

Systems Analysis of Gaze
Stabilisation Behaviour and
Imaging of Motor Systems in the
Blowfly *Calliphora*

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Declaration of Originality

Everything presented in this thesis is my own work except where attributed. Work done in collaboration is indicated in the respective chapters: The behavioural data I analysed in chapter 2 was collected by Karin Bierig, Francisco J.H. Heras and Gino Bolliger, and the synchrotron experiments in chapter 4 were conducted in collaboration with Rajmund Mokso, Martina Wicklein, Simon M. Walker, Michael Doube, Tonya Mueller, and Graham K. Taylor.

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Abstract

A fast and powerful gaze stabilization reflex supports self-motion estimation and flight control in flies. Changes in body posture are conveyed by a variety of sensory modalities and compensated for by fast and accurate head movements. This thesis aims to further our understanding of the behavioural basis of compensatory head movements, and presents a first foray into the *in vivo* imaging of the motor systems that actuate these control reflexes.

Major sensors that contribute to gaze stabilisation are the visual ocelli and compound eyes on the head, and the mechanosensory halteres on the thorax. The integration of visual feedback and mechanosensory feedforward control gives rise to a two-degree-of-freedom controller, a design which is extensively used in engineering applications. I performed a linear systems analysis of compensatory head roll in response to forced thorax oscillations in the fly. The feedforward pathway exhibited a high bandwidth and constant gain and reduced the response delay of the reflex. Large stability margins in the feedback pathway supplied by the compound eyes guaranteed stable behaviour in the face of response variability. The occlusion of the ocelli did not change the gain of the feedback pathway, but significantly reduced the latency.

I investigated the use of iodine-enhanced computed x-ray microtomography (microCT) to perform fast three-dimensional imaging of the neck and flight motor systems. Virtual dissections of major functional units illustrate the possibilities and limitations of microCT. To observe the configuration of motor systems in behaving flies I performed gated microtomography using hard x-rays at the TOMCAT

beamline of the Swiss Light Source, a third generation synchrotron. While 3D tomograms of the neck motor system proved elusive, this thesis presents the first *in vivo* tomograms of the flight motor and wing hinge during tethered flight.

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Chapter 1

Introduction

Achieving and maintaining stable operating conditions is a crucial task for technical systems and biological organisms alike. How is sensory information used to compensate for disturbances in a fast, stable and robust fashion? How may we investigate the motor systems mediating stabilisation reflexes in living, behaving animals? This thesis aims to address these questions in the blowfly through the use of a range of techniques. I applied linear systems analysis to elucidate the systems architecture underlying a specific compensatory reflex, fly gaze control. Key to the functional organization of the gaze control system are the biomechanics of the fly neck motor, which have not yet been studied *in vivo*. I will introduce x-ray based 3D microscopy as a promising method to observe the motor systems underlying reflex control both *in situ* and *in vivo*, which may be applicable to a great number of animal model systems far beyond flying insects. This introductory chapter presents the blowfly *Calliphora vicina* as a model system to discover general principles of sensorimotor control. In subsequent chapters I will focus on two stabilisation reflexes I studied in the blowfly and provide further context on the methods developed and applied during my PhD project.



Figure 1.1: Overlay of a high-speed video sequence of a blowfly before and during initiation of a saccade. Subtle head movements around the yaw axis are visible. Arrows mark frames with strong head movements.

1.1 The fly as a model system for sensorimotor control

The blowfly *Calliphora vicina* combines a short generation time, which benefits a constant supply of experimental animals, with remarkable neural and morphological adaptations enabling extraordinary aerial manoeuvrability (Boeddekker et al. [2003], Fig. 1.1). Its comparatively large size facilitates behavioural experiments as well as electrophysiological measurements, and - as shall be shown in chapters 3 and 4 - makes it amenable to a range of imaging techniques, both *in situ* and *in vivo*. The main attraction for the use of flying insects in sensory neuroscience and motor control is the limited and well-defined set of behaviours performed by a comparatively small nervous system that consists of less than 4×10^5 neurons (Strausfeld et al. [1987]). This is, however, not to say that the reflexes themselves are limited and simple: the aerial performance, for example, of male flies during chasing flight of potential mates remains unmatched by any man-made contraption (Floreano et al. [2009]). In spite of its comparatively small nervous system, the blowfly uses a large set of sensors to stabilize flight by means of an intricate flight motor, the functional organization of which is still not fully understood (Dickinson [2005]; Dudley [2000]; Taylor [2001]).

1.2 Fly gaze control

1.2.1 Compensatory head movements

Flies move their heads in response to visual and mechanical stimuli. Gaze stabilisation refers to the process of keeping the head, and therefore the eyes, aligned with the inertial system through compensatory head rotations. These have been observed in both walking and flying flies (Kress and Egelhaaf [2012]; Schilstra and van Hateren [1999b]). Several functions of gaze control have been suggested: Not only do compensatory head movements reduce motion blur caused by fast rotations, they also align the visual scene of the fly with the principal axes of head-based visual sensors. This mechanical alignment of the external reference frame with sensory measuring axes may simplify and facilitate sensory processing, and the transformation of sensory feedback signals into motor commands. Furthermore, compensatory head rotations reduce the rotational components of optic flow, leaving translatory optic flow nearly uncontaminated (Koenderink and van Doorn [1987]). The resulting optic flow contains parallax information, and therefore relative distance information on the structure of the environment. All these benefits of gaze stabilisation can only be exploited if head movements occur at rates fast enough to efficiently counter aerial disturbances and saccadic attitude changes in excess of $1000^\circ/\text{s}$ (Schilstra and van Hateren [1999a]). The following subsections review the sensory, neuronal and motor bases of this reflex.

1.2.2 Sensory basis of gaze control

I will discuss the three major sensors investigated in chapter 2 of this thesis: Two visual modalities, the compound eyes and the ocelli, and a mechanosensor, the halteres. Their positions on the animal are depicted in Fig. 1.2.

The compound eyes, or facet eyes, consist of a large number of ommatidia, or facets (Petrowitz et al. [2000]) and feed into a well-studied motion vision pathway, where local photoreceptor signals are processed by means of Reichardt-type correlation detectors (elementary motion detectors, EMDs) (Reichardt [1987]). Each of these EMDs detects local optic flow along a preferred axis across a small portion of the visual field. Lobula Plate Tangential Cells (LPTCs) integrate the

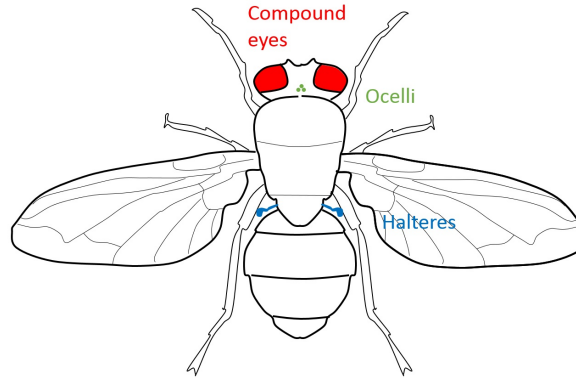


Figure 1.2: Three sensory modalities involved in gaze control and studied in this thesis. The compound eyes are large facet eyes on the head of the fly. The ocelli are three small lens eyes positioned dorsally on the head. The halteres are small club-like structures on the thorax beating in anti-phase with the wings and are thought to encode angular velocity. (See also (Hengstenberg [1991]).)

output of many EMDs and respond to motion around or along preferred axes of motion, thus signalling self-movement of the fly in a population code (Franz and Krapp [2000]; Krapp and Hengstenberg [1996]). The ocelli, three simple lens eyes on the head of the fly, integrate light intensities over a large part of their visual fields and, in locusts, were hypothesized to detect changes in attitude (Simmons [2002]). The lack of an EMD-like, slow processing stage increases the sensitivity to faster movements compared to the dynamic range of the motion vision pathway. L-neurons integrate the signals from the photoreceptors in the three ocelli (Rev: Mizunami [1994]) and output to descending interneurons that mediate information to motor neurons in the flight and neck motor systems. The halteres are a remarkable specialisation of true, or dipteran, flies. These club-shaped organs which evolved from hind wings beat in anti-phase to the wings and serve as equilibrium organs that detect angular rates of motion. They input directly to the steering motoneurons in the wing hinge which control wing kinematics, and therefore flight behaviour (Dickinson [1999]; Sandeman and Markl [1980]), as well as compensatory head movements (Milde et al. [1987]; Nalbach and Hengstenberg [1994]). A range of further sensory modalities is involved in compensatory head movements, where the information obtained is processed in

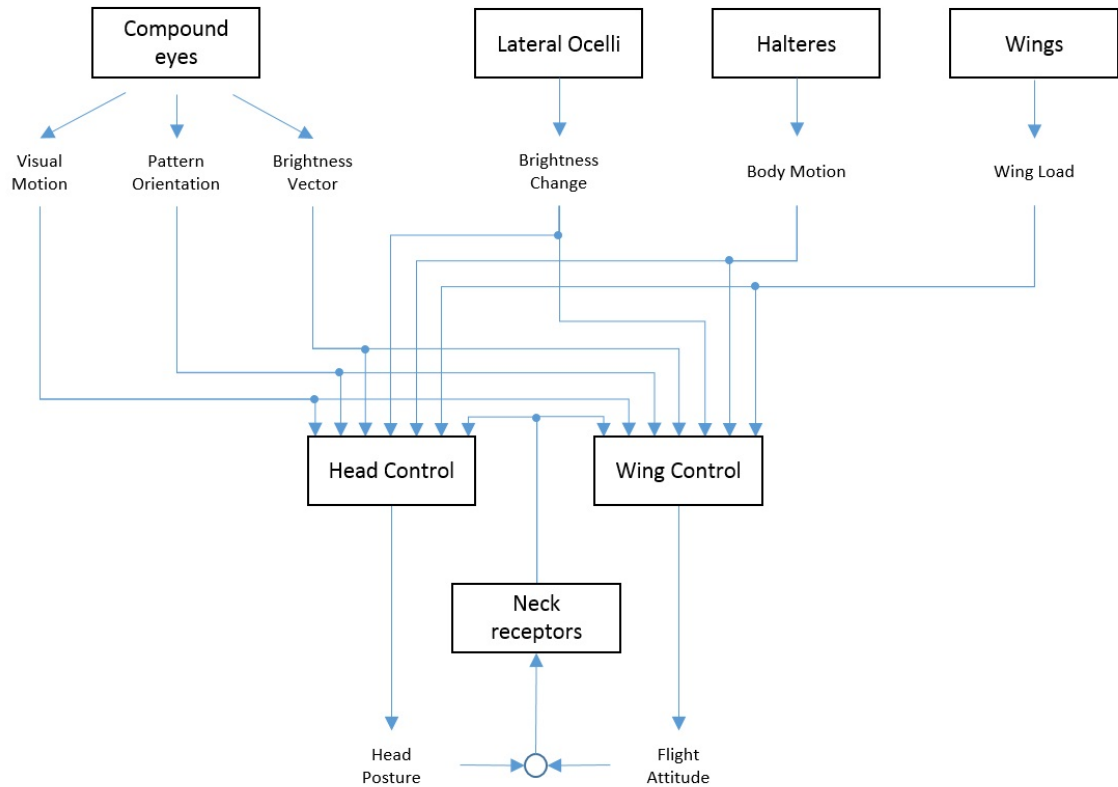


Figure 1.3: Hypothesized inputs to the gaze control system, according to Hengstenberg [1991]. External, physical signals are sensed by sensory organs including leg receptors, compound eyes, wings and halteres. The resulting visual and inertial control signals are integrated to control not only gaze but also walking and flight attitudes. The investigation of individual sensory contributions and their integration is expected to support the study of biological control strategies.

multiple, distinctly different pathways. An early attempt by Hengstenberg [1991] at representing these different pathways involved in gaze and flight control is depicted in Fig. 1.3.

