers, and foragers were more likely to have fully developed ovaries (containing at least one developed egg) than nonforagers (Wald $\chi^2 = 9.216$, n = 73, df = 1, p = 0.002). In addition, bees that were marked in the act of laying were as likely to be later observed foraging as bees that did not lay (Wald $\chi^2 = 0.300$, n = 30, df = 1, p = 0.5839; see Figure S1 available online). For these analyses, ovary development was scored on a five-point scale following [22]. Collapsing these data to a binary scale considering levels 1 and 2 as inactive and levels 3+ as active (a common convention for these data), we found that ovary development was significantly influenced by the presence or absence of the queen (generalized mixed model assuming binomial error: analysis of deviance: $p < 0.001$) and varied between colonies in our study ($p = 0.038$), but there was still no significant difference in levels of ovary activation between foragers and nonforagers ($p = 0.426$). A similar mode of analysis confirmed no difference in ovary development between foragers and nurses

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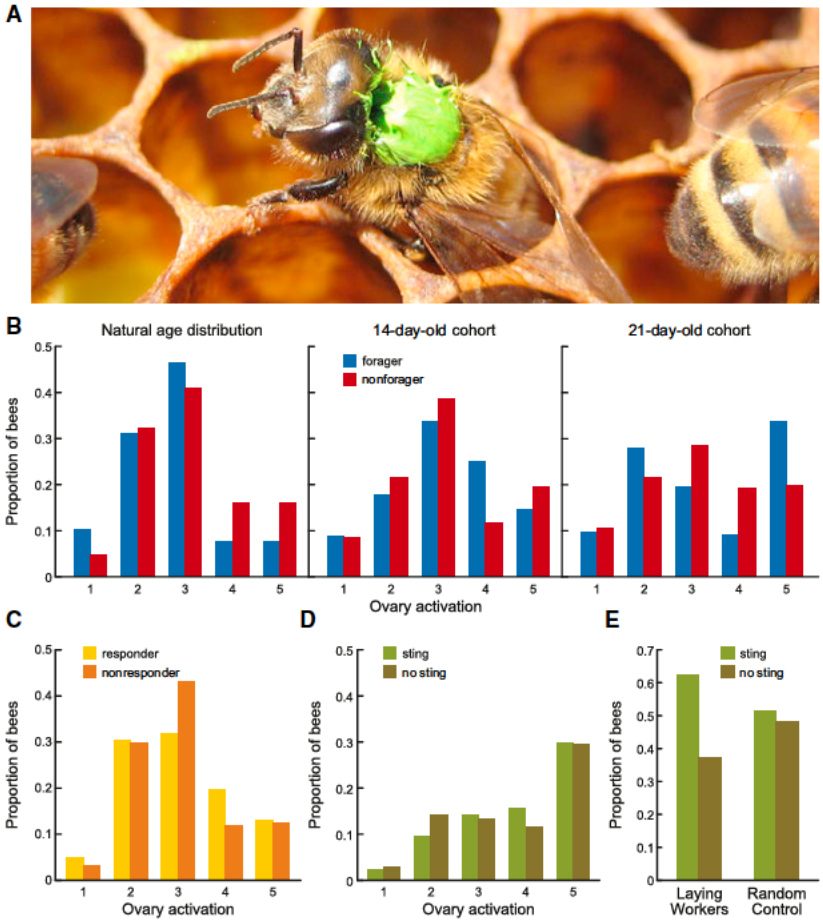

Figure 1. Reproductive Worker Honeybees Do Not Avoid Risky Behaviors

(A) A worker honeybee, previously marked as a forager, laying an egg.

(B) Distribution of mixed-age foragers and nonforagers with different levels of ovary activation (n = 119 foragers and 125 nonforagers; ordered logit, pseudo R^2 = 0.0305, p = 0.287) and age-matched cohorts of 14 days (n = 68 foragers and 153 nonforagers; ordered logit, pseudo R^2 = 0.0027, p = 0.590) and 21 days (n = 133 foragers and 140 nonforagers; ordered logit, pseudo R^2 = 0.0269, p = 0.042).

