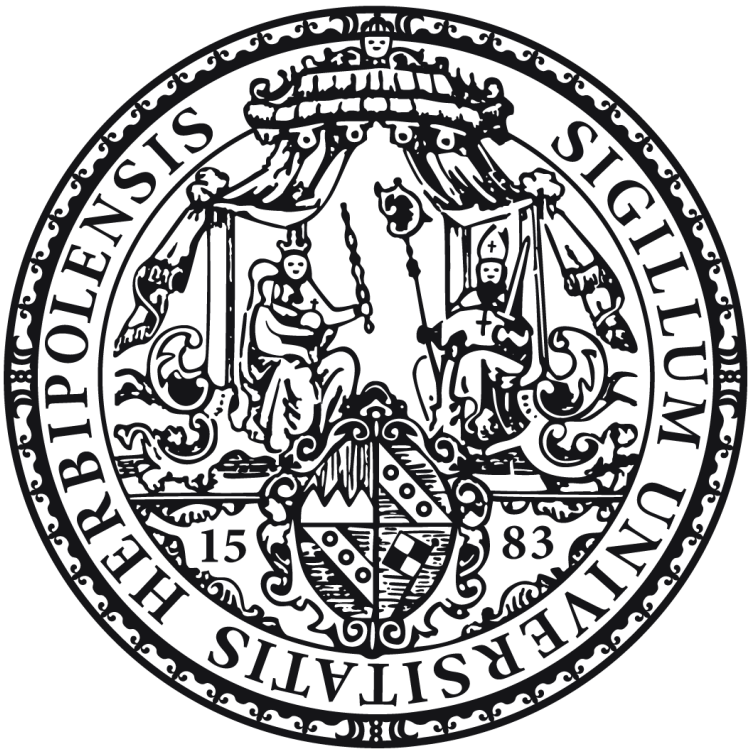
Octopaminergic Signaling in the Honeybee Flight Muscles

A Requirement for Thermogenesis

Octopaminerge Signalwege in der Flugmuskulatur der Honigbiene

Eine Voraussetzung für die Thermogenese



Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Julius-Maximilians-Universität Würzburg

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Würzburg

Würzburg 2022

"A painter should begin every canvas with a wash of black, because all things in nature are dark except where exposed by the light."

Leonardo da Vinci

Für Tine.

Submitted on: .....

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SUMMARY

For all animals the cold represents a dreadful danger. In the event of severe heat loss, animals fall into a chill coma. If this state persists, it is inevitably followed by death. In poikilotherms (e.g. insects), the optimal temperature range is narrow compared to homeotherms (e.g. mammals), resulting in a critical core temperature being reached more quickly. As a consequence, poikilotherms either had to develop survival strategies, migrate or die. Unlike the majority of insects, the Western honeybee (*Apis mellifera*) is able to organize itself into a superorganism. In this process, worker bees warm and cool the colony by coordinated use of their flight muscles. This enables precise control of the core temperature in the hive, analogous to the core body temperature in homeothermic animals. However, to survive the harsh temperatures in the northern hemisphere, the thermogenic mechanism of honeybees must be in constant readiness. This mechanism is called shivering thermogenesis, in which honeybees generate heat using their flight muscles.

My thesis presents the molecular and neurochemical background underlying shivering thermogenesis in worker honeybees. In this context, I investigated biogenic amine signaling. I found that the depletion of vesicular monoamines impairs thermogenesis, resulting in a decrease in thoracic temperature. Subsequent investigations involving various biogenic amines showed that octopamine can reverse this effect. This clearly indicates the involvement of the octopaminergic system. Proceeding from these results, the next step was to elucidate the honeybee thoracic octopaminergic system. This required a multidisciplinary approach to ultimately provide profound insights into the function and action of octopamine at the flight muscles. This led to the identification of octopaminergic flight muscle controlling neurons, which presumably transport octopamine to the flight muscle release sites. These neurons most likely innervate octopamine β receptors and their activation may stimulate intracellular glycolytic pathways, which ensure sufficient energy supply to the muscles.

Next, I examined the response of the thoracic octopaminergic system to cold stress conditions. I found that the thoracic octopaminergic system tends towards an equilibrium, even though the initial stress response leads to fluctuations of octopamine signaling. My results indicate the importance of the neuro-muscular octopaminergic system and thus the

need for its robustness. Moreover, cold sensitivity was observed for the expression of one transcript of the octopamine receptor gene *AmOARβ2*. Furthermore, I found that honeybees without colony context show a physiological disruption within the octopaminergic system. This disruption has profound effects on the honeybees protection against the cold.

I could show how important the neuro-muscular octopaminergic system is for thermogenesis in honeybees. In this context, the previously unknown neurochemical modulation of the honeybee thorax has now been revealed. I also provide a broad basis to conduct further experiments regarding honeybee thermogenesis and muscle physiology.

ZUSAMMENFASSUNG

Kälte stellt für alle Tiere eine lebensbedrohliche Situation dar. Erleiden sie einen schwerwiegenden Wärmeverlust, stellt sich der Zustand eines Kältekomas ein. Hält dieser Zustand über einen längeren Zeitraum an, folgt unweigerlich der Tod. Poikilotherme (z.B. Insekten) weisen ein schmaleres optimales Temperaturfenster als Homoiotherme (z.B. Säugetiere) auf, wodurch sie ihre kritische Körpertemperatur schneller erreichen. Dadurch waren Poikilotherme gezwungen entweder Überlebenstrategien zu entwickeln, abzuwandern oder zu sterben. Im Gegensatz zu den meisten anderen Insektenarten, ist die Westliche Honigbiene *Apis mellifera* in der Lage einen Superorganismus zu bilden, in dem Arbeiterbienen durch den koordinierten Einsatz ihrer Flugmuskeln für Erwärmung oder Abkühlung sorgen. In Analogie zur Körpertemperatur von Homoiothermen, ermöglicht dies die exakte Kontrolle der Kerntemperatur des Bienenstocks. Um unter den rauen Bedingungen in der nördlichen Hemisphäre bestehen zu können, muss eine ununterbrochene Einsatzbereitschaft des thermogenen Mechanismus der Honigbiene garantiert werden. Dabei ist die Honigbiene in der Lage durch Zittern der Flugmuskulatur Wärme zu erzeugen.

In dieser Dissertation stelle ich die molekularen und neurochemischen Grundlagen des thermogenen Muskelzitterns bei Honigbienenarbeiterinnen vor. In diesem Zusammenhang habe ich die Signalwege von verschiedenen biogenen Aminen untersucht und konnte demonstrieren, dass eine Erschöpfung vesikulärer Monoamine den Prozess der Thermogenese beeinflusst und zu einem Absinken der Thoraxtemperatur führt. Unter Einbeziehung veschiedener biogener Amine, konnten Folgeuntersuchungen zeigen, dass dieser Effekt durch Octopamin rückgängig gemacht werden kann. Dies weist eindeutig auf eine Beteiligung des octopaminergen Systems hin. Auf Basis dieser Erkenntnisse folgte die Erforschung des thorakalen octopaminergen Systems der Honigbiene. Dabei erforderte es einen multidisziplinären Ansatz, um weitere Einblicke in die Funktion und Wirkung von Octopamin in der Flugmuskulatur zu gewinnen. Im Zuge dessen, konnten flugmuskelinnervierende octopaminerge Neuronen identifiziert werden, die mutmaßlich die Flugmuskeln mit Octopamin versorgen. Es sind höchstwahrscheinlich diese Neuronen, die für eine Stimulation von Octopamin-β-Rezeptoren verantwortlich sind und wordurch intrazelluläre glykolytische Prozesse eine ausreichende Muskelversorgung

gewährleisten. In den darauffolgenden Experimenten habe ich das Ansprechen des thorakalen octopaminergen Systems auf Kältestress untersucht und konnte zeigen, dass dieses System nach einem Gleichgewichtszustand strebt. Dies trifft selbst nach einer starken initialen Stressantwort zu. Meine Ergebnisse verdeutlichen die Bedeutsamkeit des neuromuskulären octopaminergen Systems und zeigen seine erforderliche Resilienz gegenüber exogenen Faktoren. Es konnte die Kälteempfindlichkeit eines Transkriptes des Octopaminrezeptorgens *AmOARβ2* nachgewiesen werden. Zusätzlich konnte ich zeigen, dass Honigbienen ohne den sozialen Kontext der Kolonie eine starke physiologische Störung innerhalb des untersuchten Systems und damit auch in Bezug auf ihre Kälteresilienz aufweisen.

Meine Dissertation verdeutlicht die enorme Bedeutung des neuromuskulären octopaminergen Systems im Kontext der Thermogenese im Organismus Honigbiene. In diesem Rahmen konnte die bisher unerforschte neurochemische Modulation des Honigbienenthorax aufgeklärt werden. Darüber hinaus bietet meine Arbeit eine Grundlage für künftige Experimente zur Thermogenese und Muskelphysiologie der Honigbiene.

CONTENTS

LIST OF ABBREVIATIONS

Notation	Description	Page List
3-MT	3-Methoxytyramine	96, 97
AmDAR	*Apis mellifera* dopamine receptor	95
AmDAT	*Apis mellifera* dopamine transporter	90
AmGAPDH	*Apis mellifera* glyceraldehyde 3-phosphate dehydrogenase	92
AmOAR	*Apis mellifera* octopamine receptor	10–12, 15, 87, 88, III, V
AmTAR	*Apis mellifera* tyramine receptor	10, 15
ATP	adenosine triphosphate	5, 92
BAT	brown adipose tissue	5
C-terminus	carboxy terminus	9
cAMP	cyclic adenosine monophosphate	10, 12, 97
CNS	central nervous system	11, 12, 14
COMT	catechol-O-methyltransferase	89–92, 96, 97
CYP2D6	cytochrome P450 2D6	8, 9, 90
DADH	dopamine-dehydroxylating enzyme	8, 9
DAT	dopamine transporter	90
DDC	DOPA decarboxylase	8, 9, 91
DL	dorsolongitudinal wing depressor	6, 15, 87, 92
DUM	dorsal unpaired median	7, 13
DV	dorsoventral wing elevator	6, 15, 87
ECD	electrochemical detection	87, 97, 98
FFA	free fatty acid	5, 13

Notation	Description	Page List
GPCR	G protein-coupled receptor	9, 10, 12
HPLC	high-performance liquid chromatography	87, 94, 97, 98
HSP	heat shock protein	14, 93, 94
IPC	insulin producing cell	12
MAO	monoamine oxidase	89–91
MAOI	MAO inhibitor	91, 97
MAT	monoamine transporter	90
MMTG	mesometa-thoracic ganglion	15, 87, 96
MS	mass spectrometry	93, 94
NAT	N-acetyltransferase	90
NMR	nuclear magnetic resonance spectroscopy	93
OAR	octopamine receptor	10
OAT	octopamine transporter	90, 91
PAH	phenylalanine hydroxylase	8, 9, 91
PD	Parkinson's disease	91, 92, 96
PFK	phosphofructokinase	92
PK	pyruvate kinase	92
qPCR	quantitative polymerase chain reaction	92–94
RNA	ribonucleic acid	88, 89, 91–94
RNA-Seq	RNA sequencing	93, 94
RNAi	RNA interference	91
ROS	reactive oxygen species	94, 96
SERT	serotonin transporter	90
TBH	tyramine β-hydroxylase	8, 9, 91
TDC	tyrosine decarboxylase	8, 91
TH	tyrosine hydroxylase	8, 9, 91, 95
UCP1	uncoupling protein 1	5
VMAT	vesicular monoamine transporter	90, 95
VUM	ventral unpaired median	7, 13
WAT	white adipose tissue	5

Chapter

ONE

INTRODUCTION

"Those who are late will be punished by life itself."

This famous quote simply states reacting too quickly, too slowly, too weakly, or too strongly can be devastating. This rule applies to all organisms, from the smallest bacteria to the human body. Survival relies on physiological processes to encounter the challenges caused by environmental alterations (Singh et al., 2010; Torda et al., 2017). In times of increasingly fluctuating ambient factors, a suitable physiological response is of growing importance for all organisms (Rosenzweig et al., 2008; Briga and Verhulst, 2015). Especially timing and magnitude of the response to exogenous stressors are of particular significance (Concordet and Ferry, 1993; McMichael and Lindgren, 2011; Kagias et al., 2012; Wenzel et al., 2016; Walker et al., 2020).

Physiological reactions to a changing environment can be manifold. Changes in environmental parameters trigger physiological stress responses because they threaten the equilibrium of metabolic processes. The metabolism may be altered in the short term and sometimes even permanently (Porter and Gates, 1969; Kajimura et al., 2015; Sepa-Kishi et al., 2017; Chouchani et al., 2019). Additionally, this is often accompanied and supported by adaptations of behaviour (Blair-West et al., 1968; Lagerspetz, 2000; Lagerspetz and Vainio, 2006). The following paragraphs discuss various physiological mechanisms that animals have evolved to defend themselves against abiotic stressors. In this context, representatives of deuterostomia and protostomia are compared whenever possible.

1.1 Physiological Response to Abiotic Stressors

Abiotic environmental factors can be of physical or chemical origin. Humidity and air oxygen content are important key indicators for the quality of a habitat (Ackerman et al., 2004; Harrison et al., 2010; Alizadeh and Sanche, 2013). Furthermore, before other necessary nutrients, water is an essential component for the survival of all living beings. Water quality and usability strongly depends on the abiotic parameters salinity and pH (Carlucci and Pramer, 1960; Dudgeon et al., 2006; Hong et al., 2011). But the most important parameter is the ambient temperature (Toolson and Hadley, 1987; Hadley et al., 1991; Heinrich, 1993). The speed of all physiological processes is temperature-dependant. A simplified version of the Arrhenius' equation predicts the impact of a given temperature change and assumes a doubled reaction rate by an increase of 10 K (Hegarty, 1973; Reyes et al., 2008; Manogaran et al., 2019; Mundim et al., 2020). The temperature of the various compartments (e.g. air, water, soil) differs in its significance as an abiotic factor dependant on the species.

Organisms had to evolve ways to cope with temperature fluctuations. Two main strategies prevailed within the animal kingdom: Homeothermy and poikilothermy. Birds and mammals are deuterostomial representatives for homeothermic animals. They maintain a stable core body temperature and therefore can keep their metabolic activity constant over a broad range of ambient temperatures. In contrast, protostomes and herewith also insects are poikilothermic animals and are physiologically unable to keep a constant body temperature. Consequently, their body temperature is extremely dependent on the ambient temperature (Bartholomew, 1981; Josephson, 1981; Vinogradov, 1995; Geiser, 1998; Crnokrak and Roff, 1999; Wojda, 2017). In simplified terms, whereas homeotherms react to heat or cold with altered physiological processes, poikilotherms have to flee from it or endure it. Under extreme temperature conditions, poikilotherms fall into a coma from which they cannot recover independently. The persistence of this state can lead to death (Mellanby, 1939, 1954; MacMillan and Sinclair, 2011; Hazell and Bale, 2011).

The honeybee *Apis mellifera* as an insect can be considered as poikilothermic. An individual worker bee cannot maintain a constant core temperature. However, its eusociality allows it to form a homeothermic 'superorganism' that includes all worker bees. Using diverse strategies, the colony is able to keep the in-hive temperature constant, especially during breeding season. This precise temperature control also ensures survival during winter and in extreme habitats. Therefore eusociality of the honeybee enables its nearly worldwide distribution in a variety of habitats and a broad range of environmental conditions. This requires a number of temperature regulation mechanisms. Thus, the honeybee is a valuable model organism to study various physiological aspects of adaptation to different climatic conditions (Kronenberg and Heller, 1982; Stabentheiner et al., 2003; Barchuk et al., 2007; Stabentheiner et al., 2010; Wallberg et al., 2014, 2017; Stabentheiner et al., 2021).

1.2 Physiological Response to Heat Stress

In Western Australia, soil temperatures in extreme habitats reach up to 78 °C in mid summer (Mott, 1972). Most representatives of deuterostomia and protostomia are not exposed to such extreme temperature conditions, but have to cope with heat stress in their habitats. Physiological protective strategies are therefore indispensable. Although the strategies are based on different mechanisms, their intended target is thermostasis or tolerance of a higher core temperature and disablement or failure of these mechanisms endangers survival (Toolson and Hadley, 1987; Hadley et al., 1991; Moseley, 1997; Ooi et al., 2009; Baker, 2019).

The process of evaporative cooling is a cardinal principle for lowering body temperature. Thermal conductivity of water and the cooling effect due to evaporation are hereby harnessed. In both deuterostomia and protostomia, evaporative cooling confers to heat loss and therefore acclimatization processes. The honeybee uses this process not only to reduce its own body temperature, but also to cool the entire hive. This is of particular importance for survival of the colony. The primary focus of thermoregulation in the hive is to ensure proper progeny development. For self-cooling, the honeybee uses a mechanism reminiscent of panting. In this process, vomited liquid is caught on the extended proboscis and evaporates (Heinrich, 1980; Barchuk et al., 2007; Jarimi et al., 2020). However, these are not the only mechanisms for cooling purposes. Evaporative cooling effects can also take place in the branches of tracheal system (Heinrich, 1980). In addition, worker bees control the air quality inside their hive by coordinated fanning, which provides air circulation and thus heat, gas and moisture exchange. Abiotic parameters (temperature, humidity) in the hive are decisive for the motivation of fanning (Cook and Breed, 2013; Cook et al., 2016, 2017; Peters et al., 2019). Furthermore, the bodies of worker bees can absorb heat and subsequently carry it away from local hot spots. In analogy with the human cardiovascular system, worker bees represent blood and vessels through which heat is transported between core and limbs (Collins, 1981; Southwick and Heldmaier, 1987; González-Alonso, 2012; Bonoan et al., 2014; Peters et al., 2019).

Convergent developments of evaporative cooling can be found in a variety of animals. Important evaporative cooling mechanisms of deuterostomia are sweating and panting. Increased blood circulation in the evaporative tissue can concomitantly amplify heat loss. Panting is of particular importance for small mammals, but also occurs in birds and some reptiles. In contrast, larger mammals and humans rely on a greater amount and spread of their sweat glands, which contribute to a more effective temperature regulation (Nadel et al., 1974; Hudson and Dawson, 1975; Goldberg et al., 1981; Fruth and Gisolfi, 1983; Robertshaw, 2006; Baker, 2019; Guhl et al., 2019; Malgoyre et al., 2020). Some insects have evolved abilities similar to mammalian sweating. Here, *Diceroprocta apache* protrudes through its specialized evaporative cooling. These desert cicadas can tolerate higher temperatures than mammals

and birds (thoracic temperatures up to 45-46 °C). Continuous evaporation of water through dorsal cuticular pores enables them to survive in conditions too harsh for their predators (Toolson and Hadley, 1987; Hadley et al., 1991; Heinrich, 1993).

1.3 Physiological Response to Cold Stress

1.3.1 Hibernation and Diapause

Besides heat, cold is a particularly threatening environmental situation. Likewise protection against hyperthermia, protection against hypothermia benefits from a state of thermostasis by preventing heat loss and increasing the core temperature of the individual. Additionally, a tolerance development is also possible to a certain extent (Benzinger et al., 1961). In case of life-impairing situations, animals evolved different forms of dormancy to outlast harsh environmental conditions (Denlinger, 1974; Dausmann et al., 2004; Jansen et al., 2019). 'Hibernation' (deep torpor) is a mammalian mechanism mainly to cope with the winter coldness. The duration can range from a few months to more than half a year and takes place in a 'hibernaculum', a shelter in which the animals hibernate. Periodically, hibernation can be interrupted by interbout arousals, euthermic states with 'normal' activity levels (Morin and Storey, 2009; Halsall et al., 2012; Geiser, 2013). Physiological characteristics of mammalian dormancy include substantially decreased heart rate, metabolic rate and respiratory rate (apnea-like breathing), as well as controlled hypothermia and decreased water loss (Lyman and Chatfield, 1955; Mamady and Storey, 2008; Morin and Storey, 2009; Klug and Brigham, 2015). During this process, altered protein modifications and gene expression pattern consistently downregulate the metabolism and transcription processes. In contrast, torpor specific genes are upregulated by dedicated transcription factors (e.g. cAMP response element-binding protein). Increased levels of serum albumin, macroglobulin, peroxisome proliferator-activated receptors in adipose and muscle tissue, and fatty acid carrier proteins occur as result of this torporal state (Lyman and Chatfield, 1955; Srere et al., 1992; Heldmaier et al., 2004; Eddy et al., 2005; Mamady and Storey, 2008; Morin and Storey, 2009; Geiser, 2013).

Insects can also enter a dormancy state. In this context, the diapause represents the analogue of hibernation. This state serves as a survival mechanism for hostile (recurrent) seasonal influences and therefore not only occurs during winter (Denlinger, 2002; Geiser, 2013). Diapause is often associated with a specific developmental stage and undergoes various phases. Each of these phases is characterized by specific physiological aspects and metabolic alterations, which include gene expression shifts, cell cycle arrest, altered metabolic rates and reduced water loss (Yoder et al., 1992; Zhang and Denlinger, 2010; Ikeno et al., 2010). Both, insects and mammals can accumulate fat and energy stores before they enter the

diapause or hibernation (Denlinger, 2002; Koštál, 2006; Geiser, 2013). Many insects use the diapause strategy, but the honeybee remains active throughout the winter. Thereby worker bees provide coordinated heat production via thermogenesis to ensure survival (Tauber and Tauber, 1973; Rinehart et al., 2007; Stabentheiner et al., 2003; Musolin, 2012; Tougeron, 2019).

1.3.2 Thermogenesis

During a hyperphagic period in preparation for diapause or hibernation, animals store a large proportion of their body weight as fat tissue. In mammals, there is not only an increase in white adipose tissue (WAT), but also in brown adipose tissue (BAT). WAT is primarily associated with energy storage and hormonal regulation, whereas BAT can generate heat via non-shivering thermogenesis. Thermogenesis is the ability to generate heat individually and provides an elegant yet energy-consuming protection method against the cold. In contrast to diapause and hibernation, the metabolic rate can remain high due to thermogenesis-induced euthermia. The ability of endogenous heat generation poses a major requirement for the survival of homeothermic animals. Here, thermogenic BAT plays a decisive role in mammals and is linked to cold stress adaption in rodents. The detection of BAT in adult humans has significantly pushed the field of thermogenesis research forward, although it was found in other species as well (Himms-Hagen, 1976; Cousin et al., 1992; Palou et al., 1998; Cypess et al., 2009; Wu et al., 2012; Lidell et al., 2013; Geiser, 2013; Klug and Brigham, 2015). BAT is involved in important metabolic functions and can occur throughout the whole lifespan. Factors like sex, medication (ab)use, smoking, diet, obesity, cold stress exposure and the overall health status correlate with presence and amount of BAT. The expression, presence and activity of uncoupling protein 1 (UCP1), also known as 'thermogenin', is a molecular characteristic of BAT. UCP1 is located at the interior membrane of the mitochondria and functions as a proton carrier. Purine nucleotides inhibit UCP1, whereas free fatty acids (FFAs) activate it (Srere et al., 1992; Cousin et al., 1992; Palou et al., 1998). The activating FFAs are released by adrenergic β_3-mediated lipolysis and bind to UCP1 at its intracellular side, where they alter the conformation of UCP1 and thus support proton insufficiency of the inner membrane. The influx of protons into the mitochondrial matrix ultimately releases heat. The lack of protons in the intermembrane space slows down the nearby adenosine triphosphatase (ATPase). Simultaneously, the oxidation of substrates (amino acids, carbohydrates, fats) causes an increasing release of additional protons. This process continuously releases heat as long as the state of UCP1 activation persists (Palou et al., 1998; Cypess et al., 2009; Saito et al., 2009; Fedorenko et al., 2012; Yoneshiro et al., 2016). In addition to BAT-mediated thermogenesis, other forms of heat producing processes including adaptive thermogenesis or futile cycles should be mentioned. Adaptive thermogenesis is

highly dependent on the individual nutritional state and plays a decisive role in weight regain after a significant reduction of body mass (Rosenbaum and Leibel, 2010; Dulloo et al., 2012; Chouchani et al., 2019). Futile cycles are a group of physiological mechanisms, that also occur under the metabolic state of adaptive thermogenesis. Characteristics are energy-consuming processes in which biochemical reactions run simultaneously in opposite directions. This includes metabolic as well as transportation pathways. The name futile cycle was given because there is no net metabolic turnover and the re-release of the consumed energy provides heat (Katz and Rognstad, 1978; Samoilov et al., 2005; Gamu and Tupling, 2017; Brownstein et al., 2021). Substrates of futile cycles range from ions (Ca^{2+}; Gamu and Tupling, 2017) to small molecules directly linked to energy metabolism (creatine, glucose, lipids). A viable glucose-based futile cycle has been reported in bumblebees (*Bombus rufocinctus*). Due to its relationship to the bumblebee, the honeybee possesses the necessary genetic bases, but there is no evidence of a functional futile cycle in honeybees (Newsholme et al., 1972; Surholt et al., 1990; Staples et al., 2004; Gamu and Tupling, 2017; Brownstein et al., 2021).

The mechanisms discussed above relate to non-shivering thermogenesis, whereas, birds, mammals and insects mainly use shivering thermogenesis during cold stress. Birds and mammals rely on their skeletal muscles to achieve this, while insects rely on their flight muscles. The energy consuming process of shivering thermogenesis consists of involuntary muscle contractions (Heinrich, 1974; Hohtola, 2004; Kajimura et al., 2015; Sepa-Kishi et al., 2017; Blondin and Haman, 2018). Therefore, the mammalian physiology contributes energy through various available pathways such as carbohydrate, lipid and protein metabolism (Haman and Blondin, 2017).

Honeybees exclusively use shivering thermogenesis. This highlights the importance of the flight muscles (Stabentheiner et al., 2003, 2021). Honeybee flight muscle formation consists of two separate thoracic muscles, the dorsolongitudinal wing depressor (DL) and dorsoventral wing elevator (DV). Flight muscles are also subject to various other activities like flight initiation (Esch and Goller, 1991; Pflüger and Duch, 2011), flight (Suarez et al., 1996), fanning (Peters et al., 2019) and communication (buzzing) (Esch and Goller, 1991; Tsujiuchi et al., 2007). It has been shown that the contraction ratio (DL/DV) of honeybee flight muscles differs during buzzing (1.08), flight phase (0.86) and shivering thermogenesis (1.34). These discrepancies, especially between shivering thermogenesis and flight, also occur in bumblebees, suggesting differential modulation of the flight apparatus (Bastian and Esch, 1970; Esch et al., 1991; Esch and Goller, 1991). However, it is still unclear how this modulation is accomplished.

1.4 Octopamine

1.4.1 Octopamine and Shivering Thermogenesis

Decades ago, a study indicated that adrenergic substances impact the core temperature of honeybees (Belzunces et al., 1996). Since thermogenesis in honeybees is exclusively based on flight muscle shivering, administration of adrenergic substances seems to have an influence on this process. However, this raises the question how the effect is mediated since the honeybee is not equipped with the deuterostomial adrenergic system (Roeder, 2005). In locusts (*Schistocerca gregaria*, *Locusta migratoria*) and other athropods, octopamine plays an essential role in the initial flight phase by modulating the muscle activity. Here, efferent dorsal unpaired median (DUM) and ventral unpaired median (VUM) octopaminergic neurons innervate the flight muscles (Pflüger and Duch, 2011). A genome analysis of *Apis mellifera scutellata* and *Apis mellifera monticola* shows striking high mutation rates in all octopamine β receptor genes, suggesting a putative adaptation mechanism to high altitudes and the prevailing colder climate in their habitat (Wallberg et al., 2017). Furthermore, a change in octopaminergic gene expression under cold stress conditions was investigated in *Apis cerana cerana* (Xu et al., 2017a). Fluctuating octopamine levels were found in brains of *Apis mellifera* under cold stress (Chen et al., 2008). These findings indicate an involvement of octopamine in honeybee shivering thermogenesis. Nevertheless, there is still a lack of information regarding a direct role of octopamine in shivering thermogenesis in both deuterostomia and protostomia. In this context, the first step should be to understand the relation between the protostomial octopaminergic and the deuterostomial adrenergic system. Since both systems share a common evolutionary origin, they are considered as homologues. This is reflected by pronounced similarities in terms of structure, function, receptors and transmitter molecules between the two systems. It is therefore plausible, that adrenergic compounds might exhibit effects in the octopaminergic system of insects and vice versa (Belzunces et al., 1996; Roeder et al., 2003; Roeder, 2005; Pflüger and Stevenson, 2005; Verlinden et al., 2010; Fuchs et al., 2014; Bauknecht and Jékely, 2017; Gainetdinov et al., 2018). The origin of octopamine and how it is formed will be explained in the following.

1.4.2 Octopamine Biosynthesis

Octopamine was first described as a compound in the mollusc species *Octopus vulgaris* (Erspamer, 1948, 1952; Hirashima and Huang, 2008). Research on octopamine and related substances was vigorously pursued in the following decades. This was additionally supported by an improvement of analytical methods. Octopamine contains one phenolic alcohol group in the para position to a side chain with a terminal primary amine. This side chain is hydroxylated at the benzylic β position. It displays a close chemical and structural

resemblance to the mammalian norepinephrine. The structure of norepinephrine differs from that of octopamine in its catechol functionality and stereoinformation of the chiral carbon in β position (Erspamer, 1952; O'Neil et al., 2006; Hirashima and Huang, 2008). The biogenic amine octopamine is synthesized as follows (Figure 1.1). The biosynthesis pathway of octopamine originates from the amino acid L-phenylalanine. Since phenylalanine cannot be produced by many deuterostomia and protostomia, including honeybees, it represents an essential dietary component and must be supplied exogenously (de Groot, 1953; Feldhaar et al., 2007; Solorzano et al., 2009; Arts et al., 2017; Nogales-Mérida et al., 2019; Rihani et al., 2019; Ennis et al., 2020). In the first step the amino acid L-tyrosine derives from L-phenylalanine via a hydroxylation of the aromatic ring catalyzed by phenylalanine hydroxylase (PAH). The second step involves a decarboxylation of L-tyrosine through tyrosine decarboxylase (TDC), yielding in the biogenic amine tyramine (Phan et al., 1983). Subsequently, octopamine is a product of an aliphatic hydroxylation enzymatically catalyzed by tyramine β-hydroxylase (TBH; Roeder et al., 2003; Roeder, 2005; Cole et al., 2005; Blenau et al., 2020).

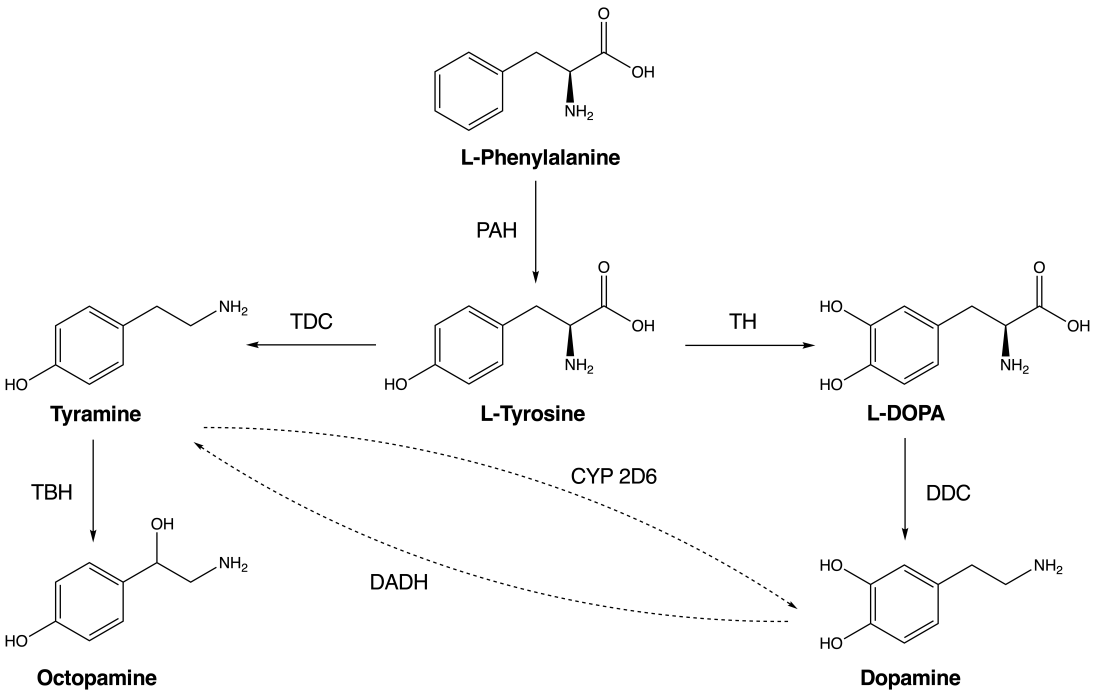

Figure 1.1: **Biosynthesis of octopamine and dopamine.** Biosynthesis pathways of octopamine and dopamine linked by a salvage pathway, which is known from others species but may exist in insects as well. The first step of both biosyntheses consists of the formulation of L-tyrosine from L-phenylalanine by PAH. L-tyrosine can then be converted into tyramine via TDC or into L-DOPA via TH catalysis. Tyramine yields octopamine through TBH and L-DOPA yields dopamine through DDC. In turn, tyramine can be produced from dopamine by DADH and the reversion is catalyzed by CYP2D6. Figure modified after Roeder (1999) & Fuchs et al. (2014).

An alternative pathway interconnects the octopamine biosynthesis with that of dopamine. This pathway also starts with the formation of L-tyrosine from L-phenylalanine via PAH. In turn, L-DOPA is formed out of L-tyrosine, catalyzed by tyrosine hydroxylase (TH). In a final step of the dopamine biosynthesis, dopamine yields enzymatically via DOPA decarboxylase (DDC) from L-DOPA. Dopamine itself can then be degraded to tyramine. This step is catalyzed by dopamine-dehydroxylating enzyme (DADH). Tyramine can then fuel octopamine formation by TBH. In a reverse reaction dopamine can be formed from tyramine by cytochrome P450 2D6 (CYP2D6). However, it is questionable whether these interconnecting enzymatic sequences have a physiological relevance and occur in insects at all (Hiroi et al., 1998; Roeder, 2005; Fuchs et al., 2014; Jameson and Hsiao, 2019; Rekdal et al., 2020). For an overview of the octopamine/dopamine biosynthesis pathways see Figure 1.1.

