Neural correlates of diverse navigational strategies

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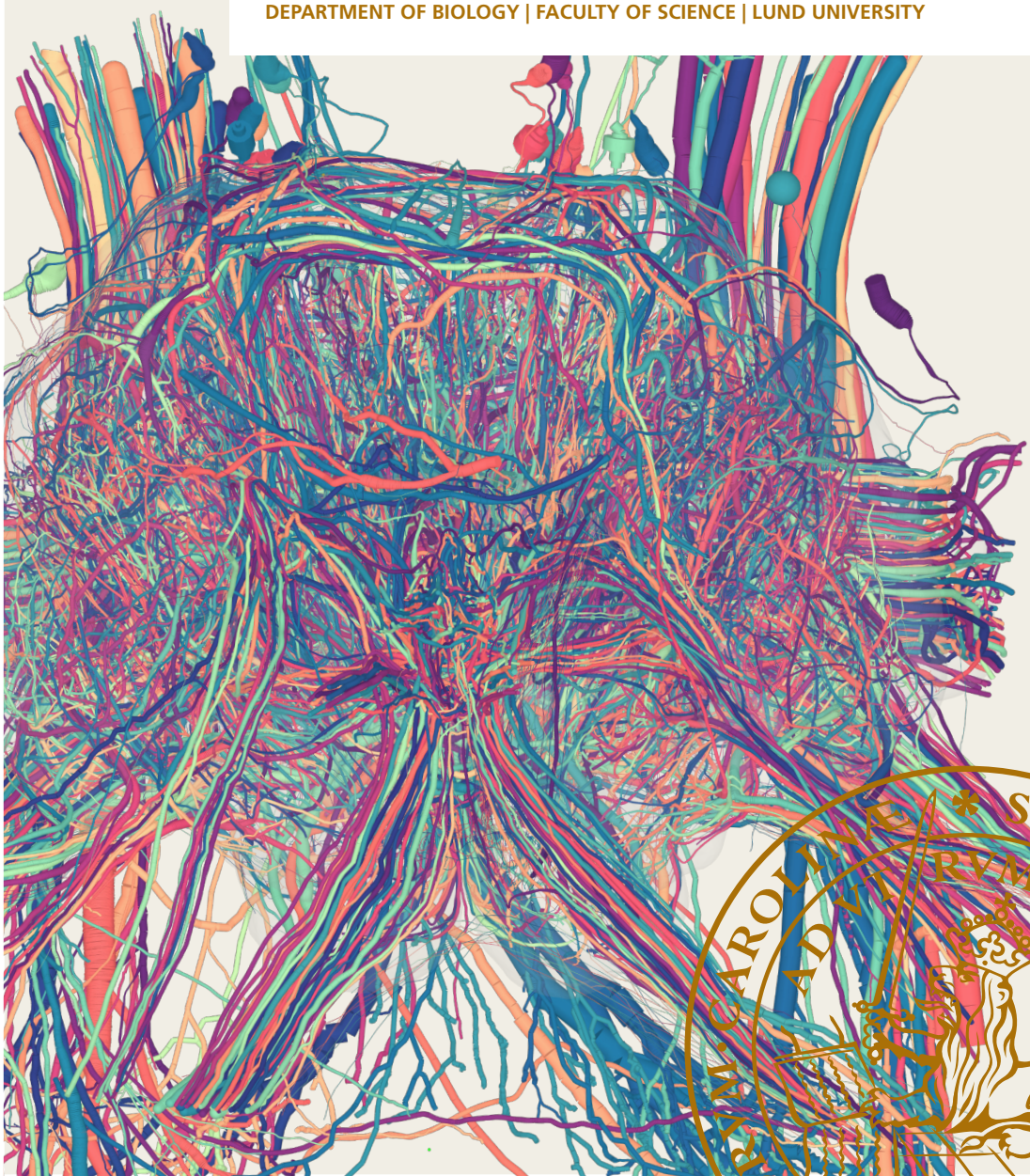

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DOCTORAL DISSERTATION

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Abstract
Insects have evolved diverse and remarkable strategies for navigating in various ecologies all over the world. In particular, central place foragers, such as bees and ants, have become renowned for their fascinating navigational capabilities. At the heart of insect navigation lies a brain area known as the central complex (CX). Functionally, the CX integrates allothetic sensory information with self-motion cues to generate an internal map of angular position. It plays a role in driving motor commands and has been suggested as the neural substrate for encoding travel direction as well as navigational vectors theorized to be involved during path integration. Interestingly, the CX appears to have been highly anatomically conserved, even across insect species that diverged hundreds of millions of years ago. The conserved nature of the CX stands in juxtaposition to the fascinating diversity of insect behavior. How does a highly conserved brain area give rise to such diverse navigational behavior?

Using block-face electron microscopy combined with neuron segmentation and synapse annotation, I analyzed CX circuits in six species of bees and ants: the honeybee, the bumblebee (Paper 1), the sweat bee, the army ant, the desert ant, and the bull ant. Our data suggests that there are core circuits that have been exceptionally well preserved across evolutionary time. Namely, the head direction circuit (Paper 2) which contains neurons that share total numbers, projectivity, and connectivity motifs from flies to bees and ants. In contrast, inputs from sensory areas vary to a much larger degree. Our data suggests that the relative contribution of parallel input pathways depends strongly on the information available in the habitat of a species. Also variable are the circuits that encode self-motion, something which is fundamental for building navigationally relevant internal representations (Paper 3). Altogether, these neuroanatomical maps provide the framework for future functional and modeling studies that seek to understand how sensory information is transformed into behavioral decisions within the context of navigation.

Key words
central complex, connectomics, path integration, head direction, bees, ants, neuroethology

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Cover illustration front: Comprehensive reconstruction of neurons innervating the bumblebee central complex (color profile by Valentin Gillet)

Cover illustration back: The central complex of six species of insects organized along a circular phylogenetic tree (color profile by Valentin Gillet).

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To my family and friends

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List of papers

I **Sayre ME**, Templin R, Chavez J, Kempenaers J, & Heinze S (2021). **A projectome of the bumblebee central complex**. *Elife*, *10*, https://doi.org/10.7554/eLife.68911

II **Sayre ME**, Gunnarsson ES, Szadaj F, Gillet V, Honkanen A, Ellendula S, Narendra A, & Heinze S. **The head direction circuit of ants and bees**. (*Manuscript*)

III **Sayre ME***, **Gillet V***, Zadel A, Nerme V, Narendra A, & Heinze S. **A novel navigation circuit in the hymenopteran central complex**. (*Manuscript*)

Author contributions

Paper 1 **Marcel E. Sayre**: Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing - original draft, Writing - review and editing; **Rachel Templin**: Investigation, Methodology, Writing - review and editing; **Johanna Chavez**, **Julian Kempenaers**: Investigation, Writing review and editing; **Stanley Heinze**: Conceptualization, Methodology, Investigation, Resources, Data Curation, Validation, Writing – Review and Editing, Supervision, Project administration, Funding acquisition.

Paper 2 **Marcel E. Sayre**: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Visualization, Data Curation, Writing - Original Draft, Writing - Review and Editing; **Ebba S. Gunnarsson**, **Felicia Szadaj**: Investigation; **Valentin Gillet**: Investigation, Software, Writing - Review and Editing; **Anna Honkanen**: Resources; **Saroja Ellendula**: Investigation; **Ajay Narendra**: Resources, Writing - Review and Editing, Supervision; **Stanley Heinze**: Conceptualization, Methodology, Investigation, Resources, Data Curation, Validation, Writing – Review and Editing, Supervision, Project administration, Funding acquisition.

Paper 3 **Marcel E. Sayre***: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Visualization, Data Curation, Writing - Original Draft, Writing - Review and Editing; **Valentin Gillet***: Conceptualization, Methodology, Software, Formal Analysis, Investigation, Data Curation, Writing - Review and Editing; **Ana Zadel**, **Vytautas Nerme**: Investigation; **Ajay Narendra**: Resources, Writing - Review and Editing, Supervision; **Stanley Heinze**: Conceptualization, Methodology, Investigation, Resources, Data Curation, Validation, Writing – Review and Editing, Supervision, Project administration, Funding acquisition.

*Shared first authorship

Publications not included in this thesis

Sayre ME, Strausfeld NJ. Mushroom bodies in crustaceans: Insect-like organization in the caridid shrimp Lebbeus groenlandicus. *Journal of Comparative Neurology*. 2019; 527:2371–2387. https://doi.org/10.1002/cne.24678

Strausfeld NJ, Sayre ME. Mushroom bodies in Reptantia reflect a major transition in crustacean brain evolution. *Journal of Comparative Neurology*. 2020; 528(2):261–282. https://doi.org/10.1002/cne.24752

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Chou A, Sayre ME, Lin C, Cronin TW. Neuroanatomy of stomatopod central complexes offers putative neural substrate for oriented behaviors in crustaceans. *bioRxiv*. 2022; https://doi.org/10.1101/2022.06.10.495695

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Popular summary

Insects have evolved diverse and remarkable strategies for navigating in various ecologies all over the world. In particular, central place foragers, such as bees and ants, have become renowned for their fascinating navigational capabilities. At the heart of insect navigation lies a brain area known as the central complex (CX). Functionally, the CX integrates world-centric sensory information with self-motion cues to generate an internal map of angular position. It plays a role in driving motor commands and has been suggested as the neural substrate for encoding travel direction, as well as navigational vectors theorized to be involved in path integration. Interestingly, the CX appears to have been highly anatomically conserved, even across insect species that diverged hundreds of millions of years ago. The conserved nature of the CX stands in juxtaposition to the extraordinary diversity of insect behavior. How does a highly conserved brain area give rise to such diverse navigational behavior?

Using block-face electron microscopy combined with neuron tracing and synapse annotation, we analyzed CX circuits in six species of bees and ants: the honeybee, the bumblebee (Paper 1), the sweat bee, the army ant, the desert ant, and the bull ant. Our data suggests that there are core circuits that have been exceptionally well preserved across evolutionary time. Namely, the head direction circuit (Paper 2) which contains neurons that share total numbers, projectivity, and connectivity motifs from flies to bees and ants. In contrast, inputs from sensory areas vary to a much larger degree. Our data suggests that the relative contribution of parallel input pathways depends strongly on the information available in the habitat of a species. Also variable are the circuits that encode self-motion, something which is fundamental for building navigationally relevant internal representations (Paper 3). Altogether, these neuroanatomical maps provide the framework for future functional and modeling studies that seek to understand how sensory information is transformed into behavioral decisions within the context of navigation.

Populärvetenskaplig sammanfattning på svenska

Insekter har utvecklat många olika och anmärkningsvärda strategier för att kunna navigera i världens vitt skilda miljöer. Särskilt bin och myror har blivit välkända för sin fascinerande förmåga att navigera. När man pratar om navigering hos insekter kommer ofta ett område i hjärnan på tal; det centrala komplexet (CX). Detta komplex sammankopplar yttre sinnesintryck med självrörelse hos insekter för att skapa en inre karta över djurets position. Det spelar en stor roll vid kommandon för rörelse och har föreslagits vara det neurala substratet som kodar för rörelseriktning såväl som navigeringsvektorer som man tror är involverade i ”path integration”. Det centrala komplexet verkar också vara mycket anatomiskt välbevarat, även när det gäller olika insektsarter som utvecklades åt olika håll redan för flera hundra miljoner år sedan. Komplexets välbevarade anatomi står i kontrast till den stora beteendevariationen hos insekter. Hur kan ett sådant oförändrat område i hjärnan ge upphov till så skilda navigeringsbeteenden?

Genom att använda “block-face” elektronmikroskopi, samt genom att spåra nervceller och synapser kunde vi analysera CX-kretsar i sex olika arter av bin och myror: honungsbin, humlor (artikel 1), vägbin, vandrarmyror, Cataglyphis och bulldoggsmyror. Vår data visar att det finns kärnkretsar som har varit exceptionellt välbevarade över lång tid, till exempel huvudriktningskretsen (artikel 2). Denna krets har samma antal nervceller, projektivitet och sammankopplingar hos flugor, bin och myror. Däremot skiljer sig signalerna från olika sinnesintryck åt. Vi kan visa att detta till stor del beror på hur mycket information som finns tillgänglig i artens habitat. Något som också skiljer sig åt är kretsarna som kodar för självrörelse, vilket är otroligt viktigt för den inre representationen av navigeringen (artikel 3). Sammanfattningsvis bidrar dessa neuroanatomiska kartor med stommen för framtida funktionella studier och modellering som vill förstå hur sinnesinformation omvandlas till beteende inom navigering.

Chapter 1

Navigation in diverse ecologies

Consider the remarkable life of an army ant. In contrast to most ant species that maintain a fixed nest, army ants live a nomadic lifestyle whereby their colony frequently moves from location to location in swarms known as mass raids. During mass raids, army ants collectively forage, devouring any living arthropod (or small animal) that is unfortunate enough to cross their path. During phases in which an army ant colony is stationary they form "bivouacs", dense and spherical structures that they create by interlinking the bodies of thousands of individual worker ants (Kronauer, 2020). Army ant mass raiding behavior is initiated by small numbers of individuals that slowly fan out from the colony in greater and greater distances, leaving a pheromone trail each time they do (Chandra et al., 2020). Importantly, species of army ants have greatly reduced eyes (Bulova et al., 2016) and are likely to rely on non-visual cues, such as olfaction and proprioception, while navigating. New world army ants, like *Eciton hamatum*, inhabit rainforests that are cluttered with dense vegetation. And while this deluge of visual information may not be an issue for nomadic army ants, this may pose something of a challenge for other insect species that keep a permanent nest which they must locate after a foraging trip.