1.2.3 Neck motor system

The neck motor system controls the attitude of the head, a rigid body, in mainly 4 degrees of freedom (three rotational degrees of freedom and retraction/protraction), resulting in a system with ostensibly simple kinematics. Anatomical studies, however, indicate that a greater number of direct and indirect neck muscles are involved in head movements than there are steering muscles in the wing hinge (e.g. Strausfeld et al. [1987]; Wisser and Nachtigall [1984]). Most of the 21 pairs of neck muscles are innervated by a single neck motor neuron (NMN), and all NMN only innervate an individual muscle (Strausfeld et al. [1987]). Recordings from individual NMNs in response to visual motion stimuli show similar receptive field properties as those found in tangential cells (LPTCs), but a higher preference for rotational movement axes and, consequently, a higher degree of binocularity (Huston [2008]).

1.2.4 Multisensory integration

How is information from this variety of sensors used to actuate and ultimately control head position based on a comparatively large number of direct and indirect muscles which set up a high-dimensional motor system? Pioneering behavioural experiments by Hengstenberg [1988, 1991] proposed a dynamic complementation of the frequency ranges that individual sensory modalities respond to. While compound eyes signal mainly responses in the low dynamic range, halteres occupy the higher frequency range (also cf. Taylor and Krapp [2007]). Huston and Krapp [2009] studied the integration of haltere- and compound eye- mediated information in neck motor neurons and found that a sub-group of neck motor neurons responded to visual inputs only under simultaneous stimulation of the halteres. Furthermore, Rosner et al. [2010] found that the gain of head pitch responses to visual stimuli was related to the state of the system, especially the movement of halteres. Haag et al. [2010] revisited the integration of stimuli from

a variety of sensors in an identified fly neck motor neuron and reported that it received input from a central neuron hypothesized to signal flight state information as well as from two LPTCs, the halteres, and the wind-sensitive Johnston's organ located on the antennae. These studies highlight the complexity of the control system underlying gaze stabilisation in flies where the investigation of the design is hampered by the large number of sensors, their non-linear interaction and our limited knowledge of the biomechanics and dynamics of the underlying actuating system.

1.3 Imaging studies of motor systems

This thesis presents methods that allow for an integrative approach to sensorimotor research, rather than a treatise of motor system and neural processing in isolation. The main argument for an integrative approach has been made by Nishikawa et al. [2007] and by Huston [2008], and will be further discussed in section 2.4. Our understanding of behavioural control relies greatly not only on the analysis of its performance at the systems level, but also on the investigation of individual components involved. In flies, many of these can be observed in behaving flies or at least in stationary flies *in vivo*. These components, or building blocks, include sensors (e.g. Halteres: Sandeman and Markl [1980]), interneurons (e.g. LPTCs: Krapp and Hengstenberg [1996], descending neurons: Haag et al. [2010]), motor neurons and muscles (Neck: Huston and Krapp [2009], Steering muscles: Balint and Dickinson [2001]) as well as the wing kinematics, flight behaviour (Schilstra and van Hateren [1999a]) and head movements (Schilstra and van Hateren [1999b]). However, so far it has not been possible to observe the displacements of muscles and cuticle in the complex motor systems mediating flight and gaze control. I will discuss x-ray transmission tomography as a potential methodology to acquire 3D volume representations in chapters 3 and 4. The geometry of a motor system is an important part of its functional anatomy, as the pulling planes and actions of muscles contribute to the overall physiology of the system. The technique, first results, and its limitations are discussed in chapters 3 and 4.

1.4 Structure of this thesis

This thesis is structured as follows: In chapter 2, I use linear systems theory for an input-output analysis of the sensory contributions to performance and stability of compensatory head roll. This study treats the head roll reflex as a grey box and results will be deduced from behavioural experiments. In chapter 3, I present a method to elucidate the anatomical properties of the neck motor - a key element of the gaze stabilisation system - and discuss the potential of contrast-enhanced x-ray microtomography for a three-dimensional reconstruction of its functional anatomy. In chapter 4, I discuss how I used *in vivo*, two-dimensional x-ray image data from a dedicated synchrotron beamline endstation to observe flight biomechanics in behaving flies. In chapter 5, I will discuss the implications of the methods and results for our general understanding of fly reflex control.

Chapter 2

Linear Systems Analysis of Compensatory Head Roll

2.1 Introduction

This chapter aims to identify the dynamic properties of sensorimotor pathways involved in gaze stabilisation using linear, time-invariant (LTI) systems theory. The main motivation behind this analysis can be roughly summarised in three points. Firstly, gaze stabilisation serves as a model reflex to study the control principles underlying a wider range of compensatory and equilibrium reflexes, such as yaw rate (flight) control (Elzinga et al. [2012]). Secondly, reflexes for the stabilisation of gaze exist in many visually-oriented animals, including primates and humans (Rev:Angelaki [2004]). Thirdly, as we shall see, the architecture underlying gaze control in the fly is consistent with design principles used in technical control applications, namely so called two-degree-of-freedom controllers (Doyle et al. [2006], Application: Kerhuel et al. [2007]).

Here, I analyse head roll control in the fly using behavioural data. The main focus is on the contributions of individual sensorimotor pathways on the stability of the reflex. To perform compensatory head movements blowflies use information from a variety of sensors as described in chapter 1. Not only do these sensory systems have different intrinsic properties, such as pathway-specific latencies, bandwidths and sensor noise levels, their anatomical location on the animal also

matters in the context of whether they convey feedback or feedforward information to the control system: A feedback signal carries information on the parameter to be controlled, while a feedforward sensor provides information about a reference signal or an external disturbance and does not convey information on the system's state or output signal.

It has been argued that the nonlinear slip speed tuning curve of the elementary motion detectors in the fly's visual system is responsible for the stability of optomotor reflexes (Warzecha and Egelhaaf [1996]). Their findings have been employed in an artificial visual servo, where a visual speed sensor was replaced by insect-inspired Reichardt-type correlation detectors (Borst et al. [2009]). I will explicitly examine the marginal stability of this visual feedback control loop through an estimation of the maximally tolerable increases in gain and phase. These values are often referred to as stability margins (Doyle et al. [2006]; Guzzella [2009]). In addition, I will further investigate the role of haltere-mediated feedforward control from a linear systems perspective and compare the control design employed here to those found in other biological control systems.

Finally, I investigate ocellar contributions to head movements by using frequency responses to estimate changes in gain and delay after occlusion of the ocelli. While it has been shown that the ocelli are involved in the control of head movements in locusts (Taylor [1981]) and dragonflies (Stange [1981]), it has been suggested only recently that ocellar information decreases the latency of head movements in the blowfly (Parsons [2008]). My results show that ocelli indeed decrease the latency in the visual feedback control loop in the frequency range around 1 Hz. This delay reduction, though small, may have important consequences for the stability of the control system.

Note that a similar analysis as presented here may be found in Schwyn et al. [2011]. The differences between the studies are briefly outlined in the conclusions of this chapter.

2.1.1 Concepts regarding linear systems theory

This subsection gives a brief overview over important definitions in the remit of linear systems theory for readers unfamiliar with LTI systems analysis and

identification.

A range of characterisation formalisms and identification methods exist for dynamic systems. Ideally, the system under consideration can be approximated as linear time-invariant, i.e. fulfilling

$$S(\alpha \mathbf{u} + \beta \mathbf{v}) = \alpha S(\mathbf{u}) + \beta S(\mathbf{v}), \quad (2.1)$$

and

$$S(\mathbf{u}(t)) = \mathbf{y}(t) \Rightarrow S(\mathbf{u}(t + T)) = \mathbf{y}(t + T) \quad (2.2)$$

where S is the system operator, $\mathbf{u}$, $\mathbf{v}$, and $\mathbf{y}$ are vectors (often called signals), α and β are scalars, t is a time axis and T is a real number. The behaviour of a linear time-invariant system can be completely defined by its frequency response, i.e. the gain and phase at which individual input frequency components are transferred through the system. Although few, if any, real physical systems fulfil either of these conditions, approximating biological systems as linear and time-invariant systems (LTI systems) has proved useful time and again (e.g. French [2009]; Theobald et al. [2010]).

Two architectures have to be distinguished in the analysis and synthesis of control systems: Feedforward control (Fig. 2.1A) uses a reference signal to control a plant without feedback. The error of the system is determined by how accurately the plant's dynamic properties are known. Any parametric uncertainty, i.e. uncertainty about the characteristics of the actuating system and noise affecting the control signal, the system cannot compensate for. Feedback control (Fig. 2.1B), on the other hand, is the control of the actuator's output through the use of an error signal. The two control designs can be combined to form a two-degree-of-freedom control system, the general form of which is shown in Fig. 2.1C.

The performance of feedback control loops is governed by a trade-off between gain and bandwidth of the feedback controller. In the presence of processing

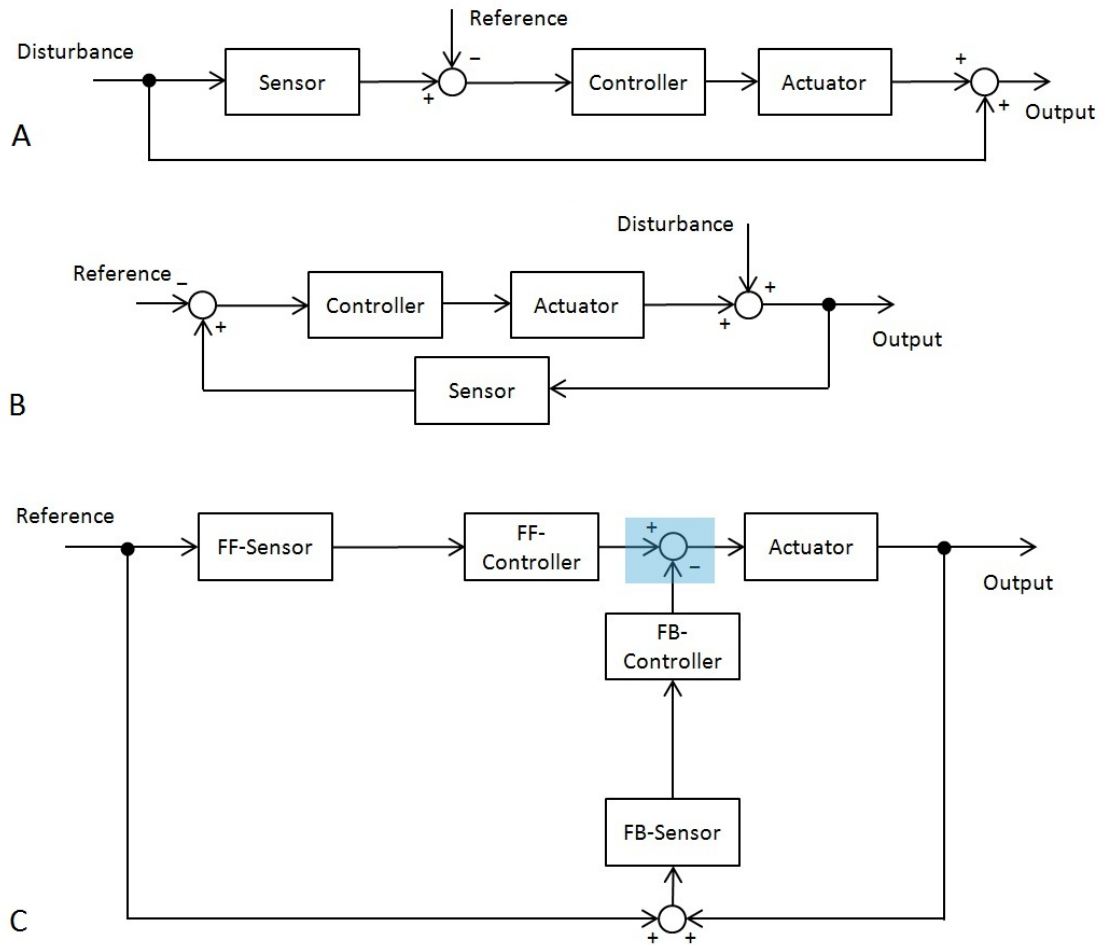


Figure 2.1: Basic control-architectures used in linear control systems. (A) Feedforward control aims to reach equilibrium in the face of a measurable disturbance. (B) Feedback control involving a reference signal and an output disturbance. (C) Two-degrees-of-freedom control, a combination of feedforward and feedback control. FF: Feedforward, FB: Feedback.