1.4.3 Octopamine Receptors in Insects

Neurotransmitters exhibit physiological effects by an interaction with respective receptors. Receptor structure and functionality is very heterogeneous, ranging from membrane-bound or intracellular proteins to the formation of ion channels as well as coupling to various enzymes or G proteins (Jensen and DeSombre, 1973; Unwin, 1993; Porter and Vaillancourt, 1998; Foye, 2008; Aktories et al., 2022). In general, the family of G protein-coupled receptors (GPCRs) may account for one of the most important and largest group of membrane-bound receptors (Lagerström and Schiöth, 2008; Smith et al., 2018; Aktories et al., 2022). GPCRs typically consist of seven transmembrane domains, three intracellular and three extracellular loops, whereby the receptor protein endings are oriented in opposite directions. The polypeptide amino terminus protrudes into the extracellular space, while the carboxy terminus (C-terminus) remains at the cytosolic side of the bilayer (Latorraca et al., 2017; Hilger et al., 2018). The C-terminus forms a target of specific interactions with various proteins, allowing post-translational modifications, which can transient or permanently alter the function of the receptor protein. Thus, phosphorylation of the C-terminus can lead to biased signaling via β-arrestin and may trigger receptor desensitization processes and internalization (e.g. clathrin-mediated receptor endocytosis). These regulatory mechanisms allow the cell to respond to physiological extremes, such as overstimulation (Hausdorff et al., 1990; Pitcher et al., 1992; Warne et al., 2008; Liu et al., 2017; Zhang and Kim, 2017; Sente et al., 2018). GPCRs are categorized into different subclasses, depending on the coupled G protein type. Highly specific interactions of ligand and receptor are formed at the extracellular binding site, leading to a discrimination between an active and inactive state of the receptor (two-state model) (Vernier et al., 1995; Seifert and Wenzel-Seifert, 2002). Upon receptor activation, the associated heterotrimeric (α, β, γ) G protein dissociates into two subunits: α-subunit and βγ-subunit. Both may contribute via different pathways and

mediate the following signal transduction by intracellular messengers. The first steps of the intracellular cascade are determined by the GPCR subclass through interactions between cellular enzymes and the specific subunit (α, $\beta\gamma$). The following steps are dependent on the host cell equipment. Therefore, an effect mediated by the same type of receptor can trigger completely different outcomes. In this context, constitution of tissue, cell type and physiological state at the time of stimulus play a decisive role (Seifert and Wenzel-Seifert, 2002; Lagerström and Schiöth, 2008; Smith et al., 2018; Capper and Wacker, 2018; Hilger et al., 2018; Aktories et al., 2022).

The group of protostomial octopamine receptors (OARs) consists exclusively of GPCRs. Due to the close functional and structural relationship, amine receptors in honeybee have been named after their analogues in mammals (Roeder et al., 2003; Roeder, 2005). The honeybee genome harbors five different AmOARs. Their putative receptor proteins form classes which can be distinguished by their respective second messengers. For instance, all octopamine β receptors (AmOARβ1, AmOARβ2, AmOARβ3 and AmOARβ4) cause an increase of intracellular cyclic adenosine monophosphate (cAMP) level upon activation. Since AmOARβ3 and AmOARβ4 are putative splice variants of the same gene, the term AmOARβ3/4 will be used in the remainder of this thesis. AmOARα2 exhibits a decrease of intracellular cAMP concentration and can therefore be considered as a functional counterpart of the AmOARβ group. AmOARα1 triggers an enzymatic release of inositol-1,4,5-trisphosphate and diacylglycerol, resulting in an elevated intracellular Ca^{2+} level (Blenau et al., 2000; Grohmann et al., 2003; Balfanz et al., 2013; Reim et al., 2017; Blenau et al., 2020). The experimental part of this thesis (Chapter 2 & Chapter 3) will focus in particular on *AmOARα1*, *AmOARβ2* and *AmOARβ3/4*.

Due to the high grade of chemical and structural similarity of tyramine and octopamine, both molecules harbor affinities to each other's receptors. Tyramine, being the precursor of octopamine, represents a discrete neurotransmitter with own dedicated receptors (AmTAR1, AmTAR2) in protostomia (Erspamer, 1948; Blenau et al., 2000; Roeder et al., 2003; Roeder, 2005; Cole et al., 2005; Alkema et al., 2005; Reim et al., 2017; Blenau et al., 2020).

1.4.4 Octopaminergic Effects in Insects

In deuterostomia, octopamine serves as a neuromodulator, neurotransmitter and neurohormone (Axelrod and Saavedra, 1977; Evans, 1980; David and Coulon, 1985; Roeder, 1999; Blenau and Baumann, 2001; Scheiner et al., 2002; Evans and Maqueira, 2005; Verlinden et al., 2010). Accordingly, octopamine modulates, drives and controls various physiological processes and control circuits. Most of the known effects relate to the insect nervous system and associated organs (Scheiner et al., 2002; Barron et al., 2007; Verlinden et al., 2010; Aonuma and Watanabe, 2012; Pauls et al., 2021; Schilcher et al., 2021). The octopaminergic system has a variety of different effects in insects. Particularly in flies (*Drosophila*), locusts (*Locusta migratoria*, *Schistocerca americana*, *Schistocerca gregaria*) and bees (*Apis mellifera*) numerous studies have been conducted so far. In the following, the focus will be primarily on representatives of these genera.

Octopaminergic effects in *Apis mellifera*

Most information about octopaminergic effects and its regulatory circuits in honeybees has been gathered in context of the central nervous system (CNS). Here, octopamine effects individual task performance and colony task performance. Octopamine brain titres are usually greater in forager bees than in nurse bees. Given its eusocial lifestyle, worker bees must be able to perform a variety of tasks. The main tasks in the life of a worker bee includes caring for the brood (nursing) and foraging. Younger bees are generally located inside the hive, while older bees (> three weeks) fly out to ensure water and food supply (nectar and pollen) for the whole colony (Winston, 1991; Wagener-Hulme et al., 1999; Schulz et al., 2003; Reim and Scheiner, 2014). The interplay of octopamine and associated gene expression (AmOAR genes) affects honeybee foraging in many ways. This includes the onset of foraging, foraging efficiency, maintenance of foraging activity, preference during foraging and also the sensitivity to stimuli relevant for foraging. Nurse bees take care of the brood and ensure the welfare of the offspring. There is a correlation between an intensification of the interindividual hygienic behavior during brood care and a specific anti-octopamine labeling in the CNS. Consequently, octopamine is heavily involved in the complex modulation of task performance and division of labour in *Apis mellifera* (Hammer, 1993; Schulz and Robinson, 1999; Barron et al., 2002; Scheiner et al., 2002; Spivak et al., 2003; Schulz et al., 2003; Barron and Robinson, 2005; Giray et al., 2007; Reim and Scheiner, 2014). Octopamine also impacts locomotion behaviour as well as light response and phototaxis. Furthermore, an octopamine-induced change in gene expression of the clock gene *period* was found. *Period* is associated with circadian rhythm and locomotor activity (Hardin et al., 1990; Dunlap, 1999; Bloch et al., 2001; Fussnecker et al., 2006; Bloch and Meshi, 2007; Schilcher et al., 2021). There are also manifold octopaminergic effects on behavioural plasticity and the

waggle dance, a communication tool to exchange information about food sources among worker bees. Finally, when honeybees are exposed to various stress conditions (e.g. chilling, spinning), a significant octopaminergic response in the bee brain is elicited (Hammer, 1993; Scheiner et al., 2006; Giurfa, 2006; Barron et al., 2007; Chen et al., 2008). For nurse bees and forager bees, olfactory and gustatory neuronal circuits play an important part during their task performance. Increased gustatory and olfactory sucrose responsiveness, associated appetitive and aversive learning performance as well as memory is mediated by octopamine. This was shown for different age cohorts of *Apis mellifera* and various application types (e.g. peroral, parenteral) (Mercer and Menzel, 1982; Scheiner et al., 2002, 2003; Unoki et al., 2006; Scheiner et al., 2006; Giurfa, 2006; Barron et al., 2007; Agarwal et al., 2011; Behrends and Scheiner, 2012).

Octopaminergic Effects in *Drosophila*

Congruent to honeybees, octopaminergic neurons in flies are associated with learning processes and also memory. The octopamine profile is close to that found in honeybees (Schwaerzel et al., 2003; Wu et al., 2013; Iliadi et al., 2017). Additionally, the *Drosophila* larval development is mediated by octopamine. In this case, octopamine acts as an inhibitory signal on the neural circuitry in the CNS (Dasari and Cooper, 2004). Olfactory-associated memory and learning in the mushroom bodies are based on adenylate cyclase activity and intracellular cAMP signaling, caused by octopaminergic GPCR cascades (Tomchik and Davis, 2009; Wu et al., 2013).

Drosophila holds a special position as a model organism due to its enormous molecular manipulation potential (Feany and Bender, 2000; Schneider, 2000; Yamaguchi and Yoshida, 2018; Su, 2019). Using molecular tools in *Drosophila*, octopamine was found to regulate various hormonal transduction pathways. It intervenes in specific cascades of neuropeptide producing cells (e.g. insulin producing cells; IPCs) and triggers presynaptic vesicular neuropeptide release of motoneurons. In addition, octopamine alters IPC insulin release (e.g. *Drosophila insulin-like peptide*) and links classic metabolic pathways with the physiology of sleep (Crocker et al., 2010; Shakiryanova et al., 2011; Nässel et al., 2013; Luo et al., 2014). Furthermore, studies indicate that octopamine is required for stimulating the synthesis of steroidal prohormone ecdysone, which is involved in insect molting. The backbone of ecdysone can be modified and thereby enzymatically converted to the active compound 20-hydroxyecdysone catalyzed by ecdysone 20-monooxygenase. The increase of this enzymatic turnover is fueled by autocrine AmOARβ3 stimulation. Consequently, octopamine is involved in regulating the metamorphic development in insects by influencing essential hormone production (Rauschenbach et al., 2007, 2008; Ohhara et al., 2015).

It has been shown that octopaminergic pathways are even associated with the gut microbiome in *Drosophila*. Symbiotic interactions between the host and its gut microbiome may differ

interindividually and can have a major impact on the host organism. Variation in microbiome composition allows differentiation between individuals and even determination of kinship within a group. By relating octopaminergic pathways and the metabolic capacity of the gut microbiome, it appears that locomotion and aggressive behaviour are also influenced by octopamine (Arumugam et al., 2011; Yatsunenko et al., 2012; Putignani et al., 2014; Lizé et al., 2014; Schretter et al., 2018; Jia et al., 2021).

Ultimately, octopamine is associated with muscle innervation in *Drosophila* and contributes to muscle activity and contractility. Octopamine-dependent modulation causes flight initiation and increases the probability of flight occurrence. During insect's flight phase, octopamine is released and a flight situation can also be stimulated by octopamine administration (Brembs et al., 2007; Ache et al., 2019). Moreover, there are inhibitory effects of octopamine on oviduct contraction, which is mediated by neurons derived from the innervating ganglion (Rodríguez-Valentín et al., 2006; Ormerod et al., 2013). Sujkowski et al. (2017) showed that octopamine can induce receptor-mediated stimulation of exercise-like metabolic changes, in muscle tissue as well as in adipose tissue. Studies in *Drosophila* clearly demonstrate, that octopamine plays a key role in almost all compartments and activities of the body.

Octopaminergic Effects in Locusts

Considering the role of octopamine in many metabolic pathways and particularly muscle physiology and energy supply, it is not surprising that muscle activity is consistently accompanied by octopaminergic innervation in locusts as well. This indicates a high degree of conservation of octopaminergic effects within the clade of insects. In the neurophysiology of protostomia, locusts serve as muscle physiology prototypes. In particular, locomotion and flight as well as aminergic modulation of the periphery are well-studied areas. Specific types of octopaminergic neurons (DUM/VUM neurons) are integral to the connection between flight muscles and thoracic ganglia in locusts (Bräunig and Pflüger, 2001; Pflüger and Duch, 2011). Moreover, octopamine alters muscle physiology, allowing peak motor performance (e.g. flying). This is achieved by boosting glycolysis turnover, mediating the metabolism and releasing FFAs along with carbohydrates from the abdominal fat body reservoir (Orchard et al., 1981, 1982; Orchard and Lange, 1984; Leitch et al., 2003; Pflüger and Stevenson, 2005; Pflüger and Duch, 2011). As flying is one of the most physically demanding and energy-consuming processes, it is necessary to establish the required metabolic state in preparation for flight and during flight (Duch and Pflüger, 1999; Bräunig and Pflüger, 2001; Leitch et al., 2003; Pflüger and Duch, 2011). The role of octopamine in locusts is not only to modulate metabolism, but octopamine directly triggers excitatory responses. Consequently, octopaminergic neurons are heavily involved in insect's flight and flight-supporting physiological processes, including the respiratory system (Ramirez and Pearson,

1991b,a; Rillich et al., 2013). Octopaminergic effects in the locust brain are consistent with effects in the CNS of flies and bees. In the locust CNS, octopamine also serves as a regulatory neurotransmitter involved in the olfactory system and the visual system as well as learning processes. Here, multimodal neurons in the visual tract of locusts are chemically modulated by a specific octopaminergic neuron, reducing the neuronal response to visual inputs (Hammer and Menzel, 1998; Stern, 2009; Cassenaer and Laurent, 2012; Xu et al., 2017b). In addition, octopamine stimulates cellular transcription factors to boost the expression of heat shock proteins (HSPs) to protect the CNS through β octopamine receptors in *Locusta migratoria* (Armstrong et al., 2006).

Assessment of the Current State of Knowledge

Although the circumstances differ in each model organism, octopaminergic effects overlap within various species and reveal an general pattern regarding the purpose of octopamine in insects. This strengthens the hypothesis that the octopaminergic system is highly conserved within the clade (Verlinden et al., 2010). Considering the publication gap of muscle physiology and peripheral innervation in honeybee, it can be expected that octopamine also fulfils similar functions as it does in flies and locusts, due to high consistency across species. For this reason, octopamine may also play a central role in shivering thermogenesis.
There are notably fewer studies on octopaminergic effects outside the CNS in honeybee. This is not because octopamine does not exert a modulatory function in this area, but because insect research in the past has focused primarily on the neurobiology of the brain. Many known octopaminergic effects often lack pharmacological interpretation or structure-activity relationship and therefore corresponding receptor interactions are still poorly known. Hence, research on other body compartments and organs has been largely neglected (Scheiner et al., 2006; Verlinden et al., 2010; Gainetdinov et al., 2018). In this context, a new area of research on physiological and neurochemical aspects of the honeybee octopaminergic thoracic system is presented in the following chapters.

1.5 Thesis Outline

Honeybees gained a unique set of skills to preserve thermal homeostasis within the superorganism. While there are several mechanisms for cooling, there is only one for heating and fighting the cold. Due to the fact that flight muscles are the source of thermogenesis, my research focused on the DL and DV as well as the mesometa-thoracic ganglion (MMTG). The aim of my study was to elucidate the thoracic octopaminergic system to discover the role of octopamine in honeybee thermogenesis. At the beginning of my work, the octopaminergic thoracic system and its molecular background in honeybee thermogenesis appeared to be a black box. In addition, the information was scarce on neurochemical modulation of shivering thermogenesis in honeybee. My work in this area fills precisely these research gaps and ties up the loose ends of both of these areas.

During my doctoral studies, I conducted experiments to test a sequential series of hypotheses. In order to perform these experiments, a unique combination of methods from a variety of research areas were required, including methods of immunochemistry, behavioural physiology, thermography, pharmacology, pharmaceutical analytics and molecular biology. By combining these methods in a logical sequence, I was able to provide novel information on the precise role of octopamine in honeybee thermogenesis, to address questions in a multidimensional manner and to integrate them into an overall scheme (Figure 4.1).

In the beginning of my project, I faced the question, how honeybee thermogenesis is physiologically modulated. Based on evidence from literature, modulation by octopamine was a plausible explanation. In the second chapter (Chapter 2) of this work, a connection between octopaminergic modulation and thermogenesis is established for the first time. I hypothesized that octopaminergic signaling, in particular octopamine β receptor stimulation is involved in honeybee thermogenesis. To examine this hypothesis, the first step was to determine the basis for an octopaminergic effect by screening amines (dopamine, octopamine, serotonin, tyramine) and receptor gene expression patterns (*AmOARα1*, *AmOARα2*, *AmOARβ1*, *AmOARβ2*, *AmOARβ3/4*, *AmTAR1*, *AmTAR2*) in the corresponding tissues of naive animals. Different biogenic amines and receptor modulators were tested in bees with and without induced hypothermia. Moreover, various intracellular processes were elucidated by analytical and pharmacological methods to further confirm the initial hypothesis.

After uncovering the thoracic octopaminergic system and its indispensability for native thermogenesis, the response of this system to cold stress was tested in Chapter 3. Here my hypothesis was, that the discovered neuro-muscular octopaminergic system must be sustained regardless of abiotic stressors and therefore tends to an equilibrium. In Chapter 4, certain results and their implications are discussed in detail. These findings are placed in the context of other studies and the current state of knowledge on the respective topic. Several approaches are outlined to continue and expand this area of pioneering research in the future.

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Chapter

TWO

OCTOPAMINE DRIVES HONEYBEE THERMOGENESIS

Manuscript was originally published in:
Sinan Kaya-Zeeb, Lorenz Engelmayer, Mara Straßburger, Jasmin Bayer, Heike Bähre, Roland Seifert, Oliver Scherf-Clavel, Markus Thamm (2022).
Octopamine drives honeybee thermogenesis *eLife* **11**:e74334

This article can be downloaded from https://doi.org/10.7554/eLife.74334.

RESEARCH ARTICLE

Octopamine drives honeybee thermogenesis

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Abstract In times of environmental change species have two options to survive: they either relocate to a new habitat or they adapt to the altered environment. Adaptation requires physiological plasticity and provides a selection benefit. In this regard, the Western honeybee (*Apis mellifera*) protrudes with its thermoregulatory capabilities, which enables a nearly worldwide distribution. Especially in the cold, shivering thermogenesis enables foraging as well as proper brood development and thus survival. In this study, we present octopamine signaling as a neurochemical prerequisite for honeybee thermogenesis: we were able to induce hypothermia by depleting octopamine in the flight muscles. Additionally, we could restore the ability to increase body temperature by administering octopamine. Thus, we conclude that octopamine signaling in the flight muscles is necessary for thermogenesis. Moreover, we show that these effects are mediated by β octopamine receptors. The significance of our results is highlighted by the fact the respective receptor genes underlie enormous selective pressure due to adaptation to cold climates. Finally, octopamine signaling in the service of thermogenesis might be a key strategy to survive in a changing environment.

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Competing interest: The authors declare that no competing interests exist.

Funding: See page 18

Received: 29 September 2021
Preprinted: 06 November 2021
Accepted: 15 February 2022
Published: 15 March 2022

Reviewing Editor: K VijayRaghavan, National Centre for Biological Sciences, Tata Institute of Fundamental Research, India

Editor's evaluation

This study is of broad interest to researchers in the field of entomology and physiology. These findings may shed light on at least one mechanism underlying selective advantages conferred to insect species on evolutionary timescales. Though the chemical signal, its source, and recipient tissues underlying thermogenesis are elucidated, hypotheses regarding their downstream effects remain to be substantiated.

Introduction

The Western honeybee (*Apis mellifera*) owns incredible thermoregulation strategies, which allow the colony to keep the brood area constantly at 34 °C (***Simpson, 1961***). Due to this special feature, honeybees are relatively independent of the ambient temperature (T_A), which may contribute decisively to their almost worldwide distribution (***Wallberg et al., 2014***). In contrast to other ectotherms, honeybee thermoregulation includes thermogenesis. Here, primarily workerbees actively increase their thorax temperatures (T_{THX}, ***Kovac et al., 2009***; ***Stabentheiner et al., 2010***). This thermogenesis is of immense social importance, because it enables foraging at T_A below 10 °C (***Bujok et al., 2002***; ***Stabentheiner et al., 2003***) and a proper brood development (***Himmer, 1932***; ***Weiss, 1962***; ***Tautz et al., 2003***; ***Wang et al., 2016***), reduces parasite infections (***Starks et al., 2000***; ***Campbell et al., 2010***), and is a powerful defense mechanism against predatory hornets (***Ken et al., 2005***; ***Baracchi et al., 2010***).

The individual heating pattern of workerbees consists of a wave-like rise and fall in T_{THX} (***Kronenberg and Heller, 1982***) and is realized exclusively by the activation of the indirect flight muscles, formed by the dorsoventral wing elevators (DV) and the dorsal-longitudinal wing depressors (DL), even if wing and thorax vibration are not visible (***Esch et al., 1991***; ***Esch and Goller, 1991***). However, these muscles are utilized in various other behaviors, which includes flight (***Esch et al., 1975***; ***Esch, 1976***), fanning (***Simpson, 1961***) and communication during the waggle dance (***Esch, 1961***; ***Wenner, 1962***). In order to perform these various tasks, diverse contraction mechanisms exist which must be controlled differently (***Esch and Goller, 1991***). Some evidence indicates a crucial role of octopamine in the insect flight muscles (***Blau and Wegener, 1994***; ***Blau et al., 1994***; ***Wegener, 1996***; ***Duch et al., 1999***). Unfortunately, it remains unknown whether octopamine is used as a neurochemical in honeybee flight muscles or whether an octopamine receptor gene is expressed in these tissues. However, DL and DV are under control of the mesometa-thoracic ganglion (MMTG, ***Markl, 1966***) and the octopaminergic innervation of the flight muscles seems to be a conserved feature in insects (***Duch et al., 1999***; ***Schlurmann and Hausen, 2003***; ***Pauls et al., 2018***). It was further demonstrated that the brain octopamine concentration of workerbees is significantly decreased due to cold stress (***Chen et al., 2008***), which indicates the temperature sensitivity of the neuronal octopaminergic system. In this context, ***Wallberg et al., 2017*** made the observation that honeybee β octopamine receptor genes (*AmOARβ1-3/4*) are subject to altitudinal adaptation processes in honeybees. Yet, the physiological significance of this result has not been investigated so far. One important parameter that decreases significantly with increasing altitude is T_A. Consequently, honeybee thermogenesis is essential for colony survival, and the adaptive pressure on *AmOARβ1-3/4* may indicate the involvement of octopamine in this process.

We hypothesize that honeybee thermogenesis relies on octopamine signaling and that β octopamine receptors are crucially involved in this process. We have investigated systematically the honeybee thoracic octopaminergic system. Moreover, we have tested our hypothesis and we can show that octopamine promotes thermogenesis by directly affecting the flight muscles.

Results

Honeybee flight muscles are innervated by octopaminergic neurons

First of all, we investigated whether octopamine can be a potential regulator of flight muscle functions in honeybees. Thus, we analyzed which monoamines are actually present in these tissues using

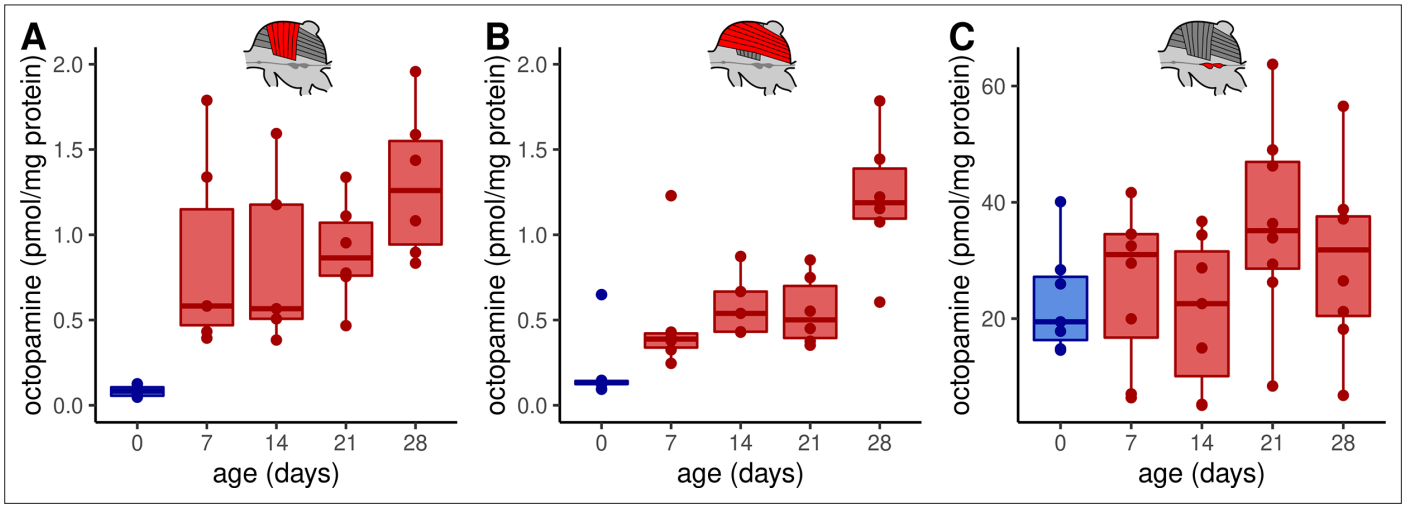

Figure 1. Octopamine concentrations in thoracic tissues across age. Octopamine concentrations differ significantly between different workerbee age groups in DV (**A**) and DL (**B**) but not in the MMTG (**C**). blue = no active heating, red = active heating. Shown is median ± interquartile range (IQR). For statistics see ***Table 1***.

The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. Monoamine quantification in workerbee thoracic tissues.

high-performance liquid chromatography (HPLC) together with an electrochemical detector (ECD). We can detect octopamine and dopamine in both, DV and DL, whereas serotonin and tyramine are not detectable (***Figure 1A***, ***Figure 1—figure supplement 1A***). We further compared the flight muscle octopamine concentration in differently aged workerbees. Newly emerged bees which cannot perform thermogenesis have the lowest octopamine concentration in DV and DL (***Figure 1A–B***) and the octopamine concentration increases with the age of the workerbee (***Figure 1A–B***). In contrast to octopamine, the concentrations of dopamine have a different time course in DV and DL (***Figure 1—figure supplement 1B-C***). We have further analyzed the MMTG. In addition to octopamine, serotonin, dopamine, and tyramine are also detectable, but no age-related differences can be observed for any of these monoamines (***Figure 1C***, ***Figure 1—figure supplement 1D-F***).

Nerves originating from the MMTG exclusively innervate the honeybee flight muscles (***Markl, 1966***; ***Pan, 1980***). To answer whether octopamine in DV and DL can be delivered directly by octopaminergic neurons from the MMTG we used an octopamine specific antibody to analyze the octopamine distribution in these tissues. Octopamine-like immunoreactivity (OA-IR) is observable in four individual cell clusters, with most of the cell bodies being found at the ventral midline (***Figure 2A–E***). Some OA-IR positive cell bodies are also located at the dorsal midline (***Figure 2D–E***). Most MMTG leaving nerves show OA-IR (***Figure 2G–I***), as varicose fibers in IIN1 and a thicker axonal bundle in IIN3 demonstrate (***Figure 2G1***). Finally, finest OA-IR positive varicose structures can be found directly at muscle fibers (***Figure 2J–K***).

AmOARβ2 is expressed in the flight muscles

We next determined which octopamine receptor genes are expressed in the workerbee flight muscle. The honeybee genome harbors five different genes that code for octopamine receptors and two additional genes encoding tyramine receptors. The respective receptor proteins are functionally characterized (***Blenau et al., 2000***; ***Grohmann et al., 2003***; ***Balfanz et al., 2014***; ***Reim et al., 2017***; ***Blenau et al., 2020***). We observe strong signals for PCR products for *AmOARα1* and *AmOARβ2*, weak DNA bands for *AmOARβ1* and *AmOARβ3/4*, and no amplification product in the case of *AmOARα2* and both tyramine receptor genes (*AmTAR1* & *AmTAR2*; ***Figure 3A***). In addition, PCR products indicate the expression for all known honeybee octopamine and tyramine receptor genes in neural tissues (brain, MMTG).

We further determined the relative gene expression of the most promising candidates by quantitative Real Time PCR (qPCR, ***Figure 3B–E***). *AmOARα1* and *AmOARβ2* expression can be observed in DV and DL in all age groups of workerbees. Here, relative expression increases with age, as shown by significant differences between newly emerged bees (0 days) and the three oldest groups.

Octopamine is mandatory for honeybee thermogenesis

To investigate the consequences of octopamine missing in the flight muscles, we fed workerbees with reserpine. This drug has the ability to deplete vesicles on monoaminergic synapses (***Plummer et al., 1954***; ***Cheung and Parmar, 2020***). The octopamine concentrations in DV and DL are significantly decreased due to our treatment (***Figure 4A–B***). In contrast, the dopamine concentration in the flight muscle seems not to be affected (***Figure 4—figure supplement 1***). The same is true for the concentrations of octopamine (***Figure 4C***) and of the other monoamines in the MMTG (***Figure 4—figure supplement 1***).

The reserpine feeding additionally causes hypothermia in both, nurse bees and forager bees (***Figure 4D***, ***Table 3***). A preliminary screen with serotonin, dopamine, octopamine and tyramine revealed, that octopamine may reverse the reserpine effect (***Figure 4—figure supplement 3***). We were able to show that this octopamine effect is robust. We reversed the reserpine-induced hypothermia by injecting octopamine directly into the flight muscles (***Figure 4***, ***Table 3***).

As stated above, we hypothesize that β octopamine receptors are crucially involved in honeybee thermogenesis. Via $G\alpha_s$ proteins, these receptors are positively coupled to membrane-bound adenylyl cyclases (mAC), which leads to an increase of the intracellular adenosine 3′,5′-cyclic mono-phosphate (cAMP) concentration upon receptor activation (***Balfanz et al., 2014***). To control our hypothesis, we have repeated the reserpine experiment reported above. The reserpine induced hypothermia as well as the octopamine reversion of this effect are again clearly observable (***Figure 4E***, ***Table 3***). We stopped thermography after 5 min and the bees were immediately flash-frozen to subsequently

Table 1. Statistical analysis of HPLC analysis of the octopamine content.

ns = not significant.

Analysis	Test	Groups (n)	Result	
HPLC octopamine DV ***Figure 1A***	Kruskal-Wallis test		$\chi^2 = 15.772$, df = 4, p = 0,0033	**
	Dunns test	0 days (6) vs. 7 days (6)	Z = –2.4593, $p_{adj} = 0.1392$	ns
		0 days (6) vs. 14 days (6)	Z = –2.8856, $p_{adj} = 0.0391$	*
		0 days (6) vs. 21 days (6)	Z = –2.7217, $p_{adj} = 0.065$	ns
		0 days (6) vs. 28 days (6)	Z = –3.7382, $p_{adj} = 0.0017$	**
		7 days (6) vs. 14 days (6)	Z = 0.4263, $p_{adj} = 1.0$	ns
		7 days (6) vs. 21 days (6)	Z = 0.2623, $p_{adj} = 1.0$	ns
		7 days (6) vs. 28 days (6)	Z = 1.2789, $p_{adj} = 1.0$	ns
		14 days (6) vs. 21 days (6)	Z = 0.164, $p_{adj} = 1.0$	ns
		14 days (6) vs. 28 days (6)	Z = –0.8526, $p_{adj} = 1.0$	ns
		21 days (6) vs. 28 days (6)	Z = –1.0165, $p_{adj} = 1.0$	ns
HPLC octopamine DL ***Figure 1B***	Kruskal-Wallis test		$\chi^2 = 16.292$, df = 4, p = 0.0027	**
	Dunns test	0 days (6) vs. 7 days (6)	Z = –1.3117, $p_{adj} = 1.0$	ns
		0 days (6) vs. 14 days (6)	Z = –2.6561, $p_{adj} = 0.0791$	*
		0 days (6) vs. 21 days (6)	Z = –1.9019, $p_{adj} = 0.5718$	ns
		0 days (6) vs. 28 days (6)	Z = –3.8038, $p_{adj} = 0.0014$	**
		7 days (6) vs. 14 days (6)	Z = 1.3444, $p_{adj} = 1.0$	ns
		7 days (6) vs. 21 days (6)	Z = 0.5902, $p_{adj} = 1.0$	ns
		7 days (6) vs. 28 days (6)	Z = 2.4921, $p_{adj} = 0.127$	ns
		14 days (6) vs. 21 days (6)	Z = 0.7542, $p_{adj} = 1.0$	ns
		14 days (6) vs. 28 days (6)	Z = –1.1477, $p_{adj} = 1.0$	ns
		21 days (6) vs. 28 days (6)	Z = –1.9019, $p_{adj} = 0.5718$	ns
HPLC octopamine MMTG ***Figure 1C***	Kruskal-Wallis test		$\chi^2 = 5.4912$, df = 4, p = 0.2405	ns
	groups (n): 0 days (7), 7 days (8), 14 days (7), 21 days (8), 28 days (8)			

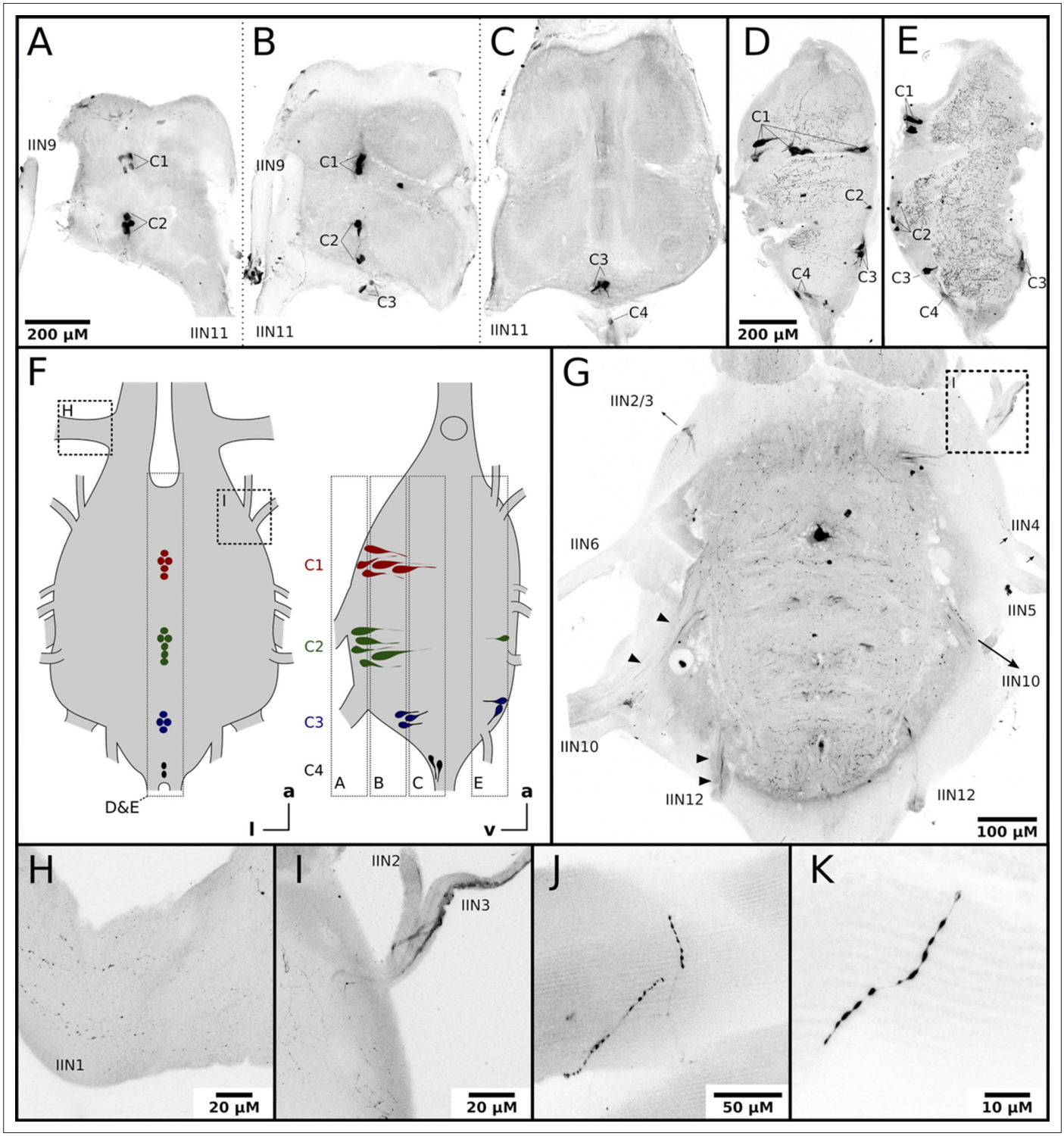

Figure 2. Honeybee flight muscles are innervated by octopaminergic neurons. (**A–E**) Different cell clusters with OA-IR are observable. Consecutive frontal sections of the MMTG of the same workerbee (**A–C**) beginning with the most ventral section (**A**) showing clusters of OA-IR positive cells (**C1–C4**). Sagital sections (**D–E**) in the midline area of the MMTG of two individual bees display the same OA-IR positive cell clusters. (**F**) Schematic interpretation of the location of the cell clusters found in A-E. Additionally, the approximate location of frontal sections (**A–C, G**), the sagital sections (**D–E**), and the detailed images (**H–I**) are indicated by dashed boxes. (**G**) Dorsally located frontal section of the MMTG in showing several nerves which are leaving the ganglion. Strong OA-IR-positive fibers run into the nerves IIN3, IIN10, and IIN12 (arrowheads). (**H**) Within the nerve IIN1 fine varicose

Figure 2 continued on next page

Figure 2 continued

structures with OA-IR are observable. (**I**) An OA-IR-positive axon bundle runs through the nerve IIN3. (**J–K**) Flight muscle preparations reveal fine varicose structures with OA-IR closely attached to muscle fibers.

quantify the tissue cAMP concentrations of their flight muscles. The tissue cAMP concentration is significant lower in reserpinized bees when compared with control (***Figure 4D***). Furthermore, octopamine injection into the flight muscles of reserpinized bees leads to a strong increase of the tissue cAMP concentration (***Figure 4D***). The tissue guanosine 3',5'-cyclic monophosphate (cGMP) concentrations seem not to be affected by our treatment (***Figure 4—figure supplement 4***). Further cyclic nucleotides in the flight muscles were either below the lower limit of quantification (cytidine 3',5'-cyclic monophosphate, cCMP) or were not detectable at all.