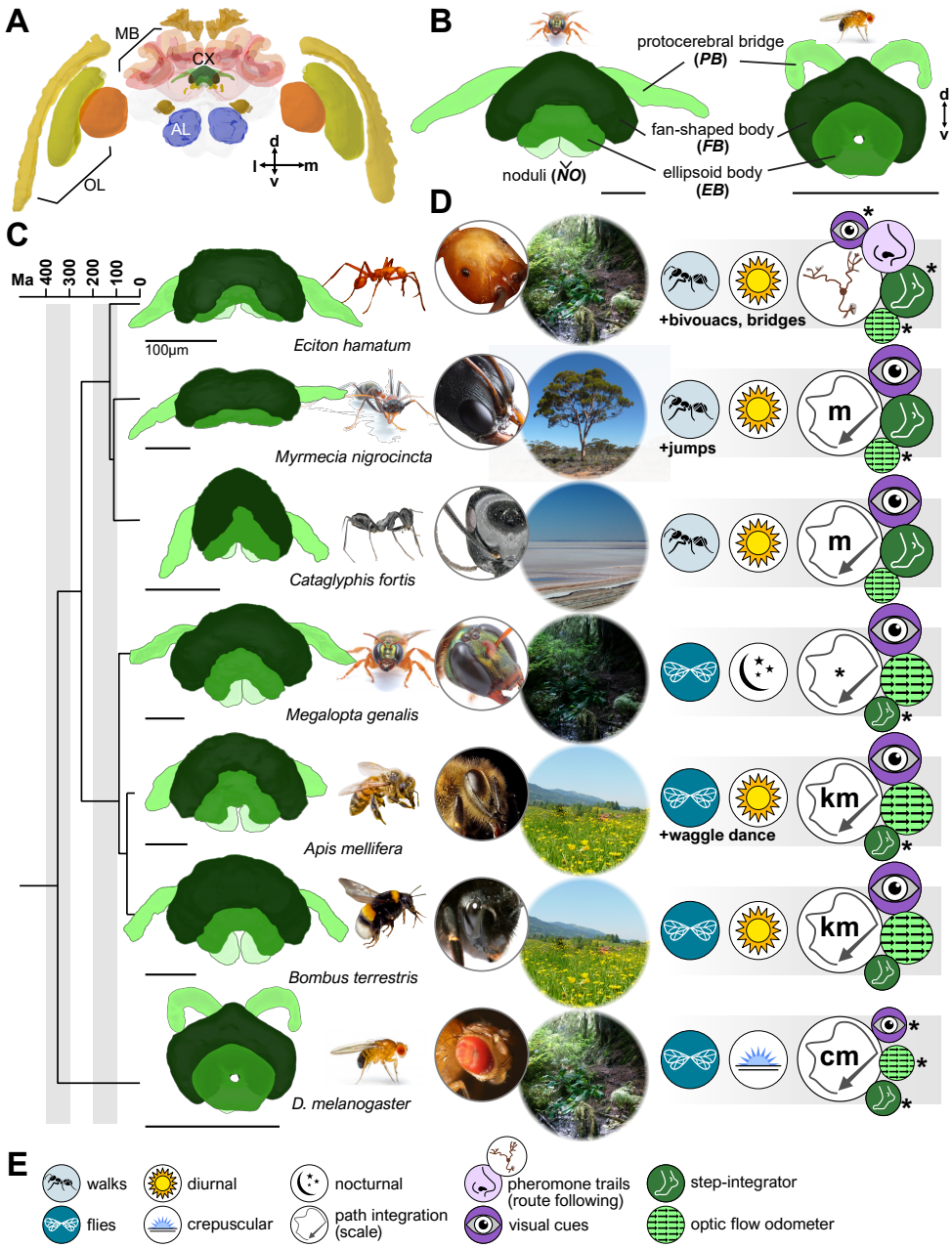
The Panamanian sweat bee, *Megalopta genalis*, is a great example of such a species. Sweat bees establish themselves in small holes made from hollowed out wood where they live alone or with few other individuals (Warrant et al., 2004). While it is not known exactly how far sweat bees travel when they forage, returning to an obscure hole in such a cluttered environment is an impressive feat, especially considering that sweat bees are nocturnal and manage this using visual landmarks under the canopy in extremely dim lighting conditions (Warrant et al., 2004; Chaib et al., 2021).

In terms of visual sensory information, *Cataglyphis fortis* desert ants face the opposite problem of the sweat bee. Desert ants are thermophiles that inhabit barren and inhospitable

salt plains in the Tunisian desert. While the sweat bee must navigate through what must be visual sensory overload, desert ants forage in a landscape that is nearly completely devoid of distinct terrestrial visual features (Wehner, 2020). Further, the temperature gets so hot that pheromone trails become vaporized, making route following by olfaction impossible. Still, desert ants regularly forage hundreds of meters from their nest which, like the sweat bee nest, is an obscure hole, not visible along the foraging path. They do this using a navigational strategy known as path integration (Chapter 2). When path integrating, desert ants use a combination of directional cues, such as the pattern of polarized light across the sky (Fent, 1986; WEHNER, 2001), combined with self-motion cues, such as the number of steps they have taken (Wittlinger et al., 2006), to maintain an internal representation of where they are relative to their starting point. Path integration enables desert ants to return to their nest in a straight line, regardless of how convoluted their path may have been during their outbound journey.

Army ants, sweat bees, desert ants, and all other insects share the presence of a navigational brain area called the central complex (CX). Remarkably, despite differences in ecology, lifestyle, and navigational behavior, the CX appears to be exceptionally well conserved across species (Figure 1). As will be further explored in Chapter 3, the CX plays fundamental roles in sensory integration, action selection, motor coordination, and sleep. The CX contains neural circuitry that tracks heading direction like a biological compass, receives cues about self-motion, and propagates commands for steering and forward movement (Honkanen et al., 2019). Neuroanatomical evidence suggests that the CX has been preserved across hundreds of millions of years of evolutionary time (Homberg, 2008). Phenotypic homologues of CX cell types that share morphology and projection pattern have been identified across insect orders, including in locusts (Heinze and Homberg, 2008; Heinze et al., 2009; Hadeln et al., 2020), monarch butterflies (Heinze and Reppert, 2011; Heinze et al., 2013), dung beetles (Jundi et al., 2018), bees (Homberg, 1985; Milde, 1988; Stone et al., 2017; Hensgen et al., 2020), and fruit flies (Wolff et al., 2015; Wolff and Rubin, 2018; Hulse et al., 2021).

There is a juxtaposition between the conserved nature of the CX and the fascinating diversity of insect navigational behavior. Such a paradox poses an interesting question: how does a highly conserved brain area give rise to such diverse navigational behavior?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km
+waggle dance
km
cm
E
walks
flies
diurnal
crepuscular
nocturnal
path integration
(scale)
pheromone trails
(route following)
visual cues
step-integrator
optic flow odometer

(a)

Figure 1 : Neural correlates of diverse navigational behavior: CX anatomy and highlights of navigational behavior in six species of bees and ants, as well as the fruit fly.
(**A**) 3D reconstruction of the insect brain (*M. genalis*; retrieved from (insectbraindb.org; Heinze et al., 2021). (**B**) Comparison of CX neuropil identities between a bee (*M. genalis*) and the fruit fly (*D. melanogaster*; data from Scheffer et al., 2020). The NO are posterior to the FB/EB in the fly and are therefore not visible from this orientation (note also the differences in scale). (**C**) CXs of the species compared throughout this study organized by phylogeny (Ward, 2014). Divergence estimates in millions of years (Ma) are based on those reported in Misof et al. (2014) and Peters et al. (2017). (**D**) Notable, but non-exhaustive list of commonalities and differences between each species in the context of navigational behavior. Images of compound eyes provides a rough basis for qualitative insight into their visual capabilities (i.e., compare the relatively tiny eyes of the army ant to those of other species; note however that images are not drawn to scale). Landscapes show snapshots of each species ecology. Icons indicate some interesting species-specific attributes related to the foraging journey or mode of locomotion. Asterisks indicate the attribute is likely but, to the best of my knowledge, has not been shown experimentally. (**E**) Icon legend. Images (full insect): *E. hamatum*, *M. nigrocincta*, and *M. genalis*: Ajay Narendra, *C. fortis*: Estella Ortega (www.antweb.org), *A. mellifera*: David Cappaert, and *B. terrestris*: Holger Uwe Schmitt. Images (eye): *E. hamatum* and *M. genalis*: Ajay Narendra, *M. nigrocincta*: Matt Inman, *C. fortis*: Estella Ortega (www.antweb.org), *A. mellifera*: Jon Sullivan, *B. terrestris*: Holger Uwe Schmitt, *D. melanogaster*: Thomas Wydra. Abbrev: OL; optic lobe, AL; antennal lobe, MB; mushroom body, CX; central complex, d; dorsal, v; ventral, l; lateral, m; medial. Scale bars: 100 µm.

One possible explanation is that perhaps the CX is not as conserved as current evidence would suggest. While all insects studied thus far have a CX that is made up of the same arrangement of neuropils with similar morphology and homologous cell types, it is possible that differences exist at the level of connectivity. Homologous neurons may form different connectivity patterns or microcircuits that are not observable using standard light microscopy-based methods. Alternatively, it could be that the neural connectivity underlying the CX is retained across species, and differences in behavior arise from species-specific neuromodulation of identical circuits. A third possibility is the CX is functionally and anatomically well conserved, and perhaps divergent navigational behaviors are attributable to differences in signalling from regions upstream or downstream of the CX.

Building from this, navigational behavior that appears diverse may arise from the same basic behavioral repertoire that all insects likely inherited from some Devonian common ancestor (i.e., the "devonian toolkit"; Dickinson, 2014). In this scenario, different behaviors could be encoded using the same internal mechanism (Honkanen et al., 2019). For instance consider menotaxis, an essential component of navigational behavior during which an organism maintains a constant angle relative to some external stimuli (Warren et al., 2019; Heisenberg and Wolf, 1984). Long distance migration (Reppert et al., 2016; Warrant et al., 2016; Homberg, 2015), homing during path integration, dispersal in fruit flies (Leitch et al., 2021), and straight-line orientation in beetles (El Jundi et al., 2016), all involve holding a constant angle relative to some external stimulus. Menotaxis can even be exploited for

non-navigational behaviors, such as target interception in predatory dragonflies (Olberg et al., 2000).

Resolving this paradox requires comprehensive and comparative mapping of CX neurons and their connectivity across insects with diverse navigational lifestyles. The work presented in this thesis is the beginning of such an investigation.

More specifically, research within the scope of this thesis is aimed at mapping the CX neural circuitry of six species of bees and ants: the honeybee (*Apis mellifera*), the bumblebee (*Bombus terrestris*), the sweat bee (*Megalopta genalis*), the desert ant (*Cataglyphis fortis*), the army ant (*Eciton hamatum*), and the bull ant (*Myrmecia nigrocincta*; Figure 1C). These six species were chosen for numerous reasons, including that their navigational behavior has been studied in detail, they use differing strategies for navigating in their respective ecologies, and they are evolutionarily relatively close.

So far, a comprehensive map of all CX neurons and their connections (a "connectome") has only been established in the fruit fly, *Drosophila melanogaster* (Scheffer et al., 2020). The fly connectome revealed new cell types and produced novel findings that pose interesting questions which pave the way for future functional studies. The fly CX connectome also provides a comprehensive starting point from which to compare the neural circuits in other insect species, and will therefore be referred to frequently throughout this text (Hulse et al., 2021).

The chapters that follow include necessary background, beginning with an overview of navigational behavior in bees and ants, focusing in particular on path integration (Chapter 2). Chapter 3 discusses the general organization of insect brains and the CX. Chapter 4 includes an introduction to the insect head direction circuit and neural pathways underlying the integration of self-motion. In Chapter 5, I introduce recent models of the CX and focus on a proposed role for the fan-shaped body (FB; Figure 1B) as a navigational "vector calculator" (Hulse et al., 2021). Then, in Chapter 6, I turn to the methodology, where I describe the pipeline used to generate and trace multi-resolution volumetric electron microscopy datasets. Lastly, in Chapter 7, I summarize findings from the three papers that are the product of this thesis.

To the best of my knowledge, this work presents the first ever connectomic data of the central brain for any insect besides the fruit fly to date. Using a multi-resolution imaging approach that enables both the comprehensive tracing of every CX neuron as well as every neural connection in specific CX regions, we aim to close a comparative gap and shed light on the neural circuitry underlying diverse navigational behavior.

Chapter 2

The navigational behavior of bees and ants

Path Integration

I often wonder if I would make it as a bee. While avoiding more pressing work, I imagine that I am camping in a forest and that, to survive, I would need to collect food from plants nearby. Absent any GPS, trails, or landmarks, how far could I go and still manage to return to my tent? If I found food kilometers away, would I still be able to point in the direction of my tent and know how far I need to travel to reach it? Take it as an indication of my confidence in the matter that I will avoid finding out for now.

Yet again to be outdone by insects, central place foragers, such as bees and ants, perform such a task as part of their daily routine. As central place foragers, these insects maintain a nest or colony, often at a fixed location, where they keep their resources, raise their brood, and shelter. The survival of the colony therefore depends in large part on the navigational capabilities of its foraging workers. Some ant species will forage up to hundreds of meters from their nest while honeybees and some solitary bees, can travel distances in the tens of kilometers (Frisch, 1967; Janzen, 1971). Despite the convoluted twists and turns of their outbound path naive foragers will return to their nest in the most efficient way possible: along a straight line. This is a navigational strategy known as path integration (Heinze et al., 2018).

During path integration, a foraging insect (or other animal) maintains a constant estimate of its position relative to its nest or starting point (Heinze et al., 2018; Wehner, 2003; Collett and Collett, 2000). The benefit of path integration is that such a technique enables animals to travel to areas they have never explored and still return to their nest or other salient

location. During an outbound journey, path integrating insects monitor direction and distance travelled along their path to generate a 'homing' vector; an internal representation of the distance and direction of their "home" or starting point.

Historically, studies have focused on insects that excel at path integration over long distances, such as honeybees and desert ants. However recent studies have shown that path integration is also used on a much smaller scale by fruit flies to relocate fictive food patches in the absence of other sensory stimuli (Kim and Dickinson, 2017; Titova et al., 2022). Another recent study showed that wingless bumblebees use path integration while walking in an enclosed arena, suggesting that the use of path integration in bees is flexible and perhaps always active as a backup strategy when other, more reliable navigational cues (such as visual landmarks) are not available (Patel et al., 2022).

Some animals, like fruit flies (Kim and Dickinson, 2017), have been shown to path integrate in complete darkness. Under these circumstances, the only cues available for angular and translational velocity are self-motion (idiothetic) cues, generated from proprioception and motor feedback. Purely idiothetic path integration may suffice for short range navigation, but such a system quickly accrues error and is unreliable over longer distances (Heinze et al., 2018).

Directional cues

Long distance foraging requires the use of external compass cues that can reliably inform the insect about its heading direction. The ability to use polarized skylight and the azimuthal position of celestial bodies to maintain a heading direction is widespread across insects (Wehner, 1984; Homberg et al., 2011; Homberg, 2015; Warrant et al., 2016; Reppert and de Roode, 2018; Warren et al., 2019; Dacke et al., 2020). Importantly, insects that migrate or rely on the same foraging vector at different times of the day must be able to account for the moving position of the sun across the sky and are known to do so using a 'time compensated' sun compass (Froy et al., 2003; Gould, 1980).

Many insects have also been shown to use other, less reliable, sensory cues to guide heading direction, including the Earth's magnetic field (Dreyer et al., 2018; Fleischmann et al., 2018; Guerra et al., 2014), wind (Dacke et al., 2019; Müller et al., 1997), and odor plumes (Murlis et al., 1992). In the same way that you can never really turn your nose or ears off, insects that can sense the above-mentioned cues likely do so during the entirety of their foraging trip, however cue conflict experiments show that they are likely suppressed when more reliable cues are available. Insects are therefore flexible navigators, able to make use of many senses which are internally organized in a hierarchy based on reliability (Dacke et al., 2019).