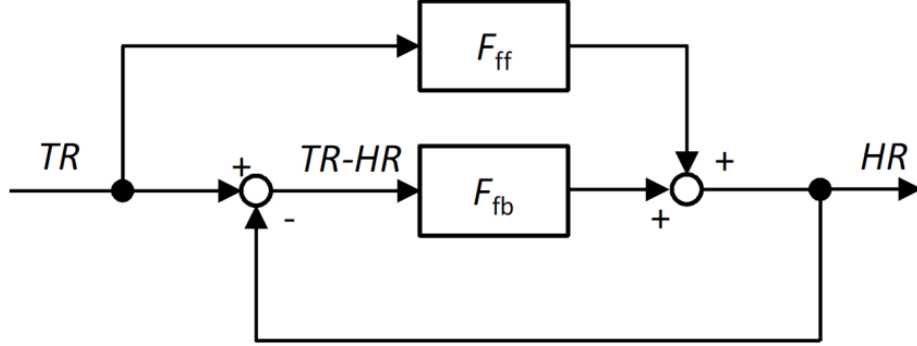


Figure 2.2: Simplified head roll control architecture. Serial combinations of sensors, controllers and actuators have been replaced by abstract sensorimotor pathways.

delays, frequency components of the feedback mediated by the sensor may be phase-shifted such that they are amplified rather than compensated for. In this case, the system becomes unstable. Particularly in the presence of parametric uncertainty and sensor noise, stability margins provide more information than the simple classification into "stable" and "unstable" systems, and will therefore be used in the analysis of the head roll reflex in this chapter. The phase margin refers to the maximal increase in phase lag in the open loop system before a 180° phase shift is introduced at frequencies where the open-loop gain is greater than 1. That is, the phase margin defines how much additional processing delay may be added to the sensor before the condition described above takes effect and the closed-loop control system becomes unstable. Similarly, the gain margin describes the maximum admissible increase in constant gain before the system becomes unstable.

In the context of fly gaze stabilisation, feedback control is mediated by all sensory modalities located on the head itself. These convey information on head attitude, which may be interpreted as the error signal.

While head roll stabilisation in the fly may be seen as a control design with a reference input of zero (perfect compensation), the nature of the control architecture underlying this reflex is equally well represented by a different interpretation: If the input signal is defined as the disturbance acting on the thorax (thorax roll

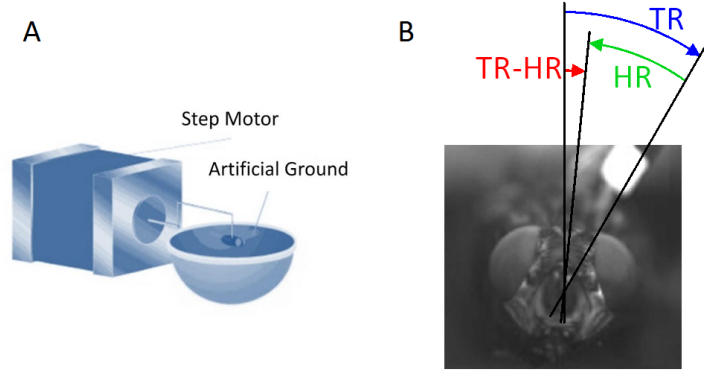


Figure 2.3: Experimental setup and angle definitions. (A) Stimulation apparatus. Sinusoidal thorax roll is imposed on the fly by a step motor, while a fixed, dark hemisphere provides a visual stimulus. (B) Sample frame and definition of angles. Head deflection (TR-HR, red) is minimal if compensatory head roll (HR, green) closely follows thorax roll (TR, blue). (Fig. 2.3B adapted from Parsons [2008])

angle over time), and the output of the system is defined as the actuation of head position by the neck motor system (head roll), compensation is readily achieved by the output (HR) following the input (TR) as closely as possible such that the compensation error (head deflection, TR-HR) is close to zero for all times (Fig. 2.3).

The terms feedforward control and feedback control are well established and widely used in the control engineering community (Doyle et al. [2006]). The terms feedforward and feedback sensors and signals are used here to emphasize that the properties of the sensors, especially their location on the fly, determines the feedback or feedforward nature of the signals available to the animal's nervous system.

2.2 Methods and Material

These experiments were performed by Karin Bierig, a visiting research technician from the Max Planck Institute for Biological Cybernetics in Tuebingen, Germany. The set-up used has been described previously by Parsons [2008]. In the following a short summary of the setup, experimental paradigm, and stimulus parameters

are given for reference.

2.2.1 Behavioural experiments

Five female flies were subjected to the experiments comparing head roll compensation with and without halteres (conditions C1 and C2, respectively). The experiments comparing visually mediated compensation with and without ocelli were conducted in eight female flies (conditions D1 and D2, respectively).

2.2.1.1 Contribution of the halteres

The fly's ocelli were occluded and the animal was attached to a motor with a piece of cardboard waxed dorsally to the metathorax such that the fly could be rotated around its longitudinal (roll) body axis. A black hemisphere was positioned ventrally. This provided a high-contrast edge at an elevation close to the compound eye equator and stimulated the fly's motion-vision pathways supplied by the compound eyes. Flies were subjected to moderate frontal airflow which encouraged prolonged periods of flight. They were stimulated by thorax oscillations of 30 amplitude at 9 logarithmically spaced frequencies from 0.06 to 15 Hz for at least 5 cycles per frequency/trial. High-speed videography of the head movements was performed using a Fastcam SA3 high speed camera (Photron, San Diego, CA). The cameras frame rate was adjusted to the stimulation frequency to acquire at least 50 frames per cycle for each trial. To identify individual sensory contributions to head roll stabilisation, we used two experimental conditions. In the first condition (C1), the fly's halteres were left intact. In the second condition (C2), the halteres were clipped as close as possible to the thorax. As the halteres measure Coriolis forces which are related to the inertial moment of their stalks (Nalbach and Hengstenberg [1994]), their removal eliminates all haltere-mediated inertial information.

2.2.1.2 Contribution of the ocelli

In order to examine the contributions of the visual sensors the fly was tethered dorsally to a stationary holder. The dark hemisphere was mounted to a step motor and placed below the fly such that it provided a high-contrast edge to

stimulate the visual-motion pathways, provided by the compound eyes, and the ocelli. Again, to encourage prolonged periods of sustained flight moderate frontal airflow was provided. During experimental trials, the visual stimulus was oscillated to simulate a roll rotation of the fly. The stimulation amplitude was 30° and stimulation frequencies of 1, 3, 6, and 10 Hz were used. Head movements were recorded using a Fastcam SA3 high speed camera (Photron, San Diego, CA). The frame rate of the camera was adjusted to the stimulation frequency to acquire at least 50 frames per cycle in all trials.

To characterize the contribution of the ocelli, two experimental conditions, D1 and D2, were used. In the first condition, D1, the ocelli of the fly were left intact. In the second condition, D2, the ocelli were occluded using black acrylic paint. In this stimulus setup the animals encountered only visual stimulation.

2.2.2 Image processing and estimation of stimulus and response angles

The recorded image sequences (snapshot: Fig. 2.3) were processed using a LabVIEW program designed by M. Parsons and described previously (Parsons [2008]) to extract the time course of thorax roll (TR) and head deflection in the laboratory frame (TR-HR). The program matched shifted and rotated templates along the borders of the fly's eyes to all frames. Note that, while TR is clockwise positive, the head roll (HR) has the opposite sign.

As mentioned above, we assumed that the control system was linear. In all experimental trials, the output amplitude spectrum showed a strong peak at the input frequency. The linearity of the system was investigated previously using a stimulus consisting of two superimposed input frequencies (Schwyn et al. [2011]). The results of these experiments showed a low level of intermodulation, suggesting a high degree of linearity of the system.

The program used to extract head and thorax roll did not always find adequate matches for all frames image sequences recorded by the high speed cameras were post-processed as follows: for each frame where no sufficiently close match of the templates was found, the angle value was interpolated between neighbouring frames, even if the either or both of the neighbouring frames was an insufficient

match. This algorithm was iteratively applied 5 times to the response data series, effectively implementing a selective, non-linear low-pass operation.

The gain and phase of both the input (TR) and output (HR) at each stimulus frequency and condition were computed based on a windowed cross correlation between TR and HR, where the window size was chosen to match the period of the respective stimulus frequency, and

$$T = F_s / f \quad (2.3)$$

$$gain(n) = \frac{\max_{k \in \{0..T-1\}} \left(\sum_{\tau=nT}^{(n+1)T-1} s(\tau)r(\tau+k) \right)}{\sum_{\tau=nT}^{(n+1)T-1} s(\tau)r(\tau)} \quad (2.4)$$

$$phase(n) = -2\pi \frac{\max_{k \in \{0..T-1\}} \left(\sum_{\tau=nT}^{(n+1)T-1} s(\tau)r(\tau+k) \right)}{T} \quad (2.5)$$

where f denotes the n^{th} stimulation frequency and $s(.)$ and $r(.)$ are the discrete response and stimulus traces sampled at frequency F_s .

The normalized cross correlation was calculated for each cycle of the input signal, except for the first and last cycles in order to avoid padding. Mean amplitudes and mean phases for each trial were then calculated as well as their intra-individual standard deviations. We estimated constant time delays from the frequency responses using the average gradient at the four highest stimulation frequencies.

2.2.3 Derivation of sampled pathway frequency responses

The following relationships are evident from the block diagram (Fig. 2.2) of the reduced head roll reflex:

$$F_{fb} = \frac{G_2}{1 - G_2} \quad (2.6)$$

$$F_{ff} = G_1 - \frac{G_2}{1 - G_2} \quad (2.7)$$

where G_1 is the frequency response of condition C1 (intact halteres) and G_2 is the frequency response of condition C2 (halteres removed). The complex gain (phasor) of the pathway frequency responses (F_{ff} and F_{fb}) could thus be calculated on a frequency-by-frequency basis using the average gain and average phase of each trial.

2.2.4 Derivation of stability margins

The sampled pathway frequency responses were interpolated linearly between frequency pairs on a log scale of 100 data points per decade. The gain and phase crossover frequencies were defined as the lowest frequency with gain greater than 1 (0 dB) and phase smaller than -180° , respectively.