Octopamine receptor antagonists also induce hypothermia

Next, we aimed to confirm the described effects of octopamine on honeybee thermogenesis and also to further narrow down the responsible receptor subtypes. Therefore, we injected different pharmacological substances directly into the flight muscles and analyzed their effect on thermogenesis. These substances antagonize various octopamine, tyramine, or adrenergic receptors. All antagonists either lead to hypothermia in both, nurse bees and forager bees, or they are not effective at all. The

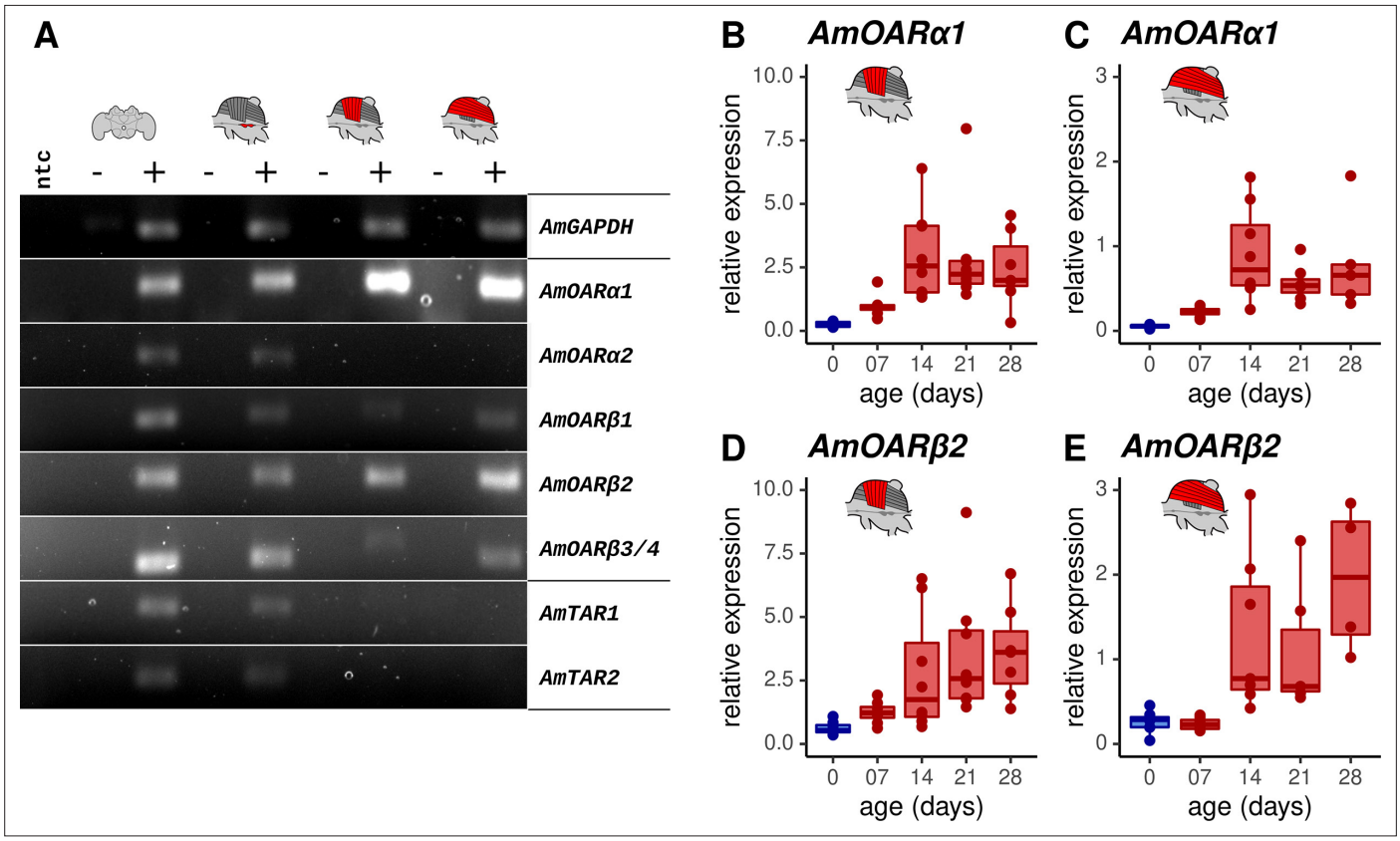

Figure 3. Octopamine receptor expression in the flight muscles. (**A**) Brain, MMTG, DV, and DL were manually dissected from workerbees and underwent subsequent RNA isolation, cDNA synthesis and PCR analysis (+). The reverse transcriptase was omitted during cDNA synthesis for negative controls (-). RNase free water serves as no template (ntc) and *AmGAPDH* as loading control. (**B–E**) *AmOARα1* and *AmOARβ2* expression in DV and DL of differential aged workerbees with (red) or without (blue) the capability for thermogenesis. Data are represented as boxplots. Shown is median ± IQR. For statistic see ***Table 2***.

The online version of this article includes the following source data for figure 3:

Source data 1. Labelled original files of the full raw unedited PCR gels.

Source data 2. Unlabelled original files of the full raw unedited PCR gels.

Table 2. Statistical analysis of the flight muscle gene expression analysis.

ns = not significant.

Analysis	Test	Groups (n)	Result	
qPCR *AmOARα1* DV ***Figure 3D***	Kruskal-Wallis test		χ^2 = 25.734, df = 4, p = 0.00004	***
	Dunns test	0 days (8) vs. 7 days (8)	Z = –1.6253, p_{adj} = 1.0	ns
		0 days (8) vs. 14 days (8)	Z = –3.9776, p_{adj} = 0.0007	***
		0 days (8) vs. 21 days (8)	Z = –3.9135, p_{adj} = 0.0009	***
		0 days (8) vs. 28 days (8)	Z = –3.8493, p_{adj} = 0.0012	**
		7 days (8) vs. 14 days (8)	Z = –2.3523, p_{adj} = 0.1866	ns
		7 days (8) vs. 21 days (8)	Z = –2.2882, p_{adj} = 0.2213	ns
		7 days (8) vs. 28 days (8)	Z = –2.224, p_{adj} = 0.2615	ns
		14 days (8) vs. 21 days (8)	Z = 0.0642, p_{adj} = 1.0	ns
		14 days (8) vs. 28 days (8)	Z = 0.1283, p_{adj} = 1.0	ns
		21 days (8) vs. 28 days (8)	Z = 0.0642, p_{adj} = 1.0	ns
qPCR *AmOARα1* DL ***Figure 3C***	Kruskal-Wallis test		χ^2 = 28.163, df = 4, p = 0.00001	***
	Dunns test	0 days (8) vs. 7 days (8)	Z = –1.5661, p_{adj} = 1.0	ns
		0 days (8) vs. 14 days (8)	Z = –4.4373, p_{adj} = 0.0001	***
		0 days (8) vs. 21 days (7)	Z = –3.6548, p_{adj} = 0.0026	**
		0 days (8) vs. 28 days (5)	Z = –3.7128, p_{adj} = 0.002	**
		7 days (8) vs. 14 days (8)	Z = –2.8712, p_{adj} = 0.0409	*
		7 days (8) vs. 21 days (7)	Z = –2.1418, p_{adj} = 0.322	ns
		7 days (8) vs. 28 days (5)	Z = –2.3392, p_{adj} = 0193	ns
		14 days (8) vs. 21 days (7)	Z = 0.6320, p_{adj} = 1.0	ns
		14 days (8) vs. 28 days (5)	Z = 0.179, p_{adj} = 1.0	ns
		21 days (7) vs. 28 days (5)	Z = –0.3844, p_{adj} = 1.0	ns
qPCR *AmOARβ2* DV ***Figure 3D***	Kruskal-Wallis test		χ^2 = 24.54, df = 4, p = 0.00006	***
	Dunns test	0 days (8) vs. 7 days (8)	Z = –1.6894, p_{adj} = 0,911	ns
		0 days (8) vs. 14 days (8)	Z = –2.8228, p_{adj} = 0.0476	*
		0 days (8) vs. 21 days (8)	Z = –3.8707, p_{adj} = 0.0011	**
		0 days (8) vs. 28 days (8)	Z = –4.3412, p_{adj} = 0.0001	***
		7 days (8) vs. 14 days (8)	Z = –1.1334, p_{adj} = 1.0	ns
		7 days (8) vs. 21 days (8)	Z = –2.1813, p_{adj} = 0.292	ns
		7 days (8) vs. 28 days (8)	Z = –2.6517, p_{adj} = 0.0801	ns
		14 days (8) vs. 21 days (8)	Z = –1.0479, p_{adj} = 1.0	ns
		14 days (8) vs. 28 days (8)	Z = –1.5183, p_{adj} = 1.0	ns
		21 days (8) vs. 28 days (8)	Z = –0.4705, p_{adj} = 1.0	ns

Table 2 continued on next page

Table 2 continued

Analysis	Test	Groups (n)	Result	
qPCR *AmOARβ2* DL ***Figure 3E***	Kruskal-Wallis test		χ^2 = 24.737, df = 4, p = 0.00006	***
	Dunns test	0 days (8) vs. 7 days (8)	Z = 0.5429, p_{adj} = 1.0	ns
		0 days (8) vs. 14 days (7)	Z = –2.9652, p_{adj} = 0.0302	*
		0 days (8) vs. 21 days (6)	Z = –2.4814, p_{adj} = 0.130	ns
		0 days (8) vs. 28 days (4)	Z = –3.1454, p_{adj} = 0.0166	*
		7 days (8) vs. 14 days (7)	Z = –3.4897, p_{adj} = 0.0048	**
		7 days (8) vs. 21 days (6)	Z = –2.9841, p_{adj} = 0.0284	*
		7 days (8) vs. 28 days (4)	Z = –3.5887, p_{adj} = 0.0033	**
		14 days (7) vs. 21 days (6)	Z = 0.3496, p_{adj} = 1.0	ns
		14 days (7) vs. 28 days (4)	Z = –0.6246, p_{adj} = 1.0	ns
		21 days (6) vs. 28 days (4)	Z = –0.9079, p_{adj} = 1.0	ns

non-selective but potent octopamine receptor antagonist mianserin leads to hypothermia (***Figure 5A***, ***Table 3***), while the effective tyramine receptor and α octopamine receptor antagonist yohimbine does not (***Figure 5B***, ***Table 3***). Finally, alprenolol causes hypothermia too (***Figure 5C***, ***Table 3***), whereas carvedilol and metoprolol did not have an observable effect on thermogenesis (***Table 3***).

Downstream metabolic pathway analyses points to glycolysis

In a final experiment series, we investigated the signaling pathway downstream of octopamine receptors in more detail. Up this point, our results indicate the activation of β octopamine receptors, leading to an increase in cAMP concentration. This second messenger has the potential to activate protein kinase A (PKA). To test whether PKA is directly involved in the cellular pathway that enables thermogenesis, we used Rp-8-CPT-cAMPS which is a potent, metabolically stable and membrane-permeable inhibitor of PKA (***Dostmann et al., 1990***; ***Gjertsen et al., 1995***). Rp-8-CPT-cAMPS negatively effects thermogenesis in both, nurse bees and forager bees (***Figure 6A***). Furthermore, we wanted to know whether octopamine release, which most likely activates PKA, could stimulate glycolysis. To test this hypothesis, we quantified pyruvate concentration in DL muscles after octopamine stimulation. Pyruvate is formed in the final step of glycolysis and its metabolites are further catabolized in the tricarboxylic acid cycle (***Zhang et al., 2019***). Pyruvate concentrations increase significantly after octopamine stimulation (***Figure 6B***). Finally, we observed that the *AmGAPDH* gene shows increased expression triggered by cold stress (***Figure 6C***). This gene encodes glyceraldehyde 3-phosphate dehydrogenase which converts glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate during glycolysis. A similar increase in *AmGAPDH* expression can be observed when the bees were treated with an octopamine injection in to the flight muscles instead of cold stress. (***Figure 6D***).

Discussion

In this study, we hypothesized that octopamine has a critical role in the shivering thermogenesis of honeybees. An important prerequisite is that this monoamine can be used as a neurochemical substance at the flight muscles, which seems to be a conserved feature in insects (***Duch et al., 1999***; ***Schlurmann and Hausen, 2003***; ***Pauls et al., 2018***). We can demonstrate that octopamine is present in workerbee flight muscles by independent analysis methods (HPLC-ECD, antibody labeling). This is most likely delivered via flight muscle innervating neurons from the MMTG. Here, we can detect four octopaminergic cell clusters. Those are known to derive from a single median neuroblast at the posterior border of each segment of the developing neuroectoderm and are then displaced during the fusion of ganglia to the dorsal or the ventral surface (***Bräunig and Pflüger, 2001***). We postulate that the octopaminergic cells in each cluster we found are descendants of individual neuroblasts of their neuromere. The honeybee MMTG is formed by fusion of four neuromers (mesothorax, methathorax, first and second abdominal ganglia; ***Markl, 1966***). Furthermore, the MMTG nerves IIN1 and

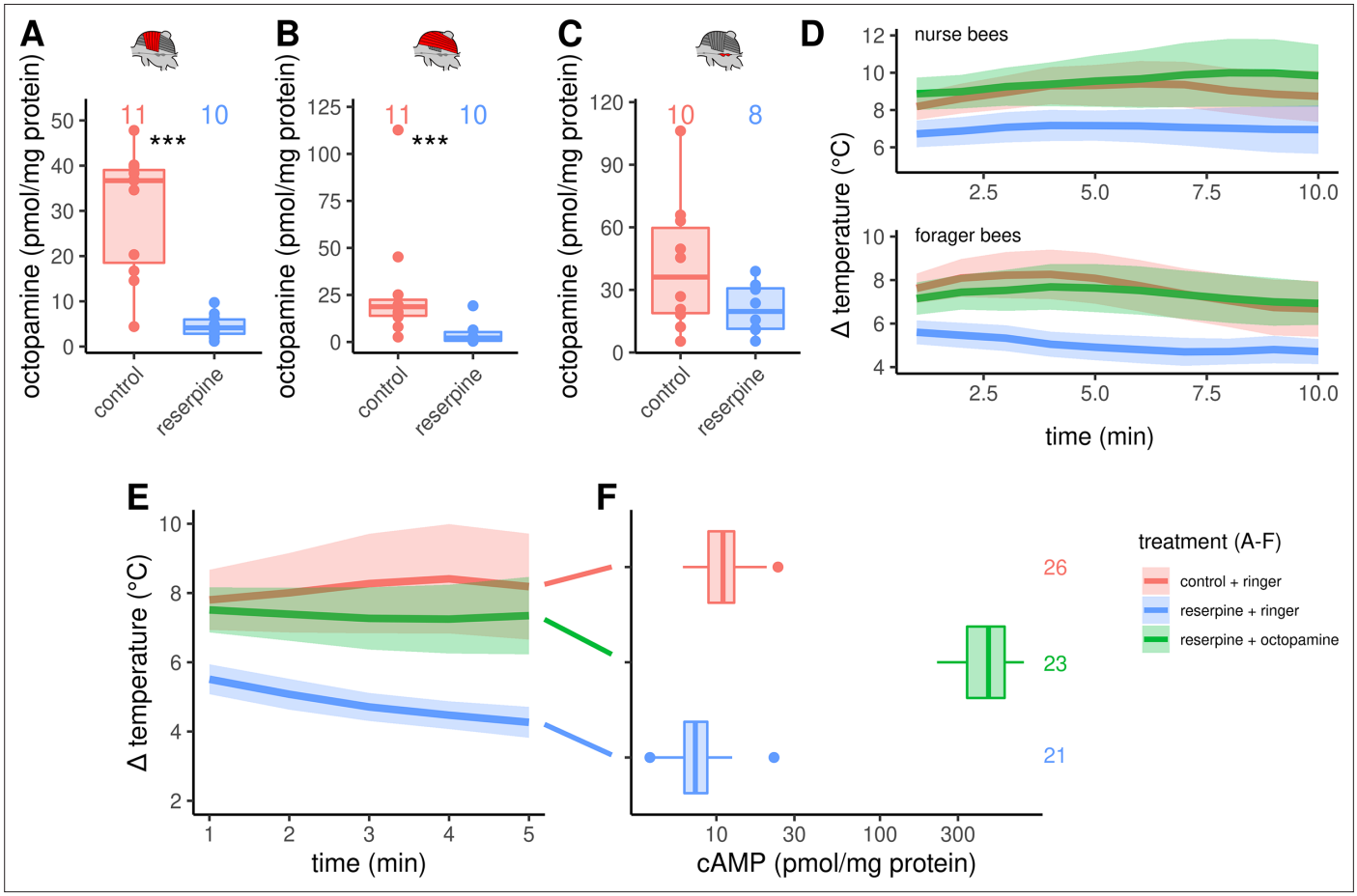

Figure 4. Octopaminergic control of honeybee thermogenesis. (**A–C**) Octopamine concentrations are decreased in DV and DL but not MMTG of reserpinezed workerbees. Data are represented as boxplots. Shown is median ± IQR. Mann-Whitney *U* test, DV(A): W = 105, z = –3.70, p < 0.001; DL(B): W = 102, z = –3.37, p < 0.001; MMTG(C): W = 56, z = –0.94, p = 0.1728. (**D**) Reserpinezed bees show hypothermia when compared with control. An injection of octopamine into the flight muscle helps the bees to recover, as no differences are observable between the control group and the recovered bees. The solid line represents the mean difference between T_{THX} and T_A and the shaded area represents the 95% confidence interval. For statistic see ***Table 3***. (**E**) Similar experiment as in (**D**) but bees were frozen in liquid N_2 after 5 min for cAMP quantification. For statistic see ***Table 3***. (**F**) The tissue cAMP concentrations in the flight muscles differ significantly due the treatment (Kruskal-Wallis test, X^2 = 52.636, df = 2, p < 0.001). Reserpinezed bees has the lowest tissue cAMP concentrations in the flight muscles when compared with controls (Dunns test, Z = 2.6383, p_{adj} = 0.025) and recovered bees (Z = 7.117, p_{adj}= < 0.001). Controls also differ from the recovered bees (Z = –4.7998, p_{adj} <0.001). Data are represented as boxplots. Shown is median ± IQR.

The online version of this article includes the following figure supplement(s) for figure 4:

Figure supplement 1. The effect of reserpine on monoamine concentrations in DV, DL and MMTG.

Figure supplement 2. Time series of thermographic recordings of thoraces of selected workerbees.

Figure supplement 3. The effect of different monoamines on thermogenesis of reserpinized bees.

Figure supplement 4. The effect of reserpine on flight muscle cGMP concentrations.

IIN3 innervate DV and DL, respectively (***Markl, 1966***; ***Pan, 1980***), while some of their neuronal structures contain octopamine. Finally, they reach DV and DL as octopaminergic varicosities suggest. We conclude, that octopaminergic neurons from the MMTG directly innervate the flight muscles and therefore influence thermogenesis.

If this is true, octopamine should be detectable at comparable concentrations in the flight muscles of workerbees capable of thermogenesis. Indeed, we found no differences in bees with ages ranging

Table 3. Statistical analysis of the thermogenesis dependent on the pharmacological treatment.

c = control, *r* = reserpine, ATS = ANOVA type statistic, ns = not significant.

Experiment	Groups (n)	ATS	Df	p	
		9.3635	1.9854	0.00009	***
Reserpine Nurse bees ***Figure 4D***	c + ringer (21) vs. *r* + ringer(23)	13.9618	1.0	0.0002	***
	c + ringer (21) vs. *r* + octopamine (23)	0.0952	1.0	0.7577	ns
	r + ringer(23) vs. *r* + octopamine (23)	14.2223	1.0	0.0002	***
		14.5704	1.9437	0.0000006	***
Reserpine Forager bees ***Figure 4D***	c + ringer (29) vs. *r* + ringer(28)	126.5492	1.0000	0.0000003	***
	c + ringer (29) vs. *r* + octopamine (29)	0.0753	1.0	0.7838	ns
	r + ringer(28) vs. *r* + octopamine (29)	21.1833	1.0000	0.000004	***
		22.8759	1.8981	0,0000000003	***
Reserpine cAMP Quantification ***Figure 4E***	c + ringer (26) vs. *r* + ringer(21)	39.9913	1.0000	0.0000000003	***
	c + ringer (26) vs. *r* + octopamine (23)	0.1155	1.0	0.734	ns
	r + ringer(21) vs. *r* + octopamine (23)	37.3015	1.0000	0.000000001	***
Mianserin Nurse bees Forager bees ***Figure 5A***	control (30) vs. mianserin (30)	9.2737	1.0000	0.0023	**
	control (30) vs. mianserin (30)	8.4638	1.0000	0.0036	**
Yohimbine Nurse bees Forager bees ***Figure 5B***	control (30) vs. yohimbine (30)	0.8011	1.0000	0.3708	ns
	control (32) vs. yohimbine (33)	0.0584	1.0000	0.8091	ns
Alprenolol Nurse bees Forager bees ***Figure 5C***	control (30) vs. alprenolol (30)	7.5516	1.0000	0.0059	**
	control (34) vs. alprenolol (33)	10.9721	1.0000	0.0009	***
Carvedilol Nurse bees Forager bees	control (30) vs. carvedilol (30)	0.1235	1.0000	0.7252	ns
	control (36) vs. carvedilol (34)	0.2650	1.0000	0.6067	ns
Metoprolol Nurse bees Forager bees	control (30) vs. metoprolol (30)	0.1031	1.0000	0.7481	ns
	control (36) vs. metoprolol (36)	0.2029	1.0000	0.6524	ns
Rp-8-CPT-cAMPS Nurse bees Forager bees ***Figure 6A***	control (25) vs. Rp-8-CPT-cAMPS (23)	4.062	1.0000	0.044	*
	control (15) vs. Rp-8-CPT-cAMPS (14)	27.7439	1.0000	0.0000001	***

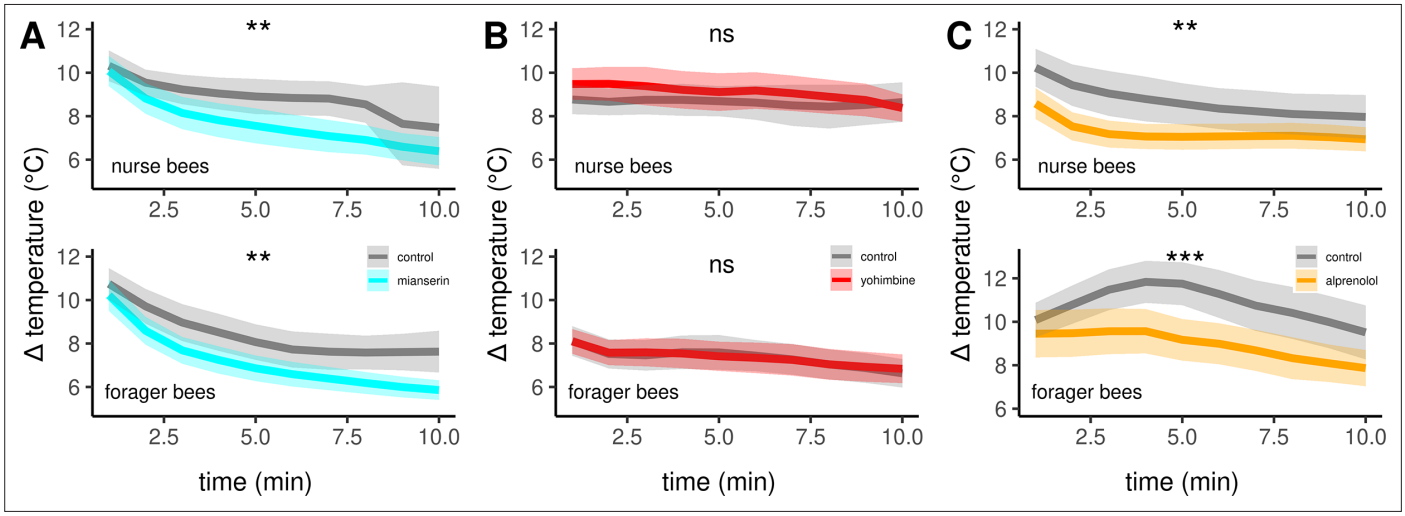

Figure 5. The effects of different antagonists on workerbee thermogenesis. Mianserin (**A**) and alprenolol (**C**) cause hypothermia in workerbees but not yohimbine (**B**). The solid line represents the mean difference between T_{THX} and T_A and the shaded area represents the 95% confidence interval. For statistic see ***Table 3***.

from 7 days up to 4 weeks. They are all similarly engaged in active heat production independent of their actual task within the colony (***Stabentheiner et al., 2010***). In contrast, newly emerged bees, which are not capable of heat production (***Harrison, 1987***; ***Stabentheiner et al., 2010***), have significant lower flight muscle octopamine concentrations. It remains uncertain whether there is a causal relationship between the low octopamine concentrations and the absence of thermogenesis in newly emerged bees or whether this observation is merely a correlation. Several factors could be responsible, such as incomplete differentiation of flight muscle tissues (***Roberts and Elekonich, 2005***; ***Correa-Fernandez and Cruz-Landim, 2010***).

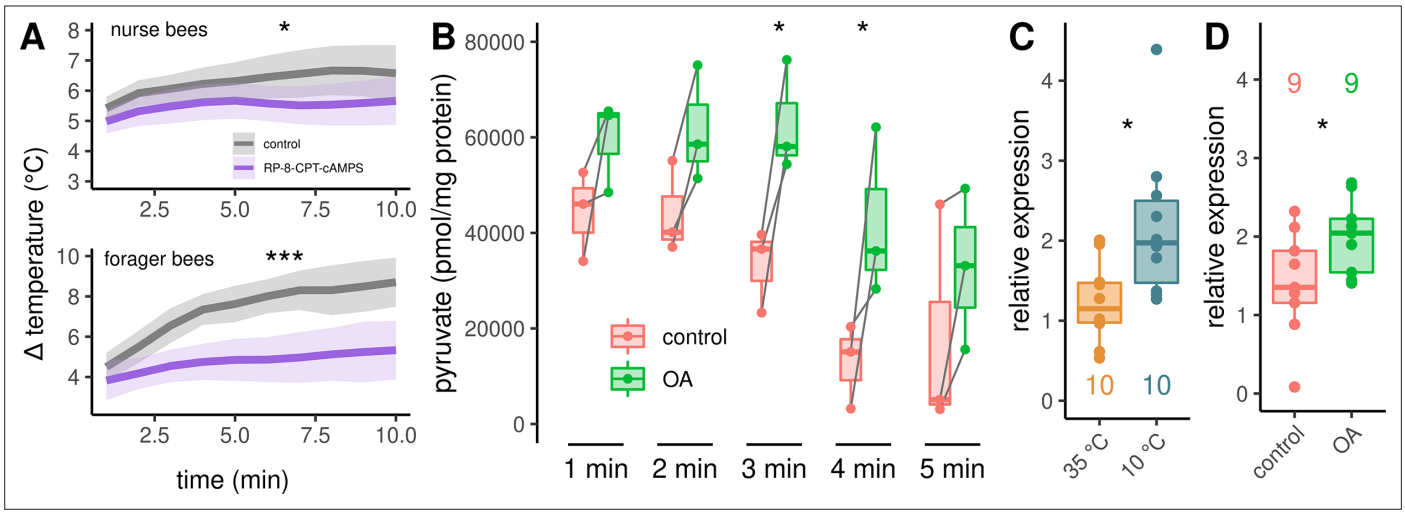

Figure 6. Analysis of the downstream pathway. (**A**) The PKA inhibitor Rp-8-CPT-cAMPS causes hypothermia in nurse bees and forager bees. The solid line represents the mean difference between T_{THX} and T_A and the shaded area represents the 95% confidence interval. For statistic see ***Table 3***. (**B**) DL muscles were separated into two mirror-similar parts and treated differently. Bath application of octopamine (**B**) leads to an difference in the mean pyruvate concentration when compared with control (two-way RM ANOVA, F(1)=38.28, p < 0.001). The simple main effect of treatment becomes significant after 3 and 4 min (Sidaks multiple comparisons test, 3 min: p = 0.016, 4 min: p = 0.017). Shown is median ± IQR. Data points of the same individual are connected by gray lines. (**C–D**) *AmGAPDH* expression in DV and DL is upregulated due to cold stress (C, Mann-Whitney *U* test, W = 83, z = –2.24, p = 0.01261). This result can be mimicked by an injection of octopamine directly into the flight muscles (D, Mann-Whitney *U* test, W = 18, z = –1.68, p = 0.04694). Shown is median ± IQR.

We determined *AmOARα1* and *AmOARβ2* as the predominant octopamine receptor genes expressed in the flight muscle, and their expression is detectable across age. The relative expression of both genes is higher in older bees, but at the same time a huge inter-individual variation is detectable. This might reflect differential demands to muscle activity in the context of the age-dependent task allocation and its neurochemical control. Workerbees perform very different tasks as a function of their age (**Seeley, 1995**). Yet, they are all similarly engaged in heat production if they are older than two days (**Stabentheiner et al., 2010**). Besides flight and thermogenesis another important function of the flight muscles is fanning for cooling purposes (**Hess, 1926**; **Hazelhoff, 1954**; **Simpson, 1961**) and octopamine is known to increase the probability of fanning when fed to workerbees together with tyramine (**Cook et al., 2017**). The two genes *AmOARα1* and *AmOARβ2* encode the octopamine receptor proteins AmOARα1 (**Grohmann et al., 2003**) and AmOARβ2 (**Balfanz et al., 2014**), respectively. We assume, that both receptors can receive and forward the signal mediated by an octopamine release at the flight muscles. Until now, we did not know in which situations this occurs and what specific role the corresponding receptors might have in this process.