As opposed to an isolated module, the neural substrate of the path integrator appears to

work in concert with environmental cues, such as the visual scenery (Heinze et al., 2018; Zeil, 2012). When an inexperienced bee or ant forager leaves its nest for the first time, it will systematically learn the visual surrounding in so-called learning walks in ants (Fleischmann et al., 2016; Jayatilaka et al., 2018) or learning flights in bees (Collett and Collett, 2018). During this process, the forager is presumed to store views of relevant visual cues including of visual patterns and features surrounding the nest, as well as of the panorama in the far-off distance (Carwright and Collet, 1983; Collett et al., 2013). Visual cues surrounding the nest are essential for pinpointing specific locations, considering that many nests are obscure holes or, in the case of ants, not visible from the horizontal visual plane. Although, it should be noted that desert ants that do not have access to such visual cues can additionally use odor plumes of CO2 emerging from their nest to detect its entrance (Steck et al., 2009). Visual information enables ants to return to their familiar routes when they are displaced to novel terrain (Narendra, 2007). *Drosophila* also use visual landmarks to return to remembered locations (Ofstad et al., 2011).

In visually rich environments, ants will use visual information to follow idiosyncratic foraging routes (Kohler and Wehner, 2005). Australian bull ants, *Myrmecia sp.*, are solitary foragers that live in visually complex environments. They rely predominantly on visual landmarks while foraging and, like *Cataglyphis fortis*, do not use pheromones trail (Freas et al., 2017, 2018; Freas and Cheng, 2019). In experiments where *Myrmecia* individuals were displaced from their normal foraging route, such that their path integrator conflicted with the visual surround, these ants tended to travel half-way between the two or follow visual cues exclusively (Reid et al., 2011; Narendra et al., 2013).

Odometer

The primary mechanism in which an insect determines distance travelled depends on its preferred mode of locomotion (Heinze et al., 2018). In a now classic experiment, Wittlinger et al. (2006) either shortened or extended the legs of ants at the location of a food source. They found that ants with longer legs overshot the location of their nest and ants with shorter legs did not travel far enough. Desert ants therefore use a 'pedometer' or step-integrator that tracks their distance using the number of steps taken along an outbound journey. Interestingly, desert ants will only integrate the horizontal component of the distance they walk when traversing over angled slopes (Wohlgemuth et al., 2001).

A step-integrator would not be of much use to an insect that forages by flying. Honeybees and sweat bees for instance instead rely on translational optic flow, the symmetric movement of visual information across the entire visual field for the estimation of distance (Esch and Burns, 1995; Srinivasan et al., 2000). Along with the directional location of a goal obtained from celestial cues, honeybees use optic-flow derived distance estimations to communicate the distance of said goal to other workers in their waggle dance (Esch et al.,

2001; Barron and Plath, 2017). Whereas desert ants will only use the horizontal component for distance estimation, honeybees, when flying through vertical L-shaped tunnels, measure their total distance travelled as derived from optic flow cues (Dacke and Srinivasan, 2007).

Step-integrator and optic flow-based estimations for distance are not necessarily mutually exclusive. Desert ants will use optic flow to return to their nest when their pedometer is unavailable but are unable to transfer optic-flow based distance estimations to their pedometer, suggesting that these two odometers are processed independently in the brain (Pfeffer and Wittlinger, 2016). Additionally, walking bumblebees have been shown to measure their distance in complete darkness, suggesting they may use a step-integrator as well (Chittka et al., 1999).

Summary

In summary, the sensory information a bee or ant uses to monitor distance travelled seems to correspond with the insect's preferred mode of locomotion, whereas the sensory cues used to determine direction tends to depend on the strength and reliability of the cue, which is itself dependent on the ecological environment. However, a reoccurring theme across several distantly related insects seems to be that insects can make use of the same repertoire of sensory cues when their preferred cue is not available. This suggests that insects have inherited the ability to use this repertoire of sensory cues from some common ancestor and perhaps cue hierarchy is a derived and species-specific property that is sculpted by the information content of the environment. Flexibility in navigational behavior is also reflected by an insect's ability to switch between navigational strategies (for instance, switching from path integration to visual homing once familiar landmarks become available).

While there are other fascinating examples of insect navigation (i.e., long distance migration, straight-line orientation), the focus of this thesis is on the brains of central place foragers. However, as we will see, the insect CX is remarkably conserved across species and it is likely that it plays similar roles in facilitating navigation across all insects (Honkanen et al., 2019). In the chapter that follows, I will explore the neural circuits that underlie such diverse insect navigational behavior.

Chapter 3

The insect brain

"There's a region in the fly called the central complex
because it is central, and it is complex."
- Michael Dickinson

General organization of the insect brain

Despite variations in size and shape, all insect brains have the same general layout*. Historically, the central brain has been considered to be composed of three fused ganglia termed the protocerebrum, the deutocerebrum, and the tritocerebrum (though see Strausfeld et al., 2022). Within this view, the most caudal of the three, the tritocerebrum, relays nerve fibers descending to and arising from the abdomen, the thorax, and suboesophageal ganglion to the deutocerebrum and protocerebrum. Of the three fused ganglia, the tritocerebrum is the smallest and perhaps least well studied. The deutocerebrum is situated rostral to the tritocerebrum and it contains areas that process and integrate olfactory information, namely the antennal lobes (AL) and the antennal mechanosensory and motor center (AMMC; Homberg et al., 1989).

The protocerebrum is furthest rostral of the three fused ganglia and is considered the domain for higher order processing. One of the most well studied areas of the insect brain are

*Historically, nomenclature used to identify cells and CX areas differed between fruit flies and other insects. Due to overwhelming support for CX homology across insects, the greater availability of functional and neuroanatomical data in Drosophila (Wolff et al., 2015; Wolff and Rubin, 2018; Hulse et al., 2021), and to make our anatomical descriptions more comparable to the *Drosophila* connectomics data, we adhere here to nomenclature used for *Drosophila* (Ito et al., 2014; Hulse et al., 2021) but will provide alternative names used for other insects whenever relevant (see Table 1).

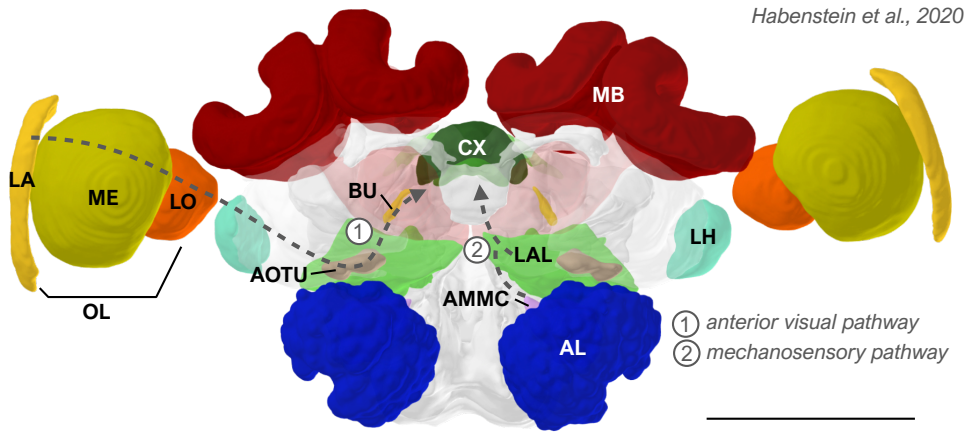

Figure 2: 3D reconstruction of a Cataglyphis nodus brain from Habenstein et al. (2020) highlighting two major sensory pathways to CX. Retrieved from the InsectBrainDB (insectbraindb.org; Heinze et al., 2021). Abbrev: OL, optic lobe; LA, lamina; ME, medulla; LO, lobulla; AOTU, anterior optic tubercle; LAL, lateral accessory lobes; BU, bulbs; AMMC, antennal mechanosensory and motor center; AL, antennal lobes; LH, lateral horn; MB, mushroom body; CX, central complex. Scale bar; 400 µm.

the mushroom bodies, which occur as a set of two and play fundamental roles in learning and memory as well as visual navigation (Figure 2; Mizunami et al., 1998; Strausfeld, 1999; Modi et al., 2020; Buehlmann et al., 2020; Kamhi et al., 2020; Li et al., 2020; Strausfeld, 1976). Also key in olfactory processing are the lateral horns (LH), which play a role in innate olfactory behavior. The protocerebrum also houses the CX, the function and anatomy of which will be explored further below.

Except for a few species with absent or reduced eyes (Bulova et al., 2016), the protocerebrum is typically flanked by optic lobes (OL) that in some insect species make up nearly 2/3 of the entire central brain (Figure 1A). Visual information captured by photoreceptors in the retina travels from the optic lobes into the ventrolateral protocerebrum, where it is relayed to the anterior optic tubercle (AOTU), the posterior optic tubercle (POTU; in non-dipteran insects), and, in some species, the mushroom bodies along separate pathways (Homberg et al., 2003; Held et al., 2020). The visual tract that passes through the AOTU projects into a group of microglomerular synaptic complexes known as the bulbs (BU) and is from there relayed primarily to the EB in the CX. This particular pathway is thought to be well-conserved across insects and is known as the anterior visual pathway (Homberg et al., 2011; Jundi et al., 2014; Honkanen et al., 2019; Pfeiffer et al., 2005; Pfeiffer and Kinoshita, 2012; Mota et al., 2011; Held et al., 2016; Hardcastle et al., 2020; Seelig and Jayaraman, 2013, Figure 2;).

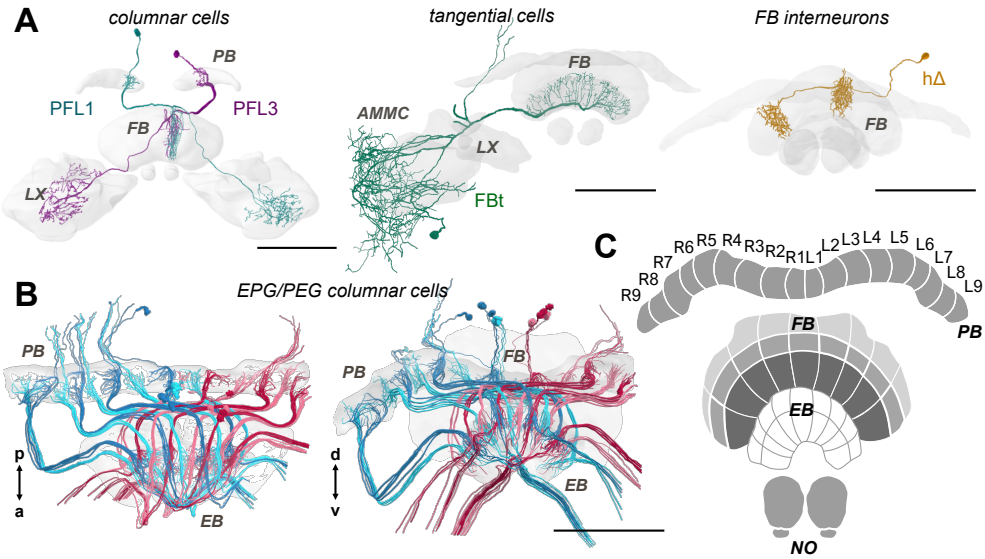

Figure 3: The projection domains of three major cell classes structure the CX into an arrangement of columns and layers.
(**A**) Examples of each CX cell class reconstructed from intracellular dye injections. All images generated from www.insectbraindb.org (Heinze et al., 2021). Data (left to right): Monarch butterfly (*Danaus plexippus*; Heinze et al., 2013), dung beetle (*Scarabaeus satyrus*; Dacke et al., 2019; Jundi et al., 2018), and sweat bee (*Megalopta genalis*; Dacke et al., 2019; Jundi et al., 2018). (**B**) Columnar cells form bundles of fibers that project from the PB to other regions in the CX. The discrete domains occupied by their branches form the columnar layout of the CX. Horizontal (left) and frontal view (right) of bumblebee (*B. terrestris*) EPG/PEG cells are shown here (Paper 1; Sayre et al., 2021). (**C**) Schematic showing columns and layers in the CX of the bumblebee. Scale bars: 200 µm.

The BU are part of a group of neuropils that are together known as the lateral complex (LX). This includes the lateral accessory lobes (LAL), the gall (GA), and several other areas, like the round body (ROB) and rubus (RUB), which to the best of my knowledge have only been clearly delineated in the cockroach (Althaus et al., 2022) and the fruit fly (Figure 2; Lin et al., 2013; Hulse et al., 2021; Wolff et al., 2015). Neurons projecting to the CX from the LAL carry mostly self motion cues, such as angular velocity, translational velocity, proprioceptive cues, and motor feedback (Currier et al., 2020; Matheson et al., 2022; Stone et al., 2017; Lyu et al., 2022; Lu et al., 2022; Westeinde et al., 2022; Pires et al., 2022). The LALs contain arborizations from descending neurons and CX output neurons, and have been implicated as regions that relay motor commands related to steering (Stone et al., 2017; Rayshubskiy et al., 2020; Steinbeck et al., 2020; Hulse et al., 2021; Westeinde et al., 2022; Pires et al., 2022).

The above general description of the organization of the insect brain is non-exhaustive.

Table 1: List of corresponding neuropil and neuron names in *Drosophila m.* and other insects. Asterisk: see Paper 1 for potential homologues in the bumblebee.

Neuropils

Drosophila m.	Other insects
PB	PB
FB	Upper division of the CB (CBU)
EB	Lower division of the CB (CBL)
NO	NO

Columnar neurons

Drosophila m.	Other insects
EPG/PEG	CL1a/b
PEN	CL2
PFL1	CPU1 type 1
PFL2	CPU2
PFL3	CPU1 type 2
PFN	CPU4
PFRa/b	*Unknown**
PFG	*Unknown*
Fx	*Unknown**

FB interneurons

hΔ	Pontines
vΔ	*Unknown**

Tangential neurons

Δ7	TB1
FBx	TU
ER	TL
LNO	TN

However, detailed reconstructions of insect brains and their constituent neuropils can be found online at the Insect Brain Database (insectbraindb.org; Heinze et al., 2021), an interactive and open-source data repository.

Anatomical layout of the insect CX

As mentioned in Chapter 1, all insects possess a CX (Figure 1C), regardless of ecology or navigational lifestyle. The CX is one of the most well studied regions in the insect brain (for review see; Pfeiffer, 2022; Turner-Evans and Jayaraman, 2016; Honkanen et al., 2019; Pfeiffer and Homberg, 2014), which I would argue is at least partially due to the fact that it is very aesthetically pleasing (Strausfeld, 2012). Situated on the midline, the CX is the only bilaterally symmetric region in the entire insect brain (Figure 2). It is formed by neural connections between four to five adjacent neuropils: the protocerebral bridge (PB), the fan-shaped body (FB), the ellipsoid body (EB), the noduli (NO), and, in the fruit fly, the asymmetric body (Figure 1B; Wolff and Rubin, 2018; Hulse et al., 2021). The FB and EB together are referred to as the central body (CB).