2.3 Results

2.3.1 Integration of mechanosensory feedforward and visual feedback information

2.3.1.1 Frequency responses with and without halteres

Flies subjected to forced thorax roll oscillations responded with sinusoidal head movements phase-locked to the stimuli (Fig. 2.4). The individual traces exhibit higher-frequency oscillations with amplitudes larger than those which could be expected to be introduced by the limited accuracy of the image processing (Parsons [2008]). Closer inspection of the image sequences indeed revealed not only

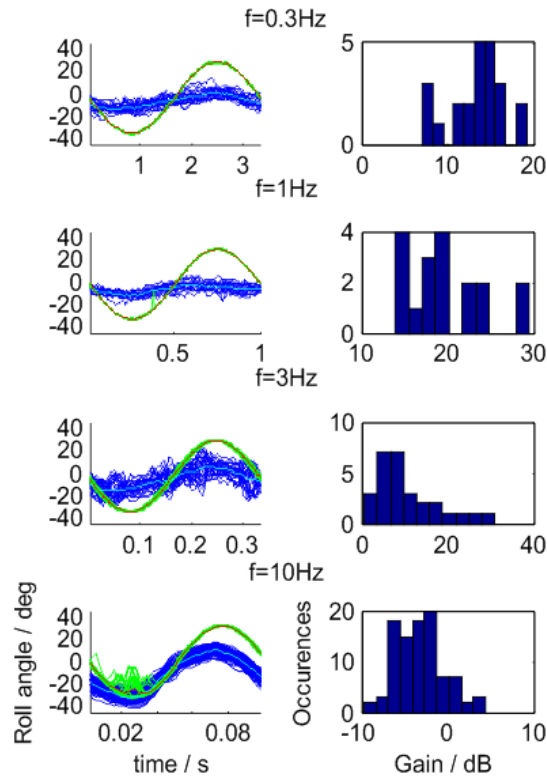


Figure 2.4: Individual head roll responses to oscillation of the thorax around the roll axis in one fly at selected frequencies. Left column: The individual response cycles of the fly at each frequency demonstrate response variability (individual responses: dark blue, mean: light blue). Right column: the gain and phase for each response cycle were calculated and offer evidence of the response variability. Experiments performed by Karin Bierig.

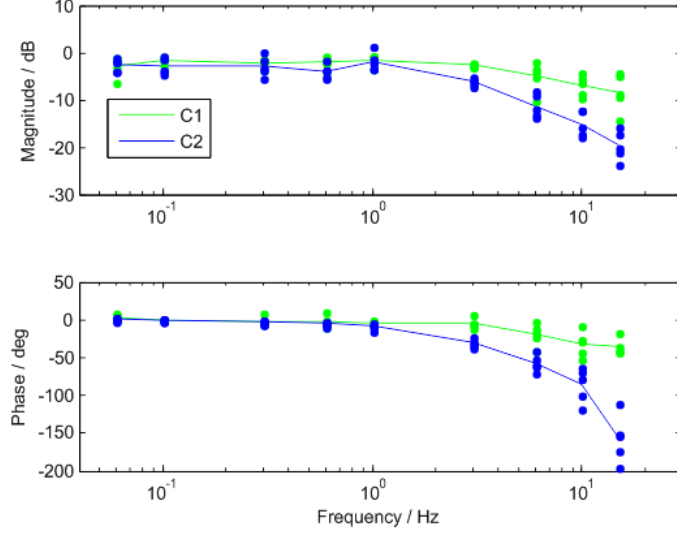


Figure 2.5: Frequency response of head roll with (green) and without (blue) the halteres.

the fly's compensatory responses at the stimulus frequency, but also head jitter seemingly unrelated to the input signal. In the following systems analysis, I only focus on the response of the fly at the stimulus frequency, i.e. on the linear approximation of the system.

The measurements of head deflection in response to experimenter-induced thorax roll oscillations at discrete frequencies served to sample the frequency response of the head roll reflex before and after removal of the halteres. Fig. 2.5 shows the resulting Bode plots of frequency response gain and phase for five female flies.

The average gain and phase varied smoothly across the frequency range of 0.06 Hz to 15 Hz. For the lower stimulation frequencies ($f < 1$ Hz) the average gain ranged from -2.2 to -5.3 dB, which is close to unity gain (0 dB). The phase in this range was close to zero degrees. Unity gain and no phase lag result in perfect compensation. At higher frequencies, both gain and phase decreased in accordance with the low-pass characteristics of the system. These decreases were more pronounced in flies without halteres (condition C2) than in flies with halteres intact (condition C1).

The decrease in the intra-individual averages of gain and phase was accompanied by an increase in response variability. The strong rolling-off of the phase indicated the presence of a constant-time delay term in processing pathway. Using the phase values at the four highest frequencies, the response delay of the system with halteres was 7.3 ± 6.3 ms, without halteres the delay was 25.0 ± 6.5 ms.

2.3.1.2 Pathway frequency responses

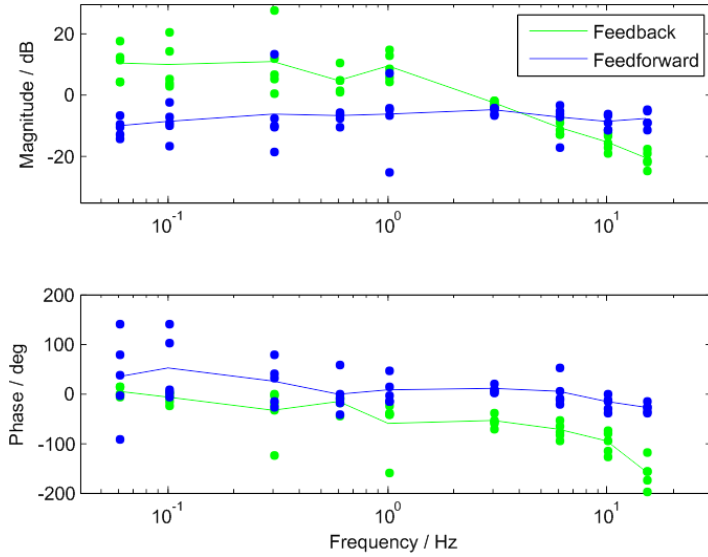


Figure 2.6: Sensorimotor pathway frequency responses. The Bode plot of the feedback pathway appeared to be consistent with low-pass filter characteristics, while the feedforward gain was relatively constant across the frequency range.

From the Bode plots in Fig. 2.5 I could calculate the frequency responses of the sensorimotor pathways in Fig. 2.2. The derivation of F_{ff} and F_{fb} was not very well conditioned and the variability of pathway gains is high, especially at lower frequencies (Fig. 2.6). The phase of the feedforward pathway at the lowest frequency appeared to be completely random. The feedback pathway exhibited high magnitudes exceeding 10 dB for lower frequencies, corresponding to a gain of 10 in linear units. In the higher-frequency range, both feedback gain and feedback

phase decreased strongly. While the feedback pathway exhibited low-pass-filter properties, the feedforward pathway seemed to have a constant gain across the frequency range studied. The rolling-off of the phase for higher frequencies was more pronounced for the visual feedback pathway, indicating a greater delay.

2.3.1.3 Marginal stability of the feedback pathway

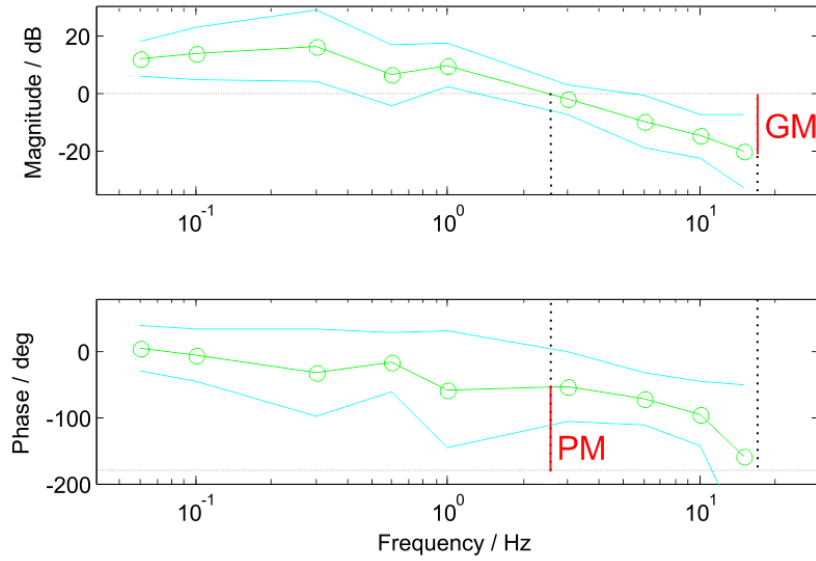


Figure 2.7: Marginal stability of the compound-eye-mediated head roll response. The phase margin (PM) and gain margin (GM) indicate large increases in gain and delay would still result in a stable system. The response variability is indicated by a range of three standard deviations (light blue lines).

The feedback pathway F_{fb} is also the open-loop equivalent of the visual response G_1 . The marginal stability of the visual feedback controller can thus be read directly from the feedback pathway Bode plot (Fig. 2.6). The feedback pathway Bode plot is reproduced in Fig. 2.7 and the average, intra-individual variability has been added (± 3 S.D., light blue lines). The gain margin of the system was 20 dB and the phase margin 132° . At the phase-crossover frequency, this corresponds to an admissible increase in delay of 153 ms.

2.3.2 Ocelli decrease latency of the head roll response

The frequency responses of head roll before (D1) and after (D2) occlusion of the ocelli were sampled for the four highest frequencies, where the rolling off of the phase was most pronounced. The frequency responses were plotted in linear units on a linear frequency scale (Fig. 2.8, mean $\pm$ standard deviation) to illustrate the difference in gradient of the phase, which corresponds to a constant-time delay.

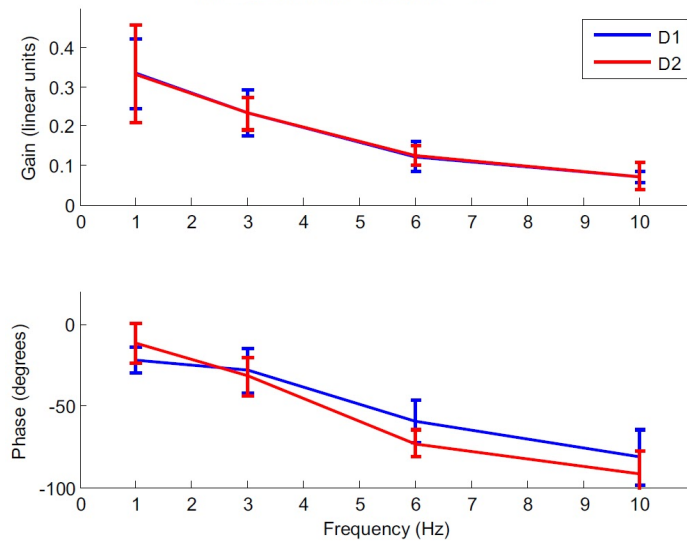


Figure 2.8: Behavioural head roll frequency responses before (D1) and after (D2) occlusion of the ocelli, plotted with linear gain and frequency scales. There is no significant change in response amplitude after occlusion of the ocelli, but the (negative) gradient of the phase decreases.

There is no significant change in gain, but there is a significant change in gradient ($p < 0.02$, paired t-test). I estimated the delay from the phase gradient as 22.4 ± 6.4 ms for D1 and 25.3 ± 8.1 ms for D2.