Our reserpine experiments solve this problem, because it makes octopamine no longer usable at the flight muscle. As direct consequence, we observe hypothermia. Moreover, if we supply the system with octopamine again we can restore heat generation. We conclude that octopamine signaling is necessary for honeybee thermogenesis. This interpretation is supported by the fact that the potent octopamine receptor antagonist mianserin (**Grohmann et al., 2003**; **Balfanz et al., 2014**; **Blenau et al., 2020**) causes hypothermia, too. Moreover, our cAMP quantification result suggests that at least one β octopamine receptor subtype mediates the octopamine signal in the service of thermogenesis. The decreased octopamine availability in the flight muscles of reserpinezed bees likely causes the loss of octopamine release if necessary. In the end, this results in a reduction of octopamine receptor activation events. In the case of β octopamine receptors, consequently, no cAMP is produced. Indeed, we observe a decrease in tissue cAMP concentrations in combination with reserpine induced hypothermia. Octopamine-induced reversal of this effect is accompanied by a tremendous increase in tissue cAMP concentrations. Unfortunately, honeybee cAMP concentrations from muscle tissues are not available, but our results are consistent with analysis in locust flight muscle (**Baines et al., 1990**; **Lange and Nykamp, 1996**). Furthermore, the lack of an octopamine effect on cGMP concentrations and the absence of the other cyclic nucleotides clearly suggests that mACs mediate the observed octopamine effects. **Hasan et al., 2014** could show that mAC activation leads to exclusive cAMP increase. Our results strongly suggests that β octopamine receptor activation is necessary for honeybee thermogenesis, since these receptors are known to be positively coupled mACs (**Balfanz et al., 2014**). Our explanation again receives support by pharmacological thermography. Due to the lack of subtype-specific octopamine receptor antagonists, we made use of well-established adrenoceptor antagonists. Deuterostome adrenoceptor and arthropoda octopamine receptors are very closely related (**Roeder, 2005**; **Spindler et al., 2013**; **Fuchs et al., 2014**; **Hochman, 2015**; **Roeder, 2020**), which also applies to receptor subtypes as supported by phylogenetic analyses (**Qi et al., 2017**). The reserpine and mianserin effects described above can be mimicked by alprenolol (**Figure 5G**). This antagonist is active at both, β1 and β2 adrenoceptors (**Åblad et al., 1973**; **Åblad et al., 1972**). Therefore, it represents a putative antagonist of AmOARβ1 and AmOARβ2 and was already used in insects in other studies (**Belzunces et al., 1996**; **Cossío-Bayúgar et al., 2012**). Contrastly, carvedilol and metoprolol did not cause any effect. Carvedilol antagonizes preferably α1 and β1 adrenoceptors (**Hansson and Himmelmann, 1998**), whereas metoprolol antagonizes βone adrenoceptors in the human heart (**Benfield et al., 1986**). We assume that both substances antagonize the corresponding octopamine receptors. Several studies show that metoprolol is effective in species belonging to all major protostome phyla (**Dzialowski et al., 2006**; **Spindler et al., 2013**; **Jungmann et al., 2017**; **Buchberger et al., 2018**). However, an expansion of the pharmacological profiles of honeybee octopamine receptors (**Grohmann et al., 2003**; **Balfanz et al., 2014**; **Blenau et al., 2020**) is needed to confirm whether the compounds we used actually antagonize the desired receptor proteins. Combining the information stated above with our results that alprenolol causes hypothermia but not yohimbine, which does not antagonize honeybee β octopamine receptors (**Balfanz et al., 2014**; **Kovac et al., 2009**), further supports the hypothesis that at least one β octopamine receptor subtype is crucially involved in honeybee thermogenesis. Since *AmOARβ2* is predominantly expressed in the flight muscles (when compared with *AmOARβ1* and *AmOARβ3/4*), AmOARβ2 is the most promising candidate. This

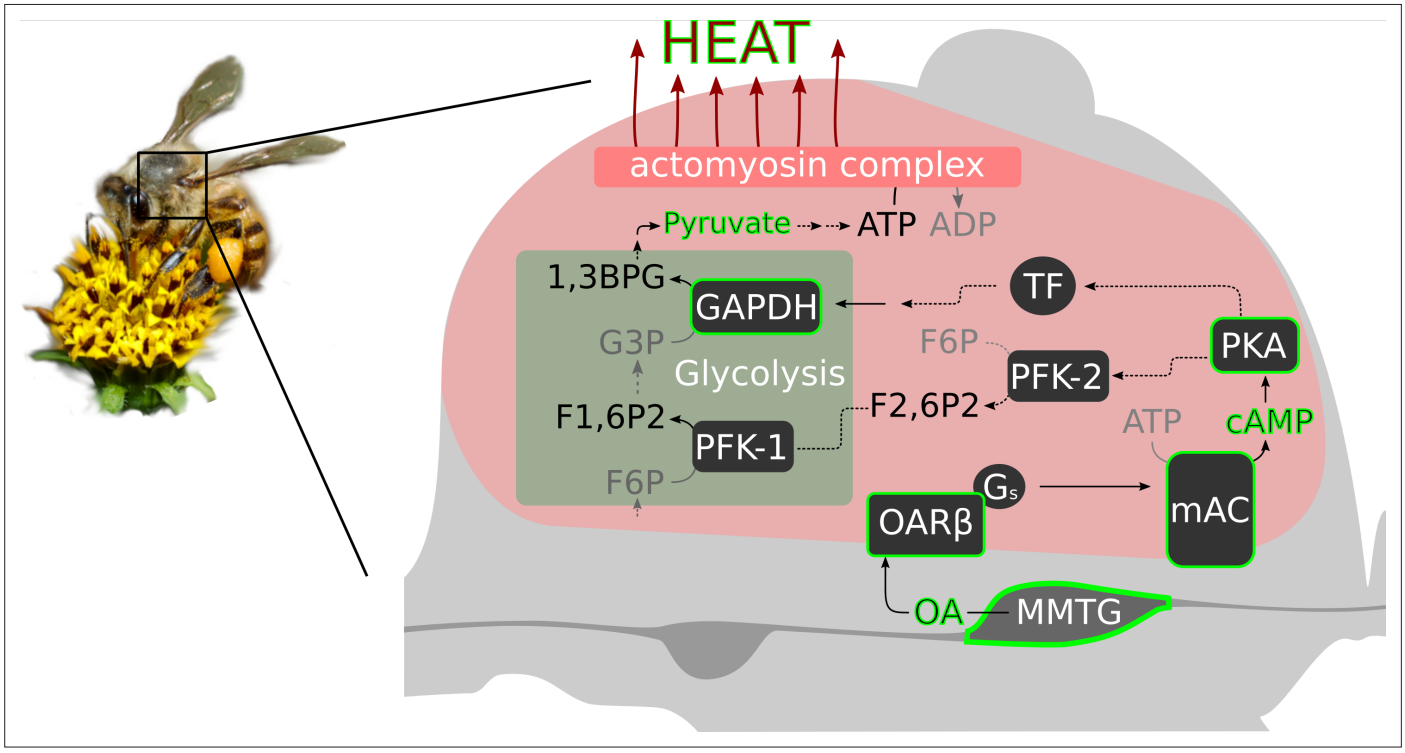

Figure 7. Octopamine and honeybee thermogenesis. The scheme summarizes our findings, with the solid lines and green borders representing interpretations supported by our results and the dashed lines representing hypothetical pathways. Muscle innervating neurons in the MMTG release octopamine (OA) directly to the flight muscles. By this, AmOARβ two receptors are activated which in turn activate the membrane-bound adenylyl cyclase (mAC) via G_s proteins. The resulting increase in the intracellular cAMP concentration leads to the activation of Proteinkinase A (PKA) which phosphorylates and by this activates phosphofructokinase 2 (PFK-2). Consequently, this enzyme produces fructose-2,6-bisphosphate (F2,6P2) which increases the activity of phosphofructokinase 1 (PFK-1). An alternative pathway is the PKA mediated activation of transcription factors (TF) which might enhance expression of *GAPDH* which encodes glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This enzyme converts glyceraldehyde 3-phosphate (G3P) into 1,3-bisphosphoglyceric acid (1,3BPG). All together, this increases the glycolysis rate so that a greater amount of pyruvate is available for ATP production. Finally, heat is generated in the actomyosin complex under ATP consumption.

assumption is supported by studies in mammals showing the predominant expression of the βtwo adrenergic receptor in skeletal muscle tissue, which is a similar receptor subtype (***Liggett et al., 1988***; ***Kim et al., 1991***).

Our PCR analysis further revealed the prevalent expression of *AmOARα1*. However, yohimbine does not cause hypothermia. This substance was shown to bind and antagonize αone octopamine receptors receptors in a wide range of insects (***Bischof and Enan, 2004***; ***Enan, 2005***; ***Ohtani et al., 2006***; ***Huang et al., 2010***). Thus, we hypothesize that this receptor is not in the service of thermogenesis.

Tyramine is also capable to reverse the reserpine induced hypothermia. However, we could observe neither tyramine nor any tyramine receptor gene expression in the flight muscles. One might argue that the tyramine effect is mediated via tyramine receptors that are expressed in the MMTG. In that case, the potent tyramine receptor antagonist yohimbine (***Ohta et al., 2003***; ***Fussnecker et al., 2006***; ***Reim et al., 2017***) should have an effect on thermogenesis, but this is not the case. Based on our results and the fact that tyramine is able to activate octopamine receptors (***Grohmann et al., 2003***; ***Balfanz et al., 2014***; ***Blenau et al., 2020***), we classify this tyramine effect as artificial and physiologically not relevant.

The data of our study supports the hypothesis that octopaminergic signaling in the flight muscle is necessary for honeybee thermogenesis. Most likely, this monoamine acts directly at the indirect flight muscles via the activation of β octopamine receptors. We speculate, that their role is to boost glycolysis (see scheme ***Figure 7***). Cold stress will induce an octopamine release directly at the flight muscles. The subsequent β octopamine receptor mediated generation of cAMP will activate proteinkinase A (PKA, ***Müller, 2000***). That PKA is in service of thermogenesis is supported by our experiments in which

bees are hypothermic as a result of PKA inhibition. PKA in turn might phosphorylates and activates phosphofructokinase 2 (PFK-2), which is the enzyme that produces fructose-2,6-bisphosphate ($F2,6P_2$, ***Rider et al., 2004***). $F2,6P_2$ is an activity increasing modulator of phosphofructokinase 1 (PFK-1, ***Hue and Rider, 1987***; ***Bartrons et al., 2018***). The PFK-1 mediated phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate ($F1,6P_2$) is a key step in glycolysis, at its end ATP is provided (***Fothergill-Gilmore and Michels, 1993***). Finally, heat is generated by the hydrolysis of ATP at the actomyosin complex (***Zhang and Feng, 2016***). Our pyruvate quantification results support this hypothesis. We can detect higher quantities of the glycolysis final product after octopamine stimulation. Another possibility is that PKA is involved in the activation of certain transcription factors. As a consequence, the expression of genes of important glycolysis enzymes may be enhanced. Here, we provide the *AmGAPDH* gene as one example whose gene product is essential in glycolysis (***Burke et al., 1996***). Its expression can be increased by both cold stress and octopamine injection. The alternative futile cycle (***Newsholme et al., 1972***), which is based on high fructose-1,6-bisphosphatase (FbPase) activity in certain bumblebee species, must be doubted, at least for honeybees. Honeybees and many other bumblebee species have comparable low FbPase activity (***Newsholme and Crabtree, 1970***; ***Staples et al., 2004***) and FbPase-PFK cycling rates are not sufficient for heat production (***Clark et al., 1973***; ***Kammer and Heinrich, 1978***; ***Newsholme and Crabtree, 1976***). Our hypothetical cascade is supported by the results of other studies. $F2,6P_2$ levels increase in locust flight muscles due to octopamine stimulation (***Blau and Wegener, 1994***; ***Blau et al., 1994***) and by this controls the rate of carbohydrate oxidation in flight muscles (***Wegener, 1996***). In mammals, adrenaline stimulates increasing $F2,6P_2$ levels and thus glycolysis (***Narabayashi et al., 1985***). This effect is achieved by β adrenoreceptor activation followed by stimulation of PKA (***Rider et al., 2004***). Chronic exercise causes stereotypical adaptations in several tissues of *Drosophila melanogaster*, which requires the activation of octopaminergic neurons (***Sujkowski et al., 2017***). In muscles, those effects are dependent on the activation of β octopamine receptors (***Sujkowski et al., 2020***). If cold stress becomes chronic, such as in cold climate at high altitude or during winter, there will probably be a similar pattern in honeybees. It is conceivable that the octopaminergic system in the flight muscles is permanently active to enable persistent heat production. If this system is compromised, it will endanger the survival of the colony due to the lost of individually performed heating, which enables foraging, breeding, and diverse defense mechanisms (***Himmer, 1932***; ***Weiss, 1962***; ***Starks et al., 2000***; ***Bujok et al., 2002***; ***Stabentheiner et al., 2003***; ***Tautz et al., 2003***; ***Ken et al., 2005***; ***Baracchi et al., 2010***; ***Campbell et al., 2010***; ***Wang et al., 2016***). This may explain the enormous selective pressure on β octopamine receptor genes (***Wallberg et al., 2017***). Issues to be addressed are how the octopaminergic system responds to cold stress. But also heat stress, and in this context adaptations to warm climate in the course of climate change can become very important. With our important contribution to the understanding of thermogenesis in honeybees we provide a solid basis to analyze these issues.

Materials and methods

Animals

Honeybee workers (*Apis mellifera carnica*) were collected from colonies of the department next to the Biocenter at the University of Würzburg, Germany. We declared bees that returned to the hive with pollen loads on their hind legs as forager bees. As nurse bees, we defined bees, that were sitting on a brood comb and were actively heating (thorax temperature, $T_{THX} \geq 32$ °C). T_{THX} was monitored with a portable thermographic camera (FLIR E6, FLIR, Wilsonville, USA). Pollen forager were collected for the gene expression analysis from the same hives and were immediately flash-frozen in liquid nitrogen and stored at –80 °C. For the age-series analysis (monoamine quantification, gene expression analysis), a queen was caged on a brood comb for 3 days. Shortly before the bees started to emerge, we transferred the brood comb into an incubator (34 °C, RH = 65 %). The first group (0-day-old bees) consisted of newly hatched bees and were collected directly from the brood comb. The remaining newly hatched bees were color-marked and then inserted into a standard hive. Those bees were collected from the hive after 7, 14, 21, and 28 days, respectively. For the *AmGAPDH* expression analysis, 7-day-old age-marked bees were collected from a hive and distributed equally into two identical cages. For the cold stress experiment, one cage was placed in an incubator at 10 °C for 120 min, while the other served as a control (120 min, 34 °C). For the octopamine injection experiment, bees of the

control group receive an injection of saline solution (270 mM sodium chloride, 3.2 mM potassium chloride, 1.2 mM calcium chloride, 10 mM magnesium chloride, 10 mM 3-(N-morpholino) propanesulfonic acid, pH = 7.4; ***Erber and Kloppenburg, 1995***) into their flight muscles. The test group was injected with octopamine (0.01 M in saline). Subsequently, both groups were incubated for 120 min at 34 °C. All collected bees (expression analysis, monoamine quantification) were immediately flash-frozen in liquid nitrogen and subsequently stored at –80 °C.

Immunohistochemistry

For octopamine immunolabeling, we used a polyclonal rabbit anti-octopamine antibody (IS1033, ImmuSmol, Bordeaux, France) together with the STAINperfect immunostaining kit A (SP-A-1000, ImmuSmol, Bordeaux, France). We have analyzed ten individual MMTGs in three independent experiments for frontal sections and additionally three individual MMTGs for sagital sections. Four individual DVs and DLs, respectively, were analyzed in two independent experiments. Due to non optimal tissue permeability, we have slightly adopted the manufacturers protocol for whole mount preparations to perform analysis with vibratom sections. In brief, tissues (MMTG, flight muscles) were micro-dissected and subsequently fixed in fixation buffer for 3 hr at 4 °C while shaking. Afterwards, the fixed tissues were washed five times for 30 min with *Wash Solution 1*, embedded in 5% (w/v) agarose and were cut into 100-µm-thick sections. Then, the tissue sections were treated consecutively: 1 hr in *Permeabilization Solution* at RT followed by two times *Wash Solution 1* for 3 min, 1 hr in *Stabilization Solution* followed by three times *Wash Solution 1* for 3 min, and 1 hr in *Saturation Solution* at RT. Afterwards, the *Saturation Solution* was replaced by the primary antibody (1:500, in *Antibody Diluent*) and the tissue sections were incubated at 4 °C while shaking for at least for 72 hr. After five times washing cycles with *Wash Solution 2* for 30 min at RT the secondary antibody (1:200 in *Antibody Diluent*, goat anti-rabbit Alexa Fluor 568; Molecular Probes, Eugene, USA) was applied for 24 hr (4 °C). After the final washing with *Wash Solution two* and *Wash Solution 3* (both 3 times for 30 min at RT) the slices were mounted in 80% Glycerol (in *Wash Solution 3*) on microscope slides. Preparations were imaged by confocal laser scanning microscopy using a Leica TCS SP2 AOBS (Leica Microsystems AG, Wetzlar, Germany). HC PL APO objective lenses (10 x/0.4 NA imm; 20 x/ 0.7 NA imm and 63 x/1.20 NA imm) with additional digital zoom were used for image acquisition. ImageJ (1.53 c, ***Schindelin et al., 2012***) was used to process images (maximum intensity projection, optimization for brightness and contrast) and Inkscape (1.1, ***Inkscape Developer Team, 2021***) was used to arrange images into figures. MMTG nerve terminology is based on the nomenclature used by ***Markl, 1966***.

Monoamine quantification

The DV and DL were dissected under liquid nitrogen. Afterwards, we thawed the remaining thoracic tissue in ice-cold ethanol to immediately dissect the MMTG. The separated tissues were kept at –80 °C until extraction. For high-performance liquid chromatography (HPLC) analysis of the monoamines we used a slightly modified protocol as described by ***Cook et al., 2017***. For extraction, 120 µL (DV, DL) or 60 µL (MMTG) of extraction solution (10.0 pg/µL 3,4-dihydroxy-benzylamine (DHBA) in 0.2 M perchloric acid) was added in the first step. After a short centrifugation (21,130 g, 2 min, 0 °C) the tissues were disintegrated via sonication (10 min, 0 °C), followed by an incubation (20 min, 0 °C). After a final centrifugation (21,130 g, 14 min, 0 °C), the supernatant was analyzed via HPLC-ECD (Thermo Fisher Scientific, Waltham, USA) and the pellet was stored at –80 °C for protein quantification. A 3 µm reverse phase column (BDS-Hypersil-C18, 150 × 3 mm, pore size 130 Å, Thermo Fisher Scientific, Waltham, USA) and an ECD-3000RS configuration with two coulometric cells (6011RS ultra-analytical cell, Thermo Fisher Scientific, Waltham, USA) were connected to a biocompatible Dionex Ultimate 3,000 UHPLC focused (Thermo Fisher Scientific, Waltham, USA). The mobile phase contained 15% (v/v) methanol, 15% (v/v) acetonitrile, 85 mM sodium phosphate monobasic, 1.75 mM sodium dodecyl sulfate, 0.5 mM sodium citrate and ultrapure water. Phosphoric acid was used for accurate pH adjustment (pH 5.6 ± 0.01). We used a flow rate of 0.5 mL/min. Two detector channels were connected in series with working potentials of 425 mV (DHBA, dopamine, serotonin) and 800 mV (octopamine, tyramine), respectively. Quantification was performed via an external calibration. The raw data analysis was carried out with the program Chromeleon (7.2.10, Thermo Fisher Scientific, Waltham, USA).

Quantitative analysis of cyclic nucleotides

Individual flight muscle tissues were dissected under liquid nitrogen. Individual DV and DL were pooled and 800 µL homogenization buffer (40% (v/v) acetonitrile, 40% (v/v) methanol, 20% (v/v) H_2O) was added and homogenized as described above. Samples were incubated at 95 °C for 10 min and then stored in the freezer (–80 °C) until further processing. After centrifugation (10 min, 21,130 g), the supernatant was transferred to mass spectroscopic analysis (HPLC-MS) as described by ***Beste et al., 2012***. The residual pellet was used for the protein quantification.

Pyruvate quantification

Workerbees were killed by decapitating and then the intact DL muscle was carefully dissected and separated into mirror-identical parts. Subsequently, both parts were incubated with different solutions using bath application. One part was treated with saline solution whereas the other part was treated with 0.01 M octopamine (in saline). After flash freezing in liquid nitrogen pyruvate was quantified using the pyruvate assay kit (MAK071, Sigma Aldrich). The muscles were homogenized in 100 µL Pyruvate Assay Buffer and in a tissue mill at 35 Hz for 3 min. After centrifugation (10 min, 21,130 g), 25 µL of the supernatant were used per reaction. Each reaction setup (50 µL) additionally contained 23 µL Pyruvate Assay Buffer, 1 µL Pyruvate Probe Solution and 1 µL Pyruvate Enzyme Mix. After incubation at room temperature for 30 min the absorption at 570 nm was measured for each sample and each external calibrator (0, 2, 4, 6, 8, 10 nmol per reaction).

Protein quantification

To compensate possible differences in the accuracy of tissue dissection for the HPLC-ECD, HPLC-MS and the pyruvate quantification analysis, we additionally measured the protein content in the samples after Bradford (***Fic et al., 2010***) and normalized amine or cyclic nucleotide concentration to protein content. The pellet (see above) was resuspended in 120 µL (HPLC-ECD: DV, DL), 30 µL (HPLC-ECD: MMTG), or 500 µL (HPLC-MS: DV+ DL) 0.2 M NaOH. After an incubation (15 min, 0 °C), the insoluble material was sedimented (9391 g, 5 min). Finally, 2 µL (HPLC-ECD: DV, DL), 10 µL (HPLC-ECD: MMTG), or 2,5 µL (HPLC-MS: DV+ DL) of the supernatant were transferred into a final volume of 1 mL 1 x ROTI-Nanoquant solution (Carl Roth, Karlsruhe, Germany). All samples and the external calibrator (1, 2, 3, 5, 10, 20 µg/mL Albumin Fraction V, Carl Roth, Karlsruhe, Germany) were analyzed with a plate reader (Infinite 200 Pro, Tecan, Männedorf, Switzerland).

Gene expression analysis

Individual flight muscle tissues were dissected under liquid nitrogen. For the MMTG, we have used RNAlater ICE (Thermo Fisher Scientific, Waltham, USA) to prevent RNA degradation during the dissections. The GenUP Total RNA Kit (biotechrabbit, Henningsdorf, Germany) was used to extract total RNA following the standard protocol provided by the manufacturer including an extra DNase I digestion step. After binding of the RNA to the Mini Filter RNA, we added a 50 µL DNase mix containing 30 U RNase-free DNase I (Lucigen Corporation, Middleton, USA) together with the appropriate buffer and incubated for 15 min at room temperature. For the polymerase chain reaction (PCR) experiment, we pooled total RNA from one individual of each age (7, 14, 21 and 28-day-old bees) per tissue (brain, MMTG, DV, DL). 400 ng total RNA of each tissue were used for cDNA synthesis using the Biozym cDNA Synthesis Kit (Biozym, Hessisch Oldendorf, Germany). The cDNAs were then analyzed in 20 µL PCR reactions (1 µL cDNA, 8.2 µL H_2O, 10 µL 2 x qPCR S'Green BlueMix (Biozym, Hessisch Oldendorf, Germany)), 0.4 µL of each primer (0.2 µM) using the following protocol: 95 °C for 2 min and 35 cycles at 95 °C for 5 s and 30 °C for 30 s. Finally, 10 µL for each PCR reaction was analyzed on a 1.5% agarose gel. For the qPCR experiments, we used individual total RNA per tissue. Here, for each sample 70 ng (DV) and 30 ng (DL) RNA were used. All cDNA synthesis reactions were performed with the Biozym cDNA Synthesis Kit (Biozym, Hessisch Oldendorf, Germany). PCR triplicates of each cDNA (5 µL) were analyzed in a qPCR on a Rotor-Gene Q (Qiagen, Hilden, Germany) in a total reaction volume of 20 µL. Every reaction contains 4.2 µL H_2O, 10 µL 2 x qPCR S'Green BlueMix (Biozym, Hessisch Oldendorf, Germany), 0.4 µL of each primer (0.2 µM) and 5 µL cDNA. Finally, octopamine receptor gene expression was determined relative to the reference genes *AmGAPDH* and *AmRPL10* using the R package 'EasypcR' (v1.1.3) which uses the algorithm published by ***Hellemans et al., 2007***. For the *AmGAPDH* relative expression analysis *AmRPL32* and *AmRPL19* served as reference genes.

Pharmacological thermography

For the reserpine experiments, forager bees and nurse bees were collected as described above. The bees were kept and fed in equal proportions in two adjacent cages (34 °C, RH = 65 %) for 3 days. The reserpine group was fed with 500 µM reserpine solution (in 30% sucrose solution) ad libitum and the control group with 30% sucrose only. To enhance the solubility, the reserpine was pre-dissolved in acetone. For the experiments with receptor antagonists, the day before each measuring day, 20 bees were collected from the same hive and kept overnight in a cage at 34 °C (RH = 65 %). In the incubator, the bees were fed ad libitum with 30% sucrose solution. All injection solutions were freshly prepared every experimental day. All biogenic amines (Sigma-Aldrich), receptor antagonists (Sigma-Aldrich) or Rp-8-CPT-cAMPS (Biolog) were used in a concentration of 0.01 M in saline solution (see above). For solubility reasons, a 10:1 volume mixture of saline solution and dimethyl sulfoxide was used for carvedilol instead of pure buffer. Each bee was immobilized on ice until no more movement could be detected. The thorax was then punctured centrally to inject 1.0 µL testing solution using a 10.0 µL Hamilton syringe. Directly before the start of every measurement, the control group received an injection of the pure saline solution and the treatment group an injection of 0.01 M of the biogenic amine or the respective antagonist directly into their flight muscles. To enable optimal conditions for thermogenesis and thermographic recordings, we adapted the method of a tethered animal that walks upon a treadmill (***Moore et al., 2014***). This allows the bee to seemingly move freely, while at the same time the camera always monitors the same area of the bees thorax. This setup was located inside an incubator (18.5 °C, RH = 65 %) together with a thermographic camera (FLIR A65 camera, lens: 45°, *f* = 13 mm, FLIR, Wilsonville, USA). A thermal imaging video with 30 frames/min was recorded of each bee over 10 min. We converted the thermographic videos using the R package Thermimage (4.1.2, ***Tattersall, 2020***) to subsequently read out the thoracic temperatures with ImageJ (1.53 c, ***Schindelin et al., 2012***).

Statistical analysis

All statistical analyses were performed using R (4.0.4 including 'stats', ***R Development Core Team, 2020***) and the R packages 'rstatix' (0.7.0, ***Kassambara, 2020b***) and 'FSA' (0.9.1, ***Ogle et al., 2021***). We performed a Shapiro-Wilk test to check the data for normality distribution. Since most data subsets did not display a normal distribution, we analyzed the data using either the Mann-Whitney *U* test or the Kruskal-Wallis test followed by Dunns post hoc analysis if significant differences were observable. For the statistical analysis of the pharmacological thermography experiments, we calculated the mean value per min for T_{THX} and T_A, respectively. Afterwards, the Δ temperature ($T_{THX} - T_A$) for the total time span of the experiment (five or 10 min) was subjected to nonparametric analysis of longitudinal data using a F1 LD F1 model of the R package 'nparLD' (2.1 ***Noguchi et al., 2012***). Visualization of the data was performed with the R packages 'ggplot2' (3.3.3, ***Wickham, 2016***), 'ggpubr' (0.4.0, ***Kassambara, 2020a***), 'png' (0.1–7, ***Urbanek, 2013***), 'cowplot' (1.1.1, ***Wilke, 2019***), and 'magick' (2.7.0, ***Wilke, 2019***).

Acknowledgements

We thank Dirk Ahrens for beekeeping; Annette Garbe, Anna Senker, Feriba Ghanbari, Karin Möller, Lena Wolf, Linnéa Jürgensen, Saskia Delac and Valentina Vey for technical assistance during the experiments; Flavio Roces, Johannes Spaethe, Marcus Gutmann, Petra Högger, Ricarda Scheiner and Robin Grob for supplying technical equipment and Michael G.K. Brunk for his help with ImageJ. Usage of the Leica SP2 confocal Laser Scanning Microscope was made possible by the the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - HBFG-133–612. A special thanks goes to Christian Wegener, Jens Schlossmann, Anna Stöckl and Basil el Jundi for fruitful discussions and/or reviewing earlier versions of the manuscript. This publication was supported by the Open Access Publication Fund of the University of Wuerzburg.

Additional information

Funding

Funder	Grant reference number	Author
Deutsche Forschungsgemeinschaft	TH2264/2-1	Markus Thamm

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

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Decision letter and Author response

Decision letter https://doi.org/10.7554/eLife.74334.sa1
Author response https://doi.org/10.7554/eLife.74334.sa2

Additional files

Supplementary files

- Transparent reporting form

Data availability

All data generated or analyzed during this study are included in the manuscript and supporting file; Source Data files have been provided for Figures 1,3,4,5,6.

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RESEARCH ARTICLE

Figures and figure supplements

Octopamine drives honeybee thermogenesis

Sinan Kaya-Zeeb *et al*

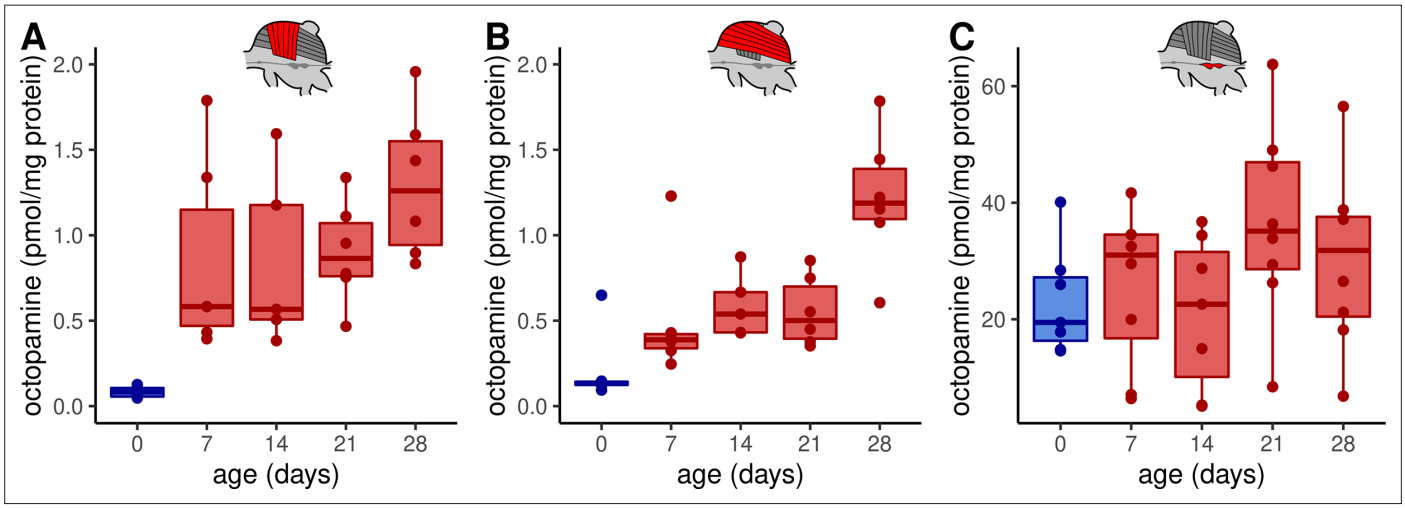
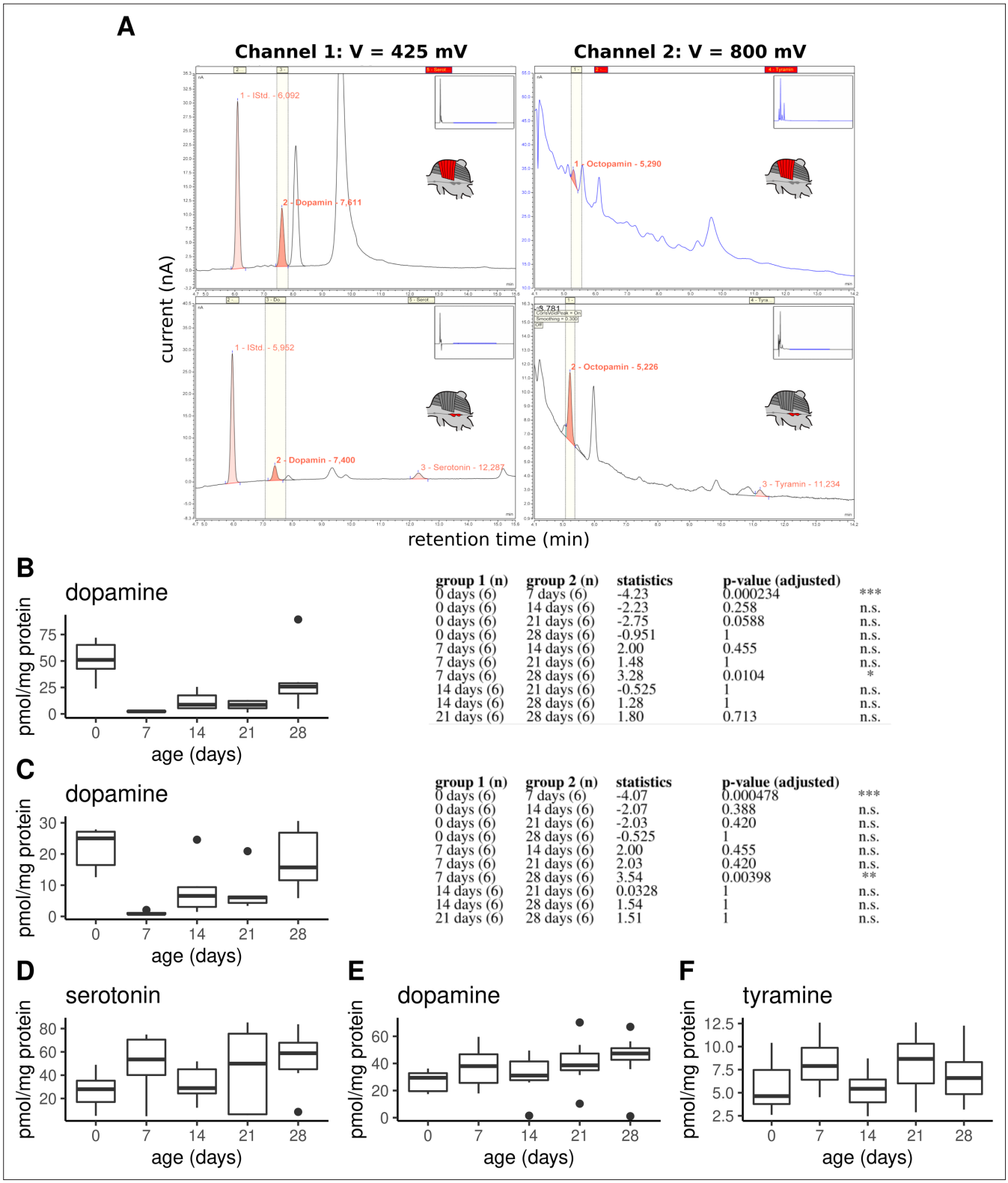


Figure 1. Octopamine concentrations in thoracic tissues across age. Octopamine concentrations differ significantly between different workerbee age groups in DV (**A**) and DL (**B**) but not in the MMTG (**C**). blue = no active heating, red = active heating. Shown is median ± interquartile range (IQR). For statistics see ***Table 1***.



group 1 (n)	group 2 (n)	statistics	p-value (adjusted)	
0 days (6)	7 days (6)	-4.23	0.000234	***
0 days (6)	14 days (6)	-2.23	0.258	n.s.
0 days (6)	21 days (6)	-2.75	0.0588	n.s.
0 days (6)	28 days (6)	-0.951	1	n.s.
7 days (6)	14 days (6)	2.00	0.455	n.s.
7 days (6)	21 days (6)	1.48	1	n.s.
7 days (6)	28 days (6)	3.28	0.0104	*
14 days (6)	21 days (6)	-0.525	1	n.s.
14 days (6)	28 days (6)	1.28	1	n.s.
21 days (6)	28 days (6)	1.80	0.713	n.s.

group 1 (n)	group 2 (n)	statistics	p-value (adjusted)	
0 days (6)	7 days (6)	-4.07	0.000478	***
0 days (6)	14 days (6)	-2.07	0.388	n.s.
0 days (6)	21 days (6)	-2.03	0.420	n.s.
0 days (6)	28 days (6)	-0.525	1	n.s.
7 days (6)	14 days (6)	2.00	0.455	n.s.
7 days (6)	21 days (6)	2.03	0.420	n.s.
7 days (6)	28 days (6)	3.54	0.00398	**
14 days (6)	21 days (6)	0.0328	1	n.s.
14 days (6)	28 days (6)	1.54	1	n.s.
21 days (6)	28 days (6)	1.51	1	n.s.

Figure 1—figure supplement 1. Monoamine quantification in workerbee thoracic tissues. (**A**) Example chromatograms of individual DV (top) and MMTG samples (bottom). (**B–F**) Dopamine concentrations in dorsoventral (B; Kruskal-Wallis test: $X^2 = 21.381$, df = 4, p = 0.0003) and dorsal-longitudinal flight muscles (C; $X^2 = 20.215$, df = 4, p = 0.0005) are high in newly emerged bees and dramatically decrease until the age of 7 days. Afterwards, dopamine concentrations increases with aging. Group comparison results (Dunns test) are shown to the right of each graph. Additionally, serotonin (D;

Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

X^2 = 6.489, df = 4, p = 0.166), dopamine (E; X^2 = 7.4, df = 4, p = 0.116), and tyramine (D; X^2 = 6.226, df = 4, p = 0.183) are detectable in the mesometathoracic ganglia, wheras no age-related differences are observable. Shown is median ± IQR.