(C) No differences were observed in ovary activation between bees that defended the hive against a simulated vertebrate predator (n = 207) and those that did not (n = 195) (ordered logit, pseudo R^2 = 0.0065, p = 0.084).

(D) No differences were observed in ovary activation between bees that responded to a 9V electric shock by stinging (n = 75) and those that did not (n = 60) (ordered logit, pseudo R^2 = 0.0065, p = 0.159); bees observed laying eggs were purposefully oversampled.

(E) No differences were observed in likelihood of stinging in response to 9V electric shock between bees observed laying eggs (n = 48) and randomly sampled hivemates (n = 87) (Fisher's exact test, p = 0.279).

(p = 0.785), source colony (p = 0.226), or age (p = 0.724) in the age-matched samples collected at 14 and 21 days old. In summary, several experiments conducted with seven different colonies showed that reproductive workers in queenless colonies are as likely to forage as bees with less-developed ovaries.

Foraging is individually costly, but participating in colony defense is suicidal because the act of stinging causes the death of the individual worker. To test whether laying workers altruistically engage in colony defense, we disturbed a queenless colony by removing the hive cover and shook a black lure over the exposed honeycombs to then sample bees that attacked the lure and bees that did not. There was no difference in the level of ovary activation between attackers and nonattackers (Figure 1C). We also tested the likelihood to sting in response to an electric shock in a laboratory assay for aggressiveness [23]. Level of ovary activation had no effect on the likelihood of stinging in response to the shock stimulus (Figure 1D). Furthermore, there was no difference in the likelihood to sting for bees with fully developed ovaries compared to those without (Fisher's exact p = 1.0), or those observed to have laid an egg compared to randomly sampled control bees (Fisher's exact p = 0.279; Figure 1E). Taking these results together, multiple experiments conducted with five colonies indicated that reproductive workers in queenless colonies are as likely to engage in colony defense as bees with less developed ovaries.

In addition to engaging in personally risky behaviors that benefit the colony, reproductive workers in queenless colonies also metabolically invested in brood food and wax production for the good of their colony. Queenright honeybees show precise task-related physiological specializations, with a negative association between ovary development and development of the brood-food-producing hypopharyngeal glands (HPGs) [16], demonstrating a physiological trade-off between personal reproduction and investment in colony maintenance. By contrast, we observed a significant positive correlation between ovary development and HPG development in bees from queenless colonies (Figure 2A). There was also a significant positive correlation between ovary development and the number of fully formed wax flakes produced by the abdominal wax glands (Figure 2B).

The observed coactivation of HPGs and wax glands in queenless bees deviates markedly from the precise task-related physiological specializations typically seen in workers from queenright colonies, which have a predictable age-based system of division of labor [1, 24]. To explore this further, we performed direct comparisons of bees in queenright and queenless colonies of similar population sizes. After marking all foragers over the course of at least two days, we collected them along with samples of nonforaging hivemates and measured development of the ovaries, HPGs, and wax glands. Foragers in queenless colonies (N = 4) had greater HPG (ordered logit, n = 194, pseudo R^2 = 0.1772, p < 0.0001; Figure 2C) and wax gland development (ordered logit, n = 195, pseudo R^2 = 0.1445, p = 0.001; Figure 2D) than those in queenright colonies (N = 3). Similar results were obtained for a sample of queenless foragers that were 8 weeks old and were known to have been foraging since 3 weeks of age (Figure S2).