2.3.3 Conclusions

I have performed a behavioural, linear systems analysis of head roll in response to forced thorax oscillations. This stimulus corresponds to a change in attitude brought about by a sudden gust of wind, rather than a saccadic body rotation. I

tested the contribution of different sensory modalities on the dynamic properties of the reflex.

The removal of the halteres increased the response delay in the system by a factor of three and strongly decreased the system's gain at higher frequencies. The ocelli had a more subtle, but nonetheless significant effect on the phase at higher frequencies. Surprisingly, there was no difference in gain between D1 (with ocelli) and D2 (without ocelli). The neural mechanisms underlying this reduction in phase, but not gain, will require further investigation. In similar experiments at a single frequency, Parsons [2008] reported that ocelli contributed to changes in gain as well as in phase of head responses. The comparatively long delay in the visual pathway limits the gain and bandwidth for stable operation. However, the stability margins I estimated from the pathway frequency responses were surprisingly large: The delay in the system could be increased by another 153 ms before instabilities occur.

My findings are consistent with previous reports that the sensory modalities cover different, but partially overlapping dynamic ranges (Hengstenberg [1991]). An extension of this interpretation leads us not only to consider the dynamic properties of the individual sensory modalities, but also whether they convey feedback- or feedforward information. In past studies (Hengstenberg [1991]; Parsons [2008]), it was assumed that, if the head roll response is indeed linear and time-invariant, the frequency response of the intact system should be equal to the sum of the frequency responses of the individual, parallel sensorimotor pathways. This is, however, not the case if a subset of the pathways provide feedback control, as can easily be seen from the block diagrams and frequency responses in this chapter.

I did not attempt to identify the transfer functions of individual subsystems using analytical functions (cf. Schwyn et al. [2011]), but rather focused on the behaviourally relevant consequences of the interplay of feedforward and feedback control. Overall characteristics of the system as a whole, such as stability margins, are directly related to the behaviour of the animal. The open-loop and closed-loop frequency responses presented in this chapter may also be identified

by rational transfer functions. The resulting lumped parameters, such as the damping constant and natural frequency in the case of a second-order system, would not directly correspond to any physiological property of the animal. Such analysis may be used in future studies where additional data on the responses of individual subsystems is to be included.

Why does the compound eye pathway operate under such high stability margins? One possible explanation is the high variability in visually induced head movements (Rosner et al. [2010]), which has been suggested to be modulated by the internal state of the animal. Another possible explanation requires us to consider the effect of non-linearities in the processing chain. The saturation of the motor system, for example, is a candidate for a decrease in bandwidth.

The dynamic properties of the feedback loop depend on the individual components of the processing chain. While behavioural experiments allowed me to arrive at a phenomenological picture of head roll control, further insight into individual subsystems will be required to integrate the non-linear aspects of information processing, multisensory integration and motor output. In future studies, additional information on the magnitude of the response variability may be gathered using electrophysiological recordings and further behavioural experiments. If such data is available, stochastic systems theory may be applied to investigate the optimality of the reflex across species and with respect to different norms (Davis [2004]).

Chapter 3

Static X-ray Imaging of Motor System Morphology

The second part of this thesis focuses on the imaging of the motor system, an essential part of sensorimotor control as discussed in the previous chapter. I examine the potential of three-dimensional x-ray imaging for comparative studies *in situ* (this chapter), and functional studies *in vivo* (chapter 4). The aim is to develop and evaluate imaging techniques that may in the future support the design of functional, geometric models of the neck and flight motor systems.

3.1 Introduction

The study of animal morphology is a backbone of the biological sciences with applications to taxonomy, evolution, development and ecology (Rev:Friedrich and Beutel [2008]). But the fixing, staining and sectioning of material is time-consuming, often involves toxic or hazardous materials, results in the destruction of the sample and can lead to distortion artefacts (e.g. Huetteroth and Waddell [2011]; Saalfeld et al. [2012]). Studies in natural variability (e.g. Kovacević et al. [2005]) and broad comparative investigations (e.g. Ott and Rogers [2010]) are set to profit from faster, more robust data acquisition methods.

A range of techniques allow the acquisition of the full, three-dimensional volume data set of the specimen *in situ*, i.e. without physical sectioning of the ani-

mal. These methods include optical coherence tomography (OCT), optical transmission tomography, magnetic resonance imaging (MRI), and x-ray computed tomography (x-ray CT). In optical transmission and coherence tomography Fercher [1996]; Hielscher et al. [2004], the attenuation and reflection, respectively, of visible light or near-infrared radiation by structures inside the sample are measured. These techniques rely on the (at least partial) translucence of the tissue under consideration to optical light. MRI and CT, on the other hand, are well suited to the imaging of dense and thick specimens (Rudin [2006]). MRI relies on nuclear magnetic resonance, the alignment of the magnetization of (mostly Hydrogen-) nuclei in response to targeted radio frequency pulses (Lauterbur [1973]). The relaxation of the echo signal, the decay of spin alignment, is sensed using body coils. MRI is a technique with high soft tissue contrast, but the decay rates measured are on the order of milliseconds, and these generally need to be sampled across the frequency space for each individual slice. This makes MRI a relatively slow modality for high resolution-imaging (Rev:Ziegler et al. [2011]), although constant progress is made in reducing acquisition time, mainly through the use of higher field strengths and parallel acquisition coils (e.g. SENSE:Pruessmann et al. [1999]). Computed x-ray tomography, on the other hand, is a fast modality that routinely achieves spatial resolutions of few micrometres (Kalender [2006]) and thus is the imaging technique used in this study.

3.1.1 A brief introduction to computed x-ray microtomography

Computed tomography (CT) describes the imaging of a three-dimensional volume by means of calculating individual slices, in general from transmission radiographs, or projections, of the object. Without prefix, CT usually implies the use of x-rays to acquire the projections, however, computed tomography may also involve electrons (electron tomography) and is sometimes used to refer to the clinically relevant positron emission tomography (PET) or single photon emission computed tomography (SPECT). X-ray CT has been in clinical practice since 1972 (Rev:Kalender [2006]) and has become a workhorse of clinical radiography despite increased radiation doses compared to planar radiography or MRI

(Brenner and Hall [2007]). Like in MRI and OCT, the object is sectioned virtually rather than physically. Because x-rays penetrate dense materials such as metal and bone, they are widely used in non-destructive materials testing in engineering and investigations of the skeletal system in the biomedical sciences. Contrast is defined by the attenuation of the x-rays passing through the tissue. Soft (or lower-energy) x-rays are mainly absorbed and scattered by electron-dense materials. Contrast agents such as iodine and barium are in routine clinical use for investigations of the gastro-intestinal and vascular system (Kalender [2006]).

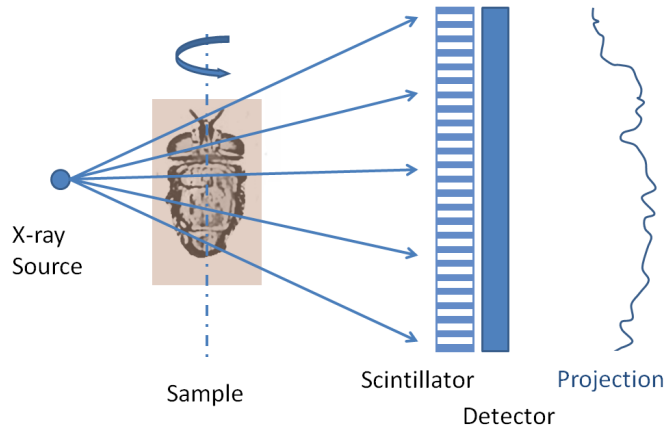


Figure 3.1: Simplified schematic of a laboratory-based microCT scanner. Electrons are accelerated in a vacuum and hit an anode target, often copper or molybdenum, at high energies. As the electrons hit the target and are decelerated, they release x-rays by means of Bremsstrahlung. The size of the focal spot determines the maximally achievable spatial resolution. The sample is rotated in the x-ray beam, while a scintillator-detector arrangement acquires the projections. A profile of the two-dimensional radiograph is displayed behind the detector for illustrative purposes. Note that only cross-sections through the scintillator, detector and obtained projections are displayed. These three elements are two-dimensional in the actual system.

In laboratory applications like the one presented in this chapter, CT systems consist of an x-ray source that generates high energy x-rays by means of Bremsstrahlung (Haug and Nakel [2004]), a sample rotation and manipulation stage, a scintillator converting x-ray photons to visible light, and a detector cam-

era (Fig. 3.1). In this fan-beam configuration, the sample can be magnified by increasing the source-to-sample distance. However, the spatial resolution is ultimately limited by the size of the target. Imaging systems with small focal-spot sizes are often referred to as x-ray microtomography systems, or microCT scanners (Flannery et al. [1987]).

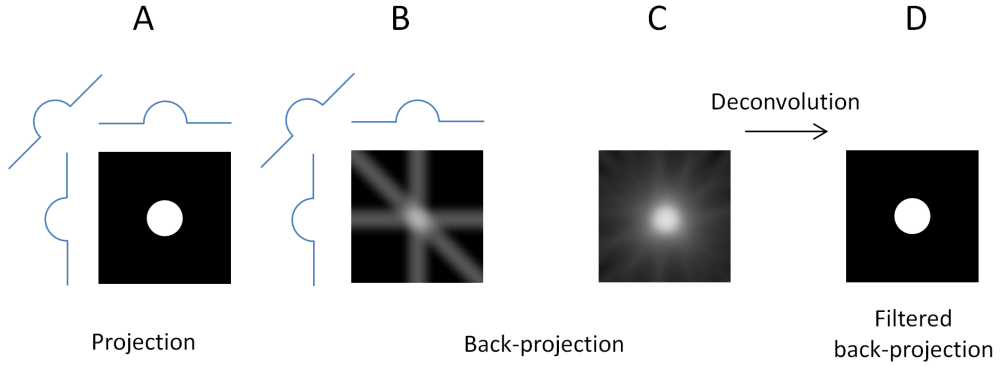


Figure 3.2: Reconstruction of volume data from a projection set. As an example, a slice containing a round object is projected on a screen from multiple angles (A). Backprojection of three profiles (B) and eight profiles indicates slow convergence to a blurred version of the original object. If the backprojected slice is deconvoluted with the point-spread-function, an accurate image of the object is obtained (D).

If sufficiently many projections have been collected, the volume imaged can be accurately reconstructed (Radon [1917]). In modern scanners, this reconstruction is implemented using filtered backprojection (Fig. 3.2). For a given slice, the profile acquired at each angle is smeared out across all pixels (object points) that were between source and detector at that angle. The resulting image is a blurred version of the object slice, that is, the convolution of the object slice with the point spread function (PSF) of the imaging paradigm. The PSF is the image generated by an object consisting of a single point. The backprojected image is deconvoluted with the PSF to obtain the original image. Note that the process involves an additional parallelisation step for fan-beam-scanners like the one used in this chapter, as x-rays travel through multiple slices, not just one like in the parallel-beam case (Slaney and Kak [1988]). In this chapter, I

study the potential of microCT for functional and comparative studies of motor systems. As mentioned, microCT lacks intrinsic soft tissue contrast. Iodine has been demonstrated to be a potent but easy-to-use contrast-agent for various soft tissues (Jeffery et al. [2011]; Metscher [2009a]). I therefore stained ethanol-fixed blowflies *Calliphora vicina* using varying concentrations of iodine and measured image contrast after imaging. I further investigated the use of iodine before critical-point-drying, a widely used conservation method.