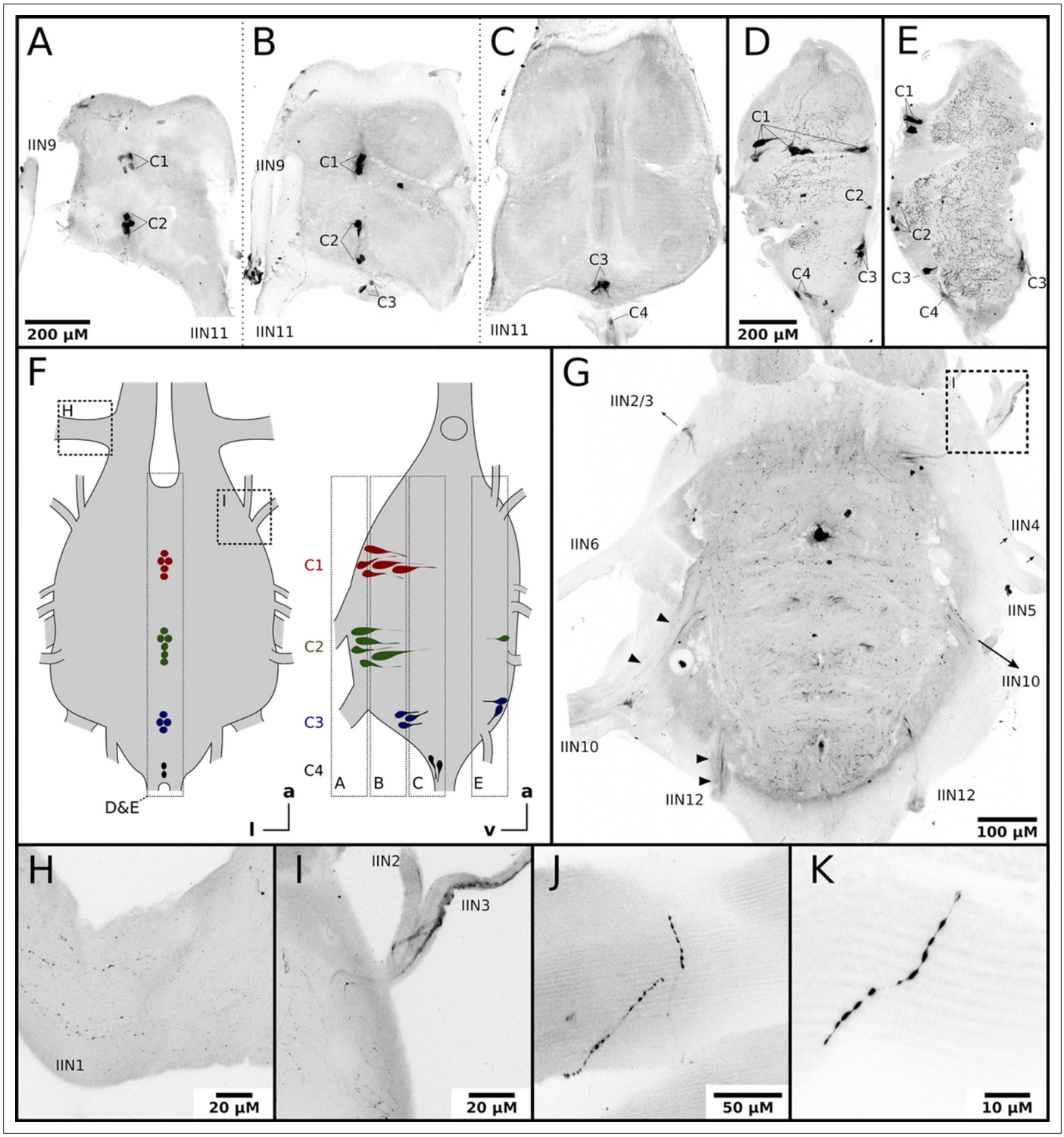


Figure 2. Honeybee flight muscles are innervated by octopaminergic neurons. (**A–E**) Different cell clusters with OA-IR are observable. Consecutive frontal sections of the MMTG of the same workerbee (**A–C**) beginning with the most ventral section (**A**) showing clusters of OA-IR positive cells (**C1–C4**). Sagital sections (**D–E**) in the midline area of the MMTG of two individual bees display the same OA-IR positive cell clusters. (**F**) Schematic interpretation of the location of the cell clusters found in A-E. Additionally, the approximate location of frontal sections (**A–C, G**), the sagital sections (**D–E**), and the detailed images (**H–I**) are indicated by dashed boxes. (**G**) Dorsally located frontal section of the MMTG in showing several nerves which are leaving the ganglion. Strong OA-IR-positive fibers run into the nerves IIN3, IIN10, and IIN12 (arrowheads). (**H**) Within the nerve IIN1 fine varicose

Figure 2 continued on next page

Figure 2 continued

structures with OA-IR are observable. (**I**) An OA-IR-positive axon bundle runs through the nerve IIN3. (**J–K**) Flight muscle preparations reveal fine varicose structures with OA-IR closely attached to muscle fibers.

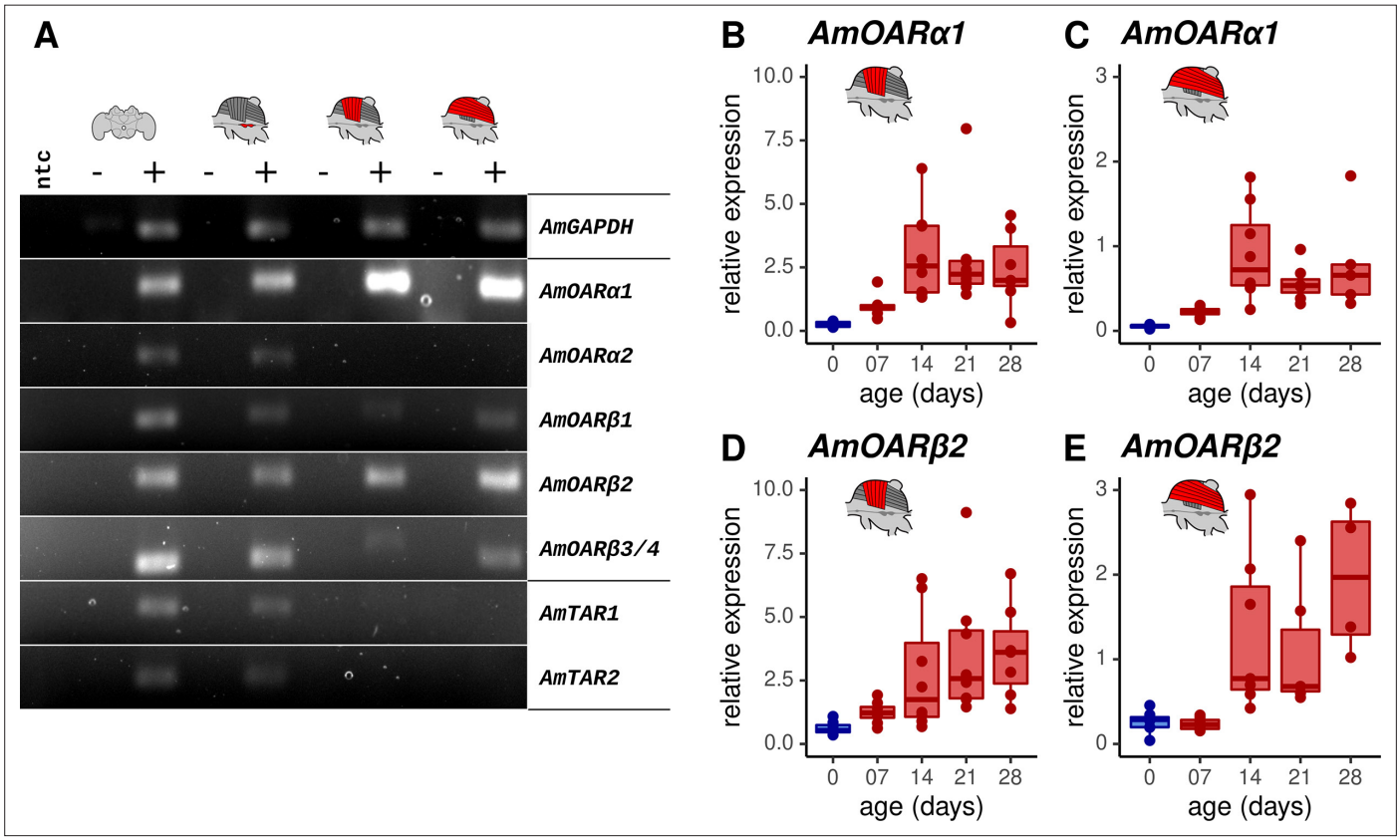


Figure 3. Octopamine receptor expression in the flight muscles. (**A**) Brain, MMTG, DV, and DL were manually dissected from workerbees and underwent subsequent RNA isolation, cDNA synthesis and PCR analysis (+). The reverse transcriptase was omitted during cDNA synthesis for negative controls (-). RNase free water serves as no template (ntc) and *AmGAPDH* as loading control. (**B–E**) *AmOARα1* and *AmOARβ2* expression in DV and DL of differential aged workerbees with (red) or without (blue) the capability for thermogenesis. Data are represented as boxplots. Shown is median ± IQR. For statistic see ***Table 2***.

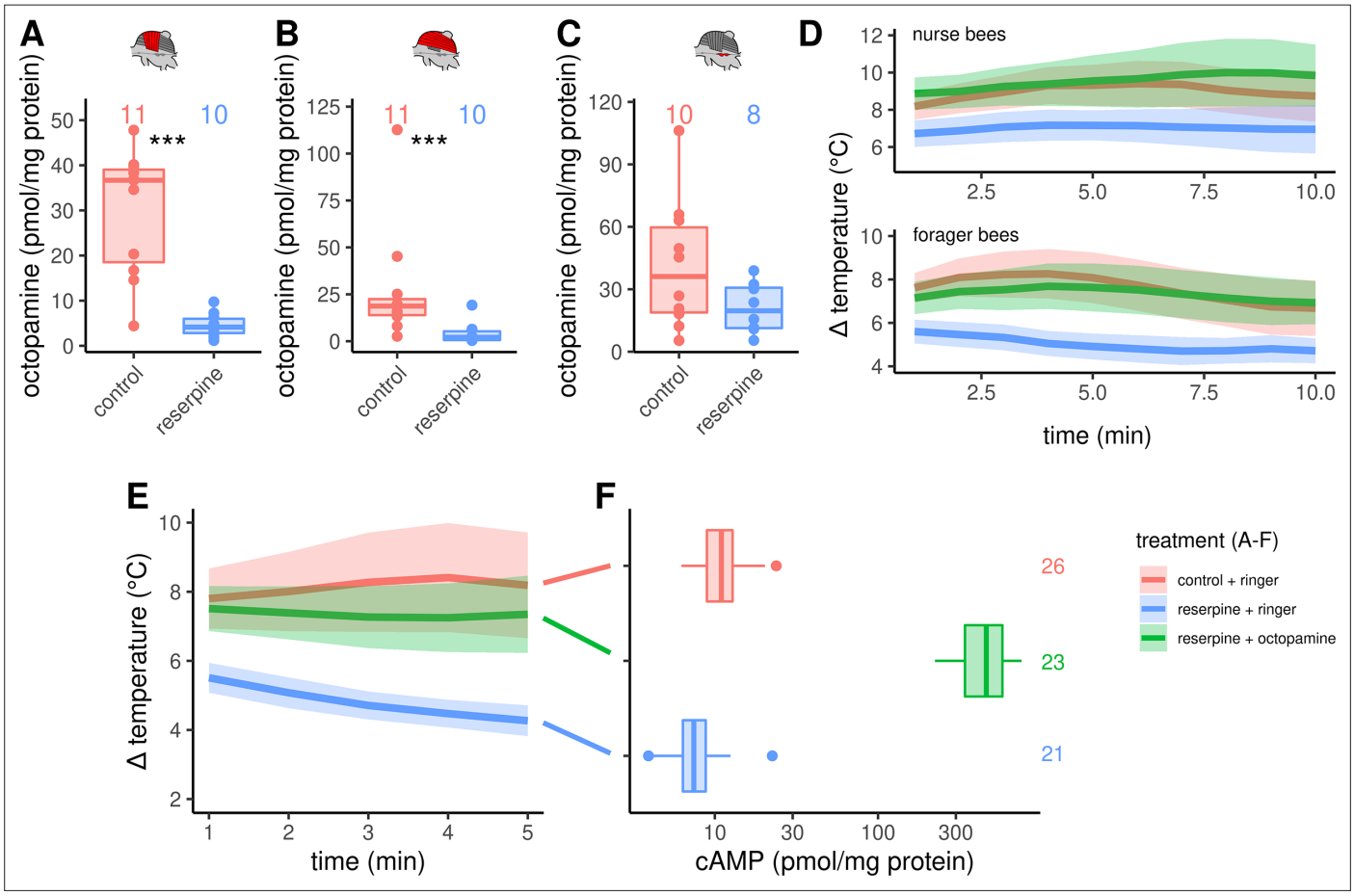


Figure 4. Octopaminergic control of honeybee thermogenesis. (**A–C**) Octopamine concentrations are decreased in DV and DL but not MMTG of reserpinezed workerbees. Data are represented as boxplots. Shown is median ± IQR. Mann-Whitney *U* test, DV(A): W = 105, z = –3.70, p < 0.001; DL(B): W = 102, z = –3.37, p < 0.001; MMTG(C): W = 56, z = –0.94, p = 0.1728. (**D**) Reserpinezed bees show hypothermia when compared with control. An injection of octopamine into the flight muscle helps the bees to recover, as no differences are observable between the control group and the recovered bees. The solid line represents the mean difference between T_{THX} and T_A and the shaded area represents the 95% confidence interval. For statistic see ***Table 3***. (**E**) Similar experiment as in (**D**) but bees were frozen in liquid N_2 after 5 min for cAMP quantification. For statistic see ***Table 3***. (**F**) The tissue cAMP concentrations in the flight muscles differ significantly due the treatment (Kruskal-Wallis test, $X^2 = 52.636$, df = 2, p < 0.001). Reserpinezed bees has the lowest tissue cAMP concentrations in the flight muscles when compared with controls (Dunns test, Z = 2.6383, $p_{adj} = 0.025$) and recovered bees (Z = 7.117, p_{adj}= < 0.001). Controls also differ from the recovered bees (Z = –4.7998, p_{adj} <0.001). Data are represented as boxplots. Shown is median ± IQR.

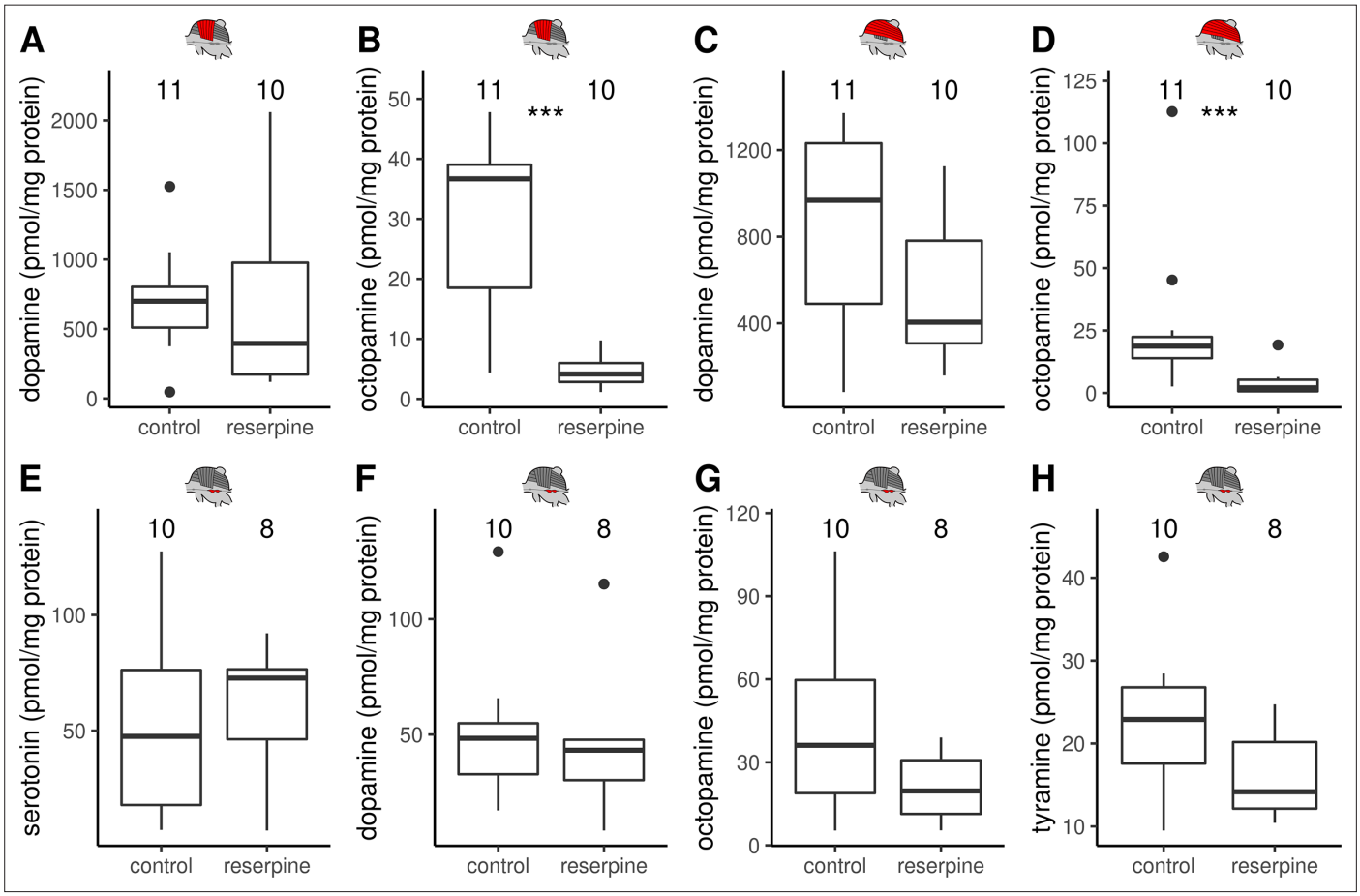


Figure 4—figure supplement 1. The effect of reserpine on monoamine concentrations in DV, DL and MMTG. (**A–H**) Monoamine concentrations in the thoracic tissues quantified by HPLC-ECD. Reserpinezed bees have significant lower octopamine (**B,D**) but not dopamine concentrations (**A,C**) than control animals in both dorsoventral (**A–B**) and dorsal-longitudinal flight muscles (**C–D**). In the mesometa-thoracic ganglia serotonin (**E**), dopamine (**F**), octopamine (**G**), and tyramine (**H**) were detectable but no reserpine effect was observable. Mann-Whitney *U* test: DV & octopamine, W = 105, z = –3.70, $p < 0.001$; DL & octopamine:, W = 102, z = –3.37, $p < 0.001$; all others: $p > 0.05$. Shown is median ± IQR.

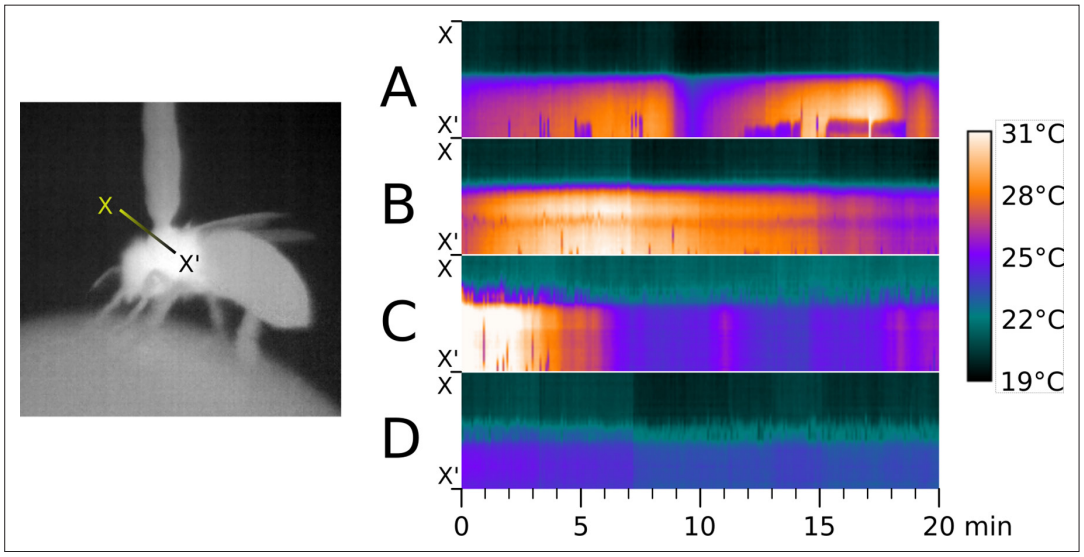
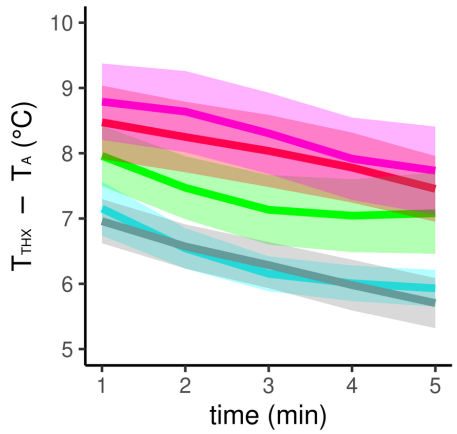


Figure 4—figure supplement 2. Time series of thermographic recordings of thoraces of selected workerbees.
To show differences in individual heating patterns, the thermographically determined temperatures along the line X - X′ (left) were plotted over time and exemplied for four bees (right, **A-D**).



		ATS	df	p-value	
		3.413	3.914	0.0089	**
group 1 (n)	**group 2 (n)**	**ATS**	**df**	**p-value**	
reserpine (20)	octopamine (21)	5.913	1.000000	0.0150	*
reserpine (20)	dopamine (19)	2.4137	1.000000	0.1203	n.s.
reserpine (20)	tyramine (22)	7.4217	1.000000	0.0064	**
reserpine (20)	serotonin (21)	0.0083	1.000000	0.9275	n.s.
octopamine (21)	dopamine (19)	0.8596	1.000000	0.3539	n.s.
octopamine (21)	tyramine (22)	0.0452	1.000000	0.8316	n.s.
octopamine (21)	serotonin (21)	6.2233	1.000000	0.0126	*
dopamine (19)	tyramine (22)	1.3761	1.000000	0.2408	n.s.
dopamine (19)	serotonin (21)	2.4608	1.000000	0.1167	n.s.
tyramine (22)	serotonin (21)	7.9104	1.000000	0.0049	**

Figure 4—figure supplement 3. The effect of different monoamines on thermogenesis of reserpinized bees.
Screen for monoamines that are able to equalize the negative impact of reserpine on the workerbee thermogenesis. Injection of octopamine and tyramine lead to significantly increased thorax temperatures of reserpine-fed bees, wheres serotonin and dopamine does not. The solid line represents the mean difference between the thorax temperature (T_{THX}) and ambient temperature (T_A) and the shaded area represents the 95% confidence interval.

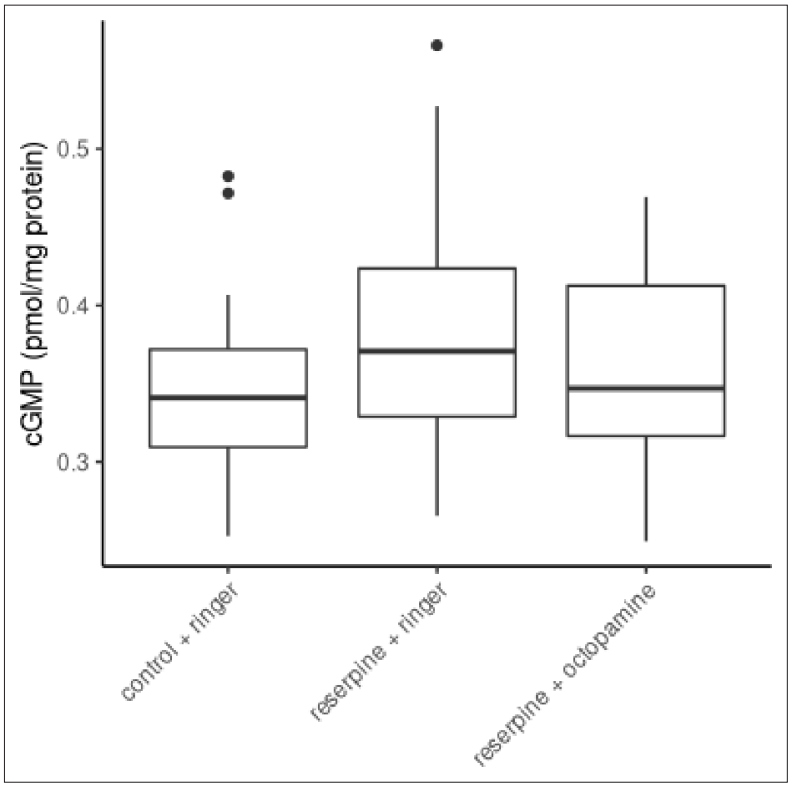


Figure 4—figure supplement 4. The effect of reserpine on flight muscle cGMP concentrations. Control bees, reserpinized bees and recovered reserpinized bees does not differ in their tissue cGMP concentrations of their flight muscles (Kruskal-Wallis test, x2 = 2.212, df = 2, p = 0.331).

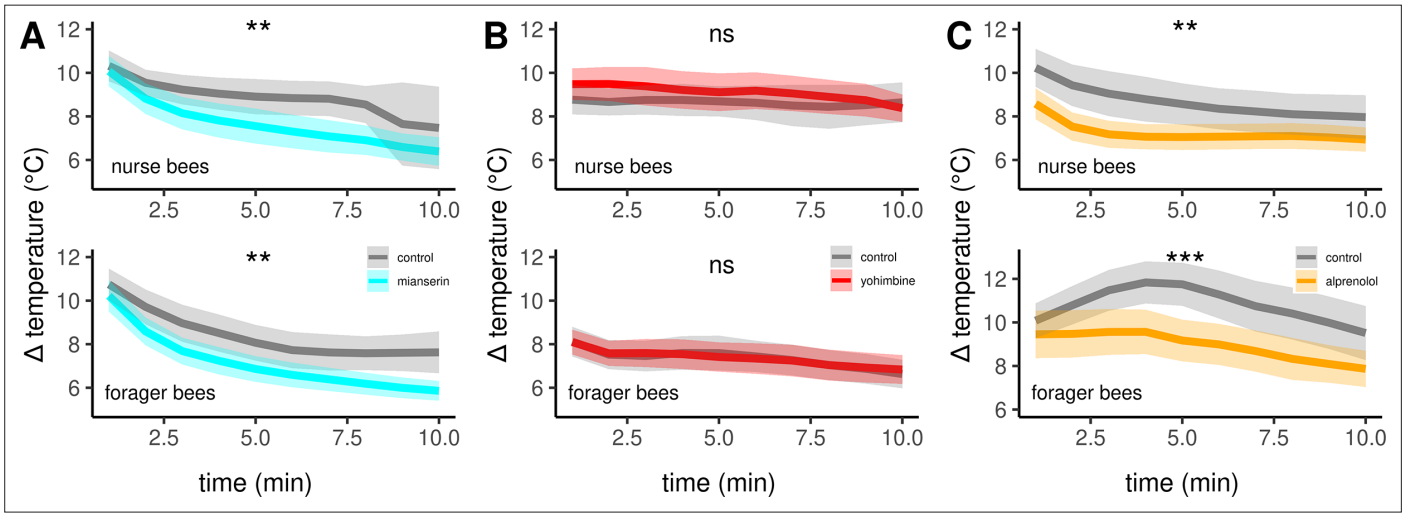


Figure 5. The effects of different antagonists on workerbee thermogenesis. Mianserin (**A**) and alprenolol (**C**) cause hypothermia in workerbees but not yohimbine (**B**). The solid line represents the mean difference between T_{THX} and T_A and the shaded area represents the 95% confidence interval. For statistic see ***Table 3***.

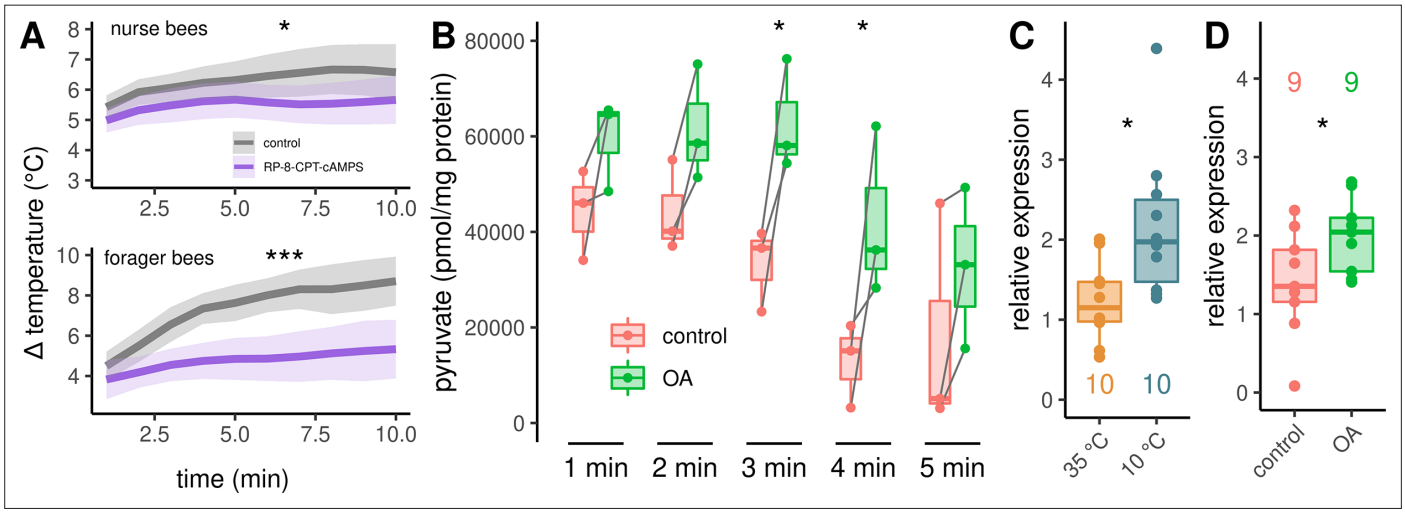


Figure 6. Analysis of the downstream pathway. (**A**) The PKA inhibitor Rp-8-CPT-cAMPS causes hypothermia in nurse bees and forager bees. The solid line represents the mean difference between T_{THX} and T_A and the shaded area represents the 95% confidence interval. For statistic see ***Table 3***. (**B**) DL muscles were separated into two mirror-similar parts and treated differently. Bath application of octopamine (**B**) leads to an difference in the mean pyruvate concentration when compared with control (two-way RM ANOVA, $F(1)=38.28$, $p < 0.001$). The simple main effect of treatment becomes significant after 3 and 4 min (Sidaks multiple comparisons test, 3 min: $p = 0.016$, 4 min: $p = 0.017$). Shown is median ± IQR. Data points of the same individual are connected by gray lines. (**C–D**) *AmGAPDH* expression in DV and DL is upregulated due to cold stress (C, Mann-Whitney *U* test, $W = 83$, $z = -2.24$, $p = 0.01261$). This result can be mimicked by an injection of octopamine directly into the flight muscles (D, Mann-Whitney *U* test, $W = 18$, $z = -1.68$, $p = 0.04694$). Shown is median ± IQR.

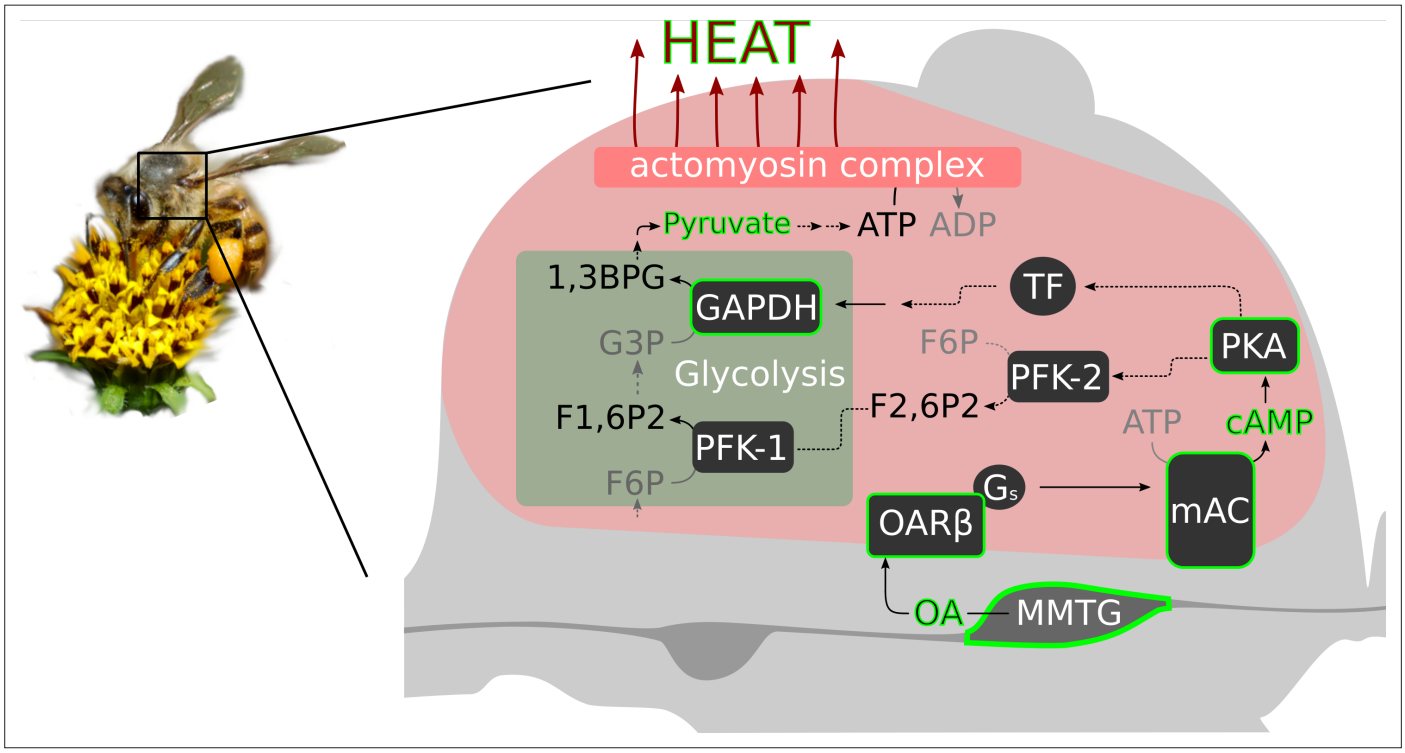


Figure 7. Octopamine and honeybee thermogenesis. The scheme summarizes our findings, with the solid lines and green borders representing interpretations supported by our results and the dashed lines representing hypothetical pathways. Muscle innervating neurons in the MMTG release octopamine (OA) directly to the flight muscles. By this, AmOARβ two receptors are activated which in turn activate the membrane-bound adenylyl cyclase (mAC) via G_s proteins. The resulting increase in the intracellular cAMP concentration leads to the activation of Proteinkinase A (PKA) which phosphorylates and by this activates phosphofructokinase 2 (PFK-2). Consequently, this enzyme produces fructose-2,6-bisphosphate (F2,6P2) which increases the activity of phosphofructokinase 1 (PFK-1). An alternative pathway is the PKA mediated activation of transcription factors (TF) which might enhance expression of *GAPDH* which encodes glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This enzyme converts glyceraldehyde 3-phosphate (G3P) into 1,3-bisphosphoglyceric acid (1,3BPG). All together, this increases the glycolysis rate so that a greater amount of pyruvate is available for ATP production. Finally, heat is generated in the actomyosin complex under ATP consumption.