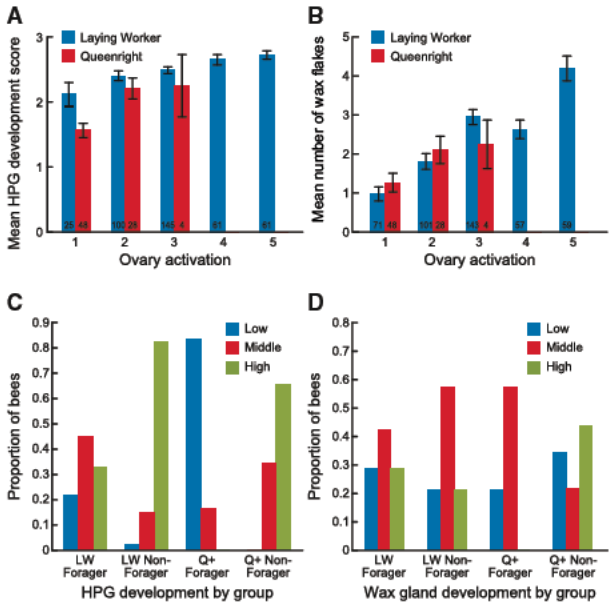

Figure 2. Reproductive Worker Honeybees Maintain Physiology for Hive Tasks

(A and B) The level of hypopharyngeal gland (HPG) development (A) and wax production (B) is positively correlated with the level of ovary activation in queenless colonies (least-squares regression, n = 392, R^2 = 0.049, p < 0.0001 for HPG; least-squares regression, n = 431, R^2 = 0.163, p < 0.0001 for wax). Workers in queenright colonies rarely have highly activated ovaries. Sample sizes are shown at the base of the bars; error bars represent SEM.

(C and D) Workers in colonies with laying workers were more likely to maintain HPGs (C; ordered logit, n = 194, pseudo R^2 = 0.1772, p < 0.0001) and wax glands (D; ordered logit, n = 195, pseudo R^2 = 0.1445, p = 0.001) while foraging than workers in queenright colonies.

Principal component analysis based on ovary, HPG, and wax gland development measurements (N = 6 colonies, n = 165 individuals) revealed that whereas queenright workers differentiated into separate forager and nonforager clusters, queenless workers did not (Figure 3). These results indicate that task specialization has broken down in queenless colonies, with forager bees maintaining the capacity to engage in brood care and colony maintenance tasks as well as personal reproduction.

Previous reports have shown a negative association between level of ovary development and level of foraging activity [17, 19, 25] and HPG development [16] in queenright colonies, where full ovary activation and worker reproduction are very rare. Although in this study we did not directly compare worker activity levels in queenright and hopelessly queenless colonies, we have conclusively shown that queenless workers split investment between both their own personal reproduction and the altruistic behaviors of foraging, colony defense, and maintenance, and that engaging in personal reproduction does not reduce the likelihood of bees engaging in colony defense and foraging roles.

In a queenright colony, worker task specialization is organized by temporal polyethism, with bees beginning life engaged in in-hive tasks and delaying high-risk colony defense and foraging tasks until later in life [26–28]. Elements of this pattern were preserved in queenless colonies, in that most queenless workers commenced foraging when >2 weeks old (comparable to behavioral development in queenright colonies; Figure S3). Beginning high-risk foraging tasks later in life is a common pattern across social insects and appears to be an evolved strategy to maximize lifespan, lifetime colony investment, and personal reproduction [26, 29]. This basic pattern was preserved in queenless colonies, but unlike in queenright colonies, bees did not then exclusively specialize on foraging.

The generalist behavior of reproductive workers in queenless colonies that forage and defend the hive while maintaining the ability to care for brood, build comb, and lay eggs is similar to solitary or primitively social bees. Queenless honeybee colonies resemble a communal form of social organization called "quasisociality," defined as individuals of a single generation that share a nest and exhibit cooperative brood care [5]. This type of sociality is exhibited by many euglossine orchid bees [5], the most closely related extant taxon to the honeybees [30]. The queenless state thus exposes heretofore unrealized plasticity in honeybee social organization, with queenless bees manifesting an atavistic social structure typical of many primitively social species.