3.1.2 Major anatomical structures and the neck and flight motor systems

Virtual dissections presented in section 3.3 serve to illustrate the potential and limitations of iodine-enhanced microCT at short scan times. In the next two paragraphs I will briefly introduce the anatomical terms used.

The head of the fly supports the compound eyes and the ocelli (see section 1.2). The photoreceptors in the retina of the compound eye are the site of photo-transduction. The lamina lies beneath the retina and is the first part of the optic lobes, which also include medulla, lobula and lobula plate (Gullan [2005]).

A focus of this thesis is on the fly neck and flight motor systems. The flight motor consists of two distinct types of muscles, indirect power muscles and direct steering muscles (Rev: Dickinson and Tu [1997]). The large indirect flight muscles generate the power needed to sustain flight and span the thorax along the antero-posterior (dorsal longitudinal muscle, DLM) and dorsoventral axes (dorso-ventral muscle, DVM). These antagonistic, stretch-activated muscles fill much of the fly's thoracic volume and generate strong deformations of the exoskeleton. These in turn cause the sweeping motion of the wing, which are connected to the thorax by a complicated joint, the wing hinge, e.g. (Wisser and Nachtigall [1984]). The steering muscles, or control muscles, attach to sclerites in the wing hinge and influence the wing kinematics. There are eighteen steering muscles, two of which attach to the basalar apophysis, a conspicuous sclerite which will be used as a benchmark of tissue differentiation in the virtual dissections.

The individual units of the power muscles, the steering muscles and neck muscles present challenges of different degrees for fast microCT imaging.

3.2 Methods and Material

Male and female adult blowflies *Calliphora vicina* were taken from the laboratory stock at the Department of Bioengineering, Imperial College London, which was maintained at approximately 22 °C and a day:night cycle of 12h:12h. Flies were between 5 and 15 days old at the start of specimen preparation as described below.

3.2.1 Ethanol-fixed specimen preparation

21 flies were sacrificed by briefly freezing to -20 °C. Legs, wings and the abdomen were removed and the animals were fixed in absolute ethanol. After between six and seven days, three flies each were transferred into solutions prepared from absolute ethanol and 10 % aqueous iodine, such that the iodine concentrations were 0.3%, 0.5%, 1%, 3%, 5%, 10%, and 0% (control). Specimens were kept in these solutions for 2 days before they were washed and scanned. The washing consisted of three washes in absolute ethanol, 10 minutes apart, for solutions with 1% or less iodine and was followed by microCT scanning. Flies that were stained in the solutions with higher iodine concentrations were washed 12 hours before the scan as well as within one hour before the imaging.

3.2.2 Dried specimen preparation

Two blowflies were sacrificed by briefly freezing to -20 °C. Wings, legs and abdomen were removed and the animals were dehydrated in absolute ethanol. One of the specimens was stained in 1% iodine as described above for 48 hours. Both samples were washed in absolute ethanol and dried in a BAL-TEC CPD 030 critical point dryer according to standard procedure: The specimens were flooded with liquid CO_2 and left to immerse until the meniscus between liquid and gaseous phase disappeared, after which the CO_2 was vented. This filling-venting cycle was repeated 10 times before specimens were finally removed from the dryer.

3.2.3 MicroCT imaging

X-ray microtomography was performed using a Nikon/Metris XTek HMX ST 225 fan beam scanner at the Natural History Museum, London. The x-ray energy spectrum was the same for all scans, Bremsstrahlung created by a $155\mu A$ current of electrons with energy of 140 keV incident on a Molybdenum target. Medium-long scans were performed on one 1%-iodine-stained sample and one unstained sample. These lasted 104 minutes, during which 3142 projections were acquired (exposure: 2 s), and only one sample was scanned at a time (voxel size $(6.7\mu m)^3$). Short scans were 26 minutes of duration and encompassed the same number of radiographic acquisitions (exposure: 500 ms). These were performed on all samples listed above. Three ethanol-fixed samples were imaged in a single scan (voxel size $(7.2\mu m)^3$). The two dried specimens were imaged in one scan with the same spatial resolution.

3.2.4 Quantification of tissue contrast

Image quality was measured by means of the contrast-to-noise ratio, which describes the distance of object and background intensity distributions with respect to image noise. In order to avoid classifying the micro-structure of soft tissues as image noise, we only used the background noise in our calculations. The CNR of a specific tissue type was thus derived as

$$CNR_{tissue} = \frac{\mu_{tissue} - \mu_{background}}{\sqrt{2} * \sigma_{background}} \quad (3.1)$$

where $\mu_{background}$ and $\sigma_{background}$ were the mean and standard deviation of the background intensity distribution, respectively, as defined by the ethanol inside the exoskeleton of the fly, and μ_{tissue} was the mean of the intensity distribution of the respective tissue type. The constant factor in the denominator was introduced for results to be consistent with the definition of CNR used in chapter 4, where the root sum square of tissue and background standard deviations is used. An area (size: 100 pixels) of the image of the dorsal longitudinal muscle was used in the calculation of the muscle CNR and compared to an adjacent, ten-by-ten

pixel background patch. This way the CNR of muscle tissue was calculated for 8 transverse orthoslices. The CNR of the lobula (representing neuropil tissue), and the tracts between medulla and lobula was calculated in the same way in three slices. A volume of 7x7x3 voxels in the pronotal apodeme, a large volume of cuticle in the neck, was used to calculate the mean and standard deviation for cuticle.

3.2.5 Renderings and virtual dissections

The reconstructed image data was aligned using Fiji (NIH). Volume rendering was performed either with VGStudioMAX 2.1 (VolumeGraphics), or with Drishti, a scientific visualisation engine that is open-source and freely available online (Limaye [2006]). Transfer functions from voxel intensity to opacity were chosen automatically in VGStudioMAX 2.1 (isosurface tool), but manually in Drishti.

3.3 Results

3.3.1 Iodine-based enhancement in ethanol-fixed specimens

Medium-long (104 minutes) x-ray microtomographic imaging in an ethanol-fixed, unstained fly resulted in very low contrast between tissue and fixative. The contrast-to-noise, as measured in the dorsal-longitudinal flight muscle and in the background ethanol, was 0.33 ± 0.08 (mean $\pm$ standard deviation). CNR-values for other tissues were not determined, as the quality of the scans did not allow clear distinction of object and background voxels for nervous tissue or cuticle.

Iodine improves the soft-tissue contrast in medium-long microCT scans of *Calliphora vicina* and allows important anatomical structures to be distinguished (Fig. 3.3 A, B and C). Nervous tissue (e.g. brain, thoracic ganglion), muscles (e.g. dorsal-longitudinal and dorso-ventral flight muscle, steering muscles in the wing hinge) and cuticular sclerites (e.g. the basalar apophysis, an attachment site of three steering muscle) are visible in this scan of 104 minutes duration. Note that the consistency of structures in the frontal plane was achieved without manual alignment of slices, a common post-processing step in the conventional

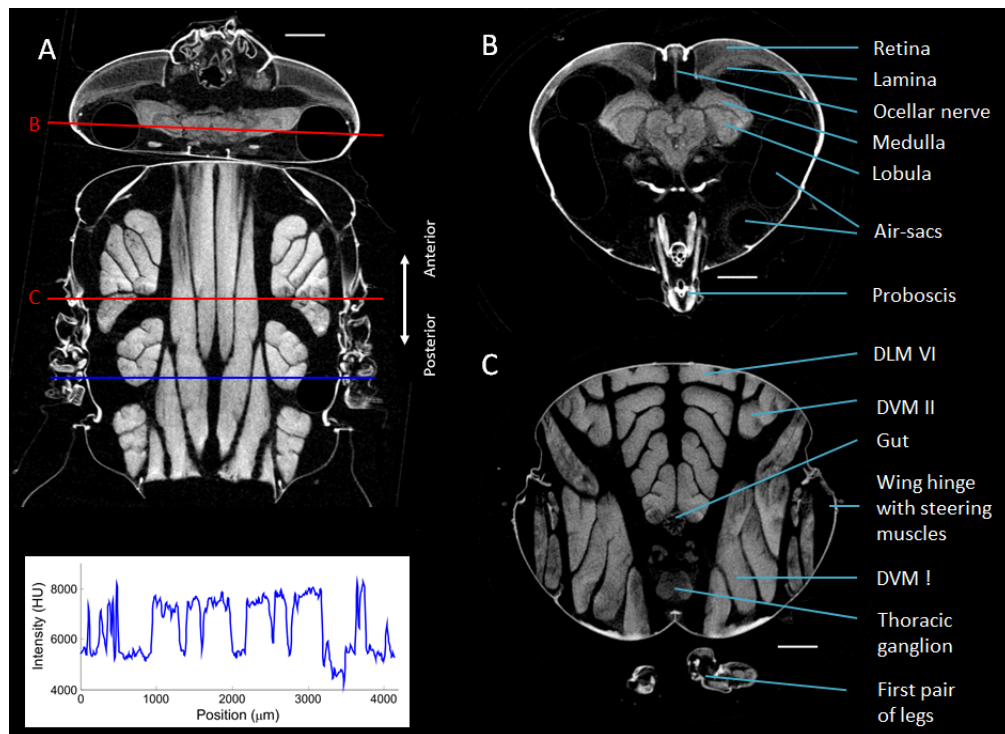


Figure 3.3: Virtual sections through the blowfly head and thorax using microCT. Indirect flight muscles can clearly be seen in a frontal section of the thorax (A). The positions of transverse orthoslices (B) and (C) are indicated in (A). A line profile through the thorax (blue line) is shown in the inset. The spectrum of intensity values was compressed such that background (ethanol and air) appeared black, and the different tissue types occupied the whole dynamic range. Scalebars 500 μm .

histology of sectioned specimens. The internal structure of the fly was clearly differentiated from the background (ethanol and air, Fig. 3.3 A). The CNR of muscle tissue was 11.2 ± 2.8 . The exoskeleton generally has a higher attenuation coefficient, and therefore exhibits higher intensity in the scans, than muscle tissue. The staining of muscle tissue is not uniform, however: the image of the dorsal longitudinal muscle, for example, becomes lighter as it approaches its anterior attachment site, and the distinction between cuticle and muscle tissue on the basis of voxel brightness values alone becomes impossible. Major brain areas can be distinguished as well: The neuropiles of the optic lobes, for example, appear denser than the tracts between them. The CNR between neuropiles and tracts was 3.3 ± 1.4 , which is usually sufficient for manual segmentation. The trajectory of the ocellar nerve can be traced despite being only 6 pixels (around $40 \mu m$) thin in parts of the orthoslice in Fig. 3.3 C. The visibility of such small structures, as well as the well-defined and sharp border between tissues and background, indicates a spatial resolution approaching the size of the voxels themselves ($6.7 \mu m$ in this case).