The CX comprises three major cell classes (Figure 3A). The main computational units of the CX are 'columnar cells' that stem from dorsally situated cell bodies (Figure 3A). These cells send ventrally projecting neurites in bundles alongside other columnar cells that share the same lineage (Boyan and Williams, 2011; Farnworth et al., 2020). All columnar cells

arborize in the PB and, depending on type, the FB or EB, and either the nodulus located in the contralateral hemisphere or another tertiary neuropil outside of the CX (for instance the LAL; Figure 3A). These cells are named as such because their projection domains form an array of 16-18 vertical columns in the PB and 8-9 vertical columns in the CB (Figure 3B-C). They also serve as the main output from the CX to other brain areas. Columnar cell types are defined according to the neuropils they arborize within, with the first neuropil being the region the cell receives predominantly input from and the remaining neuropils being the regions that the cell sends output to. For instance, an "E-PG" (EPG for short) neuron has fibers that project from the EB to the PB and Gall while a PEG cell receives predominantly input in the PB and sends output to the EB and Gall. Many insect neurons contain mixed input and output fibers within the same branching field however, so it is worth noting that such naming conventions are relatively simplistic compared to reality (Scheffer et al., 2020). The second class of CX cells are tangential cells, which provide the main source of input from other brain areas to the CX (Figure 3A). Tangential cells send wide branching fibers that become splayed out across the width of their projection neuropil. Within the FB and EB, tangential cells establish discrete layers (Figure 3A; Hulse et al., 2021; Strausfeld, 1976; Hadeln et al., 2020). The third and final class of CX cells include hΔ and, in flies, vΔ interneurons whose processes exclusively innervate the FB neuropil (Figure 3A; Hulse et al., 2021).

In conclusion, insect brains are highly stereotyped across insect orders. Several key pathways carry sensory information from peripheral sensory structures to higher order integrative centers in the central brain (Figure 2). This includes the CX, a collection of four neuropils whose highly modular arrangement is structured by the neural fibers of columnar cells, tangential cells, and FB interneurons (Figure 3A). The structure of the CX is intricately linked to its functional role in mediating navigational behavior, as will be shown next in Chapter 4 and Chapter 5.

Chapter 4

Representations of space in the central complex

The head direction network

Navigation requires the brain to compute internal representations of space. This includes representations of heading direction, information that is crucial for any form of navigation. Neurons whose firing rates correspond to angular orientation (Ranck Jr, 1984; Taube et al., 1990) have been found across distantly related animals, including in rats, bats, monkeys, fish, birds, and insects (Ranck Jr, 1984; Taube et al., 1990; Hulse and Jayaraman, 2019; Petrucco et al., 2022; Seelig and Jayaraman, 2015; Heinze and Homberg, 2007; Varga and Ritzmann, 2016).

Evidence of head direction circuits in insects came initially from intracellular recording experiments in which locust tangential neurons in the PB (Δ7 cells) were shown to form a map-like representation of polarized light e-vectors that were systematically arranged across the width of the PB (Heinze and Homberg, 2007). Nearly a decade later, the presence of an insect head direction circuit was confirmed by calcium imaging experiments in the fruit fly, which revealed that a population of EB columnar cells called EPG "compass" cells tracked the rotational movements of a fly, akin to a biological compass (Figure 4A; Seelig and Jayaraman, 2015). Since its initial discovery, the fly head direction network has been the subject of extensive analysis (Turner-Evans et al., 2017; Kim et al., 2019b; Fisher et al., 2019; Haberkern et al., 2019; Turner-Evans et al., 2020; Green et al., 2017; Hulse et al., 2021; Hulse and Jayaraman, 2019; Seelig and Jayaraman, 2015; Noorman et al., 2022; Kim et al., 2017).

Early models of head direction circuits proposed that a unique heading representation could

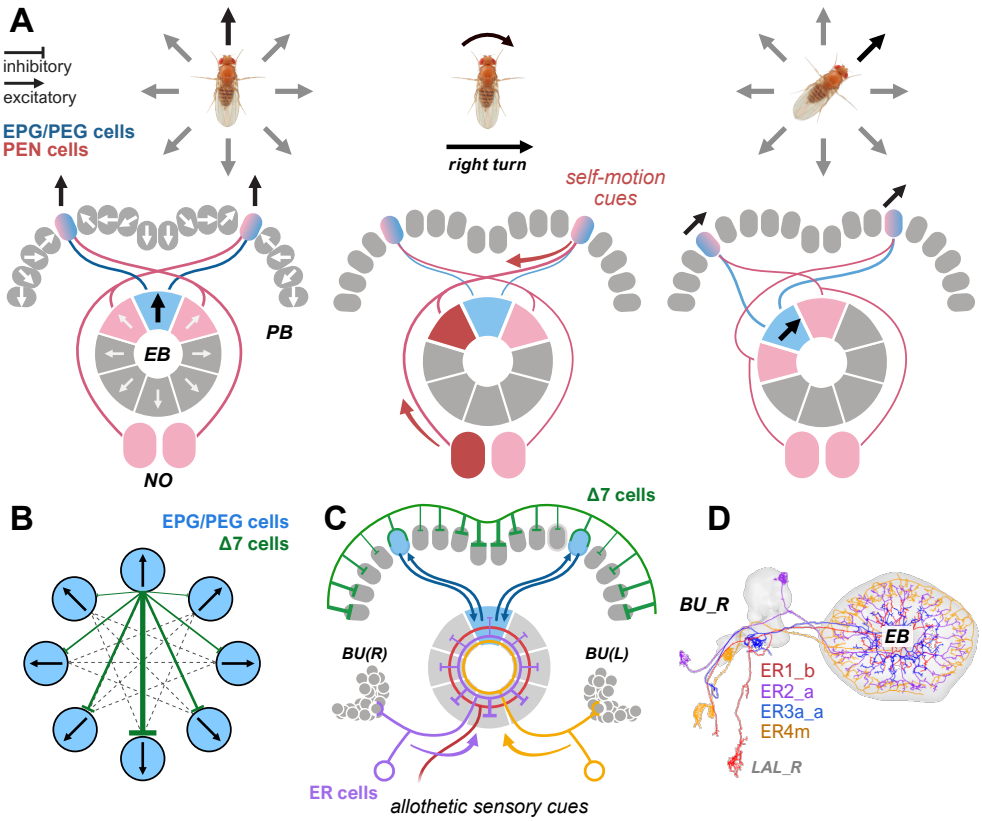

Figure 4: The fruit fly head direction circuit. (**A**) Schematic demonstrating EPG/PEG (blue) and PEN (red) population activity while a fly rotates to the right. Arrows in the CX represent azimuthal directions around the fly's body axis. The offset projection of PEN cells relative to EPG cells results in a shift of the activity bump to the left when the animal turns to the right. (**B**) An illustration of a ring attractor network. Active EPG/PEG cell populations inhibit those that are tuned to the opposite azimuthal direction via inhibition by Δ7 cells (green). (**C**) Drawing showing anatomy of EPG cells, Δ7 cells, and ER cells in the fruit fly CX. Note: this schematic shows the functional connectivity of the entire population of Δ7 cells; individual Δ7 cells have their outputs separated by 7 columns. (**D**) Fly ER cells that encode different sensory modalities innervate distinct layers in the EB (Scheffer et al., 2020; Hulse et al., 2021).

arise from a ring attractor network, whereby local excitation of a cell population of similar tuning (an activity "bump") suppresses excitation of cells that are anti-correlated in tuning via long range inhibition (Skaggs et al., 1995; Knierim and Zhang, 2012). Experimental studies have shown compelling evidence that the fruit fly head direction circuit is indeed functionally organized in this manner (Figure 4B; Kim et al., 2017; Turner-Evans et al., 2020). Local excitation arises from recurrent connectivity among EPG, PEG, and PEN

columnar cells (Figure 4A), while long range structured inhibition is supplied via PB Δ7 cells (Figure 4B-C Turner-Evans et al., 2020; Hulse et al., 2021; Franconville et al., 2018; Kim et al., 2017). PEN "angular velocity" cells play a major role in this circuit by integrating self-motion cues related to the fly's rotational velocity acquired from their presynaptic partners (LNO cells) in the NO (Green et al., 2017; Turner-Evans et al., 2017). A key feature that distinguishes EPG and PEN cells is their difference in projectivity from the PB to the EB. PEN cells are offset in the EB relative to EPG cells by a single column towards the contralateral hemisphere (Figure 4A). Thus, when PEN cells in one hemisphere increase their activity in response to rotational self-motion cues, this projection offset enables them to shift the activity bump towards the contralateral hemisphere, moving the bump laterally around the ring (Figure 4A; Green et al., 2017; Turner-Evans et al., 2017). Self-motion cues include those from rotational optic flow as well as from proprioceptive stimuli, the latter of which enable the bump to track the fly's angular position even in complete darkness (Green et al., 2017; Turner-Evans et al., 2017). In total darkness however, the activity bump begins to accumulate error relative to the true orientation of the fly due to the noisiness of internal self-motion cues (Hulse and Jayaraman, 2019).

Accuracy in heading representation is obtained via the tethering of external (allothetic) cues to the EPG population by a system of tangential neurons called ER cells ("ring" neurons; Figure 4C-D). Functional imaging experiments of ER cells in the fruit fly have revealed that subtypes of ER cells are tuned to wind information (Okubo et al., 2020), (Shiozaki et al., 2020), visual features (Seelig and Jayaraman, 2013), polarized light (Seelig and Jayaraman, 2013; Omoto et al., 2017; Sun et al., 2017; Shiozaki et al., 2020; Hardcastle et al., 2020), and also play a role in sleep structure and homeostasis (Liu et al., 2016; Donlea et al., 2018; Liu et al., 2019). ER cells receive retinotopically mapped visual information from their presynaptic partners in the BU (Seelig and Jayaraman, 2013), as well as mechanosensory input from the LAL. Both are carried to the EB where ER cell branches fan out and innervate every individual column (Figure 4D). Despite receiving organized information in the BU, retinotopy is not maintained in ER cell outputs, a feature supported by the fact that the position of the EPG activity bump is arbitrarily mapped across individuals and between different visual scenes (Haberkern et al., 2019; Seelig and Jayaraman, 2015; Kim et al., 2019a). Such remapping is thought to occur via quick Hebbian synaptic remodeling, specifically caused by associative synaptic depression between ER and EPG cells in the EB (Kim et al., 2019a; Fisher et al., 2019).

ER homologues in other insects ("TL" cells; Table 1) have been found to be tuned to polarized light, including in the locust (Vitzthum et al., 2002; Heinze and Homberg, 2009), sweat bee (Stone et al., 2017), dung beetle (Jundi et al., 2018), and the monarch butterfly (Heinze et al., 2013). The presence of several polarization pathways have been found in the locust, including via TL2 neurons that receive bilateral light information and TL3 neurons that receive only ipsilateral light information (Vitzthum et al., 2002; Heinze and Homberg,

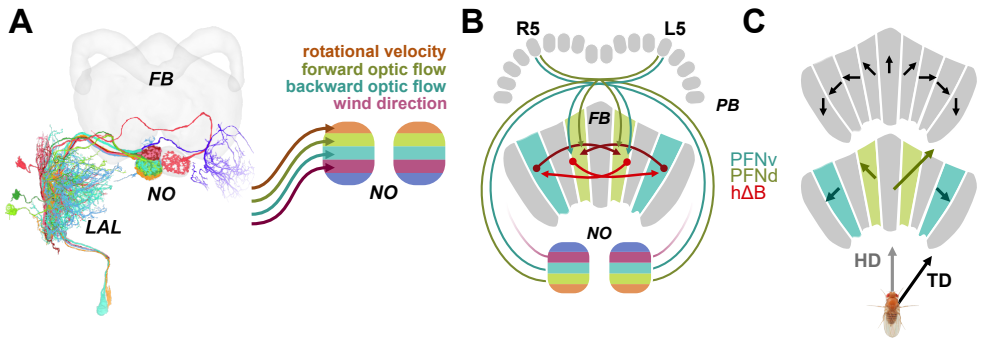

Figure 5: Self-motion cues are integrated with head direction activity to generate an internal representation of travel direction in the fly CX.
(**A**) In the fly, different LNO types encode different kinds of self-motion cues along multiple channels that are anatomically segregated in the NO. (**B**) PFN cells integrate self-motion cues from LNO cells with hΔ interneurons in the FB. PFNd and PFNv cells create axoaxonal synapses and axodendritic synapses, respectively, onto hΔB cells. PFNd activity is therefore retained in the same column it projects to while PFNv activity is shifted contralaterally by four columns (see main text; Lu et al., 2022; Lyu et al., 2022). (**C**) 360° of azimuthal space is encoded across the width of the FB with each column representing ca. 45° . Continuing with the above example, PFNd,v connectivity to hΔB cells therefore results in PFNd cell activity being maintained at an offset of +/- 45° while PFNv cells are shifted by +/- 135° . This creates a set of orthogonal vectors, the summed activity of which creates an internal representation of the fly's travel direction (TD) that is separate from, but reliant upon, activity from the fly's head direction (HD) system.

2009). Both project to the ipsilateral BU and AOTU and while it is not known where the contralateral TL2 input comes from, locust TuTu or LoTu neurons are likely candidates (Reviewed in; Heinze, 2014).

Until now, the identification of morphological homologues of head direction cells in non-dipteran insect species has relied on intracellular dye injections of single neurons. Thus, previous studies were unable to determine the exact quantities of cell types, their polarity, and how these cells were actually connected to each other, instead inferring polarity and connectivity from morphological features and overlapping fibers, respectively. In Paper 2, we generated detailed reconstructions of the head direction circuits across multiple species, enabling a comprehensive comparative analysis among species that differ in their navigational strategies.

The noduli relay self-motion information to the CX

Insects use optic flow, proprioception, and motor feedback to derive self-motion cues, which enable them to track their body's movement relative to their sensory environment.

These cues are relayed to the CX by LNO tangential cells that get input from the LAL and send output to the NO along multiple pathways segregated by sensory modality (Hulse et al., 2021; Stone et al., 2017; Hadeln et al., 2020; Currier et al., 2020; Lyu et al., 2022; Lu et al., 2022). In flies, a total of nine LNO cells arborize in neatly defined layers within the NO (Figure 5A; Wolff and Rubin, 2018). The dorsal-most layer of the fly NO contains GLNO cells (subtype of LNO) that convey rotational velocity cues to PEN cells of the head direction circuit (Figure 4A). In the ventral layers, LNO cells are presynaptic to PFN cells that innervate the PB and FB (Figure 5B). Calcium imaging experiments in the fly have shown that PFN subtypes PFNv and PFNd carry translational velocity cues from LNO1 and LNO2 tangential cells in the NO to hΔB interneurons in a manner which transforms individual vector components tethered to the head direction into a representation of the fly's travel direction (Figure 5B-C; Lyu et al., 2022; Lu et al., 2022). Like head direction, travel direction was represented by a bump of activity that moved laterally across the FB. When simulating movement using translational optic flow and a rotational bar in a virtual reality system, the head direction activity bump was correlated with the travel direction bump when the fly was facing in the same direction as it was traveling, but would become separated when the two directions differed (Lyu et al., 2022; Lu et al., 2022). Similarly, functional imaging and patch clamp recordings of another PFN subtype (PFNa cells) found these to carry wind direction signals to hΔC cells which enabled the fly to track odor sources upwind (Currier et al., 2020; Matheson et al., 2022). Both PFNv,d and PFNa occupy different layers in the NO and appear to form distinct and parallel channels to hΔ cells in the FB.