Chapter

THREE

ROBUSTNESS OF THE HONEYBEE NEURO-MUSCULAR OCTOPAMINERGIC SYSTEM IN THE FACE OF COLD STRESS

Manuscript was originally published in:
Sinan Kaya-Zeeb, Saskia Delac, Lena Wolf, Ana Luiza Marante, Oliver Scherf-Clavel, Markus Thamm (2022).
Robustness of the honeybee neuro-muscular octopaminergic system in the face of cold stress *Frontiers in Physiology* **13**:1002740

This article can be downloaded from https://doi.org/10.3389/fphys.2022.1002740.

frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 27 September 2022
DOI 10.3389/fphys.2022.1002740

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

RECEIVED 25 July 2022
ACCEPTED 30 August 2022
PUBLISHED 27 September 2022

CITATION
Kaya-Zeeb S, Delac S, Wolf L, Marante AL, Scherf-Clavel O and Thamm M (2022), Robustness of the honeybee neuro-muscular octopaminergic system in the face of cold stress.
Front. Physiol. 13:1002740.
doi: 10.3389/fphys.2022.1002740

Robustness of the honeybee neuro-muscular octopaminergic system in the face of cold stress

Sinan Kaya-Zeeb[1]*, Saskia Delac[1], Lena Wolf[1], Ana Luiza Marante[1], Oliver Scherf-Clavel[2] and Markus Thamm[1]

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In recent decades, our planet has undergone dramatic environmental changes resulting in the loss of numerous species. This contrasts with species that can adapt quickly to rapidly changing ambient conditions, which require physiological plasticity and must occur rapidly. The Western honeybee (*Apis mellifera*) apparently meets this challenge with remarkable success, as this species is adapted to numerous climates, resulting in an almost worldwide distribution. Here, coordinated individual thermoregulatory activities ensure survival at the colony level and thus the transmission of genetic material. Recently, we showed that shivering thermogenesis, which is critical for honeybee thermoregulation, depends on octopamine signaling. In this study, we tested the hypothesis that the thoracic neuro-muscular octopaminergic system strives for a steady-state equilibrium under cold stress to maintain endogenous thermogenesis. We can show that this applies for both, octopamine provision by flight muscle innervating neurons and octopamine receptor expression in the flight muscles. Additionally, we discovered alternative splicing for *AmOARβ2*. At least the expression of one isoform is needed to survive cold stress conditions. We assume that the thoracic neuro-muscular octopaminergic system is finely tuned in order to contribute decisively to survival in a changing environment.

KEYWORDS

honeybees, thermogenesis, cold stress, octopamine, octopamine receptors, gene expression

1 Introduction

Independence from ambient temperature provides a decisive competitive benefit. Insects, however, are not known to have individual physiological thermostasis as mammals or birds do (Nord and Giroud, 2020). Their activity level is highly dependent on ambient temperatures and although some insects use various strategies (passive or active) to vary their body temperature, they cannot maintain a constant core body temperature (Bartholomew, 1981; Josephson, 1981; Block, 1994; Colinet et al., 2018). Nevertheless, eusociality enables some insect species to achieve thermal homeostasis as a super-organism on the colony level (Kadochová and Frouz, 2013; Stabentheiner et al.,

2021). As an eusocial insect, the Western honeybee (*Apis mellifera*) has exactly these characteristics. Several strategies are used to keep the colony at a constant temperature during the breeding season and in winter, which also contributes to the almost worldwide distribution of this species (Simpson, 1961; Seeley and Visscher, 1985; Bujok et al., 2002; Stabentheiner et al., 2003; Wallberg et al., 2014; Buckley et al., 2015; Perez and Aron, 2020; Stabentheiner et al., 2021).

For the maintenance of the colony's thermostasis, the workerbee flight muscles are of exceptional importance. For cooling purposes, foragers collect water which is subsequently evaporated from the combs and thus cooling the hive (Kühnholz and Seeley, 1997). In addition, workerbees fan hot air out of the colony with their wings, which in turn creates an airflow that lets cool air in (Fahrenholz et al., 1989; Egley and Breed, 2013). Heat generation occurs exclusively through muscle tremors of the indirect flight musculature and can therefore be referred to as shivering thermogenesis (Stock, 1999; Stabentheiner et al., 2010). We recently demonstrated that workerbee thermogenesis depends on octopamine signaling. The dorsoventral wing elevators (DV) and dorsal-longitudinal wing depressors (DL) constitute the indirect flight muscles. During thermogenesis, DV contracts less frequently than DL, whereas this ratio is reversed during flight (Esch and Goller, 1991). These muscles are innervated by octopaminergic neurons from the mesometathoracic ganglion (MMTG, Kaya-Zeeb et al., 2022). The release of octopamine for the purpose of thermogenesis activates b octopamine receptors, presumably AmOARβ2, which most likely promotes glycolysis (Kaya-Zeeb et al., 2022). With *AmOARα1*, another octopamine receptor gene displays dominant flight muscle expression, but we did not find evidence for an involvement of the corresponding receptor sub-type in thermogenesis, yet. Disturbances of the flight muscle octopaminergic system lead to an impairment of thermogenesis and consequently, the affected bees suffer from hypothermia (Kaya-Zeeb et al., 2022). At the colony level, this would endanger the survival of the colony during winter and in extreme habitats from desserts to high mountains. Moreover, new problems arise due to man-made climate change. Increasing temperature fluctuations in short time periods are to be expected, which can lead to rapid temperature decrease (Walther et al., 2002; Rosenzweig et al., 2008; Van Asch et al., 2013). The intensification of such periods poses a major challenge to physiological processes in general and to thermoregulation in particular.

In this study, we used honeybees as a model system to investigate how the thoracic neuro-muscular octopaminergic system responds to cold stress. We applied cold stress to workerbees to force them into thermogenesis in order to avoid chill coma. Subsequently, we determined the concentration of octopamine as well as the expression of the relevant octopamine receptor genes. With this approach we tested the hypothesis, that octopamine is always provided in sufficient quantity by the neurons innervating the flight muscle and, when released, can be recognized by specific receptors. This requires that the expression of receptor genes (at least *AmOARβ2*) is maintained at a constant level or increased as needed.

2 Materials and methods

2.1 Cold stress exposure

Brood combs and adult worker honeybees (*Apis mellifera carnica*) were collected from departmental bee colonies. For age-controlled bees (1 week old), hatching bees were collected from a brood comb, color-marked and reinserted into a standard hive. Alternatively, newly emerged bees were held in cages (T = 34°C, RH = 65%) and fed *ad libitum* with sucrose solution (30% w/v). After 1 week, both groups were collected from the hive or the incubator for further experiments. As forager bees, we defined bees that returned to the hive with pollen loads on their hind legs. Prior to each cold stress experiment, bees were subjected a one (1 week old bees) or a 2 day (forager bees) run-in period with identical conditions (T = 34°C, RH = 65%). During the run-in period and the stress conditions, all groups were provided with sucrose solution (30% w/v) *ad libitum*. All bees that were exposed to cold stress conditions (30/120 min, 10°C; control: 30/120 min, 34°C) were immediately flash-frozen in liquid nitrogen after experiencing cold stress conditions and subsequently stored at −80°C until further experimental procedure (gene expression analysis, monoamine quantification).

2.2 Time-survival analysis (Kaplan-Meier estimator)

One week old workerbees (hive or cage, see above) or forager bees were transferred to cages of 30–50 bees each (with sucrose solution (30% w/v) *ad libitum*). One cage (control) was held under defined conditions (T = 34°C, RH = 65%). Two additional cages were exposed to cold stress (10°C, RH = 65%). All cages were filmed for 120 min (LifeCam Cinema HD, Microsoft, Redmond, United States) to determine if and when the bees entered chill coma. Afterwards, all the cages in which chill coma occurred were transferred warm conditions to calculate a recovery rate (percentage of bees brought back from the chill coma).

2.3 Monoamine quantification (HPLC-ECD)

For each individual, DV and DL were dissected under liquid nitrogen and merged as one sample. First, frozen thoraces were

10.3389/fphys.2022.1002740

TABLE 1 Oligonucleotides used in this study.

Analysis	gene/isoform	gene ID	direction	sequence (5′–3′)
sequencing	*AmOARβ2X1A*	412896	forward	AGACGAGAGCCGTCCGA
			reverse	AAAGGCTCTCTGTTGTTCGC
Sequencing	*AmOARβ2X1B*	412896	forward	AGACGAGAGCCGTCCGA
			reverse	TGTTTGATGTACGTCTCCGAA
Sequencing	*AmOARβ2X2*	412896	forward	TGGAATTCCCTGAACGTGA
			reverse	TCAACTCCCCGTTCAAATTG
Sequencing	*AmOARβ2X3*	412896	forward	AGACGAGAGCCGTCCGA
			reverse	TCAACTCCCCGTTCAAATTG
sequencing	*AmOARβ2X3*	412896	forward	AGACGAGAGCCGTCCGA
			reverse	TCAACTCCCCGTTCAAATTG
qPCR SYBR	*AmRPL19*	724186	forward	GGGACTTCTAGGCTCCATCATGAG
			reverse	GCTTTGACGTGAGTTTGTATTTGCAA
qPCR SYBR	*AmRPL32*	406099	forward	AGTAAATTAAAGAGAAACTGGCGTAA
			reverse	TAAAACTTCCAGTTCCTTGACATTAT
qPCR SYBR	*AmOARα1*	406068	forward	GCAGGAGGAACAGCTGCGAG
			reverse	GCCGCCTTCGTCTCCATTCG
qPCR SYBR	*AmOARα2*	726331	forward	GCGAGCATCATGAACTTGTG
			reverse	CGTAGCCTATGTCCTCTGAAAG
qPCR SYBR	*AmOARβ1*	413698	forward	GGAGTAAAGTAGCAGCCGCTC
			reverse	GTGATCTGTGGCTCCTCTGGT
qPCR SYBR	*AmOARβ2*	412896	forward	CTCGAGCGAGGAGAAGTTGT
			reverse	CCAACGCTAAAGAGACCACG
qPCR SYBR	*AmOARβ3/4*	412994	forward	CGAGGACGCTCGGAATAATA
			reverse	GAAGTCGCGGTTGAAGTACG
qPCR probe	*AmOARβ2X*	412896	forward	AGACGAGAGCCGTCCGA
qPCR probe	*AmOARβ2X1A*	412896	reverse	AAAGGCTCTCTGTTGTTCGC
	AmOARβ2X1A	412896	probe	HEX-TCTTGCAACATTGATTGCCCCAT–BHQ1
	AmOARβ2X4	412896	reverse	TTCTTTCCGCGATTACATACAGA
	AmOARβ2X4	412896	probe	6FAM-TCACGTGGTGTCGTTACATCGGT–BBQ
qPCR probe	*AmOARβ2X1B/X3*	412896	reverse	TGTTTGATGTACGTCTCCGAA
	AmOARβ2X1B	412896	probe	ROX-ACGTCGAGGTACGACGATCGC–BBQ
	AmOARβ2X3	412896	probe	Cy5-CGTCCCTAAGGTACGACGATCGC–BBQ

peeled from the cuticle using watchmaker forceps. Afterwards, the large muscles were separated from the surrounding tissue. The remaining thoracic tissue was thawed in ice-cold ethanol to undergo immediate dissection of the MMTG. All extracted tissues were kept at −80°C until extraction of monoamines. The high-performance liquid chromatography (HPLC) protocol represents a slightly modified version as described earlier (Kaya-Zeeb et al., 2022). All samples (tissues and calibrators) were processed and treated equally. We used 120 ml (DV + DL) or 60 ml (MMTG) of the extraction solution (10.0 pg/ml 3,4-dihydroxy-benzylamine (DHBA) in 0.2 M perchloric acid). The raw data was processed with Chromeleon (7.2.10, Thermo Fisher Scientific, Waltham, United States) for further statistical (see below).

2.4 Protein quantification (Bradford)

The pellet from the monoamine extraction was used for protein quantification in oder to normalize monoamine concentrations. After resuspension in 500 μL (DV + DL) or 30 μL (MMTG) NaOH (0.2 M) samples were incubated on ice for 15 min and then centrifuged (9391 g, 5 min). The supernatant (DV + DL: 5 μL, MMTG: 10 μL) were transferred into 1 ml 1x ROTI®Nanoquant solution (Carl Roth, Karlsruhe, Germany). All samples and an external calibrator (1, 2, 3, 5, 10, 20 mg/ml Albumin Fraction V; Carl Roth, Karlsruhe, Germany) were analyzed using an Infinite 200 Pro (Tecan, Männedorf, Switzerland) at 590 nm (450 nm reference).

2.5 Verification of AmOARβ2 isoforms

Sequence analysis was performed with cDNA from thoraces of 20 individual workerbees. Two individual thoraces were pooled in each case and homogenized using 1 ml peqGOLD TriFast™ (Peqlab) following a 5 min incubation at room temperature. After adding of 200 μL chloroform and phase separation, the aqueous phase was transferred to 500 μL isopropyl alcohol. After centrifugation (21,130 g, 15 min, 4°C), the RNA pellet was purified by two consecutive washing steps (1 ml, 75% (v/v) ethanol), dried, and diluted in 50 μL RNase-free water. To ensure complete DNA removal we performed DNase treatment (DNase I M0303S; New England Biolabs, Ipswich, Massachusetts) according to the manufacturer's protocol with subsequent phenol-chloroform extraction (Roti®-Aqua-P/C/I; Carl Roth, Karlsruhe, Germany). The purified RNA pellet was diluted in 50 μL RNase-free water and served as template for cDNA synthesis (AccuScript™ High Fidelity first Strand cDNA Synthesis; Agilent Technologies, Santa Clara, United States). PCR amplification was conducted using Phusion® DNA Polymerase (New England Biolabs, Ipswich, Massachusetts) on cDNA with *AmOARβ2* isoform (Table 1). After successful amplification and A-tailing (*Taq* DNA Polymerase with ThermoPol® Buffer, New England Biolabs, Ipswich, Massachusetts) the PCR products were subjected to T/A cloning (pGEM®-T Vector Systems; Promega, Fitchburg, United States) with subsequent Sanger sequencing (Sanger et al., 1977; Genewiz, Leipzig, Germany).

2.6 Octopamine injection

Additionally to cold stress, 1 week old bees (see above) were treated with octopamine injections. Individual bees were chilled on ice until no further movement could be detected. Then, the thorax was punctured centrally to either inject 1.0 μL saline solution (270 mM sodium chloride, 3.2 mM potassium chloride, 1.2 mM calcium chloride, 10 mM magnesium chloride, 10 mM 3-(N-morpholino) propanesulfonic acid, pH = 7.4; Erber and Kloppenburg 1995) or 1.0 μL octopamine solution (0.01 M in saline, Sigma-Aldrich) using a 10.0 μL Hamilton syringe as formerly described in Kaya-Zeeb et al. (2022). The different treatments were kept in different cages and were incubated for either 30 min or 120 min (T = 34°C, RH = 65%). Subsequently, all bees were flash-frozen in liquid nitrogen and stored at −80°C until further procedure.

2.7 Gene expression analysis

Individual flight muscles (DV + DL) were dissected under liquid nitrogen (see Subsection 2.3) and stored at −80°C until further processing. Following the manufacturers standard protocol, we extracted total RNA of individually pooled DV and DL using the GenUP Total RNA Kit (biotechrabbit, Henningsdorf, Germany). We included a DNase I digestion step. After the RNA was bound to the *Mini Filter RNA*, we added 50 μL DNase mix containing 30 U RNase-free DNase I (Lucigen, Middleton, United States) and incubated for 15 min at room temperature. The RNA_{total} concentration was determined photometrically and individual flight muscle cDNA was generated using Biozym cDNA Synthesis Kit (Biozym, Hessisch Oldendorf, Germany). Afterwards, quantitative real-time PCR (qPCR) was used to determine gene expression. Individual cDNAs were analyzed in triplicates per gene. Total reaction volume was 20 μL and contained 4.2 μL H_2O, 10 μL 2 x qPCR S'Green BlueMix (Biozym, Hessisch Oldendorf, Germany), 0.4 μL of gene specific forward and reverse primer (0.2 μM, Table 1) and 5 μL cDNA. For the analysis of the AmOARβ2 isoforms, we established TaqMan® based duplex assay. Each reaction (20 μL) contained 2.6 μL H_2O, 10 μL 2 x qPCR S'Green BlueMix (Biozym, Hessisch Oldendorf, Germany), 0.4 μL of each primer (0.2 μM, Table 1), 0.4 μL of each TaqMan® probe (0.1 μM, Table 1) and 5 μL cDNA. All qPCR runs were performed on a Rotor-Gene Q (Qiagen, Hilden, Germany) with following cycling conditions: 95°C for 2 min, 35 cycles at 95°C for 5 s and 30°C for 30 s; followed by a melting curve analysis (not for TaqMan® assays). Gene of interest expression was quantified relative to *AmRPL32* and *AmRPL19* (Lourenço et al., 2008; Kaya-Zeeb et al., 2022).

2.8 Statistical analysis

Statistical analyses were computed using R (4.2.0, R Core Team, 2021) and the R packages 'car' (3.0.13, Fox and Weisberg, 2019), "dplyr" (1.0.9, Wickham et al., 2022), "FSA" (0.9.3, Ogle et al., 2022), "Rcpp" (1.0.8.3, Eddelbuettel and François, 2011), "reshape2" (1.4.4, Wickham, 2007), "Rmisc" (1.5.1, Hope, 2022), "rstatix" (0.7.0, Kassambara, 2021), "tidyr" (1.2.0, Wickham and Girlich, 2022), and "xtable" (1.8.4, Dahl et al., 2019). Shapiro-Wilk testing was performed to check for normal distributions. Depending on whether the data were normally distributed or not, they were analyzed using a *t*-test or Mann-Whitney *U* test, respectively. For the analysis of the Kaplan-Meier estimator we additionally used the R packages "survival" (3.3.1, Therneau, 2022; Therneau and Grambsch, 2000) and 'survminer' (0.4.9, Kassambara et al., 2021). Octopamine receptor gene expressions and *AmOARβ2* isoform abundance were relatively quantified using the R package "EasyqpcR" (1.22.1, Le Pape, 2012). Data was visualized using the R packages 'cowplot' (1.1.1, Wilke, 2020), "ggplot2" (3.3.6, Wickham, 2016), "ggpol" (0.0.7, Tiedemann, 2020), "ggpubr" (0.4.0, Kassambara, 2020), "grid" (4.2.0, R Core Team, 2021), "ggsignif" (0.6.3, Constantin and Patil, 2021), "magick" (2.7.3,

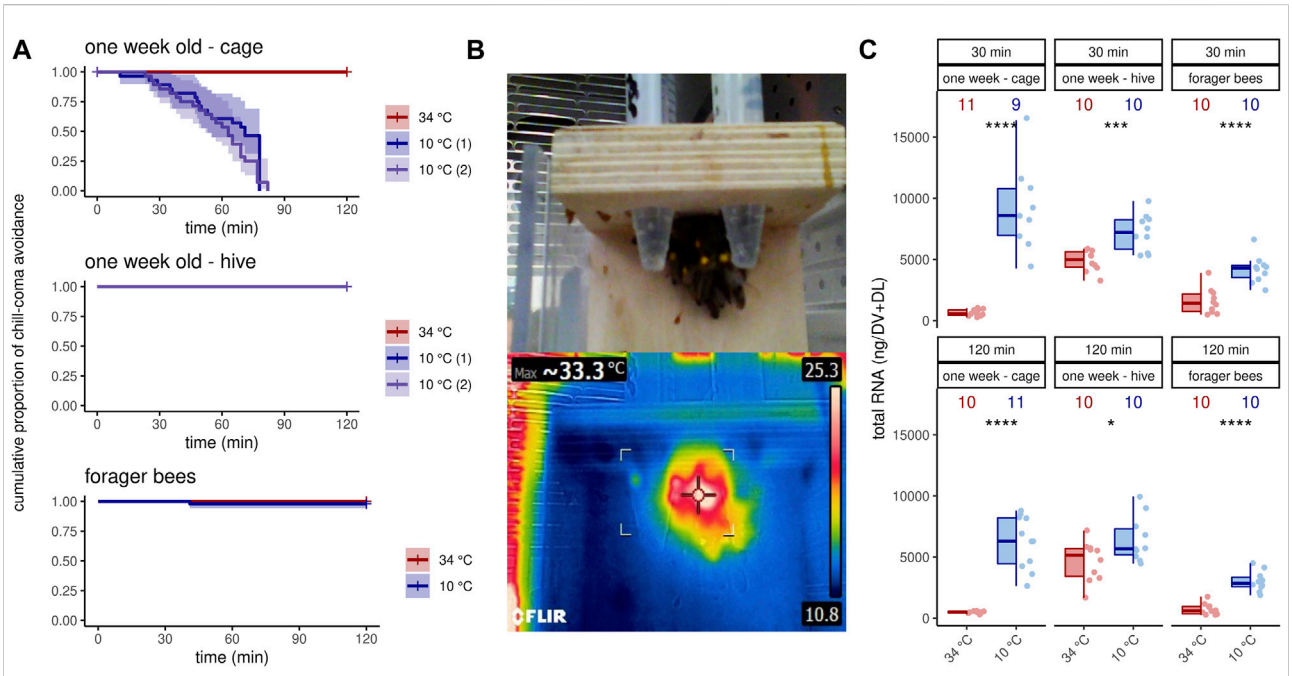

FIGURE 1
Workerbees respond differently to cold stress. **(A)** One week old workerbees from cages are not able to withstand 2 hours of cold stress without falling into chill coma (log rank test χ^2 (2) = 76.1, $p \leq 0.0001$, $N_{34\ °C}$ = 32, $N_{10\ °C\ (1)}$ = 30, $N_{10\ °C\ (2)}$ = 31). Both 10°C groups displayed a recovery rate of 100%. Not a single 1 week old workerbee from hives went into chill coma ($N_{34\ °C}$ = 31, $N_{10\ °C\ (1)}$ = 30, $N_{10\ °C\ (2)}$ = 30). When forager bees were cold stressed over 120 min, only one bee felt into chill coma (log rank test q^2 (1) = 1, p = 0.3, $N_{34\ °C}$ = 48, $N_{10\ °C}$ = 50). **(B)** Workerbees that successfully survive 2 hours of cold stress form a cluster in which they effectively perform thermogenesis. **(C)** The RNA_{total} amount significantly increases in workerbee flight muscles due to cold stress (*t*-test, 1 week - cage - 30 min: t (8.07) = −7.20, p = 0.0005; 1 week - cage - 120 min: t (10) = −8.84, p = 0.035; 1 week - hive - 30 min: t (14.3) = −4.26, p = 0.0005; 1 week - hive - 120 min: t (17.7) = −2.28, p = 0.035; forager - 30 min: t (17.9) = −5.30, $p \leq 0.0001$; forager - 120 min: t (14.3) = −7.53, $p \leq 0.0001$). For each group/data-set median ± interquartile range (IQR, left part) and individual data points (right part) are shown.

Ooms, 2021), "mdthemes" (0.1.0, Neitmann, 2020), and "png" (0.1.7, Urbanek, 2013).

3 Results

3.1 Responses of workerbees to cold exposure under laboratory conditions

We wanted to analyze the effects of cold stress on workerbees under laboratory conditions, in order to control as many factors as possible. Since our focus was on thermogenesis, these bees additionally should be able to avoid chill coma by active thermogenesis. To our surprise, the 1 week old bees from cages could not meet these conditions. All bees of this group were found in chill coma latest after 90 min (Figure 1A). The workerbees from the hives (1 week old bees, forager bees), on the other hand, withstood the cold without difficulty (Figure 1A). These bees organize themselves into a cluster to effectively perform thermogenesis (Figure 1B). The 1 week old bees from cages might be in a physiological status that does not enable them to perform thermogenesis. This is supported by the fact that the controls have very low RNA_{total} concentrations in their flight muscles. This is enormously increased by cold stress (Figure 1C). However, this is apparently not sufficient to enable thermogenesis. In hive bees, we see less pronounced but still significant differences in flight muscle RNA_{total} concentration between control and cold-stressed bees (Figure 1C). From these results we conclude the following. First, 1 week old bees from cages are not suitable for laboratory studies of cold stress and are therefore excluded from further analysis. Second, irrespective whether thermogenesis is actively performed or not, cold stress triggers an increase in transcription activity.

3.2 Octopamine receptor gene expression

We next asked if this increase in transcription activity also affects octopamine receptor gene expression. In 1 week old bees, significant differences can only be detected for *AmOARβ2* after 120 min and for *AmOARβ3/4* after 30 min cold stress (Figure 2A; Table 2). For all remaining genes and time points we observed no

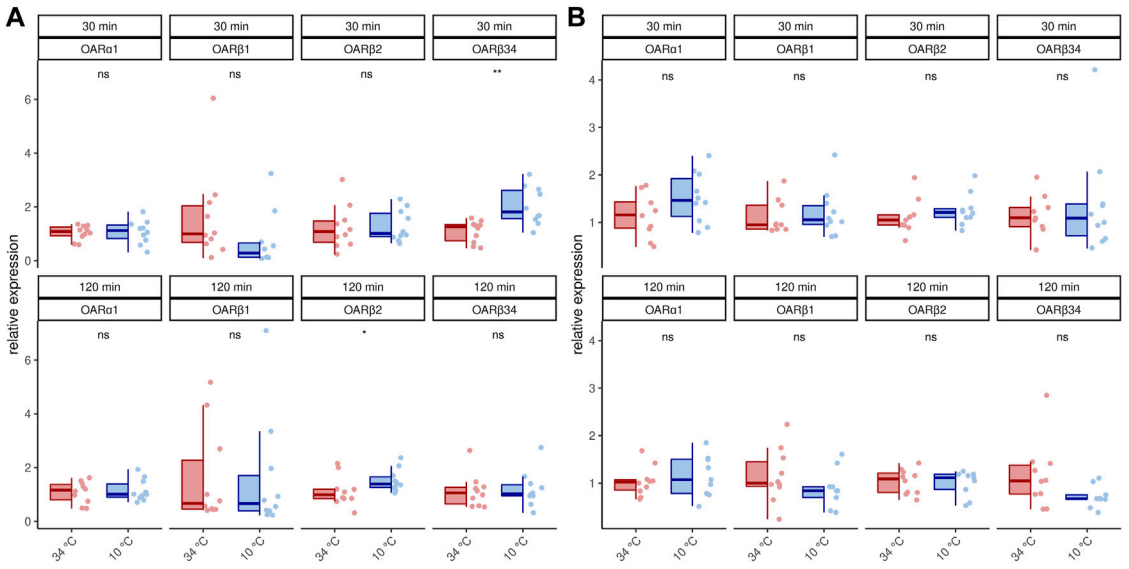

FIGURE 2
Octopamine receptor gene expression in the flight muscles workerbees under cold stress. One week old workerbees **(A)** and forager bees **(B)** were cold stressed for 30 and 120 min and subsequently, the relative octopamine receptor gene expression was quantified in flight muscles (DV + DL). * = $p < 0.05$, ** = $p < 0.01$, ns = $p \geq 0.05$, Mann-Whitney *U* test. For detailed statistics please see Table 2. For each group/data-set median ± IQR (left part) and individual data points (right part) are shown.

expression differences (Figure 2A; Table 2). Additionally, we detected no change in the expression of octopamine receptor genes in forager bees after either 30 or 120 min of cold stress (Figure 2B; Table 2).

3.3 AmOARβ2 is spliced alternatively

Publicly available transcriptome data indicate that *AmOARβ* expresses multiple isoforms due to differential splicing (NCBI, 2022) and we wanted to know, if AmOARβ2 isoform abundance is affected by cold stress. At least five *AmOARb2* isoforms exist, that differ with respect to their coding sequence (Figure 3A). Both, *AmOARβ2X1A* and *AmOARβ2X1B*, encode an identical receptor protein already functionally described by Balfanz et al. (2013). *AmOARβ2X2* encodes a truncated receptor protein with two trans-membrane domains missing. Likely, this results in impaired integrity as well as poor stability of the receptor protein (Zhu and Wess, 1998; Wise, 2012). We considered it to be unlikely, that a functional receptor protein providing correct ligand interactions could arise from such an isoform and consequently excluded *AmOARβ2X2* from further analysis. Finally we detected *AmOARβ2X3* and *AmOARβ2X4*. Both isoforms encode putative proteins that differ from each other as well as from AmOARβ2X1 by their unique C-terminus sequences.

Because our standard qPCR assay cannot distinguish different isoforms of this gene, we developed a hybridization probe based assay to analyze the *AmOARβ* isoform abundance with respect to cold stress (Figures 3B,C; Table 2). *AmOARβ2X1A* abundance is significantly increased after 30 and 12 min cold stress in 1 week old bees but not in forager bees. No differences can be observed for *AmOARβ2X1B* in 1 week old workerbees (both time points) and in forager bees after 30 min. After 120 min *AmOARβ2X1B* is significantly decreased in forager bees. *AmOARβ2X3* abundance in increased after 30 min but not after 120 min in 1 week old bees. In forager bees, no differences can be observed this isoform after 30 min whereas it is reduced after 120 min of cold stress. *AmOARβ2X4* does not reach the threshold for the most individuals and therefore was excluded from analysis.

3.4 Octopamine concentrations are stable under cold stress conditions

In addition to octopamine receptor expression, of course, octopamine is also required for thermogenesis (Kaya-Zeeb et al., 2022). We therefore wondered whether an increased thermogenic activity would require increased octopamine provision. With one exception, cold stress did not change octopamine concentrations in the flight muscles and MMTG (Figure 4). Only forager bees display higher octopamine titers in their flight muscles after 30 min at 10°C (Figure 4). Besides octopamine, we additionally detected dopamine in the flight

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TABLE 2 Results of the statistical analysis (Mann-Whitney *U* test) of octopamine receptor gene expression in workerbee flight muscles under cold stress (34°C vs 10°C). For visualization please see Figures 2, 3. * = $p < 0.05$, ** = $p < 0.01$, ns = $p \geq 0.05$.

Bees	time	gene/isoform	$N_{34\ °C}$	$N_{10\ °C}$	*U*	*p*	*Z*	*r*	—
1 week old bees	30 min	*AmOARα1*	10	10	47	0.853	-0.185	-0.041	ns
1 week old bees	30 min	*AmOARβ1*	10	10	73	0.089	-1.70	-0.380	ns
1 week old bees	30 min	*AmOARβ2*	10	10	44	0.684	−0.407	-0.091	ns
1 week old bees	30 min	*AmOARβ34*	10	10	9	0.001	−3.28	-0.733	**
1 week old bees	120 min	*AmOARα1*	10	10	49	0.971	−0.036	-0.008	ns
1 week old bees	120 min	*AmOARβ1*	10	10	58	0.579	−0.555	-0.124	ns
1 week old bees	120 min	*AmOARβ2*	10	10	22	0.036	−2.10	-0.470	*
1 week old bees	120 min	*AmOARβ34*	10	10	45	0.739	−0.333	-0.075	ns
forager bees	30 min	*AmOARα1*	9	10	28	0.182	−1.33	-0.306	ns
forager bees	30 min	*AmOARβ1*	9	10	39	0.661	−0.439	-0.101	ns
forager bees	30 min	*AmOARβ2*	9	10	30	0.243	−1.17	-0.268	ns
forager bees	30 min	*AmOARβ34*	9	10	44	0.968	−0.04	-0.009	ns
forager bees	120 min	*AmOARα1*	10	9	39	0.661	−0.439	-0.101	ns
forager bees	120 min	*AmOARβ1*	10	9	61	0.211	−1.25	-0.287	ns
forager bees	120 min	*AmOARβ2*	10	9	48	0.842	−0.199	-0.046	ns
forager bees	120 min	*AmOARβ34*	10	9	68	0.065	−1.84	-0.423	ns
1 week old	30 min	*AmOARβ2X1A*	10	10	22	0.036	−2.10	-0.470	*
1 week old	30 min	*AmOARβ2X1B*	10	10	47	0.853	−0.185	-0.041	ns
1 week old bees	30 min	*AmOARβ2X3*	10	10	21	0.029	−2.19	−0.489	*
1 week old bees	120 min	*AmOARβ2X1A*	10	10	17	0.012	−2.53	−0.565	*
1 week old bees	120 min	*AmOARβ2X1B*	10	10	58	0.579	-0.555	−0.124	ns
1 week old bees	120 min	*AmOARβ2X3*	10	10	30	0.143	−1.46	−0.328	ns
forager bees	30 min	*AmOARβ2X1A*	9	10	42	0.842	−0.199	−0.046	ns
forager bees	30 min	*AmOARβ2X1B*	9	10	40	0.72	−0.358	−0.082	ns
forager bees	30 min	*AmOARβ2X3*	9	10	29	0.211	−1.25	−0.287	ns
forager bees	120 min	*AmOARβ2X1A*	10	9	51	0.661	−0.439	−0.101	ns
forager bees	120 min	*AmOARβ2X1B*	10	9	76	0.010	−2.57	−0.59	*
forager bees	120 min	*AmOARβ2X3*	10	9	75	0.013	−2.48	−0.568	*

muscles and dopamine and serotonin in the MMTG. No differences were found here either (Supplementary Table S1, Supplementary Figure S1).

3.5 Octopamine injections cannot simulate cold stress

Octopamine is provided to the flight muscles at constant levels in order to prevent chill coma. Very likely, this octopamine is released to the flight muscles due to its essential role in thermogenesis. We finally wanted to know whether an octopamine signal could cause effects similar to cold stress. Octopamine injections into flight muscles did not increase RNA_{total} levels regardless of incubation time (Figure 5A). Similarly, octopamine receptor gene expression is not changed after 30 min (Figure 5B). However, after 120 min expression of *AmOARα1* and *AmOARβ1* is increased and expression of *AmOARβ34* is decreased (Figure 5B). *AmOARβ2* expression remains unchanged (Figure 5B). The same is true for most isoform of this gene (Figure 5C). Here, the only exception is *AmOARβ2X3* whos abundance decreases 30 min after octopamine injection (Figure 5C).