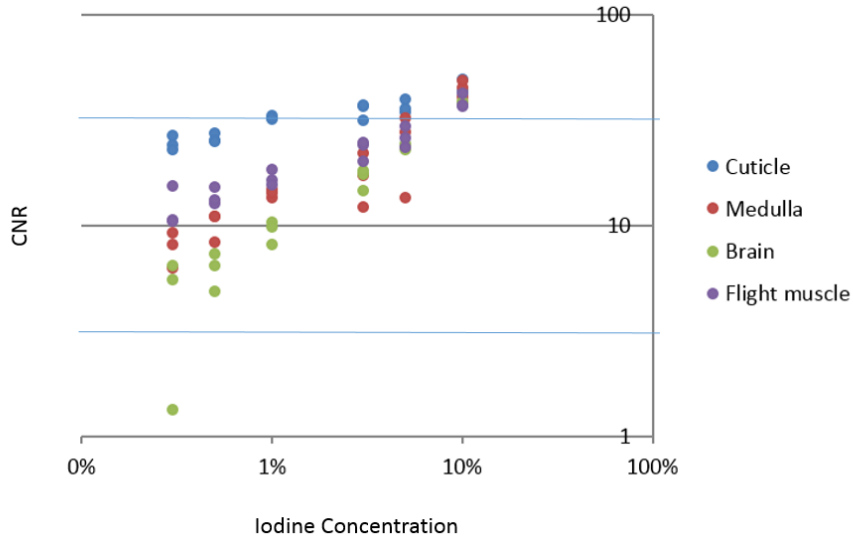


Figure 3.4: Effect of iodine-concentration on CT contrast for four major tissue types.

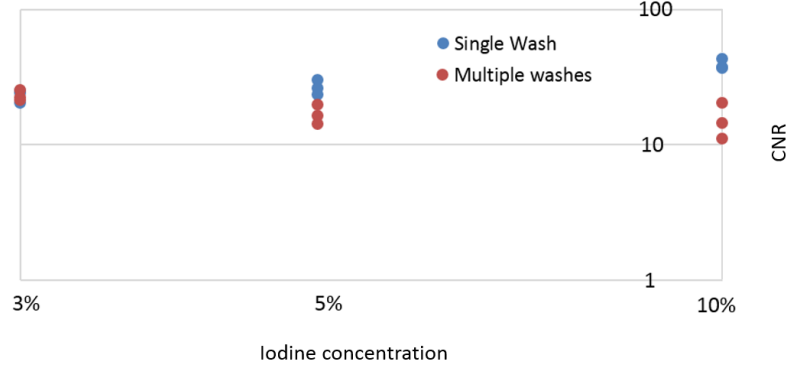


Figure 3.5: Conventional washing regime leads to a reduction in muscle-to-background contrast at higher iodine levels. The contrast-to-noise ratio of flight muscle decreases for higher iodine concentrations if the samples were only washed immediately before image acquisition (red dots). If samples were also washed the evening before the day of scanning, the CNR continues to increase at iodine concentrations of 5% and 10%.

I tested the effect of iodine concentration on the staining of different soft tissues for shorter scan durations. Scans only lasted 26 minutes, and three flies were scanned at the same time, allowing a twelvefold increase in throughput. Increasing the concentration of this contrast agent generally resulted in an increase of contrast between background and tissue (Fig. 3.4). The signal-to-noise ratio of the image data increased from around below 20 to around 50, but only if specimens stained with higher ($>1\%$) concentrations of Iodine were washed multiple times 12 hours before scanning, and again immediately before the scan. The conventional washing procedure applied to samples at lower concentrations lead to strong tissue leakage that lowered overall contrast and tissue differentiation in scans of strongly stained specimens (Fig. 3.5). The increased iodine concentrations also introduced strong shadows adjacent to stained structures, which likely represent beam-hardening artefacts: As the polychromatic beam (consisting of photons with a large range of energies) passes through the iodine-rich tissue, low-energy photons are absorbed more strongly than photons with high energies. This effect results in a shift of the beam energy spectrum towards higher x-ray energies, resulting in dark bands between light areas (e.g. Barrett and Keat

[2004]). Another side-effect of high concentrations of iodine is a lack of tissue differentiation. The CNR values of cuticle and muscle tissue are closer the more contrast agent is used.

3.3.2 Iodine-based contrast-enhancement in critical-point dried specimens

I investigated whether the use of iodine improved the tissue contrast of critical-point-dried specimens. This method of conserving the tissues is employed by a variety of collections and thus of interest in the context of comparative studies. Critical-point-dried samples generally provided sufficient tissue-to-air contrast for manual segmentation when imaged using x-ray microtomography (Fig. 3.6 A and B). Staining the animal in 1% iodine after fixation in ethanol, but before critical point drying, resulted in an improvement in contrast-to-noise ratio from 9.5 ± 4.3 to 77.7 ± 15.2 (mean $\pm$ standard deviation, $N = 3$ slices, Fig. 3.6 C and D). Volume renderings of the scans of stained and unstained specimens (Fig. 3.6 E, F) demonstrate the effect of iodine-based contrast-enhancement. In these, dark background voxels were transparent and lighter object pixels were displayed in shades of grey, with brighter voxels representing material with higher x-ray absorption. The rendering was clipped along the midsagittal plane of the fly.

subsectionVirtual dissections of neck and flight motor system

Regions of the fly neck and flight motor were rendered in Drishti from long (104 minutes) and short (26 minutes) scans of ethanol-fixed flies. The same iodine-concentration (1%) provided contrast-enhancement in both scans. In the visualisation of the medium-long scan, a virtual, sagittal clipping plane was inserted to achieve an unobstructed view of the left wing hinge (Fig. 3.7 A). For easier orientation, the pleural ridge and the anterior dorsal episternum are indicated (see also Wisser and Nachtigall [1984]). The spoon-shaped basalar apophysis, attachment site of the first and third basalar steering muscles (b1 and b3), is clearly visible and distinct from the muscles themselves. The tendon of the third basalar muscle is visible, but suffers from very low contrast. The same holds for the tendon of the pterale muscle I1. The short scan was rendered and clipped in the same way (Fig. 3.7 B). The borders between tissues and background appeared less well-

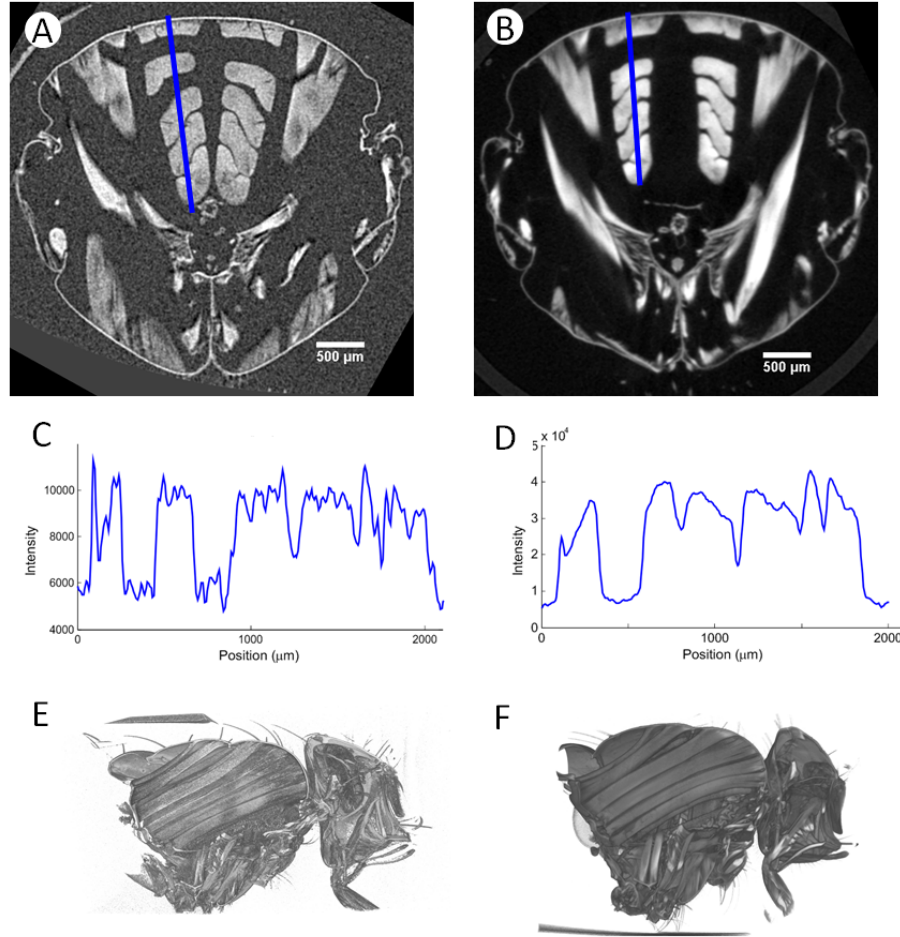


Figure 3.6: Comparison of critical-point-dried scans without (A,C,E) and with (B,D,F) iodine-enhancement (1% Iodine). The transvers slice through the thorax of the unstained fly exhibits more background noise (A) than the virtual orthoslice through the stained specimen (B). The displayed contrast range of each slice is the mean of tissue $\pm$ five times the standard deviation of the background. Profiles indicated by the blue lines in the slices show a higher signal-to-noise ratio in the case of the stained specimen (C,D). Volume rendering with VGStudioMAX 2.1 show the effect of the raw image data on 3D visualisations (E,F).

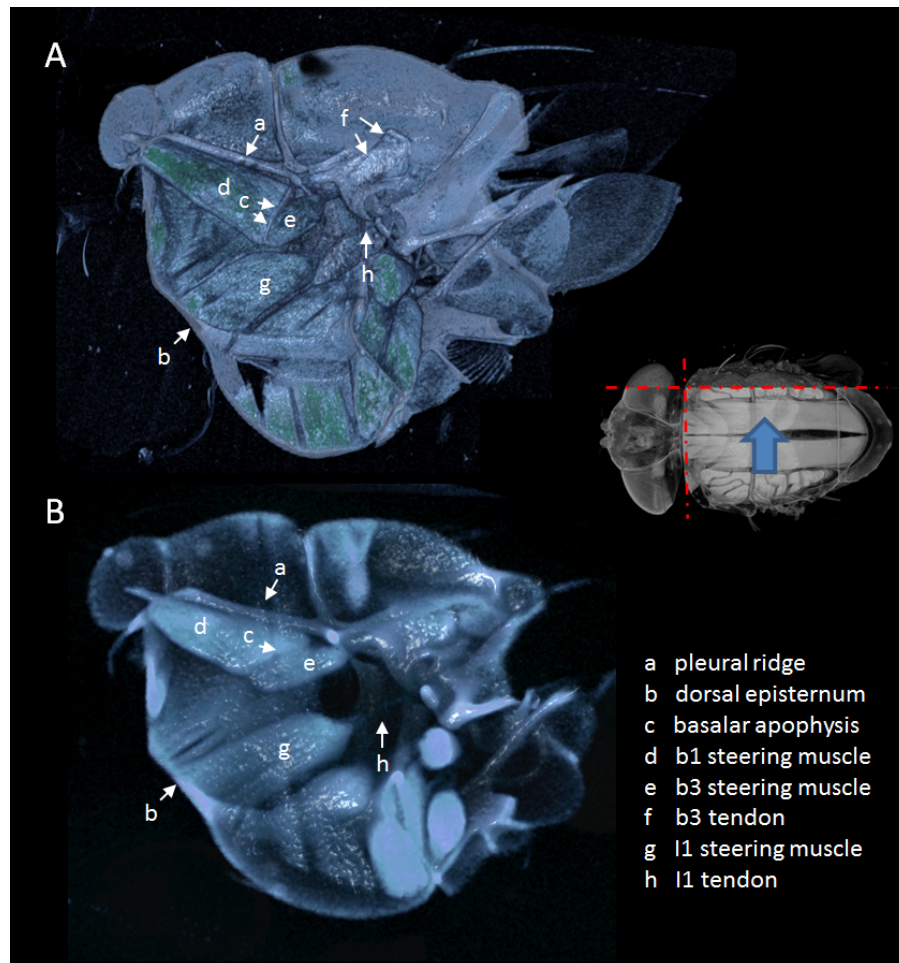


Figure 3.7: Virtual dissections of the wing hinge on the basis of a medium-long (A) and short (B) scan. In all figures, the inset of the grey fly represents the clipping planes (red) and the viewing direction (blue arrow).

defined. Nonetheless, with prior anatomical knowledge, b1, b3 and I1 muscles could be located. Only the tendon of I1 could be identified, while neither the tendon of b3 nor its attachment site could be found. These muscles are thought to play an important role in the control of wingbeat amplitude: it has been proposed that the b1 muscle is involved in a gear change mechanism (Gangschaltung, Pfau [1973]) in the fly, as well as in the initiation of flight (Wisser and Nachtigall [1984]). b3 and I1 are thought to contribute to the control of stroke amplitude.