In sweat bees (*M. genalis*), intracellular recordings of LNO1 and LNO2 homologues (previously "TN1" and "TN2" cells) were found to be tuned to forward and backward translational optic flow, with firing rates that corresponded to optic flow rates ("speed" neurons; Stone et al., 2017). Morphological homologues of LNO1 and LNO2 cells have been identified in honeybees (Hensgen et al., 2020), locusts (Hadeln et al., 2020), and fruit flies (Hulse et al., 2021). While layered NO are present in other insects, the number of layers and innervation patterns diverge across insect orders (Heinze and Homberg, 2008; Heinze and Reppert, 2012; Jundi et al., 2018; Stone et al., 2017; Hensgen et al., 2020). The question of how species-specific idiothetic cue preference (such as optic flow versus wind direction or step integration) might be reflected in the anatomical organization of the NO is addressed further in Chapter 5 and is the focus of Paper 3.

In summary, the CX contains circuits that generate internal representations of heading and travel direction, both of which integrate sensory cues relayed from peripheral brain areas to the EB and the NO, respectively. We therefore have a brain area that encodes direction and receives information about speed, both of which are prerequisites for path integration (Chapter 2). To path integrate, however, requires the assimilation of both cues into an internal representation of a "homing" vector that points back to the starting point of the

trip. Specifically, it requires the distance travelled in each direction during an outbound journey to be accumulated, summed, and then inverted.

Additionally, and perhaps at a more basic level, path integration requires contextual inputs; where does the insect want to go? How do path integrating circuits compare current heading to a goal heading and initiate steering commands if the two do not match?

While the neural mechanism for generating and storing a homing vector remains unknown, several models predict it occurs within the FB. The FB has also been implicated in integrating navigationally relevant contextual cues from neurons downstream of the mushroom bodies. Both topics are explored next, in Chapter 5.

Chapter 5

Unraveling the neural basis of vector navigation

The fan-shaped body

Of the four CX neuropils, the fan-shaped body (FB) is the largest and arguably the most complex. Cells that innervate the FB include most CX columnar cells, tangential cells, and a system of interneurons named hΔ and vΔ (historically called 'pontines'; Table 1; Hanesch et al., 1989) whose input and output processes are solely confined to the FB. The fly connectome revealed that most FB columnar cells receive head direction activity from both Δ7 and EPG cells cells (Chapter 3 Hulse et al., 2021) in the protocerebral bridge (PB). Intriguingly, this would suggest that the PB not only looks like a bridge that spans the insect protocerebrum, but also functionally acts as a bridge, reshaping and passing on directional cues from the EB to the FB (Figure 6 Hulse et al., 2021).

Similar to ER 'ring' neurons in the EB, FB tangential neurons (FBt) have wide-branching output fibers that span the full width of the FB, innervating each of the 9 vertical columns. These neurons arborize in discrete layers within the FB, of which there are 9 in the fly (Hulse et al., 2021). In contrast to the majority of ER cells which get input from a single brain area (the BU), different types of FBt cells relay information to the CX from regions all over the central brain (Hulse et al., 2021).

One of these regions includes the mushroom bodies (MB), paired associative memory centers that play a major role in curating learned behaviors (Modi et al., 2020, reviewed in). Interestingly, FBt cells are the only CX neurons to receive direct input from the MB. This input is relayed to FBt cells via MB output neurons (MBON), a group of cells that play a major role in encoding stimulus valence (Aso et al., 2014; Owald et al., 2015; Scaplen et al.,

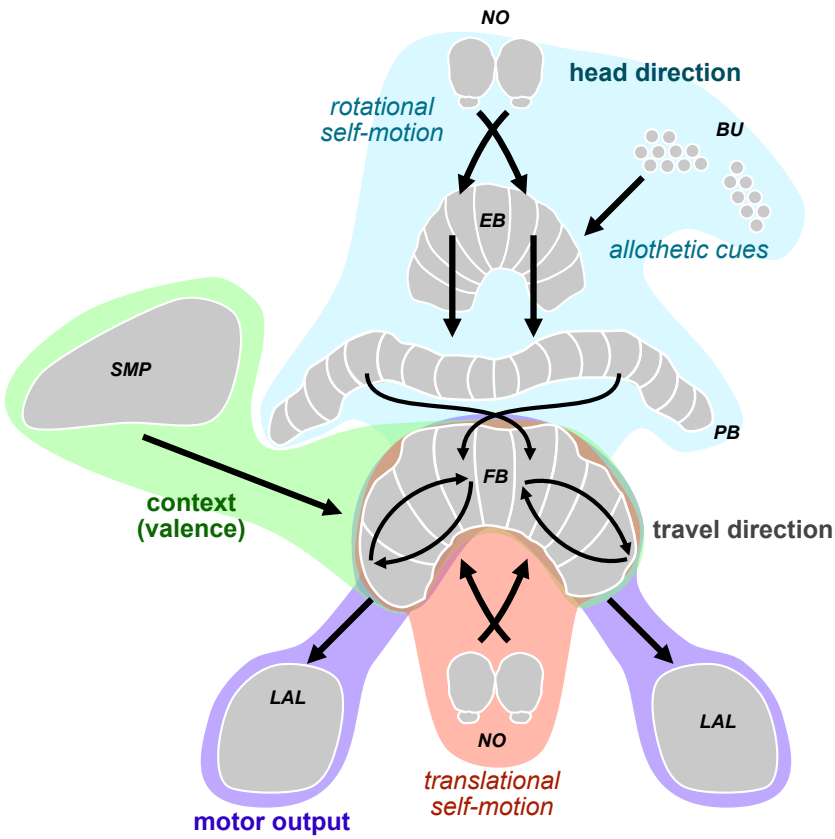

Figure 6: General patterns of information flow throughout the CX.
A schematic showing the layout of the CX when arranged according to patterns of functional connectivity (Hulse et al., 2021; Franconville et al., 2018). FB columnar cells receive head direction signals from the PB, contextual signals from tangential cells projecting from the superior medial protocerebrum (SMP), and translational self-motion input from columnar cells innervating the NO. A system of hΔ interneurons shift activity laterally across the FB, providing a neural substrate that is ideal for vector computations. FB PFL columnar cells then link the PB with the FB and LAL, making them anatomically well suited to compare directional goals with current heading and initiate motor commands (asterisk indicates uncertainty).

2020; Hulse et al., 2021). This, along with a growing body of functional evidence, suggests that FBt cells encode contextual information, in particular about stimulus salience (Sareen et al., 2021; Matheson et al., 2022) and internal state (Donlea et al., 2011).

Lastly, the FB is innervated by PFL neurons, which are one of the few major CX outputs. There are several types of PFL neurons that differ in their projectivity within the FB. Importantly, PFL cells are presynaptic to descending neurons (DNs) in the LAL. DNs regulate motor control, sending long projecting fibers to regions in the ventral nerve chord

(Borgmann and Büschges, 2015). This relationship between PFL cells and DNs together with functional imaging studies (Rayshubskiy et al., 2020; Westeinde et al., 2022; Pires et al., 2022) have provided compelling evidence that they are involved with driving motor commands (Martin et al., 2015). Additionally, their projectivity offsets in the FB make them ideal candidates for comparing a goal direction signal in the FB with a head direction signal in the PB and generating steering commands if these two directions do not match (Stone et al., 2017; Honkanen et al., 2019; Steinbeck et al., 2020; Hulse et al., 2021; Westeinde et al., 2022; Pires et al., 2022).

In summary, a growing body of evidence suggests that the FB is involved in coordinated movement that is informed by head direction and self-motion cues, and modulated by internal state as well as environmental context (Honkanen et al., 2019).

Modeling path integration

As discussed in Chapter 2, path integration is a computational strategy in which an animal maintains a constant representation of its current location relative to a starting point. This strategy requires the individual to accumulate a memory of the distance it has travelled in each outbound direction to generate a homing vector that points directly back to its starting point. A model inspired by physiology and constrained by anatomy proposed that such computations could take place in the CX (Figure 7A Stone et al., 2017).

In this model, heading direction is represented by inhibitory Δ7 cells in the PB which encode a sinusoidal activity bump inherited from EPG neurons in the EB. Intracellular recordings of NO tangential neurons in the sweat bee (*M. genalis*) revealed two cell types that encode forward and backward translational velocity from optic flow stimuli. Interestingly, these cells were tuned to optimal optic flow expansion points at +45° and -45° relative to the head direction of the bee, a tuning that can correctly represent forward speed despite of asymmetric accumulation of optic flow information during holonomic flight (Stone et al., 2017). Further, synaptic resolution reconstructions of these cells from serial block-face electron microscopy image data revealed them to be presynaptic to PFN neurons. Because of this connectivity, PFN neurons in the model integrate directional cues from Δ7 neurons in the PB with velocity cues in the NO, creating an array of "directionally locked odometers" (Stone et al., 2017) that accumulate activity in proportion to distance travelled (i.e., vector memory). Importantly, because Δ7 neurons are inhibitory, PFN activity would accumulate in directions opposite to the heading direction of the insect. Once the insect finds its goal, an internal signal could shift the goal direction from search behavior to homing behavior and use the PFN encoded trajectory back to its starting point.

PFL1 "steering cells" are assumed in the model to receive input from PFN cells. In the model, PFL1 cells project to columns in the FB that are offset from PFN projections by a

single column, enabling them to compare the current heading signal in the PB with a goal direction encoded by PFN cells in the FB in a way that automatically generates appropriate steering signals to bring the two directions into alignment.

The Stone et al. (2017) model proposes that the homing vector is accumulated by PFN neurons in two components, one in each hemisphere. Connectomic analysis of the *Drosophila* CX revealed that PFN neurons from both the left and right hemispheres synapse onto the same post synaptic cell-types making it unlikely that the vector memory is read to PFL cells in two separate components, at least in the fruit fly. Further, Hulse et al. (2021) found little evidence for 'structured' (i.e., isolated within-column and balanced) recurrent connections between PFN cells, a neural mechanism that was suggested to encode the homing vector memory in Stone et al. (2017). Hulse et al. (2021) instead propose several anatomically plausible storage mechanisms for a homing vector in the fly, including via recurrent connections between hΔ cells, graded activity of FC neurons, and via FB tangential neuron synaptic plasticity.

Despite this, the Stone et al. (2017) model made a few predictions about the bee CX that our anatomical reconstructions revealed to be true. One of these was that insects that path integrate over longer distances would require a larger capacity for the short term maintenance of the homing vector, for instance by increasing the quantity of PFN cells. Indeed, our analysis revealed that the bumblebee CX has at least twice the number of PFN cells as the fly (though there are likely more, considering that the limited resolution of our dataset prevented the identification of neuron fibers with fiber diameters below 60 µm; see Paper 1).

Additionally, through our connectomic investigation of bee PFN cells and FBt cells we found a novel sub-circuit in the bumblebee and sweat bee brain which, to the best of our knowledge, does not exist in the fly (Paper 3). If indeed there is no evidence that PFN cells accumulate directionally locked speed information as two separate components, this new sub-circuit could be well suited to provide a substrate for encoding the path integration homing vector in insects that path integrate over long distances.

Long term storage of the homing vector: a hypothesis

Following the Stone et al. (2017) model, Moël et al. (2019) proposed how such a model could be flexibly modified by the addition of an additional neuron type and a reinforcement signal, so that the circuit would be able to store a vector memory for later use (Figure 7B). The additional neuron type could, in theory, be a FB tangential neuron (Figure 7C). Anatomically, the wide-branching connectivity of FBt cells across FB columns is reminiscent of ER neurons which have the same layout in the EB, and which reorganize their outputs onto EPG head direction cells via Hebbian-like plasticity and synaptic depres-

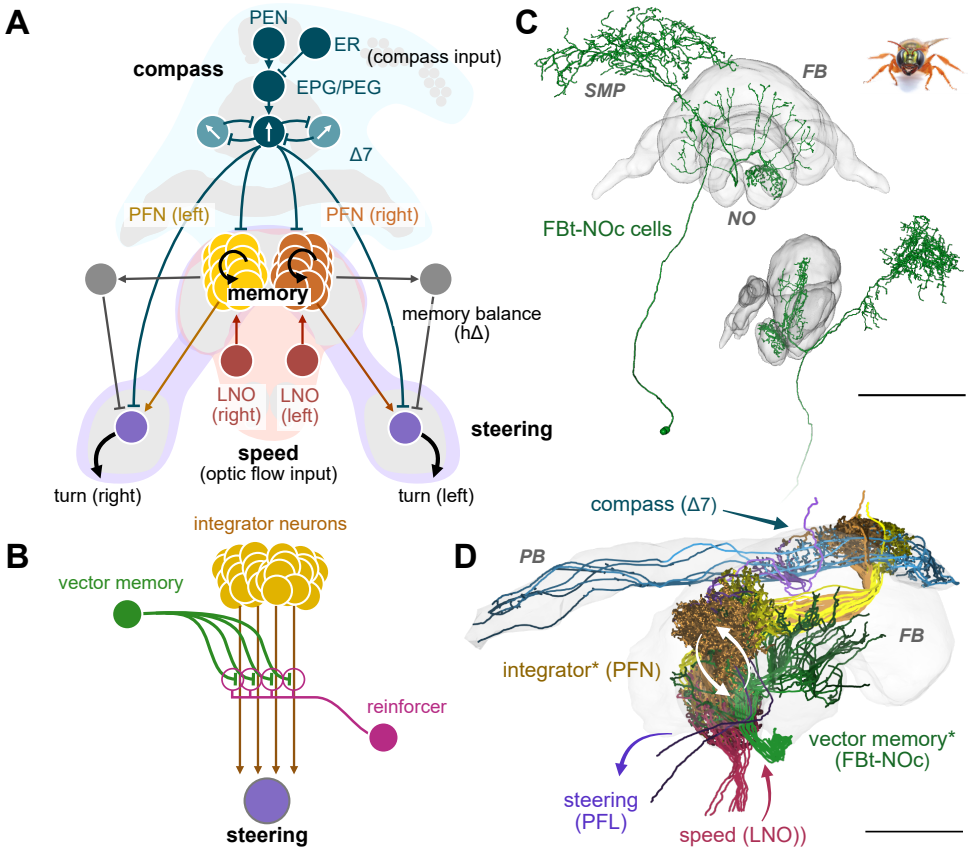

Figure 7: An anatomically plausible model for path integration in the bee.
(**A**) A simplified illustration of the Stone et al. (2017) path integration model overlaid on the information flow schematic in Figure 6. Nodes correspond to cell populations. Edges with arrows indicate excitatory connections and edges with bars indicate inhibitory connections. (**B**) Schematic showing the addition of two cell types as proposed by Moël et al. (2019) that could enable the storage and flexible use of navigational vector memories (see main text). (**C**) Intracellular dye injection of a FBt-NOc neuron in the sweat bee (Paper 1; Sayre et al., 2021). Given their uniqueness bees, their relatively large quantity (n = 15 in bumblebees; Paper 1, n = 20 in sweat bees; Paper 3) and their disposition in the NO, FB, and SNP, FBt-NOc cells are ideal candidates to play the role of 'vector memory' cells (Moël et al., 2019). (**D**) Reconstructions of neurons likely to be involved in encoding navigationally relevant vector representations in the bee CX (reconstructions of the sweat bee CX from Paper 2 and Paper 3; hΔ cells not shown here). Asterisks indicate uncertainty. Scale bar: 100 µm.

sion (Fisher et al., 2019; Kim et al., 2019a). An FBt that inherits signals from either PFN neurons or PFR neurons could theoretically form an association such that the accumulation of distance cues reorganizes the distribution of synaptic weights between tangential

cells and downstream PFL steering cells (Figure 7D). If this were true, the finding of food, for instance, would trigger a reward-like reinforcement signal from dopaminergic neurons that also selectively innervate layers in the FB (Hulse et al., 2021; Sayre et al., 2021), leading to long term storage of the homing vector (Paper 1, Paper 3).