Our data support the predictions from kin selection models that reproductive conflict is increased in queenless colonies [11], but altruism is far from eliminated, and individual bees split investment between selfish and altruistic behavior. For a hopelessly queenless colony, there may be a strong selective advantage for reproductive workers to prolong the life of their failing colony as long as possible to maximize chances of successful male production. In quasisocial species of bees, individuals benefit from cooperative group defense, "life insurance" via cooperative brood care, and other forms of reciprocity [9]. Even in colonies founded by completely unrelated individuals, seen in some ant species, there is a benefit to cooperation between the unrelated egg-laying foundresses [31]. Reproductive queenless honeybees may obtain similar fitness benefits by directing resources and costly but beneficial behaviors toward supporting their colony, raising the fitness of all [32].

The hopelessly queenless state is the terminal phase of a honeybee colony, because a colony that cannot raise workers cannot survive. But even in this late stage, reproductive workers communally maintain and defend their nest. Queenless colonies continue to function as a cooperative unit but display a simpler social order, reduced behavioral specialization, and worker investment in both colony maintenance and personal reproduction.

Experimental Procedures

Honeybee Colonies

All colonies were mixed races of *Apis mellifera*, mostly *ligustica*. Colonies 1–4 were each established on January 13, 2011 at Macquarie University, North Ryde campus, New South Wales, Australia. Each colony was started from a 1 kg "package" of bees from Australian Queen Bee Exporters. Packages are artificial swarms created by collecting young worker bees en masse from the brood nests of many different colonies and represent a mix of genotypes. They were installed into hives with five honeycomb frames: two frames of honey; one frame with a cell diameter appropriate for male larvae; and two frames that contained a mix of empty cells, pollen, honey, and worker brood. The hives were monitored for replacement queen cells, which were removed in colonies 1, 2, and 4. Colony 3 was allowed to rear a replacement queen to serve as a queenright control. After worker-laid brood appeared, frames of honeycomb containing brood were taken from queenright colonies and placed in an incubator at 34°C. One-day-old adult workers that came from these frames were marked with a paint dot on the thorax and introduced as cohorts of 1,000 individuals into each of the colonies.

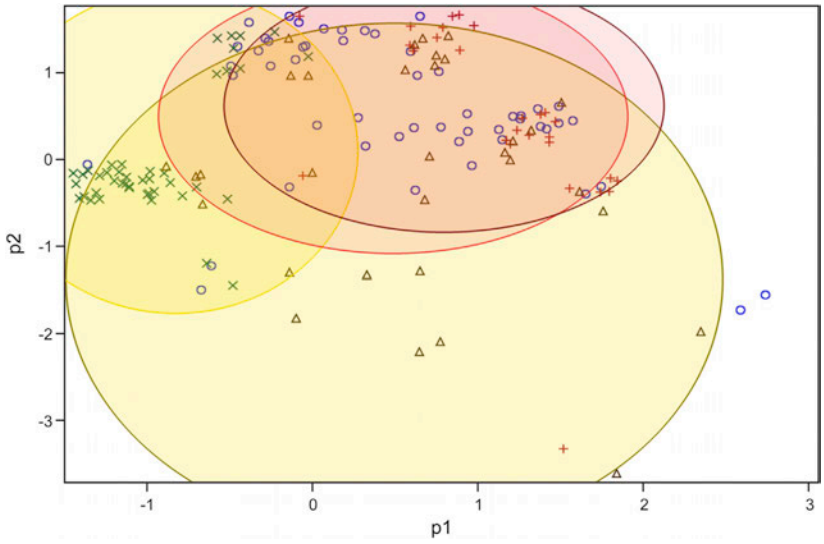

Figure 3. Honeybee Colonies with Laying Workers Lose Division of Labor

Principal component analysis of HPG, wax gland, and ovary development revealed that foragers (green, ×) and nonforagers (brown, △) from queenright colonies formed distinct clusters, whereas foragers (blue, ○) and nonforagers (red, +) from colonies with laying workers did not.