4 Discussion

Deviations from the optimal thermal state of a homoiothermic organism such as humans can have pathophysiological effects that, if they persist, lead to death (Obermeyer et al., 2017). Honeybees avoid comparable thermal stress induced consequences (Himmer, 1932; Weiss, 1962; Tautz et al., 2003; Groh et al., 2004; Wang et al., 2016) by maintaining thermostasis inside their colony as a super-organism (Simpson, 1961; Seeley and Visscher, 1985; Bujok et al., 2002; Stabentheiner et al., 2003; Buckley et al., 2015;

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TABLE 3 Results of the statistical analysis (Mann-Whitney *U* test) of the effects of octopamine injections in to workerbee flight muscles. For visualization please see Figure 5. * = $p < 0.05$, ** = $p < 0.01$, ns = $p \geq 0.05$.

Time	Subject	$N_{control}$	N_{OA}	*U*	*p*	*Z*	*r*	
30 min	RNA_{total}	10	10	48.00	0.91	−0.11	−0.02	ns
120 min	RNA_{total}	10	10	34.00	0.25	−1.16	−0.26	ns
30 min	*AmOARα1*	10	10	53.00	0.85	−0.19	−0.04	ns
30 min	*AmOARβ1*	10	10	53.00	0.85	−0.19	−0.04	ns
30 min	*AmOARβ2*	10	10	56.00	0.68	−0.41	−0.09	ns
30 min	*AmOARβ34*	10	10	58.00	0.58	−0.55	−0.12	ns
120 min	*AmOARα1*	10	10	9.00	0.001	−3.28	−0.73	**
120 min	*AmOARβ1*	10	10	17.00	0.01	−2.53	−0.57	*
120 min	*AmOARβ2*	10	10	46.00	0.80	−0.26	−0.06	ns
120 min	*AmOARβ34*	10	9	76.00	0.01	−2.57	−0.59	*
30 min	*AmOARβ2X1A*	10	10	40.00	0.48	−0.70	−0.16	ns
30 min	*AmOARβ2X1B*	9	7	46.00	0.14	−1.47	−0.37	ns
30 min	*AmOARβ2X3*	9	9	70.00	0.008	−2.66	−0.63	**
120 min	*AmOARβ2X1A*	10	10	43.00	0.63	−0.48	−0.11	ns
120 min	*AmOARβ2X1B*	9	9	18.00	0.05	−1.96	−0.46	ns
120 min	*AmOARβ2X3*	9	9	26.00	0.22	−1.22	−0.29	ns

Stabentheiner et al., 2021). We could simulate this under laboratory conditions with a small number of workerbees that manage to survive 2 hours of cold stress by effectively performing thermogenesis. This approach allowed us to analyze how the thoracic neuro-muscular system responds to cold stress at two distinct but essential levels (octopamine concentrations, expression of receptor genes) and to test the hypothesis whether this system is maintained at a constant level.

First of all, this requires the supply of sufficient amount of octopamine. In fact, we found no cold stress associated differences in octopamine levels in the MMTG and in the flight muscles. With the exception of forager bees, where the octopamine level increased in both flight muscles after 30 min but returned to equilibrium after 120 min. The MMTG harbors flight muscle innervating octopaminergic neurons, but if octopamine is not present in sufficient quantities or if binding to its receptors is prevented, thermogenesis is compromised (Kaya-Zeeb et al., 2022). Unfortunately, with our method we cannot quantify octopamine in real-time and we cannot distinguish between vesicular and released octopamine. Consequently, we do not know how much octopamine is released per release event, how often such events occur, how long it remains at the target site and if released octopamine is recycled effectively. Especially the latter would probably be an important mechanism to deal efficiently with limited resources under extreme conditions (Schroeder and Jordan, 2012) and simultaneously maintain flight muscle functionality. Future studies could address these issues by combining different methods, which may include electrochemical microsensors (Phillips and Wightman, 2003; Jarriault et al., 2018), electrophysiological recordings (Ting and Phillips, 2007) and molecular and functional analyses of honeybee monoamine transporters (Torres et al., 2003; Zhang et al., 2019).

Similar to octopamine concentrations, the overall octopamine receptor gene expression does not appear to be strongly affected by cold stress. This is remarkable because a cold stress-induced increase in the amount of flight muscle RNA_{total} points to an increased transcription efficiency and reflects the increased need for the provision of specific newly synthesized proteins. The fact that octopamine receptor gene expression is not delayed in this process underscores the importance of octopamine signaling to the flight muscles and suggests that receptors have turnover rates that require some degree of re-synthesis of receptor proteins if their function is to be maintained. The majority of G-Protein coupled receptors (GPCRs) owns ligand-activated mechanisms that cause an arrestin-mediated removal of the receptor protein from the plasma membrane (Lefkowitz and Shenoy, 2005; Kelly et al., 2008; Tobin et al., 2008) which is also true for octopamine receptors (Hoff et al., 2011). Internalized GPCRs can either be subjected to membrane reintegration or degradation. If the latter is the case in the flight muscles, *de novo* protein synthesis is required to maintain thermogenesis functionality.

This seems to be important for *AmOARβ2*, which apparently is subject to complex alternative splicing. Furthermore, the overall expression of the *AmOARβ2* gene might mask abundance differences of specific splicing isoforms. Here, only the transcript of *AmOARβ2X1A* seems to be associated with cold stress. This isoform encodes a functional described b octopamine receptor (Balfanz et al., 2013), which, according to our previous

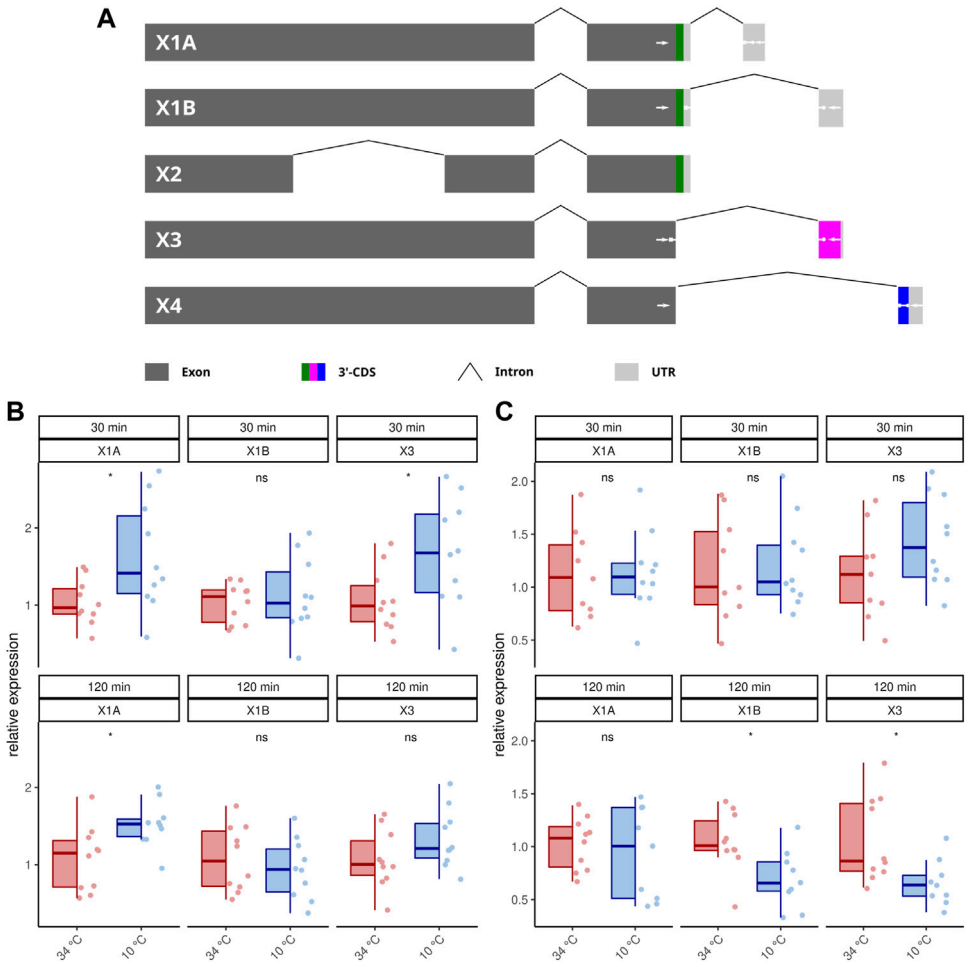

FIGURE 3
Differential splicing of *AmOARβ2*. **(A)** Schematic representation of *AmOARβ2* alternative splicing. Rectangles in dark gray represent the exons and the introns are visualized by chevron shapes. In multi color the 3′-coding sequence (3′-CDS, including the stop codon) is shown of each isoform. The untranslated region (UTR) is illustrated in light gray. White arrows demark sequences which get specifically targeted by the designed primers and probes (see also Table 1). **(B–C)** Quantification of the *AmOARβ2* isoform abundance in 1 week old workerbees **(B)** and forager bees **(C)**. For each group/data-set median ± IQR (left part) and individual data points (right part) are shown. * = $p < 0.05$, ns = $p \geq 0.05$, Mann-Whitney *U* test. For detailed statistics please see Table 2.

results, is the most likely octopamine receptor subtype in the service of thermogenesis (Kaya-Zeeb et al., 2022). The flight muscle abundance of the *AmOARβ2X1A* transcript is increased in 1 week old bees after 30 and 120 min of cold stress, even if the total expression of the *AmOARβ2* gene shows the same tendency only after 2 hours.

In contrast, in the flight muscles of forager bees cold stress does not elicit changes in *AmOARβ2* expression or *AmOARβ2X1A* transcript abundance. We assume that these expression response differences are related to the age-dependent expression of *AmOARβ2*. One-week old workerbees have a significant lower flight muscle expression of *AmOARβ2* than older workerbees (Kaya-Zeeb et al., 2022). It is possible that the flight muscle receptor expression of one-week-old workerbees are not yet at an optimal functional level and therefore receptor gene expression is up-regulated by cold stress. Octopamine signaling via b octopamine receptors is necessary for thermogenesis (Kaya-Zeeb et al., 2022) and this in turn is needed to withstand cold stress situations. If this is true, it is not surprising that in flight muscles of forager bees, which are usually older than 3 weeks (Winston, 1991), no increase of *AmOARβ2X1A* can be observed. Therefore, their receptor gene expression should already be at a functional level. The remaining *AmOARβ2* isoforms do not follow a consistent trend over time (1 week old bees) or are down-regulated after 2 hours (forager bees). For now, it is unlikely that these isoforms are important for thermogenesis, and future studies are needed to check whether the receptor proteins show the same trend as their

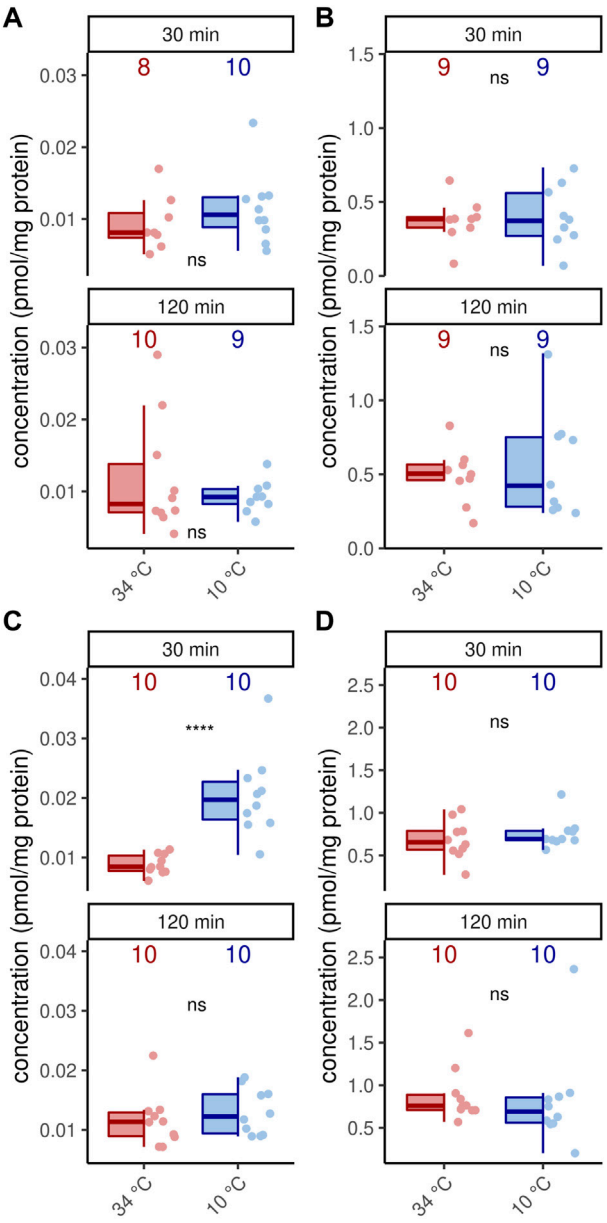

FIGURE 4
Octopamine concentration are stable under cold stress conditions. Only flight muscles after 30 min cold stress show increased octopamine concentrations (p = 0.00008, U = 8, z = −3.96, r = −0.89, Mann-Whitney U test). The remaining analysis reveal no differences in the flight muscles **(A,C)** nor the MMTG **(B,D)** in 1 week old workerbees **(A–B)** or forager bees **(C–D)**. ns = $p \geq 0.05$, Mann-Whitney U test. For each group/data-set median ± IQR (left part) and individual data points (right part) are shown.

mRNAs and to decipher their specific functions. This is also true for the remaining octopamine receptor genes, since we were not able to induce consistent expression changes by cold stress. We assume that they have important functions in flight muscle cells or associated tissues (e.g. nerves, trachea) as shown in flies (Sigrist and Andlauer, 2011; El-Kholy et al., 2015; Sujkowski et al., 2017, 2020). However, most likely they are not directly related to cold stress and thermogenesis.

Previously we could show that octopamine injections mimic cold stress induced *AmGAPDH* expression increase (Kaya-Zeeb et al., 2022). However, octopamine injections could not mimic cold-stress induced effects on RNA_{total} and *AmOARβ2* expression (including splicing). Furthermore, the changes in expression of the remaining octopamine receptor genes induced by the injections after 2 hours are probably nonspecific side effects, because comparable results are not observed under cold stress conditions. Thus, additional factors such as other neuromodulators (Salvador et al., 2021), hormones (Corona et al., 2007) or myokines (Pedersen et al., 2007; Pedersen, 2013; Schoenfeld, 2013) must be considered. It will be exciting to determine their properties and whether and how they coordinate with the octopaminergic system to maintain flight muscle function under cold stress.

Since the thoracic neuro-muscular octopaminergic system has an essential role in regulating thermogenesis, we expected a response to extreme temperature conditions. Ultimately, there are two plausible possibilities. The first option involves the up- and down-regulation of certain parts of the signaling cascade as required (Hadcock and Malbon, 1988a,b; Kim et al., 2003). Alternatively, critical components can be maintained on a constant synthesis and turnover rate (steady-state) to enable continuous operational readiness (Brodie et al., 1966; Kim et al., 2003). To date, our data suggest that the latter is the case. Constant octopamine concentrations indicate that octopamine can be released to the flight muscles if necessary, whether under optimal thermal conditions or under cold stress when thermogenesis is at full load. The same is true for octopamine receptor expression. This behavior of the thoracic neuro-muscular octopaminergic system makes perfect sense, since honeybees rely heavily on their abilities to regulate temperature (Stabentheiner et al., 2003, 2010). If they are not immediately available or even fail, the survival of the colony is very likely to be at risk. In the face of progressive climate change, the capability of thermostasis becomes increasingly important. The ability to maintain important physiological processes during increasingly frequent extreme weather events with sharply falling or rising temperatures represents a major challenge for all living organisms. Almost all relevant physiological processes are subject to a strong temperature dependence in their reaction rate (Hegarty, 1973; Reyes et al., 2008). Honeybees seem to be well equipped for such challenges and thermogenesis (and with this octopamine signaling) seems to play a crucial role here.

The question arises whether and how long the system can be kept functional if critical resources are scarce or no longer available. Our results show that 1 week old workerbees

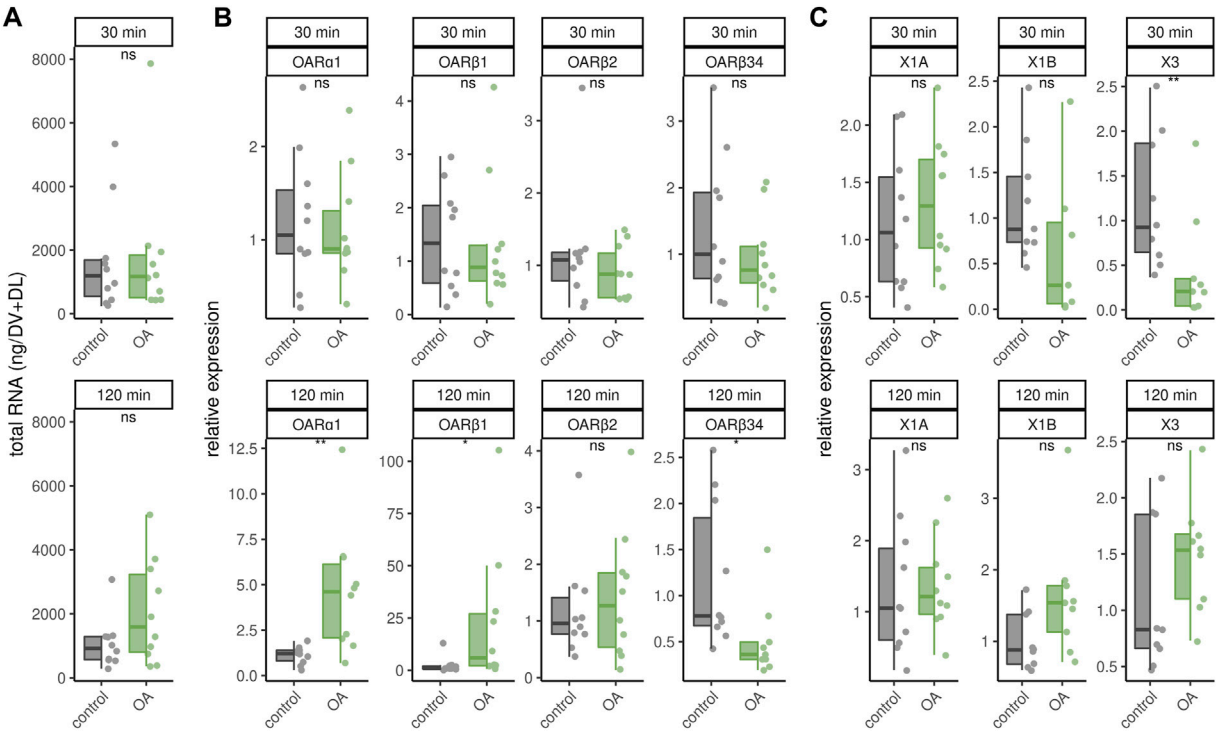

FIGURE 5
Effects of octopamine injections. Workerbees received octopamine injections in their flight muscles. After 30 and 120 min we measured RNA_{total} concentration **(A)**, octopamine receptor gene expression **(B)** and *AmOARβ2* isoform abundance **(C)**. * = $p < 0.05$, ** = $p < 0.01$, ns = $p \geq 0.05$, Mann-Whitney *U* test. For each group/data-set median ± IQR (left part) and individual data points (right part) are shown. For detailed statistics please see Table 3.

provided with only a carbohydrate source, but not amino acids or proteins, cannot maintain thermogenesis. Bees at this age are capable of thermogenesis (Stabentheiner et al., 2010). However, in our case they will lack essential amino acids (de Groot, 1953), which are required for the synthesis of octopamine and other monoamines (Roeder, 1999; Blenau and Thamm, 2011) as well as proteins (Cremonz et al., 1998). In addition, a possible deficiency of essential fatty acids, vitamins and electrolytes must be considered (Gribakin et al., 1987; Zarchin et al., 2017). Although these bees also respond to cold stress with a massive up-regulation of transcription in their flight muscles, they are incapable of effective thermogenesis and fall into a chill coma. It is conceivable that there is not enough octopamine available for thermogenesis or that the flight muscle is not optimally developed, or a combination of both. Future experiments need to show how certain aspects of nutrition affect the steady-state of the thoracic neuro-muscular octopaminergic system and how limited resource availability may disrupts thermoregulation at both the individual and colony level. This could become apparent if natural food resources are increasingly decimated by the ongoing intensification of agricultural cultivation) (Rich and Woodruff, 1996; Biesmeijer et al., 2006; Vaudo et al., 2015).

With this study, we provide an important contribution to the better understanding of the fundamental and essential physiological response of worker honeybees to cold stress. Moreover, we provide an important basis for comparative analyses with species that are not as resistant to temperature stress as honeybees. To capture both extreme possibilities of extreme weather events, future studies should definitely include heat stress. Continuing these analyses will help us gain a more comprehensive picture of strategies for adapting to changing environmental conditions, whether in the context of natural evolutionary processes or in the course of global change.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repositories and accession number(s) can be found below: www.figshare.com, doi.org/10.6084/m9.figshare.20509509.v1; https://www.ncbi.nlm.nih.gov/genbank/, ON988383-ON988387.

Author contributions

SK-Z and MT designed the study. SK-Z, AM, OS-C and MT developed the methodology. SK-Z, SD, LW and AM acquired and analyzed the data. MT contributed to data analysis and provided funding. All authors contributed to manuscript preparation.

Funding

This work was supported by a grand of the Deutsche Forschungsgemeinschaft to MT (TH2264/2-1).

Acknowledgments

We thank Dirk Ahrens-Lagast for bee keeping; Johannes Spaethe, Ricarda Scheiner and Petra Högger for supplying technical equipment. A special thanks goes to Christian Wegener, Flavio Roces, Johannes Spaethe, Felix Schilcher and Ricarda Scheiner for fruitful discussions and helpful comments. This publication was supported by the Open Access Publication Fund of the University of Würzburg.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1002740/full#supplementary-material

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frontiers

Robustness of the honeybee neuro-muscular octopaminergic system in the face of cold stress - Supplementary Material

1 SUPPLEMENTARY TABLES AND FIGURES

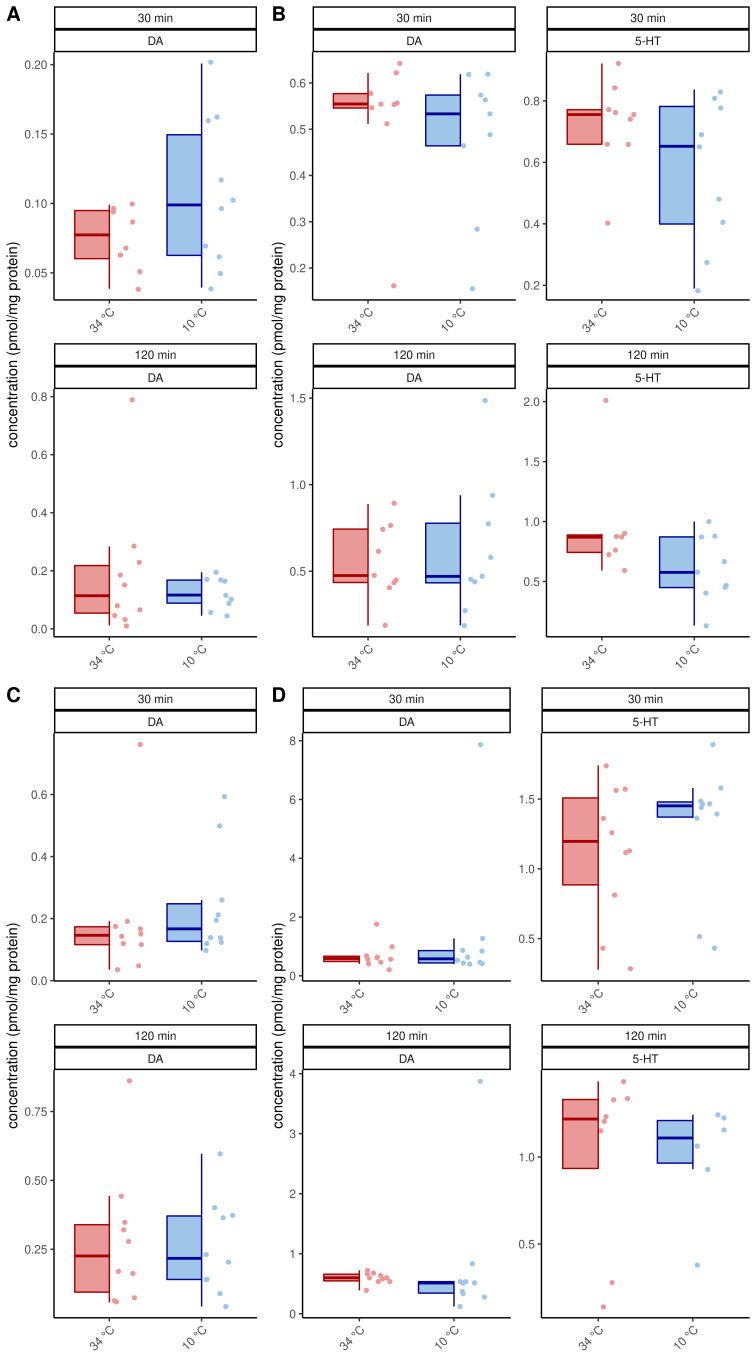
1.1 Tables

Results of the statistical analysis (Mann-Whitney *U* test) of dopamine (DA) and serotonin (5-HT) quantification in workerbee flight muscles (DV+DL) and mesometathoracic ganglion (MMTG) under cold stress. Serotonin was only detectable in the MMTG but not the flight muscles. Tyramine was not quantifiable in many samples and was therefore not considered. For visualization see Figure S1.

bees	tissue	time	amine	$N_{34\,°C}$	$N_{10\,°C}$	*U*	*p*	*z*	*r*
one week old	DV+DL	30 min	DA	8	10	26.00	0.24	-1.18	-0.28
one week old	DV+DL	120 min	DA	10	9	45.00	1.00	0.00	0.00
one week old	MMTG	30 min	DA	9	9	50.00	0.44	-0.78	-0.18
one week old	MMTG	30 min	5-HT	9	9	55.00	0.22	-1.22	-0.29
one week old	MMTG	120 min	DA	9	9	38.00	0.86	-0.17	-0.04
one week old	MMTG	120 min	5-HT	7	9	47.00	0.11	-1.58	-0.40
forager	DV+DL	30 min	DA	10	10	37.00	0.35	-0.93	-0.21
forager	DV+DL	120 min	DA	10	10	48.00	0.91	-0.11	-0.02
forager	MMTG	30 min	DA	10	10	48.00	0.91	-0.11	-0.02
forager	MMTG	30 min	5-HT	10	10	36.00	0.32	-1.00	-0.22
forager	MMTG	120 min	DA	10	10	76.00	0.05	-1.94	-0.43
forager	MMTG	120 min	5-HT	8	6	31.00	0.41	-0.82	-0.22

1.2 Figures

Dopamine (DA) and Serotonin (5-HT) concentrations in the flight muscles (A, C) and the MMTG (B, D) under cold stress conditions. For statistics see Table S1. For each group/data-set median ± IQR (left part) and individual data points (right part) are shown.



Chapter

FOUR

DISCUSSION

The work presented tells a complete and straightforward story highlighting the importance of octopaminergic signaling in honeybee flight muscle in the context of thermogenesis. Specific findings of Chapter 2 and Chapter 3 are summarized and discussed in the following discussion.

At the beginning of my doctoral studies, I hypothesized that octopamine might exhibit a regulatory function in honeybee thermogenesis. This is based on and supported by other publications (Chapter 1). I surmised that the observed octopaminergic effects are likely mediated via AmOARβs. The first hypothesis was tested and confirmed in Chapter 2 by HPLC-ECD quantification and anti-octopamine labeling. In these experiments, octopamine was detected for the first time in the DV, DL, MMTG as well as in nerve cords within the honeybee thorax (Fig. 1 & 2 in: Chapter 2). Specifically, octopaminergic neurons were found to project from the ganglion and directly innervate both flight muscles (Fig. 2 in: Chapter 2). The expression of AmOARβ2 in the muscle tissue opens the opportunity of an involvement in mediating octopaminergic effects (Fig. 3 in: Chapter 2). Additionally, octopamine and respective receptor gene transcripts showed an age-dependent increase in the flight muscles of naive animals (Fig. 1 & 3 in: Chapter 2). An in-depth investigation of the associated intracellular receptor cascade (Fig. 7 in: Chapter 2) was made possible by pharmacological manipulations combined with thermographic recordings. This could establish a direct link between the investigated octopaminergic system and shivering thermogenesis. In order to confirm the importance of octopamine signaling for shivering thermogenesis, various receptor ligands (agonists, antagonists) were used in Chapter 2. These ligands were tested on reserpinized and on naive animals (Fig. 4 & 5 in: Chapter 2). Finally, further experiments elucidated multiple steps of the intracellular cascade and substantiate the hypothesis that the octopaminergic effect derives from AmOARβs (Fig. 4 & 6 in: Chapter 2). These findings indicate that octopamine signaling fuels honeybee thermogenesis.

A link between octopaminergic signaling in the honeybee thorax and cold stress was reported

in Chapter 3. I postulated that the neuro-muscular octopaminergic system must remain unaffected or be upregulated under cold stress to ensure an adequate and required octopamine signaling. This includes octopamine, but also the expression of the associated receptor genes, particular *AmOARβ2*. Expanding on the hypotheses from Chapter 2, I anticipated that *AmOARβ2* might exhibit a cold-sensitive gene expression pattern. Subsequently, a series of experiments revealed that the octopaminergic neuro-muscular system strives for an equilibrium under cold stress (Chapter 3). This is demonstrated by constant octopamine titres and *AmOAR* gene expression in flight muscles of worker bees under cold exposure at varying time points. Furthermore, transient upregulation of individual receptor genes and octopamine titres occurred (Fig. 2 & 4 in: Chapter 3). I found evidence that *AmOARβ2* and its X1A isoform are sensitive to cold stress (Fig. 2 & 3 in: Chapter 3). This further supports the initial hypothesis from Chapter 2 about the involvement of AmOARβ2 in thermogenesis. In order to successfully cope with the cold due to shivering thermogenesis, artificial conditions appear to have a major impact on worker bees. In cage-reared worker bees, cold stress manifests in an enormous RNA_{total} increase that forms a non-physiological pattern (Fig. 1 in: Chapter 3). This indicates the importance of the physiological context for the successful execution of thermogenesis. These results further support, the requirement for an unimpaired octopaminergic neuro-muscular system. In summary, Chapter 3 reveals that the octopaminergic neuro-muscular system strives for a steady-state under cold conditions. If this system is out of balance, successful thermogenesis cannot be conducted. The major findings of Chapter 2 an Chapter 3 are condensed into Figure 4.1.

The following parts of the discussion will address specific findings in more detail. First, I will examine what might cause an enormous cold-induced RNA_{total} increase in forager bees and nurse-aged bees (Fig. 1 in: Chapter 3). In a second part of the discussion, the presence of dopamine in the flight muscles will be covered (Fig. 1/S1 in: Chapter 2 & Fig. S1 in: Chapter 3). Until this point, the physiological role of dopamine in this context remains unclear.

4.1 Metabolism of Biogenic Amines

The cold-induced increased RNA_{total} (Fig. 1 in: Chapter 3) may include transcripts that encode enzymes for biosynthesis or metabolism and membrane carriers of octopamine. The thoracic neuro-muscular octopaminergic system of the honeybee strives for an equilibrium and keeps the corresponding receptor gene expression fairly constant despite a global increase in gene expression (Chapter 3). Forager bees initially respond to cold stress with a burst of octopamine release at the flight muscle (Fig. 4 in: Chapter 3). After 120 minutes of cold exposure, the octopaminergic system has acclimatized and regained its equilibrium (Fig. 4 in: Chapter 3). This finding makes forager bees the preferred population for further investigation of octopamine-modulated thermogenesis. Due to the transient and local increase in octopamine levels, the opportunity arises to study the enzymatic formation and degradation of octopamine in more detail and interfere with these processes.

The question still remains, what happens to the extracellular transmitter molecules after their release. While several studies have focused on biosynthesis (Chapter 1), the metabolism of biogenic amines in insects remains fragmentary. Multiple physiological routes arise for the metabolism of extracellular biogenic amines. However, most importantly, an over stimulation of the corresponding amine receptors needs to be prevented, by clearing the residual receptor ligands within the synaptic cleft. This is achieved by inward transport into nearby cells (reuptake) and subsequent degradation, inactivation and recycling (Axelrod et al., 1970; Trendelenburg, 1979; Evans, 1980; Torres et al., 2003; Foye, 2008; Finberg, 2019; Aktories et al., 2022).

The monoamine oxidase (MAO) enzyme family is specialized in the deamination of monoamines. In mammals, two isoforms MAO A and MAO B exist and are localized intracellularly at the outer mitochondrial membrane. Both isoforms differ in their tissue and substrate specificity (Edmondson et al., 2004; Cho et al., 2021). MAO A shows a high affinity for the substrates (nor)epinephrine and serotonin. MAO B exhibits a higher affinity for dopamine and tyramine (Saura et al., 1992; Edmondson and Binda, 2018; Finberg, 2019; Moriguchi et al., 2019; Cho et al., 2021). Enzymatic assays demonstrate MAO-related activity in insects. However, in various body tissues examined, these analyses indicate low activity profiles compared to mammals. Blocker experiments suggest putative structural similarity to the human MAO B. Therefore, one can assume existence and relevance of MAO metabolism in insects. This is endorsed by the presence of MAO and catechol-O-methyltransferase (COMT) driven metabolites. However, neither a MAO gene nor a COMT gene have been found so far in insects (Evans, 1980; Sloley, 2004; Paxon et al., 2005; Kutchko and Siltberg-Liberles, 2013; Vavricka et al., 2014; Yamamoto and Seto, 2014).

COMT metabolism is restricted to biogenic amines that harbour a catechol function, such as dopamine and (nor)epinephrine. However, other amines can also be targeted by COMT if

they have previously been endowed with the catechol structure. In the honeybee, this applies to tyramine and octopamine when hydroxylated at the aromatic vicinal carbon. CYP2D6 can catalyze the formation of dopamine from tyramine (Figure 1.1) and octopamine can be hydroxylated to racemic norepinephrine. Formation of these products can also occur in the course of non-enzymatic oxidations (Axelrod, 1963; Evans, 1980; Hiroi et al., 1998; Jacob et al., 2005; Vavricka et al., 2014).

As the enzymatic activities of MAO and COMT are very low in insect tissues, it is discussed in the literature that other enzymes can substitute for this metabolic gap (Sloley, 2004; Paxon et al., 2005). In this context, it is striking that the activity of N-acetyltransferase (NAT) is several-fold higher in various tissues. NATs catalyze for an acetyl conjugation of amines. During this process, their chemical structure is significantly changed and they become inactive (Downer and Martin, 1987; Evans, 1980; Sloley, 2004; Paxon et al., 2005). Whereas humans harbour a low number of genes for arylalkylamine NATs, insects evolved a variety of homologues. The high activity of NATs, genetic abundance and the low enzymatic activities of MAO and COMT suggest that acetylation by NAT is a major deactivation pathway for monoamines in insects (Sloley, 2004; Vavricka et al., 2014; Yamamoto and Seto, 2014).