Such a layout would provide opportunities for flexible use of a homing vector. For example, hΔ cells downstream from FBt cells that shift activity by 180° in the FB (see next section) could potentially invert the homing vector into a 'fooding vector' that would enable the forager to return back to a foraging site at a later point. And the fact that FBt cells are downstream of MBONs in the fly may suggest that vector encoding FBt cells in the bee could be activated or inhibited by upstream MBONs when environmental or internal cues signal that it is time to forage.

Vector computations in the FB

Findings from the fruit fly CX connectome suggest a major role of the FB as a context dependent "vector calculator" (Hulse et al., 2021). As mentioned in Chapter 4, FB interneurons called hΔ play a role in transforming self-motion input into an internal representation of travel direction. hΔ cells have their inputs in one column and their outputs shifted by three columns toward the contralateral hemisphere. If the lateral width of the FB represents 360° of azimuthal space, then each of the nine columns would represent 45°, implying that hΔ cells shift FB activity by 180° (see next section; Hulse et al., 2021; Lyu et al., 2022; Lu et al., 2022; Matheson et al., 2022). A second class of FB interneurons called vΔ cells have processes that stay within a single column. vΔs have dendritic branches in one layer and their axonal outputs in a separate layer, suggesting a similar role for vΔs in transforming activity across the FB, but vertically instead of horizontally. The combined architecture of hΔ cells and vΔ cells therefore results in a "2D scaffold" (Hulse et al., 2021) along which neural activity can be either horizontally or vertically shifted, providing a neural substrate for performing vector computations.

Comparing current with desired heading to generate compensatory steering commands

FB columnar cells differ in their projection patterns from the PB to the FB. Columnar cells which follow the most common projection pattern ("default" in Paper 1, Figure 2), such as PFRa and PFG neurons in the fly, have no offset in the FB column they innervate relative to the PB column they project from. Other projection offsets include PFN cells which are contralaterally shifted by one column in the FB, PFL1 cells that are ipsilaterally shifted by one column, PFL3 cells that are ipsilaterally shifted by two columns in the fly, and PFL2 cells which are ipsilaterally shifted by four columns in the fly (for bee, see Paper 1). Im-

portantly, in contrast to PFL1 and PFL3 which only send output fibers to the contralateral LAL, PFL2 send bilateral outputs to both the contralateral and ipsilateral LALs.

Consistent with Stone et al. (2017), Hulse et al. (2021) suggest PFL neurons compare current heading in the PB with goal direction in the FB and initiate a compensatory turn if these directions do not match. However, in Hulse et al. (2021) such comparisons are proposed to be carried out by PFL3 neurons that would be most likely to be active when the fly is facing 90° either to the right or to the left of their goal direction (inbound direction; opposite of the "stored" or outbound vector direction). PFL2 neurons, with their 180° offsets in the FB and bilateral LAL projections, were suggested to drive forward velocity when the animal is facing towards their goal direction. Differences in PFL projectivity would therefore provide an elegant solution for how insects are able to compare current heading estimates with desired heading estimates and generate a steering command if there is any mismatch (or propel the animal forward if there isn't).

Summary

Navigation requires a system that can compare the navigator's current heading to the direction of some goal and initiate compensatory steering commands if those two directions do not match. Anatomical and functional studies have indicated that such a system exists in the insect FB, the largest and most numerically complex of the four CX neuropils.

The FB contains a network of interneurons that enable neural activity to be shifted both laterally and vertically, respectively corresponding to a shift in azimuth representation and between types/layers, ultimately providing an ideal substrate for performing vector computations. This includes the transformation of idiothetic translational velocity signals into an allothetic representation of traveling direction in the fruit fly. FB tangential neurons are the only CX input cells that get direct input from the MB and have been functionally implicated in providing contextual cues to the FB. They are also anatomically well suited to provide a neural substrate for the long term storage and retrieval of vectors acquired during path integration. Lastly, PFL cells that send output fibers to premotor regions have projection offsets in the FB that make them ideal for comparing directionally relevant signals in the FB to those in the PB and initiate compensatory motor commands if those two do not match. All of the above suggest that the CX, particularly the FB, is crucially involved in path integration.

And while the neural substrate underlying homing memory remains to be elucidated, connectomic maps help to constrain what is anatomically possible. The connectomic and projectomic maps produced from this thesis will shed light on such constraints within the CX of insects the rely on long distance homing behavior for survival.

Chapter 6

From histology to circuits

To compare the CX circuit structure of six insect species, I used a "projectomic" approach* (Kasthuri and Lichtman, 2007), where the acquisition and subsequent alignment of multi-resolution images enable both the tracing of every neuronal projection (i.e., "projectome") in the CX, as well as every neuronal connection (i.e., "connectome") in specific compartments of the CX (see also Hildebrand et al., 2017). Connectomic areas are scanned at synaptic resolution and placed at regions that follow the trajectory of a typical columnar cell as it projects from the PB in the left hemisphere to portions of the FB, EB, the entire NO, and, in some cases the BU in the right hemisphere (Figure 8). Compared to generating a full connectome of the entire CX, this approach makes comprehensive and comparative assessments of multiple species feasible within the span of a PhD. Further, if neuronal identity and arborization patterns are bilaterally symmetrical in the projectome, we can cautiously assume some degree of connective symmetry when analyzing the data.

What follows is an outline of this workflow, which begins with histological preparation of insect brains, followed by image acquisition using serial block face electron microscopy (SBEM), the subsequent processing of images, and finally our approach to neural tracing.

Histological preparation of insect brains for SBEM

Histology is arguably the most important element of this entire workflow. Samples which are fixed poorly, damaged, or badly stained may suffer from longer image acquisition times (noise and dwell time discussed in next section), poor contrast, or poor tissue ultrastructure, ultimately making images less reliable, more time consuming to trace, and impractical to

*EM image data for projects 2-3 were generated using a projectomic approach. The exception is Paper 1, which differed in method of image acquisition (see image acquisition section).

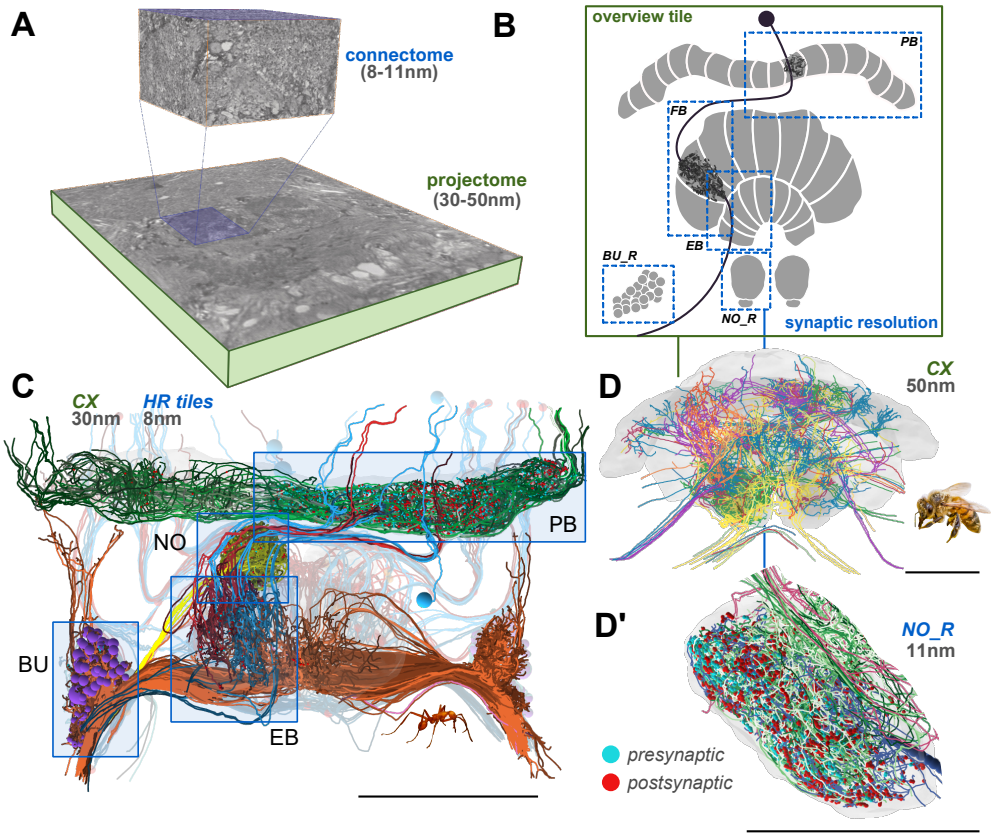

Figure 8: A multi-resolution approach to acquire SBEM images for comparative connectomics. (**A**) Volumetric rendering of a synaptic resolution EM image stack (blue) and it is relative location within a lower ("cellular") resolution overview stack (green). Values are the pixel scale in xy (i.e., 8nm x 8nm). All images were acquired with a Z-resolution of 50 nm. (**B**) Schematic identifying the CX compartments that were captured at synaptic resolutions (note that the BU_R was only imaged at synaptic resolution in the army ant and the desert ant). The trajectory of an example FB columnar cell is shown. To see exactly which regions were captured and traced for each species, see Paper 2 Figure 1 Supplement 1, and Paper 3 Figure 1 Supplement 1. (**C**) Traced data from the army ant. Blue rectangles outline the bounding boxes of synaptic resolution image stacks. (**D**) An example of neural projections from the cellular resolution overview dataset of the honeybee. (**D'**) Neurons and their synaptic partners in the NO of the same honeybee dataset, traced from synaptic resolution EM images. Scale bars: 100 um

use with automated segmention algorithms. Therefore, the initial goal of histology here is to preserve the brain tissue as closely as possible to its native state. Then, the tissue must be stained with heavy metals which provide additional fixation of fatty lipid membranes, and electron dense regions that enhance contrast and prevent charging. Finally, the tissue

must be slowly dehydrated and thoroughly embedded in plastic resin.

A protocol adapted from Hua et al. (2015) was used to stain bee and ant brains, some of which are relatively large in volume (see Paper 2, Figure 1 Supplement for comparison of CX volume across species). To begin, insects are cooled to immobility over ice (2-5 min). The head is then removed and placed directly into a low concentration fixative solution containing 0.75% glutaraldehyde, 0.75% paraformaldehyde, and 0.1M sodium cacodylate buffer (pH 7.3). Published volume EM protocols typically call for higher fixative concentrations in the range of 2-3% glutaraldehyde and 2-4% paraformaldehyde, however we experienced much better preservation of insect neural tissue with substantially lower fixative concentrations.

After fixing overnight at 4°C, brain tissue is rinsed in 0.1M sodium cacodylate then post-fixed in 2% osmium tetroxide (OsO4) for 1 h at room temp. OsO4 binds to and fixes fatty lipid membranes which are not fixed by the aldehydes, enabling visualization of cellular membranes and synaptic vesicles (Hua et al., 2015). This and all remaining steps (unless otherwise stated) are accompanied by microwave pulsation in which the tissue in solution is microwaved on a temperature-controlled bath under vacuum using finely tuned power settings. This is done to aid with solution permeation and is particularly useful for larger brains. Following post-fixation in OsO4, tissue is put directly into a solution of 2.5% potassium ferricyanide for 1 h at room temp. Potassium ferricyanide is a reducing agent thought to increase osmium deposition in membranes (Hua et al., 2015). Next, the neural tissue is rinsed in water and then transferred to a solution of 1% sodium thiocarbohydrazide where it is kept for 45 min at 40°C. Note that microwave pulsations are omitted from this step to avoid over-heating the sample. The sample is then removed from the oven, allowed to cool, and rinsed with water before being put back into a solution of 2% OsO4. Like potassium ferricyanide, sodium thiocarbohydrazide is used to enhance osmium staining, although it does so by providing an additional linkage between osmium molecules. Samples are once more rinsed in water and transferred into 1% aqueous uranyl acetate (UA) where it is left over night at 4°C. Uranyl acetate is commonly used to further aid in membrane staining (by binding to carboxyl groups) and may label proteins such as those in post-synaptic densities (Hua et al., 2015).

The next day, samples in 1% UA are transferred to an oven set at 50°C for 75 min. Hua et al. (2015) argue that there is a trade-off between permeation and reactivity since reactivity in this case implies denser membranes, which are more difficult to permeate. This two-step process combines better UA penetration (the initial overnight step) with enhanced UA reactivity (the heating step). Next, samples are rinsed in water and transferred to a lead aspartate solution for 50 min at 50°C. Note that microwave pulsations are omitted from the UA and lead aspartate steps to avoid over-heating the sample.

Finally, samples are dehydrated in an ethanol series from 20%, 50%, 60%, 70%, 80%,

90%, to 2 X 100% at intervals of 10-15 min with microwave aided permeation (vacuum off). Samples must be dehydrated because most embedding resins are hydrophobic, however if it is done too quickly the samples, which are extremely brittle at this stage, may loose structural integrity and crack or become damaged. A second purpose of dehydration is to remove all tissue that has not been fixed. Contrary to intuition, extraction of non-membrane tissue can be good in this case: it is easier to trace neurons if the only structures present are membranes and synaptic specializations. Samples are put in a solution of 100% acetone for 15 min and then through an increasing concentration series of acetone to embedding resin (Durcupan plastic polymer) at 1:3, 1:2, 1:1, 2:1, 3:1, and 2 X 100%. Samples in resin are placed in a 60°C oven for 48 h to polymerize. Lastly, samples can be removed from the oven, trimmed into a block, and then mounted to aluminum SBEM stubs using conductive epoxy (helping to reduce charging).