Colonies 5–10 were created by moving five frames of honeycomb (as above) and several thousand workers from a large colony into a new hive. Colonies 5, 6, 7, and 9 were created queenless, whereas colonies 8 and 10 had the queen moved along with her workers to the new hive. The four queenless colonies were monitored, and the rearing of replacement queens was prevented to force the colonies to become hopelessly queenless. Colonies 5 and 6 were established in Sydney, Australia, and colonies 7–10 were established at the University of Illinois Bee Research Facility, Urbana, Illinois, USA. Colonies were transported to a new location to prevent the bees from flying back to the original hive. Experiments were not started until the first worker-laid brood appeared.

Foraging Assays

To compare ovary activation between foragers and nonforagers of known age, we monitored the paint-marked cohorts in colonies 1, 2, and 4 for at least four periods of 15 min per day before midday and another four periods after midday. Foragers were identified by either a visible pollen load on the corbicula or a distended abdomen and were painted on the abdomen with a unique color for each day. This continued from day 8 to day 21 of age. During this interval, frames were occasionally removed from the hive, and bees observed in the act of laying eggs were marked with a paint dot, as were random control bees nearby. The foraging behavior of these bees was recorded. On days 14 and 21, the hive was opened, and bees marked as foragers and bees from the cohort without a foraging mark were collected. For bees of natural age demographics, all of the foragers from colonies 3 and 5–10 were marked over the course of 2–4 days. Foragers and nonforagers were then collected as they returned to the hive and from inside the hive, respectively. Additionally, 8-week-old bees were collected from colonies 1 and 2 to test whether the maintenance of developed glands into the foraging phase was a result of a younger age at first forage.

Defensive Assays

Defensive behavior was measured in colonies 1, 2, 4, 5, and 6 by removing a honeycomb frame from the colony and waving a black lure over it. The lure consisted of small leather patch with three stings on it from bees from another colony, surrounded by a ball of black feathers. Bees that flew to and attacked the lure were collected, as well as those that did not respond (considered controls). For the electric shock assays, bees from these colonies that were directly observed laying eggs as well as random nearby control bees were collected individually into vials. Bees were then transferred to a 12 × 12 cm arena with a floor composed of parallel stainless steel wires 2 mm in diameter. A BK Precision 1696 power supply was used to apply a constant 9V stimulus; this voltage was shown in pilot experiments as well as previous studies [23] to be a good discriminating voltage between bees that will versus those that will not sting. Two experimenters, blind to the behavioral group of the bee, observed whether or not the bee stung at the device. Bees were then collected into ethanol for ovary dissections.

Dissections and Gland Scoring

All dissections were performed under dissecting microscopes with the experimenter blind to the behavioral group of the bee. The level of ovary activation was scored on a 1–5 scale in accordance with [22]. HPGs were scored on a 1–3 scale, with a score of 1 representing completely underdeveloped or atrophied glands and a score of 3 representing fully developed glands that filled the internal space between the brain and anterior cuticle. Wax gland development was scored by counting the number of fully formed wax flakes on the abdominal sternites. Zero or one flakes were considered "low," two or three flakes "middle," and four or more flakes "high" in terms of gland development.

Statistical Analysis

Wald χ^2, Fisher's exact tests, and least-squares regressions were performed using MYSTAT 12 (Cranes Software International). Ordered logits were performed using STATA version 9.2 (StataCorp). For comparing ovary activation between foragers and nonforagers or defensive and nondefensive bees, level of ovary activation was analyzed with an ordered logit model with behavioral classification, colony number, and the interaction as explanatory variables. For comparing HPG activation, the level of activation was also analyzed with an ordered logit model with colony type (queenless or queenright) and colony number as explanatory variables. Principal component analysis was performed in SAS 9.2 (SAS Institute). HPG, wax, and ovary data were transformed using PROC PRINQUAL, and principal components were generated using PROC FACTOR, with jitter applied to allow multiple points occupying the same two-dimensional space to be visible.

Supplemental Information

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.045.

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