The reuptake of residual amines from the synaptic cleft is a requirement for the predominantly intracellular metabolism of MAO as well as COMT and NAT (Trendelenburg, 1979; Edmondson et al., 2004; Minchin et al., 2007; Tahay et al., 2012; Cho et al., 2021). Membrane bound reuptake transporters along with vesicular monoamine transporters (VMATs) belong to the family of solute carriers (Blakely and Edwards, 2012). Reuptake monoamine transporters (MATs) show a high substrate affinity and function in a sodium-dependent manner. They transport amines alongside a defined number of Na^+ (Pacholczyk et al., 1991; Kilty et al., 1991; Torres et al., 2003; Penmatsa et al., 2013; Martin and Krantz, 2014; Coleman et al., 2016).

Information on MATs in insects is inconsistent. Both, serotonin transporter (SERT) and dopamine transporter (DAT) have been reported in *Drosophila*, *Apis mellifera* and other insects (Corey et al., 1994; Penmatsa et al., 2013; Martin and Krantz, 2014; Zhang et al., 2019; Bombardi et al., 2022). In contrast, no octopamine transporter (OAT) has been found in *Drosophila* and *Apis mellifera* and the majority of insects. It remains unclear how the extracellular space is cleared of octopamine in species missing OATs (Martin and Krantz, 2014; Arancibia et al., 2019). Two decades ago, a functional OAT was found in the pest *Trichoplusia ni* (Malutan et al., 2002). However, our own blast analysis shows a high alignment towards the *AmDAT*. Therefore, one cannot conclusively know whether a functional OAT or DAT was found in *Trichoplusia ni*. It was hypothesized, if species lack OAT, that other MATs might carry out the task of octopamine transport. Although the interaction between octopamine and DAT has been simulated, its physiological existence

remains questionable so far (Arancibia et al., 2019). Therefore, the search for a hymenopteran OAT is still ongoing.
In direct comparison to mammals, insect metabolism of biogenic amines appears to be less precise and results in a variety of different metabolites (Sloley, 2004). The transport routes and transport proteins remain largely unrevealed. As specific amines can be detected, metabolic pathways must exist for the corresponding synthesis, degradation and specific transport.

4.2 Potential Targets of Pharmacological Intervention within Biosynthesis and Metabolism of Octopamine and Dopamine

The biosynthesis and the metabolism of biogenic amines offer several potential drug targets. Possible interventions within these pathways are described below with a focus on octopamine and dopamine. For an overview of the biosynthesis of octopamine and dopamine see Figure 1.1. PAH and TH can be inhibited by alpha-methyl-tyrosine via competitive inhibition (Spector et al., 1965; Murthy, 1975; Sotnikova et al., 2010). Additionally, TH can also be targeted and silenced by RNA interference (RNAi; Sterkel et al., 2019). Compounds from the therapeutic regimen of Parkinson's disease (PD) can be used to inhibit the DDC. Benserazide and carbidopa represent peripheral DDC inhibitors in this context (Greenacre et al., 1976; Shimizu et al., 2004). In the octopamine pathway, TDC can be inhibited by small molecules like tyrosine-methyl-esters and tyrosine amides as well as nicotinic acid (Zhu et al., 2016; Kang et al., 2018). The messenger RNA of TBH can be knocked down by RNAi and the functional protein can also be conventionally blocked by 1-arylimidazole-2(3H)thiones or targeted by antibodies (Matsuo et al., 2016; Hasan et al., 2016; Xu et al., 2018). The effects of these interventions negatively impact the affected synthesizing enzyme and restrict its activity. As a result, the endogenous level of biogenic amines in the affected tissue may decrease.
In order to increase endogenous amine titres, it is necessary to inhibit the metabolizing pathways. MAO inhibitors (MAOIs) are used in treatment regiments of various affective as well as neurological disorders (e.g. PD). Within the class of MAOIs a distinction can be made between reversible and irreversible compounds. Clorgiline is a irreversible and moclobemide a reversible inhibitor of MAO A. Rasagiline and selegiline are irreversible and safinamide is a reversible inhibitor of MAO B. All substances mentioned, may suppress the metabolism of reintroduced dopamine. These interventions allow the residual amines to remain intact longer and increase their titre (Saura et al., 1992; Edmondson and Binda, 2018; Finberg, 2019; Moriguchi et al., 2019; Cho et al., 2021). In addition to MAO, COMT can also

be inhibited. COMT inhibitors or "capones" (e.g. entacapone, tolcapone) can also cause elevated amine levels. Capones are originally developed to improve the bioavailability of L-DOPA applications in PD. Since COMT metabolism is specialized in catecholamines and (nor)epinephrine is not found in insects, the use of COMT inhibitors is limited to dopamine and related substances (Holm and Spencer, 1999; Keating and Lyseng-Williamson, 2005; Schrag, 2005; Finberg, 2019).

4.3 Involvement of Glycolytic Pathways

An unexpected high quantity of RNA_{total} occured during cold stress conditions. This indicates a participation and presence of various transcripts encoding for glycolytic enzymes (Fig. 1 in: Chapter 3). Detailed investigation of the responsible intracellular cascade revealed an increase in total pyruvate after *in vitro* octopamine bath applications in DL (Fig. 6 in: Chapter 2). In both flight muscles, the *AmGAPDH* gene expression was upregulated in response to octopamine application (i.m.) and cold stress (Fig. 6 in: Chapter 2). Since pyruvate represents the product and AmGAPDH the sixth enzyme of glycolysis, these findings suggest that muscular octopamine signaling is involved in glycolytic pathways and thus may fuel shivering thermogenesis (Shestov et al., 2014; Kaya-Zeeb et al., 2022b). Fructose-1,6-diphosphate and pyruvate levels increase transiently under cold stress in mice and then drop sharply. This indicates a massive readjustment of glycolysis turnover and therefore changes in the respective gene expression pattern can be anticipated (Liu et al., 2019). The glycolytic pathways are vital for maintaining a steady supply of ATP at a high level (Beneke and Boning, 2008). During acute or chronic cold stress honeybee shivering thermogenesis must occur for the sake of survival. Physiology may change with respect to gene expression pattern under muscle effort. Furthermore, shivering thermogenesis places enormous stress on the flight muscle, making recruitment of repair mechanisms a plausible consequence (Beneke and Boning, 2008; Schmitz et al., 2010; Ohlendieck, 2010). In *Drosophila melanogaster* and *Danio rerio*, the expression of glycolytic enzymes is directly involved in muscle development and growth. Their suppression leads to a muscular degenerated phenotype (Tixier et al., 2013). Besides pyruvate and AmGAPDH, other metabolites and enzymes of the glycolytic pathways may also be in focus of alterations.

It can be hypothesized that the energy-demanding process of shivering is accompanied by plastic changes in gene expression of the enzymatic glycolytic pathway (Beneke and Boning, 2008; Ohlendieck, 2010). This has already been shown for *AmGAPDH* in (Fig. 6 in: Chapter 2). Further investigations should focus on rate-limiting reactions. Here, phosphofructokinase (PFK) and pyruvate kinase (PK) represent enzymatic bottlenecks within glycolytic pathways (Schmitz et al., 2010). Changes in the gene expression pattern of PFK and PK during cold stress and shivering thermogenesis could be quantified by quantitative

polymerase chain reaction (qPCR) experiments. Due to the vast number of involved enzymes in the glycolytic pathways, the highly sophisticated method of RNA sequencing (RNA-Seq) offers multiple advantages compared to conventional qPCR techniques. RNA-Seq provides decisive time saving in experimental sample preparation and sample processing. Additionally, RNA-Seq analyses cover large parts or the entire transcriptome. In this way, gene expression pattern that were previously unknown or out of focus can be tracked and included in further analysis. In return, the computational and statistical follow-up is more time and energy consuming than conventional qPCR. Ideally, the RNA-Seq results should then be combined with activity assays of the respective enzymes. The high resolution and highly sophisticated methods of nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) could be harnessed for the analysis of small molecules (e.g. metabolites). NMR and MS-based metabolomics in combination are able to identify and quantify both known and unknown metabolites. Highly sensitive, state-of-the-art devices are particularly advantageous in this context. These analytical methods fulfil the necessary requirements in order to analyze desired metabolic intermediates and products of the glycolytic pathways (Smilde et al., 2005; Griffiths et al., 2010; Lei et al., 2011; Nagana Gowda et al., 2018). With the techniques described, it is possible to test whether and to what extent glycolytic turnover rates differ under cold stress and thus to assess metabolic activity during shivering thermogenesis in detail.

4.4 Heat Shock Proteins

Cold stress-related global upregulation of the RNA_{total} might indicate an increase of HSPs associated transcripts (Fig. 1 in: Chapter 3). Heat shock effects in insects were first studied in *Drosophila melanogaster* approximately 60 years ago. The subsequent discovery of HSPs and their increased formation as a cellular stress response has catapulted molecular research forward (Ritossa, 1962; Tissiéres et al., 1974; Kim et al., 1998; De Maio et al., 2012). Meanwhile, HSPs are the subject to extensive research in many species including *Caenorhabditis elegans*, *Anopheles gambiae*, *Drosophila melanogaster*, *Bombyx mori*, *Apis mellifera*, *Danio rerio*, *Mus musculus* and *Homo sapiens* (Aevermann and Waters, 2008; Waters et al., 2008; Li et al., 2009; Richter et al., 2010; Lahvic et al., 2013). HSPs are categorized according to their molecular weight and size: HSP60, HSP70, HSP90, HSP100, sHSP (Koyasu et al., 1989; Kim et al., 1998; Neckers and Ivy, 2003; Habich and Burkart, 2007; Ahamed et al., 2010; Lahvic et al., 2013). The term "small heat shock protein" (sHSP) is used for a group of smaller representatives that feature a highly conserved binding domain (Li et al., 2009; Lahvic et al., 2013). The molecular mechanism of HSPs is to maintain the structural integrity and thus functionality of endogenous proteins (carriers, cytoskeleton, enzymes, receptors) by preventing their denaturation and to ensure correct refolding (Li et al., 2009; Richter et al.,

2010; Jee, 2016). Although HSPs form a highly conserved class within clades, they may differ greatly from insects to mammals from a genetic and evolutionary perspective. Regardless of the organism, all HSPs serve a purpose in molecular defense against biotic and abiotic stressors (Li et al., 2009; Richter et al., 2010; Jee, 2016). HSPs are part of the first line of defense against various stressors (cold, heat, oxidative species, hypoxia and exercise). They serve intracellular homeostasis and thermotolerance, hence their upregulation may proceed quickly (Moseley, 1997; Aevermann and Waters, 2008; Waters et al., 2008; Li et al., 2009; Åkerfelt et al., 2010; Dimauro et al., 2016; Jee, 2016).

Under cold stress conditions, there is not only an increase in thoracic temperature due to shivering thermogenesis, but might also increased release of reactive oxygen species (ROS) and increased oxygen consumption by the musculature. HSPs are primed to protect tissues from these factors. Therefore, increased expression of HSPs can be anticipated and should be reflected within the RNA_{total} (Fig. 1 in: Chapter 3). Not only because of the cold stress, but also the accompanying factors of shivering thermogenesis, oxidative stress and hypoxia. High-resolution respirometry via O2k oxygraphs (Oroboros Instruments, Innsbruck, Austria) can be harnessed for analyzing hypoxia, ROS formation, mitochondrial membrane potential as well as Ca^{2+} flux in flight muscle homogenate (Garedew et al., 2005; Masson et al., 2017). The gene expression of HSPs can be analyzed with the same techniques as the metabolizing enzymes (qPCR, RNA-Seq). Methods that purely rely on gene expression analysis have a decisive disadvantage: they do not provide any information about the putative protein status. A change at the expression level is not necessarily reflected at the protein level. MS-based proteomic analysis can remedy this situation. The powerful combination of HPLC, MS and bioinformatical tools enable the investigation of the proteome to a scale that captures even complex post-translational modifications. The disadvantage of this method is, as with other highly sophisticated methods (e.g. RNA-Seq), the monetary effort for high performance equipment (Liang and Chan, 2007; Sinha et al., 2010; Xiao et al., 2019; Zhao et al., 2019).

4.5 Presence of Dopamine in Flight Muscle Tissue

Significant amounts of dopamine were found in individual flight muscles (Fig. 1/S1 in: Chapter 2 & Fig. S1 in: Chapter 3). Based on our current findings, a direct role of dopamine in honeybee thermogenesis is questionable. Reserpination results in a significant decrease in muscular octopamine titre, but not dopamine titre (Fig. 4/S1 in: Chapter 2). Reserpine is expected to have a potent depletion effect on dopaminergic synapses (Elverfors and Nissbrandt, 1991; Ginovart et al., 1997; Kannari et al., 2000; Metzger et al., 2002; Martínez-Olivares et al., 2006). It is unlikely that reserpine elicits no effect on honeybee putative VMATs, because of structural and functional similarities between insect and mammalian VMATs. Consistently, reserpine exhibits an effect at dopaminergic VMATs in *Drosphila* (Bainton et al., 2000; Greer et al., 2005; Vickrey et al., 2009; Martin and Krantz, 2014). The lack of an impact on dopamine levels could be interpreted as an absence of neuro-muscular dopaminergic innervation of the flight muscle. This makes haemolymph a plausible route of transmission for muscular dopamine. Appearance of physiological escape phenomena could also account for the absence of a reserpine effect on muscular dopamine levels. If the activity and expression of dopamine biosynthesis enzymes are upregulated, reserpination could be masked (e.g. TH, Figure 1.1; Cubells et al., 1995; Martínez-Olivares et al., 2006).

In order for dopamine to elicit an effect at the flight muscles, respective receptors must be present. In this context, low levels of *AmDAR* gene transcript expression in the flight muscles of both nurse bees and forager bees can be attributed to *AmDAR1*, but its physiological relevance has not been validated so far (Jasper et al., 2015). Ultimately, a role of dopamine in shivering thermogenesis remains unlikely, because no dopaminergic effect regarding thermogenesis could be validated (Fig. 4/S3 in: Chapter 2).

The detected dopamine (Fig. 1/S1 in: Chapter 2 & Fig. S1 in: Chapter 3) may not be present in the muscle tissue itself, but may originate from the surrounding tissue. This mainly refers to flight muscle supplying tracheae. In this context, the D_1 receptor exerts regulatory functions in airway smooth muscle cells of mammals (Cabezas and Velasco, 2010; Mizuta et al., 2013). Comparable functions of dopamine in the tracheal system of insects are conceivable. Here, Dopamine is involved in tracheal development and tracheal tube formations in *Drosophila* (Hsouna et al., 2007; Pérez et al., 2010). In the honeybee, muscle and tracheal tissue are inextricably linked (Hickey et al., 2022). This complicates chemical and molecular analyses as performed in Chapter 2 and Chapter 3, but questions also other studies (Margotta et al., 2012; Jasper et al., 2015; Kaya-Zeeb et al., 2022b,a). Therefore, it can be assumed that artifacts may be caused by adherent tissue (especially tracheae) during muscle dissections (Hickey et al., 2022). In summary, one possible explanation for the presence of dopamine is a function in muscle-penetrating tracheae.

Based on its physicochemical properties, dopamine may serve another role in flight muscle tissue besides being a receptor ligand. While several biogenic amines exhibit antioxidant activity, the activity of dopamine is particularly high compared to tyramine, norepinephrine, and others. This property can be exploited both *in vitro* and *in vivo* by reducing and scavenging ROS. Elevated ROS levels have a high tissue-damaging potential and are associated with a variety of diseases (e.g. neurodegenerative disorders). In PD, the therapeutic target may consist of both dopaminergic agonism and a reduction of oxidative stress. In this context, stimulation of the D_2 mediates enzymatic cell protection effects (Yen and Hsieh, 1997; Iida et al., 1999; Kanazawa and Sakakibara, 2000; Dumont and Beal, 2011; Kawagishi and Finkel, 2014; Newland et al., 2016; Finberg, 2019). Now this information can be applied to the existing data on dopamine in the flight muscles of the honeybee (Fig. 1/S1 in: Chapter 2 & Fig. S1 in: Chapter 3). During physical activity and muscle contractions, as is the case with flying and thermogenesis, a high quantity of ROS may released from mitochondria (Ferreira and Reid, 2008; Reid, 2008; Scheele et al., 2009; Westerblad and Allen, 2011). It remains unknown how the honeybee flight muscles deal with this oxidative stress (Hickey et al., 2022). In this regard, the detected dopamine may be an important myoprotective and neuroprotective agent by scavenging ROS and strengthening the cellular defense through receptor stimulation. To test the quantity of ROS release during different physiological states (flying, shivering), but also various parameters of mitochondrial activity in flight muscle homogenate, high-resolution respirometry (Oroboros Instruments, Innsbruck, Austria) represents a powerful tool (Garedew et al., 2005; Masson et al., 2017). Additionally, various direct and indirect assays for oxidative stress are commercially available (Wang and Joseph, 1999; Agarwal and Majzoub, 2017). These methods could be combined with interventions manipulating the endogenous dopamine titre or exogenous administration of dopamine. In this way, it would be possible to measure the oxidative stress in the muscles and to test whether the dopamine present had an influence on it.

The next section will address a concrete analytical problem in detail. In deuterostomia and protostomia, COMT metabolism of dopamine results in 3-Methoxytyramine (3-MT). The receptor affinity of 3-MT differs clearly from its precursor. 3-MT has an inhibitory effect on the adrenergic system and thus may prevent overstimulation (Evans, 1980; Karoum et al., 1994; Paxon et al., 2005; Antkiewicz-Michaluk et al., 2008; Sotnikova et al., 2010; Rich et al., 2022). Furthermore, changes in the levels of dopamine lead to changes in the levels of 3-MT. As we were able to detect dopamine in flight muscles and MMTG (Fig. 1/S1 in: Chapter 2 & Fig. S1 in: Chapter 3), it stands to reason that its COMT metabolite is also present (Evans, 1980; Sloley, 2004; Paxon et al., 2005; Vavricka et al., 2014; Yamamoto and Seto, 2014). The physicochemical properties of 3-MT resemble those of other biogenic amines. It can be oxidized at coulometric cells with a working potential of +400 mV. In this context, 4-hydroxy-3-methoxybenzylamine may serve as an internal standard (Heal et al., 1990;

Lenders et al., 1993; Eisenhofer et al., 2005, 2012). Both compounds are commercially available. In summary, 3-MT could be separated and quantified by the isocratic HPLC-ECD method used in Chapter 2 and Chapter 3. Detection of 3-MT alone would be an important first indication of the presence of COMT activity in flight muscles and adjacent tissues. In a second step, further experiments could be performed by pharmacological manipulation of dopamine and 3-MT levels. Administration of COMT inhibitors should result in a decrease in 3-MT levels but an increase in dopamine levels. Moreover, alternative metabolic pathways for biogenic amines could be inhibited (e.g. via MAOIs) to narrow down available routes and thus further increase the turnover rate of dopamine to 3-MT via COMT.

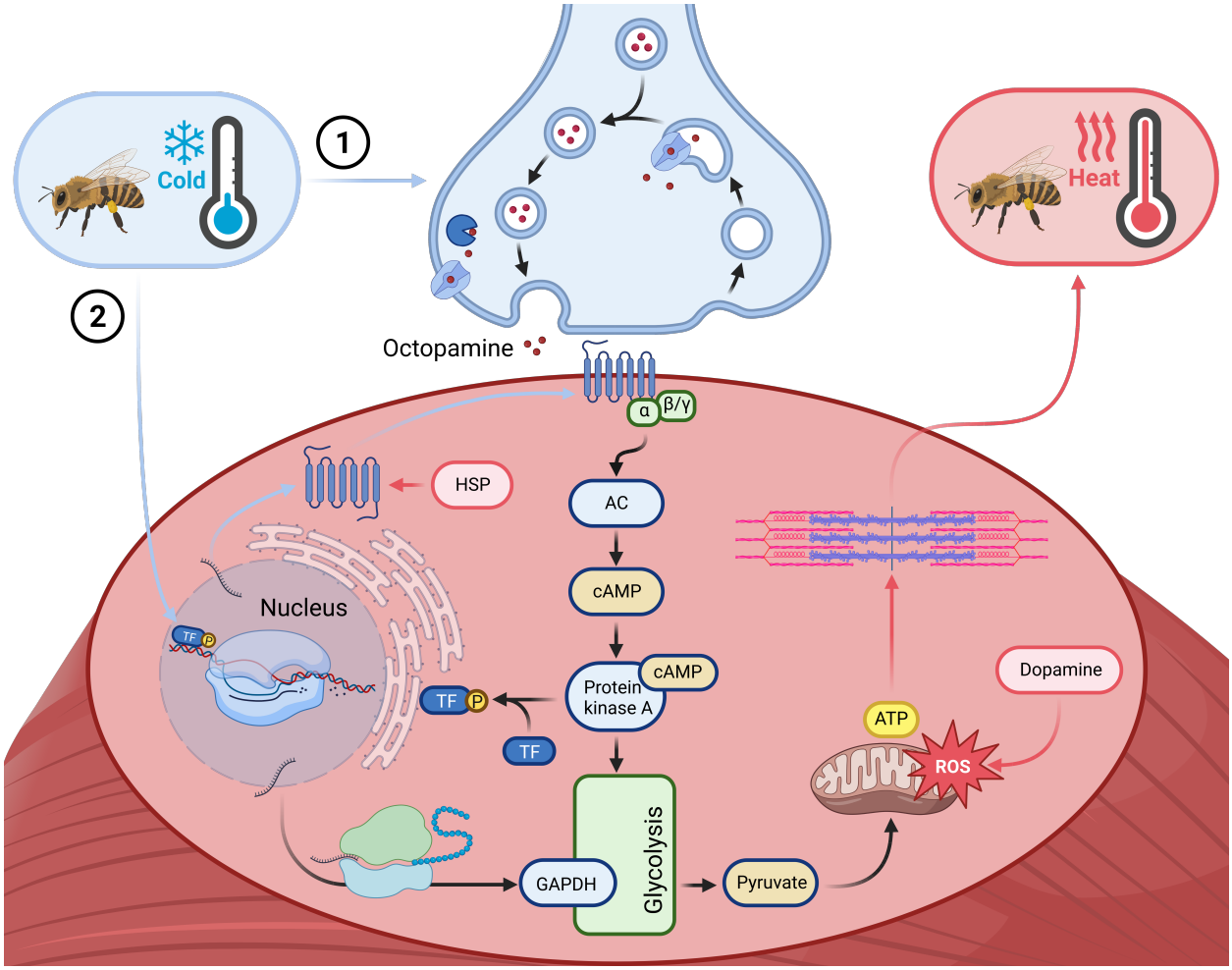


Figure 4.1: **Schematic overview of the major findings of this thesis and the discussed metabolic pathways.** Upon cold exposure, innervating neurons maintain octopamine release at the flight muscles. This is perceived by stimulatory G protein coupled octopamine β receptors. Additionally, cold stress increases overall transcription and conflicts with the strive for a stable relative expression of the associated receptor genes. Subsequent unraveled intracellular cascade driven by the second messenger cAMP leads to multiple effects, ultimately fuels thermogenesis and therefore increases the temperature within thoracic segment of the honeybee (created with BioRender.com).

4.6 Final Conclusion and Outlook

In Chapter 2 the neuro-muscular thoracic octopamingeric system was discovered and thoroughly investigated. With a multidisciplinary approach consisting of immunohistochemistry, HPLC-ECD, gene expression analysis and behavioral pharmacology the role of octopamine in honeybee thermogenesis was examined and demonstrated. Subsequently, this newly discovered system was subjected to cold stress conditions in Chapter 3, and the physiological response within the pathways revealed in Chapter 2 were assessed and interpreted (Figure 4.1). Finally, it was revealed that the discovered octopaminergic neuro-muscular system strives for an equilibrium under cold stress conditions (Chapter 3). These findings indicate that octopamine signaling not only stimulates honeybee thermogenesis but is also essential for it and thus for the survival of the colony.

The increasing intensification of agriculture and the resulting ecological decay represent a major challenge for insects (Matson et al., 1997; Raven and Wagner, 2021). In this respect, the question of the influence of food supply and availability must ultimately be assessed. Octopamine biosynthesis originates from the essential amino acid phenylalanine (Chapter 1), making the octopamine household dependent on the food availability within the habitat. In principle, pollen represents the source of amino acid supply for a honeybee colony. The impact of low pollen diversity (monocultures) on food quality and ultimately thermogenesis remains to be investigated in future studies. Finally, my thesis provides the basis for these future studies on the colony-level.

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"Wir stehen selbst enttäuscht und sehn betroffen
Den Vorhang zu und alle Fragen offen."
Der gute Mensch von Sezuan - **Bertolt Brecht**

Appendix

A

APPENDIX

Affidavit

I hereby confirm that my thesis entitled "**Octopaminergic Signaling in the Honeybee Flight Muscles - A Requirement for Thermogenesis**" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.
Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.
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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "**Octopaminerge Signalwege in der Flugmuskulatur der Honigbiene - Eine Voraussetzung für die Thermogenese**" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
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Ort, Datum Unterschrift

Danksagung

Die Liste der Menschen, die mich im Großen wie im Kleinen bei meinem Promotionsvorhaben unterstützt und begleitet haben ist lang. Ich habe habe mir größte Mühe gegeben, niemanden zu vergessen. Sollte trotz allem dieser Fall eingetreten sein, bitte ich hiermit um Entschuldigung.
An erster Stelle möchte ich meinem Doktorvater Markus Thamm für seine großartige Betreuung danken. Er war von Beginn an eine wichtige Konstante und stets ein hervorragender Ansprechpartner für Fragen und Probleme aller Art. Von ihm habe ich viel lernen können, sei es in verschiedensten Methoden der Molekularbiologie, im Zwischenmenschlichen oder auch in der Betreuung von Student*innen. Ich bewundere seinen Optimismus und positive Denkweise. Ich bin sehr dankbar, dass Du mich auf diesem Weg betreut, begleitet und unterstützt hast!
In diesem Zug möchte ich auch den beiden übrigen Mitgliedern meines Promotionskomitees meinen Dank auszusprechen. Meinen herzlichen Dank an Christian Wegener für seinen Einsatz und seine Betreuung. Dein enormer Wissensschatz war manchmal ebenso beeindrucken, wie einschüchternd.
Mein Dank gebührt auch Oliver Scherf-Clavel, der mich bereits während seiner eigenen Doktorandenzeit mit seinem Können und seinem Durchblick beeindruckt hat. Ich bin sehr dankbar für die vielen "kleinen" Crashkurse und die Zeit, die Du Dir immer bei unvorhergesehenen Problemen genommen hast.
Insgesamt kann ich mit voller Überzeugung sagen: Ich hätte mir keine bessere Besetzung für mein Promotionskomitee wünschen können. Unsere gemeinsamen Meetings haben stets für eine angenehme Atmosphäre über den Verlauf meines Promotionsvorhabens gesorgt.

Nun möchte ich Fabian und Felix danken, die mich über die Jahre mit Rat und Tat unterstützt haben. Ihr habt mir immer den Rücken freigehalten. Auf Euch konnte ich mich stets verlassen. Gemeinsam leidet es sich oft leichter! Danke, dass Ihr immer für mich da gewesen seid!
Im selben Zug will ich mich auch bei Robin, Lio, Valerie und Martin G. bedanken. Egal ob in der Pause oder bei einem wissenschaftlichen Diskurs, mit Euch konnte man immer einen regen und angenehmen Austausch führen. Danke ebenfalls allen weiteren Mitgliedern und Student*innen der AG Scheiner, die mich von Anfang an sehr herzlich aufgenommen haben. Die gemeinsamen Mittagspausen mit Euch habe ich sehr genossen. Besonderen Dank gebührt auch Karin und Conny, die versucht haben die Labore, unter der nie endenden Flut an Student*innen, am Laufen zu halten und auch immer für eine fröhliche Büroatmosphäre gesorgt haben. Vielen Dank an Prof. Ricarda Scheiner, die mich stets wie einen ihrer eigenen Doktoranden ermutigt und unterstützt hat.

Ein großes Dankeschön an alle Student*innen der AG Thamm, die mit Interesse und Begeisterung an meiner Arbeit mitgewirkt haben: Ana, Anna, Feriba, Julia, Lena, Linnéa, Lorenz, Mara, Saskia und Valentina - Danke Euch!
Danken möchte ich auch unseren Kooperationspartnern Prof. Roland Seifert und Dr. Heike Bähre, sowie Prof. Jens Schlossmann, der diese Zusammenarbeit überhaupt erst ermöglicht hat.
Danke auch an Basil, der in unerwartetsten Momenten immer einen Witz oder einen wissenschaftlichen Rat parat hatte. Ich wünsche Dir viel Erfolg in Norwegen!
Ein herzliches Dankeschön soll auch an den kompletten Lehrstuhl der Zoologie II gehen. Mit Anna Stöckl, Claudia Groh, Flavio Roces, Johannes Spaethe, Keram Pfeiffer, Thomas Münz und Prof. Wolfgang Rössler konnte man stets interessante und wissenschaftliche Gespräche führen. Egal ob man sich zufällig auf dem Gang, in der Mensa oder auf einer Konferenz getroffen hat. Danke Dirk Ahrens-Lagast für seine unermüdliche Arbeit an den Bienenstöcken und Frau Linke für ihre Hilfestellungen in dem Bürokratischen Dschungel. Ihr alle habt mir stets das Gefühl gegeben, ein Teil dieses Lehrstuhls zu sein.

Ich möchte mich bei Prof. Christoph Sotriffer und Prof. Petra Högger bedanken, dass sie mich, vor allem zu Beginn meiner Promotion, unterstützt haben. Weiter möchte ich allen Doktorand*innen des AK Högger danken, die mir oft bei meiner Aufgabe als HPLC-Verantwortlicher unter die Arme gegriffen haben. Besonderer Dank gebührt hier Jasmin, Theresa und Florian.
Bedanken möchte ich mich auch bei Christine aus der chemischen Synthese, die mich mit ihrem theoretischen und praktischen Wissen sehr unterstützt hat und sich auch die Zeit genommen hat, mir die wichtigen Details genau zu erklären. Danken möchte ich auch an Lukas aus der Analytik und Marcus aus der Technologie für den fachlichen Austausch in mancher Kaffeepause.

Ich werde noch oft an die gemeinsame Zeit mit Euch allen zurückdenken!

Schließlich möchte ich auch meinem Vater danken, dessen Unterstützung es mir ermöglicht hat zu studieren. Dadurch hat er mitunter den Grundstein für meine Promotion gelegt.
Ein Dankeschön soll auch an meine langjährigen Freunde André, Artur, Michi und Sven gehen, weil Ihr mich bereits so lange auf meinem Weg begleitet und unterstützt habt!
Und nun möchte ich einer Person danken, die einen ganz besonderen Platz in meinem Leben eingenommen hat. Beruflich, wie privat – Du warst immer für mich da und hattest den passenden Rat oder Witz für jede erdenkliche Situation zur Hand. Danke, dass es Dich gibt! Danke Tine!

Descriptions of the Specific Contributions of the PhD-Candidate to a Publication with Several Co-Authors and Confirmation by the Co-Authors

Description of the specific contributions of the PhD-candidate to a publication with several co-authors and confirmation by the co-authors

Sinan Kaya-Zeeb (Zoology II)

_____

PhD-studend and department

Title of the publication:
Octopamine drives honeybee thermogenesis

Names of Co-Authors:
Lorenz Engelmayer, Mara Straßburger, Jasmin Bayer, Heike Bähre, Roland Seifert, Oliver Scherf-Clavel, Markus Thamm

Publication details	**Description of the own contribution**
Writing of the article Which parts of the article have been written to which extent by the candidate?	Original draft Review and editing
Performed research Which experimental procedures have been conducted by the candidate?	Monoamine quantification Gene expression analysis Protein quantification Pyruvate quantification Pharmacological thermography Immunohistochemistry
Conceptual design of the research To which extent did the candidate contribute to the conceptional design of the research project?	Conceptualization was performed by Sinan Kaya-Zeeb (50%) and Markus Thamm (50%)
Data analysis To which extent did the candidate contribute to the data analysis?	Formal analysis and visualization
Overall contribution of the candidate (in%)	70 %

Confirmation by co-authors:

Name Co-author	Signature	Date
Lorenz Engelmayer		
Mara Straßburger		
Jasmin Bayer		
Heike Bähre		
Roland Seifert		
Oliver Scherf-Clavel		
Markus Thamm		

In case of co-authors who cannot be contacted, the particular confirmation of the responsible author of the publication is required:

Herewith I confirm that the above description of the specific contributions of the PhD-candidate to the publication is correct,

Name of responsible author Signature Date

_____ _____ _____

Description of the specific contributions of the PhD-candidate to a publication with several co-authors and confirmation by the co-authors

Sinan Kaya-Zeeb (Zoology II)

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PhD-studend and department

Title of the publication:
Robustness of the honeybee neuro-muscular octopaminergic system in the face of cold stress

Names of Co-Authors:
Saskia Delac, Lena Wolf, Ana Luiza Marante, Oliver Scherf-Clavel, Markus Thamm

Publication details	**Description of the own contribution**
Writing of the article Which parts of the article have been written to which extent by the candidate?	Original draft Review and editing
Performed research Which experimental procedures have been conducted by the candidate?	Monoamine quantification Gene expression analysis Protein quantification
Conceptual design of the research To which extent did the candidate contribute to the conceptional design of the research project?	Conceptualization was performed by Sinan Kaya-Zeeb (50%) and Markus Thamm (50%)
Data analysis To which extent did the candidate contribute to the data analysis?	Formal analysis and visualization
Overall contribution of the candidate (in%)	80 %

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Name Co-author	Signature	Date
Saskia Delac		
Lena Wolf		
Ana Luiza Marante		
Oliver Scherf-Clavel		
Markus Thamm		

In case of co-authors who cannot be contacted, the particular confirmation of the responsible author of the publication is required:

Herewith I confirm that the above description of the specific contributions of the PhD-candidate to the publication is correct,

Name of responsible author Signature Date

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Curriculum Vitae

List of Publications

Sinan Kaya-Zeeb, Saskia Delac, Lena Wolf, Ana Luiza Marante, Oliver Scherf-Clavel and Markus Thamm (2022)

Robustness of the honeybee neuro-muscular octopaminergic system in the face of cold stress

Frontiers in Physiology, Frontiers Media *13*, 1979.

This article can be downloaded from https://doi.org/10.3389/fphys.2022.1002740.

Sinan Kaya-Zeeb,Lorenz Engelmayer, Mara Straßburger, Jasmin Bayer, Heike Bähre, Roland Seifert, Oliver Scherf-Clavel, and Markus Thamm (2022)

Octopamine drives honeybee thermogenesis

eLife, eLife Sciences Publications, Ltd *11*, e74334.

This article can be downloaded from https://doi.org/10.7554/eLife.74334.

*Brigitte Fiala, Konstans Wells, Julia Haubenreisser, Andreas Pittroff, **Sinan Kaya-Zeeb**, Arthur Y.C. Chung, Rosli Bin Hashim, Alexander Keller (2015)

Monophyletic clades of *Macaranga*-pollinating thrips show high specifity to taxonomic sections of host plants

Biological Journal of the Linnean Society, Linnean Society of London *116*, 558-570.

This article can be downloaded from https://doi.org/10.1111/bij.12605.

* This publication is not part of the thesis.