SBEM Image acquisition

Electron microscopy is commonly referred to as the "gold standard" for visualizing tissue ultrastructure. Electron microscopy enables users to produce image data at resolutions that are unattainable using standard light microscopy and is therefore an essential tool in connectomics where neuron connectivity can only be evaluated by visualizing synaptic structures of 20-50 nm in size.

The SBEM is a standard electron microscope with a built-in ultra-microtome (Denk and Horstmann, 2004). The exposed face of the sample block (i.e., "block-face") is scanned by the electron beam in a raster-like configuration producing a 2D image and followed by a sectioning cut from a diamond knife at a thickness of usually 50 nm, although thinner or thicker cuts can be achieved. The benefit to such a setup is that it allows users to obtain 3D image data without the need to cut sections manually, a process that is notoriously difficult and time consuming for larger samples. The downside is that this is a destructive process. As the sample is imaged, it is also destroyed, so it is impossible to return to any given slice for re-imaging later.

Two SBEMs were used, both located at the Center for Microscopy and Microanalysis at the University of Queensland. A Zeiss Sigma with a built-in Gatan 3View and Digital Micrograph software was used to collect image data of the bumblebee for Project 1 and a VolumeScope SBEM with MAPS v.2 and v.3 image acquisition software (Thermo Fisher Scientific) was used for all remaining projects. What follows is a description of the imaging method using the VS with MAPs software. For a description of imaging parameters using the Zeiss microscope, see Methods in Paper 1.

To acquire multi-resolution images, an overview tile was positioned to capture the entire CX at cellular resolution. Depending on the size of the brain, a pixel scale of either 30 nm x

30 nm, 40 nm x 40 nm, or 50 nm x 50 nm was used and all were cut with a slice thickness of 50 nm. For the smaller brains (i.e., *Cataglyphis* and *Eciton*) a higher resolution was required to see the same structures that could be viewed using a lower resolution in a larger brain. The trade-off here is that, although larger brains require less resolution, there is a limitation in what can be captured within the field of view of a single tile due to physical properties of the electromagnetic lenses. Tiles which were especially large suffered from optic distortions along their outer regions, requiring the use of multiple smaller tiles which later then need to be stitched (a time-consuming process that also results in blurry imperfections along the stitched regions in some cases).

In addition to the medium resolution overview tile, higher resolution tiles were placed along specific regions of the CX to capture columns, in some cases entire hemispheres, of CX neuropils (Figure 8B-C). In all species, except for the honeybee, these tiles were captured at synaptic resolution (8 nm-10 nm pixel scale). The honeybee contained a synaptic resolution tile that covered a single NO, with medium-high resolution tiles (24 nm x 24 nm) placed over multiple columns in the PB, FB, EB, and BUs (this species served as a second pilot study for this methodology).

In most cases, the brains were imaged using a landing beam energy of 2.0 kV, a beam current of 0.1 nA, and a pixel dwell time of 3 µs. The higher the beam current the better the contrast but the more likely insulating regions in the tissue would charge, creating charging artifacts or beam related tissue damage. The longer the pixel dwell time, the less noisy the image, but at the cost of increasing scan time. As mentioned previously, samples that experience superior processing conditions withstand greater amounts of charge and reduce scan time by allowing one to increase the beam current and decrease pixel dwell time (Lu et al., 2019). This was the case for *Megalopta*, for which beam current was increased to 0.2 nA and pixel dwell time decreased to 2 µs. Lastly, although scan times were reduced significantly using this approach, each CX scan still required around 6-8 weeks of non-stop imaging, producing 2+ TB of image data.

Image registration and processing

Each scan acquires a total of 10-15,000 images which then need to be stitched (aka "montaged"), aligned, processed, and finally converted to the proper image format for tracing. This is done in a two step process. The first step is to stitch and align the overview image stack which will then serve as the template for the high resolution image stack. This is done using the open-source software TrakEM2 (Cardona et al., 2012), which is integrated as a plugin in FIJI (ImageJ; Schindelin et al., 2012). Once the overview is aligned, the second step is to register the high resolution images to the overview image stack. This is also carried out in TrakEM2 by scaling up the overview image stack to accommodate the difference in pixel scales, locking the overview image stack in place, and then running an affine align-

ment (using scale invariant feature transform, "SIFT"; Lowe, 2004) to montage the high resolution stack onto the overview. Once this is complete, the overview images are deleted from the project and the high resolution images are exported as .tif files.

To trace from one stack into the next stack, all stacks must be registered in the same 3D coordinate space. Once the high-res stacks are montaged to the overview stack, they are registered to the same 3D coordinate space manually using the Transform Editor in Amira 2019.4 (Thermo Fisher Scientific). With the overview stack set in position, the high-res stack is positioned such that each aligned slice directly overlays the same region in the overview. Coordinate values can then be extracted via the Bounding Box module.

Next, the images are processed to adjust for noise and contrast. Note that this is done following the alignment so that a backup copy of the aligned but unprocessed stack can be saved. The reasoning behind this is that it is much quicker and easier to re-process images that have already been aligned than it is to have to re-align them in order to re-process them (for instance, if we discover a better way to process our images in the future, i.e., such as finding a new denoising algorithm that works exceptionally well). To denoise the images, we simply use the Gaussian blur filter available in FIJI with a sigma value of 0.5-0.7. Depending on the image quality, we may also apply a median filter with a value of 0.5. After images have been filtered, they are then contrast adjusted using contrast limited adaptive histogram equalization in FIJI (CLAHE; Zuiderveld, 1994). Finally, .tif image files are converted to tiled image pyramids using TrakEM2, which can be used with collaborative tracing software, discussed next.

Collaborative neuron tracing and analysis

Neuron tracing is perhaps the most labor intensive and time-consuming component in generating a connectomic dataset. However, labor and time costs can be offset if images are traced and annotated collaboratively in real time. Collaborative Annotation Toolkit for Massive Amounts of Image Data (CATMAID) is a software that was built to allow users to trace datasets from any location in the world, enabling collaboration within and between research groups (Saalfeld et al., 2009). Importantly, CATMAID supports simultaneous viewing of multi-resolution stacks thanks to contributions made by Hildebrand et al. (2017). Once CATMAID is setup on a dedicated server it can be accessed via web browser on any computer with internet.

Neuron skeletons are manually traced in CATMAID by scrolling through EM images and placing nodes along the profile of a neuron. Synapses can be marked by placing special nodes ("connectors") at synaptic sites that link pre- and postsynaptic partners. CATMAID supports tagging and annotating neurons and connectors and contains numerous plugins for analysis, including graph plots, connectivity plots, and a 3D viewer for viewing neuron

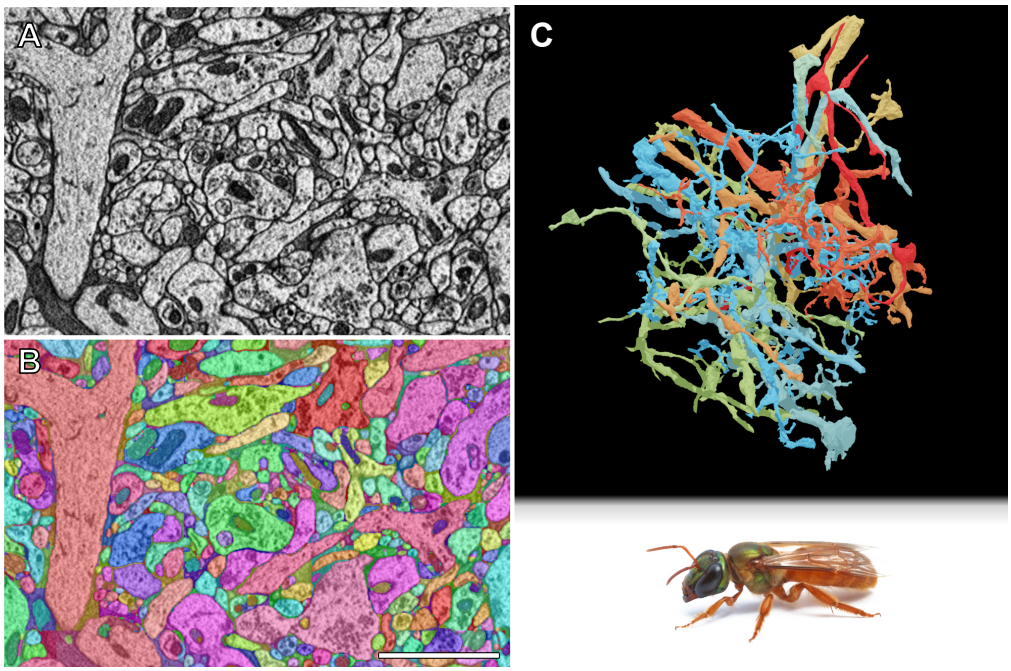

Figure 9: Automated segmentation of synaptic resolution SBEM image data.
(**A**) SBEM synaptic resolution image data of the sweat bee NO (10 nm x 10 nm pixel scale). (**B**) Neural profiles and mitochondria from (A) segmented using a convolutional neural network and local shape descriptors (Funke et al., 2019; Sheridan et al., 2022). (**C**) 3D view of segmented data rendered using Blender open-source software (Community, 2018). Segmentation data and images provided by Valentin Gillet. Sweat bee image; Ajay Narendra. Scale bar: 5 µm.

morphologies and neuropil surface volumes.

To ensure that neurons are traced accurately, there needs to be a review process whereby expert tracers verify the work done by less experienced tracers. To reduce time and avoid redundant work as much as possible, we use the CATMAID Review Widget and follow the targeted review method outlined in Schneider-Mizell et al. (2016). This includes focusing our review on the major branches which host the majority of synaptic sites, comparing inter-hemispheric symmetry of neurons with bilateral pairs, comparing our reconstructions to cell morphologies obtained using light microscopy, and comparing our cell morphologies to putatively homologous cells in other species.

For the analysis and visualization of connectomic and morphological tracing data, we use a powerful suite of R packages collectively called the natverse (NeuroAnatomy Toolbox; Bates et al., 2020), which includes packages for interfacing with CATMAID ("catmaid" and "catnat"). Also useful is the Python equivalent of natverse, NAVis (Neuron Analysis and Visualization; https://github.com/navis-org/navis. See Paper 2 and Paper 3 Methods

for more details on specific analyses that were done using these packages.

Automating the segmentation of high resolution datasets

Although collaborative tracing helps to increase efficiency by distributing labor, neural tracing is still very time intensive. To address this, major progress has been made in recent years to automate both the segmentation of EM image data (Funke et al., 2019; Scheffer et al., 2020; Sheridan et al., 2022) and the detection of synapses (Buhmann et al., 2021). Whereas skeleton tracing reveals only the trajectory of a neuron, segmentation reproduces the entire morphology of neurons in full (for instance; Scheffer et al., 2020). Such a process requires high resolution EM images that show a clear delineation of neural membranes, and is therefore usable with our synaptic resolution datasets (Figure 9A-C). At the time of writing this thesis, we have developed an automated segmentation pipeline based on (Funke et al., 2019). We trained a convolutional neural network to recognize neuron membranes and local shape descriptors (Sheridan et al., 2022) in our high resolution datasets, to segment neuron profiles and their mitochondria (Figure 9B). Due to variability in the image data, the segmented product requires human proofreading, which will be done using our own instance of PyChunkedGraph, a segmentation proofreading tool powering FlyWire (Dorkenwald et al., 2022) which allows efficient and collaborative editing. Despite the need for human input, automatic segmentation will dramatically speed up our throughput of connectomic datasets in the near future.

Summary

To comparatively map neurons and connectivity in multiple species of insects, I established a multi-resolution SBEM imaging approach whereby I target specific regions within the CX for connectomic analysis while simultaneously capturing the entire CX at lower resolution, greatly lowering image acquisition times (Figure 8 Kasthuri and Lichtman, 2007; Hildebrand et al., 2017). A crucial component of this pipeline is generating high quality histological samples that have high contrast and can withstand beam charge. In this regard, we found that using a fixative with substantially lower fixative concentrations produces better results in hymenopteran brains. Once images were acquired, I aligned and registered them using mostly open-source plugins available in FIJI. Finally, to trace neurons and annotate synapses, our group used collaborative tracing software in combination with targeted reviewing to ensure accuracy in our reconstructions. Through this methodology, we can begin to compare navigationally relevant neural circuits across multiple insect species, enabling us to start shedding light on organizational wiring principles in the light of evolution.

Chapter 7

Comparative connectomics in the CX of bees and ants

The morphological similarity of the CX across insects raises the question of how such a seemingly conserved brain area can give rise to divergent behaviors (Figure 1C). While it is certainly plausible that the CX differs at the level of circuits and neuromodulation, an alternative possibility could be that the CX is conserved even at the highest levels, and differences in species-specific behavior exist in regions peripheral to the CX. In the introduction, I proposed that the answer likely lies somewhere in-between, implying that the CX does in fact contains 'core' ancestral circuits that have persisted through evolutionary time, but also that modifications or additions could be made to these circuits which would reflect behavioral, developmental, or ecological differences across species. This provides the organizational framework for the papers that follow. Anatomical descriptions begin with neurons and corresponding structures that are morphologically shared across species, then focuses on species-specific anatomical anomalies and what they might imply based on previous research and modeling.

What are the ancestral circuits of the CX and how have they been modified?

Paper 1 Summary: A projectome of the bumblebee CX

Bumblebees *Bombus terrestris* are impressive navigators that play a fundamental role in pollination (Carreck et al., 1999; Goulson and Stout, 2001). In a pilot study, we sought to determine what kind of information could be extracted by tracing a relatively low resolution SBEM dataset (126 nm x 126 nm x 100 nm) of the entire CX of a bumblebee worker. We

paired our low resolution dataset with a higher resolution dataset of the right NO (24 nm x 24 nm x 50 nm) from a different individual to trace the fine neurites of LNO cells and PFN cells, the latter of which were previously proposed to play a role in the storage of vector memory (Stone et al., 2017). Additionally, these reconstructions were combined with immunohistochemical staining to delineate structural features of the central bodies and with intracellular dye injections to visualize detailed cellular morphology, particularly in cells whose processes extended beyond the field of view of our image data.

Altogether we mapped the projections of over 1300 neurons innervating the CX. In total, we found 11 types of columnar cells which revealed 4 distinct projection patterns from the PB to the CB. These 4 projection offsets constrain the space of computational capabilities and therefore have important implications for modeling CX function. From our reconstructions, we also identified an array of fly-like EPG and PEN head direction cells (Turner-Evans et al., 2020; Hulse et al., 2021). Bumblebee EPG and PEN cells matched those in the fly in terms of their quantity, distribution, projectivity, and their morphology, suggesting a remarkable degree of conservation within the insect head direction circuit. However, due to the limited resolution of our image data, we were unable to establish connectivity between cell types or trace key features of the circuit, such as tangential cells which stabilize the heading representation and which provide sensory input to the circuit (these uncertainties are explored further in Paper 2).

In contrast to the head direction circuit, we discovered a surprising degree of divergence from the fly in the arrangement of the bumblebee NO. In this regard, we identified a territory in the bumblebee NO termed the cap region (NOc). The NOc was unique to the bee in several ways, including that it did not receive any branching fibers from self-motion input cells (LNO cells). Instead, this region was occupied by the branching collaterals of 15 FB tangential cells (FBt-NOc cells), that sent wide projections across the width of the FB and which projected towards the superior medial protocerebrum (SMP), an area in flies that is occupied by the output fibers from MBONs. Additionally, our reconstructions revealed twice the number of PFN cells, half of which arborized exclusively in either the domain supplied by fibers of FBt-NOc cells, or LNO cells. This fulfilled a prediction made by Stone et al. (2017) who proposed a role for PFNs as candidates to encode homing vector memory. Given the potential implications of this unique bee circuit in providing a neural substrate for encoding vector memories, we revisit this circuit in much greater depth. The discovery of this circuit served as a launching-off point for Paper 3), in which we zoom in on this circuit in multiple hymenopteran species that occupy different ecologies.

In summary, while limited in resolution, our projectomic analysis of the bumblebee CX provided major insights about the quantity and projectivity of all major CX neurons in the brain of the bumblebee. This project was the first attempt at comprehensively mapping the projection patterns of all columnar cells in non-dipteran insect species, the projections of which define and constrain computations carried out by the CX. Lastly, this project was

fundamental to establishing a data collection and neural reconstruction workflow which paved the way for the projects that follow.

Paper 2: The head direction circuit of ants and bees

While our projectomic analysis of the bumblebee CX provided compelling evidence that, at least anatomically, the head direction circuit is highly conserved from flies to bees, our analysis did not include key components of the circuit nor did it include any connectivity data. In Papers 2 and 3, we greatly improved our imaging resolution (Chapter 6), expanding our analysis to include higher resolution projectomic data of five additional hymenopteran species (Figure 1C), as well as synaptic resolution data in specific regions within two of those species.

We focused our analysis first on identifying the quantity, distribution, and projection domains of EPGs and PENs in four species: the honeybee, sweat bee, army ant, and bull ant. We also broadened our investigation to include Δ7 cells that form the stabilizing component of the ring attractor system in flies (Kim et al., 2017; Turner-Evans et al., 2020). As expected, in all species EPGs and PENs were strikingly similar, sharing almost identical numbers of cells that were distributed in a manner closely approximating those of the fruit fly. We focused our tracing efforts of Δ7 cells on the sweat bee and army ant and likewise identified numbers, projections, and within-type connectivity that mirrored what has been established in the fly (Hulse et al., 2021).

However, when viewing the presynaptic terminals of Δ7 cells in the sweat bee and army ant, we identified a peculiar distinction from flies, which was the presence of a tenth column in the PB. We therefore traced the lateral most EPG and PEN cells and found that it was only innervated by the laterally extending branches of PENs innervating PB (R9/L9). Further tracing of EPGs and PENs at the innermost PB columns and outermost PB and EB columns revealed additional species specific characteristics. We proposed that these modifications may be related to the fact that hymenopterans differ from flies in having an unfurled EB (in this regard, the term "ellipsoid body" is a misnomer for all other insects). While in flies the toroidal EB provides an ideal substrate to allow a bump of activity to rotate in a full 360° , we suspect that the circuit differences we identified provide an alternative solution that would allow the bump to travel from one lateral end to the other in a morphologically open neuropil, thus functionally closing the loop.

We then turned our attention to TUBU cells that comprise the proximal end of the anterior visual pathway and their postsynaptic partners, ER cells which supply multimodal sensory input to the head direction network. Reconstructions of both cell types in the honeybee, sweat bee, army ant, bull ant, and desert ant exposed substantial divergence in cell quantities

across all species. Surprisingly however, we did not find a clear correlation between eye size (as a rough proxy of visual capacity) and the quantity of either cell type. Even army ants, with their extremely reduced eyes, had more TUBU and ER cells than highly visual species such as the honeybee for instance.

Connectivity maps detailing an invertebrate head direction circuit have so far only been carried out in *Drosophila* (Turner-Evans et al., 2020; Hulse et al., 2021). Well less detailed than the fly, findings from this project serve as a starting point for addressing a comparative void and understanding how navigationally relevant circuits have been modified over evolutionary time (Barsotti et al., 2021).

Paper 3: A novel navigation circuit in the hymenopteran central complex

In Paper 1, our detailed reconstructions of self-motion circuits in the bumblebee NO showed an anatomical organization that largely differed from the fly. Compared to the five layers that make up the fly NO, we identified only three structural layers named the NOs, NOm, and NOc (for nomenclature see Paper 1). Additionally, we discovered a new set of cells which, to the best of our knowledge, were unique to the bee, and which we hypothesized likely play a role in encoding the distance and directional memories necessary for vector navigation (Paper 1).

To determine the prevalence of this circuit and of the general bumblebee NO organization among other bees, particularly those which face different ecological pressures, we performed an in-depth analysis of NO circuits in the CX of a honeybee and a sweat bee. While honeybees share a similar ecology and navigational lifestyle with bumblebees, sweat bees are nocturnal and forage in rainforests where they must navigate through a densely cluttered habitat (Chapter 1). Previous studies have found discrepancies in the sizes of sensory processing structures that correlate with what period of the day the animal is active in (Sheehan et al., 2019; Stöckl et al., 2016). Do these discrepancies also influence downstream circuits, such as those that integrate sensory input with the CX?

We began by reconstructing the projection fields of all LNO input cells in the honeybee and sweat bee NO. Consistent with the bumblebee, we found three domains, two of which were innervated by the processes of LNO cells. While the honeybee shared the same number of LNO cells with the bumblebee (n = 5), we found 9 LNO cells in the sweat bee, each of which occupied discrete though somewhat overlapping domains. We proposed these cells may play a role in bolstering self-motion sensory input in sweat bees by providing additional cells that could strengthen or differently filter sensory input, particularly input related to visual cues which are far less reliable at night. Similar to bumblebees, honeybee mLNO

cells (LNO cells innervating the NOm subregion), were heavily overlapping and, based on morphology alone, appeared to be highly interconnected (as was predicted in Paper 1). To our surprise, sparse connectivity tracing of mLNO and their postsynaptic partners (PFNm cells) in the honeybee NO revealed the presence of multiple parallel pathways, strikingly similar to what is found in the fruit fly. Our results therefore indicated that there are at least three parallel pathways that serve self-motion information from peripheral brain regions to the CX in honeybees.

In both species, reconstructions of LNO cells left a third NO region (NOc) completely devoid of branching. Like bumblebees, this third region was instead occupied by the processes of FBt-NOc cells and PFNc cells, both of which strictly arborized within the confines of the NOc. We focused our tracing efforts next on connectomic reconstruction of both cell types in the CX of the sweat bee. We first traced all of the PFN cells innervating a single PB column (L4) and found a total of 82 PFNs, nearly half of which were PFNc (n = 43). Similar to bumblebees, this suggests that sweat bees have as many as double the total number of PFNs relative to the fly. For FBt-NOc cells, we identified 20 in the sweat bee, 5 more than were initially discovered in the bumblebee. To determine how these cells were connected, we focused our tracing analysis in the NOc and in FB column 3, the FB column in which PFN (L4) cells project to. Our results revealed a highly recurrent circuit that was segregated from PFNm cells and which formed multiple intrinsic channels that were anatomically expressed as branches innervating different layers in the FB and NOc.

That this circuit is highly recurrent, isolated, and unique to bees points towards its role in encoding navigationally relevant vector memories. In a follow up to Stone et al. (2017), Moël et al. (2019) expanded the model by adding a new neuron type and reinforcement signal to the Stone et al. (2017) path integration model and showed that this would in theory be enough to enable the storage and recall of vector memories for later use, such as for returning to previously visited foraging sites and establishing shortcuts between multiple foraging locations. Our results indicate that bee FBt-NOc cells, would be remarkably well suited anatomically to fill the role of the "vector memory cells" proposed in Moël et al. (2019). Additionally, reinforcement signals could be supplied by the processes of dopaminergic cells that, at least in bumblebees, innervate nearly half of the FB (Paper 1). While this would need to be verified in future modeling and functional recording studies, the discovery of this unique bee circuit provides a plausible neural substrate that could provide bees with the additional computational capabilities that enable them to be such impressive navigators.

Conclusions and future perspectives

The fruit fly is a formidable tool for uncovering the mechanistic function of neural circuits. This last decade has witnessed a surge of studies related to the CX, each of which pushes the field closer towards understanding its inner workings. By providing a structural framework for functional imaging studies, the recently released hemibrain connectome (Scheffer et al., 2020) seems to have only accelerated this process, with cutting-edge discoveries becoming a seemingly frequent occurrence (Westeinde et al., 2022; Pires et al., 2022; Lyu et al., 2022; Lu et al., 2022; Matheson et al., 2022; Fisher et al., 2022, from this last year alone;). In the context of navigation however, a major limitation of the fly is that it does not possess the neural circuits and behaviors that are unique to other insect species, such as those that guide the robust and flexible use of homing vectors in bees and ants.

By comparatively mapping CX circuits in a group of bees and ants with diverse navigational behaviors, we have begun to piece together the elements of this circuit that are ancestral (the "bauplan" of the CX; Barsotti et al., 2021) and those which have been modified over evolutionary time. However, the work presented here also poses many new questions. For instance, how are input pathways to the CX organized outside of the field of view of our image data? In particular, has the anterior visual pathway been retained in the army ant with its reduced eyes, or has it been modified to incorporate other more reliable sensory modalities? How do differences in the reliability of sensory cues affect the mapping from ER cells to EPG cells? Do hymenopteran species that do not rely on path integration, such as the army ant, also have the putative vector memory circuit that we have identified in Paper 3? How does this same circuit correspond from the sweat bee to the honeybee? What is the downstream organization of this circuit, and how differences in motor output are reflected by species that utilize different preferred modes of locomotion? How idiosyncratic are these circuits among individuals within the same species? By incorporating automated machine learning methodologies (such as automated segmentation and synapse detection; Chapter 6), we have begun to streamline our workflow, enabling us to pursuit the answers to these questions with unprecedented detail.

Considering the massive diversity of insect species, the detailed neural reconstructions pre-

sented here only begin to scratch the surface in understanding how CX neural circuits have evolved over time. As technological advances in connectomics make it cheaper and more efficient to acquire dense neural reconstructions, future studies should generate connectomic maps of the CX across diverse species of insects and, eventually, Arthropoda. By taking a "phylogenetic refinement" approach (Cisek, 2019), we can utilize the evolutionary history of the CX as a tool to generate ethologically relevant explanations for how the CX came to be.

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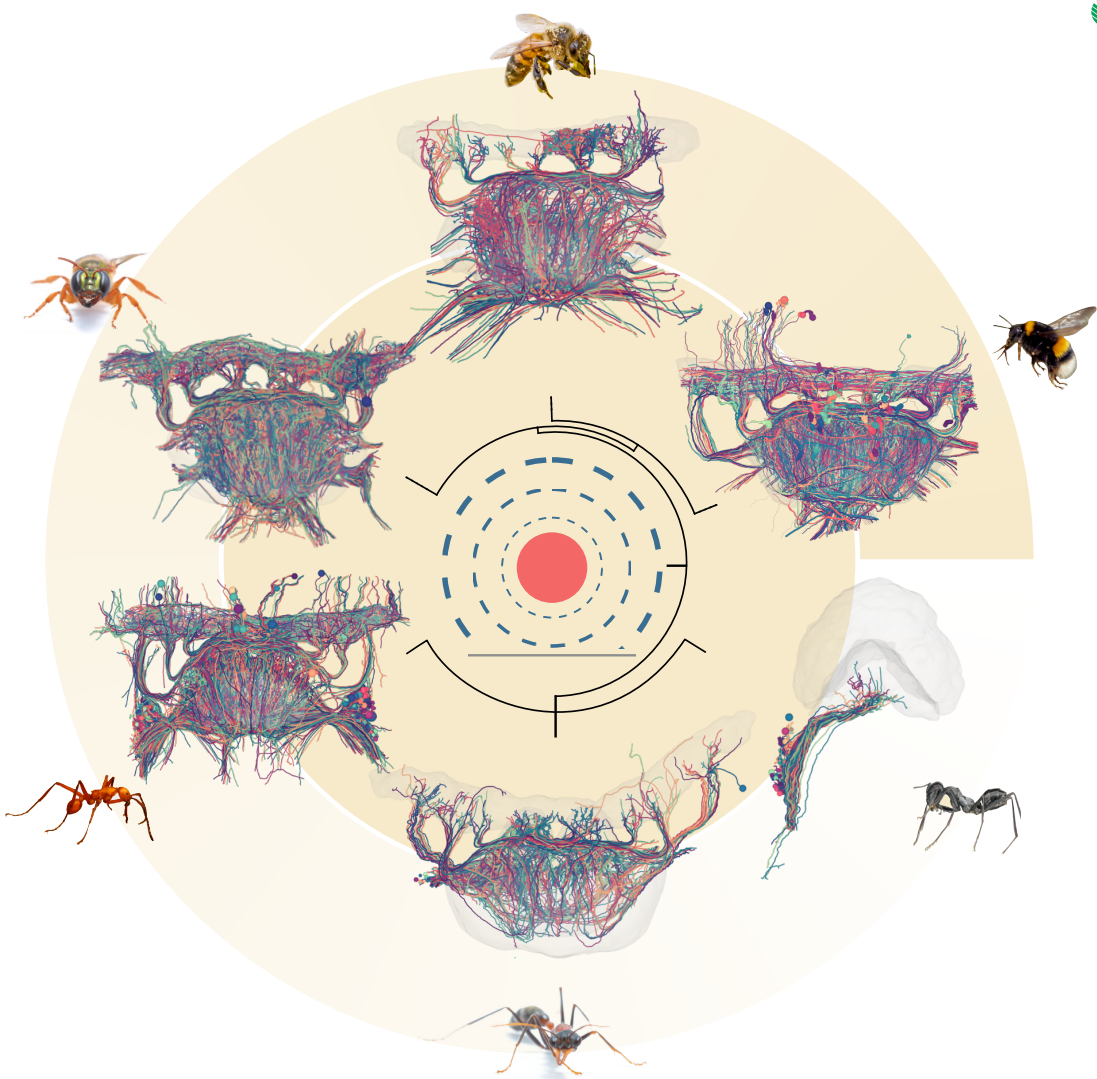
Papers

Paper I: A projectome of the bumblebee central complex

Paper II: The head direction circuit of ants and bees

Paper III: A novel circuit in the hymenopteran central complex



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