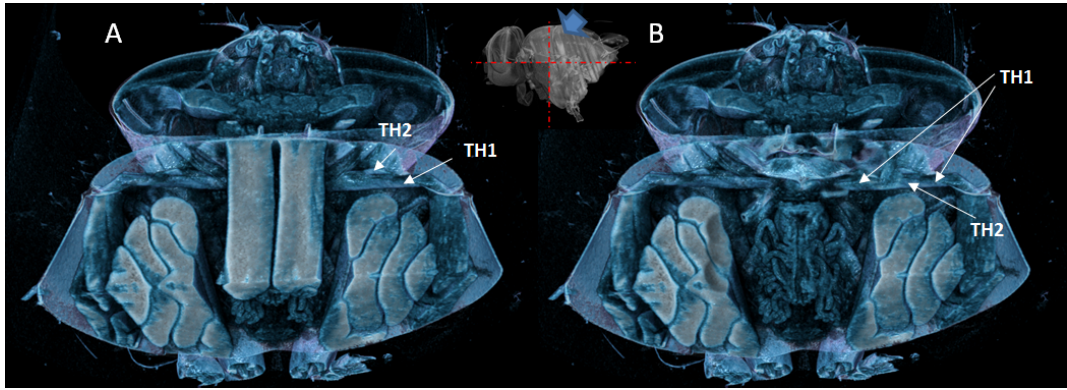


Figure 3.8: Contrast and resolution of reconstructions is sufficient for the distinction of two close neck muscles. The first transverse horizontal (TH) muscle and its lateral attachment site are well visible (A). It is accompanied by the smaller, spindle-shaped second TH muscle. The DLM was virtually removed to obtain unobstructed view of the path of TH1 from pleuron apodeme, the medial attachment site, up to the pronotum. The tendons that connect the TH muscles to the head capsule are not visible.

The medium-long scan was chosen to evaluate the suitability of microCT scanning to a three-dimensional analysis of the neck motor system (Strausfeld et al. [1987]). As an example, three groups of direct neck muscles were identified in virtual dissections (transverse horizontal (TH) muscles: Fig. 3.8, oblique horizontal (OH 3-5) and ventral longitudinal (VL) muscles: Fig. 3.9). While the two TH muscles could be individually identified, the three OH and two VL muscles could only be identified as groups. The virtual removal of individual tissue types supports the tracing of individual muscles through the surrounding tissues.

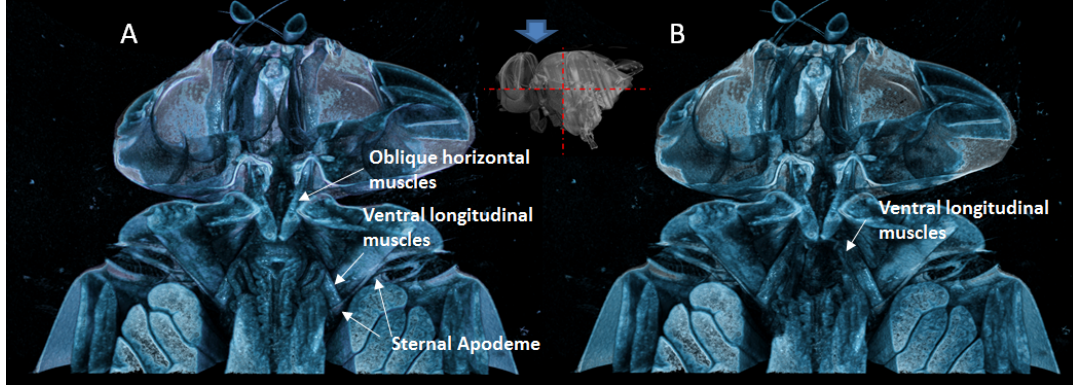


Figure 3.9: Virtual dissections of two groups of direct neck muscles. The three oblique horizontal muscles and the two ventral longitudinal muscles cannot be individually distinguished (A). The proventriculus, intestine and surrounding glands were removed to increase the visibility of the ventral longitudinal neck muscles (B). See Movie 3.1 for a animations of different virtual clipping planes.

3.4 Conclusion

Iodine-enhanced x-ray microtomography provided sufficient tissue contrast and resolution for the reconstruction of the gross morphology of insects, including brain and major nerves, indirect flight muscle and steering muscles, and the esophagus and gut (Fig. 3.4). Higher concentrations of iodine lead to increased tissue contrast, but not to superior tissue differentiation (Fig. 3.6). When applied during fixation in ethanol, iodine also increased the tissue contrast in critical-point-dried material (Fig. 3.7). Virtual dissections could be performed on data with comparatively short scan durations of 26 and 104 minutes (Fig. 3.8). Individual steering muscles and tendons were identified in scans of both durations. Groups of neck muscles were visible in the neck motor system (Fig. 3.9). Although all muscles are believed to be visible in the longer of the scans, their shape and size was often difficult to establish.

3.4.1 A simple staining procedure for universal tissue contrast

I chose to dehydrate the fly samples in ethanol, as this is readily available even in laboratories not specialized in histology, and the use of readily available, non-toxic fixatives potentially opens up the technique for a broad range of users. For the same reason, I preferred the use of aqueous iodine in solution over the potentially more harmful. Iodine is electron-dense (like many heavy metals, such as Cadmium) and therefore strongly absorbs x-rays. As has been shown previously, it is an efficient and easy-to-use CT contrast agent for a range of tissues including muscle and nervous tissue and gives good results with a range of fixatives (Metscher [2009a,b]). The staining procedure only involved a single transfer of the specimen from fixative to staining solution, and multiple washing steps depending on the chosen iodine concentration. I observed Iodine leakage to be a problem for higher concentrations ($>1\%$) of the contrast agent. I have shown that staining with iodine improves image contrast of critical-point-dried specimens. This procedure of specimen preparation is popular in entomological collections and museums (Quicke et al. [1998]). It also reduces sample shrinkage over freeze drying and allows specimens to be used in scanning electron microscopy, a high-resolution microscopic technique (Nordestgaard and Rostgaard [1985]).

Metscher [2009a] reviewed the use of iodine with a range of fixatives, including ethanol, methanol, formalin and the formaldehyde-based Bouin solution. Where iodine was used as contrast agent, the concentration was 1% and the scans lasted between 2 and 16 hours. Here I showed that even scans with lower concentrations of iodine (0.3% and 0.6%) allowed for sufficient tissue-to-air contrast. Higher concentrations of iodine lead to increased tissue contrast for short, 26-minute scans. However, the differentiation between tissues, mainly cuticle and soft tissues appeared to decrease with iodine concentrations. This may be due to saturation of the soft tissues and different uptake rates of soft tissue and cuticle. The mechanism by which iodine enhances the contrast of muscle and nervous tissue has not yet been elucidated, although glycogen has been implicated (Jeffery et al. [2011]). While I investigated the variability of tissue contrast with iodine concentrations, image quality varies with other parameters as well. The voltage between the

cathode and the target determines the energy of the electrons hitting the target, and therefore the energy spectrum of the x-ray beam. Image contrast depends on the chosen voltage because the linear x-ray attenuation coefficient of materials is energy-dependent (Hsieh [2003]).

3.4.2 Comparison to conventional morphological techniques

Imaging using computed x-ray microtomography has significant advantages over traditional sectioning methods. It offers fast and convenient methods of sample preparation and does not suffer from artefacts due to tissue distortions caused by the physical sectioning of the material. MicroCT provides isotropic spatial resolution, meaning that the voxel lengths are the same in the depth direction as in the x- and y-directions. The sectioning of the material in conventional methods, or the acquisition on a focused slice basis, often means that the resolution the depth direction, or the number of slices per unit depth, is lower than that of the individual slices themselves.

In general, though, the resolution of commercially available microCT scanners is inferior to that of confocal microscopy and electron microscopy, techniques widely used in the determination of insect morphology. Future generations of microCT scanners are expected to make use of x-ray optics, which would overcome the fundamental resolution limit of the visible light imaging system behind the scintillator (Ritman [2004]). Combination of stains may lead to an increase in tissue differentiation. Samples may be imaged multiple times, after addition of further, less harmless contrast agents, such as phototungstic acid (Metscher [2009b]), osmium tetroxide (Ribi et al. [2008]), or uranyl acetate (Terzakis [1968]). The effects of iodine have been described as readily reversible (Bock and Shear [1972]). Furthermore, a contrast mechanism discussed in the next chapter, phase contrast, may be available in commercial microCT systems soon. Phase contrast is more sensitive to soft tissue than absorption contrast (see section 4.1).

MicroCT is expected to complement conventional histological techniques such as light and electron microscopy. Non-destructive tomography can be performed on the sample before tissues deemed of interest are *removed* and investigated in more detail.

3.4.3 Towards three-dimensional models of the neck and flight motor systems

The dynamic characteristics of motor systems in insects depend on the physiological properties of muscles, the stiffness of the sclerites and processes as well as the geometric configuration of these structures (Haeufle et al. [2012]). A first step in the design of functional, geometric models is segmenting the muscles and cuticular structures involved in specific behaviours (Berry and Ibbotson [2010]). These virtual representations may support the simulation of the responses of a motor system to different patterns of muscle activation. This includes variations in the set of stimulated muscles as well as different input current waveforms. Simple models for the behaviour of individual muscles, Hill muscle models, for example, consist of three dynamical elements (two non-linear springs and one contractor) and are easily implemented (Winters JM [1987]). Optimally, we would not only acquire the static configuration of the system, but observe muscle contractions and cuticle movement while the animal is behaving as naturally as possible. In the next chapter of this thesis, I focus on such *in vivo* imaging experiments.

Chapter 4

In vivo microtomography of the fly flight motor using hard x-rays

4.1 Introduction

In the previous chapter I discussed the static imaging of invertebrate morphology using cathode tube x-ray sources which have become widely available at academic institutions. While easy-to-use and affordable, these imaging systems suffer from limited temporal and spatial resolution and are best suited to the study of dense material or fixed, stained soft tissue (Jeffery et al. [2011]; Metscher [2009a]). The function of individual muscles and their contribution to movement, however, cannot be easily deduced from static anatomical reconstructions alone.

A comparatively simple system for flight control, e.g. yaw rate stabilisation (Bender and Dickinson [2006]; Elzinga et al. [2012]) includes sensory reception and processing, the biomechanics of the flight motor as well as the resulting changes in wing kinematics which, ultimately, drive behaviour (Fig. 4.1). As outlined in section 1.4, the disposition of muscles and cuticular processes inside the fly can currently not be observed *in vivo* for methodological limitations, leaving a gap of understanding regarding the relationship between neural processing and behaviour. The aim of this chapter is to establish an imaging method that allows for non-invasive, fast and high-resolution imaging of motor systems, such as the flight motor, in behaving insects.

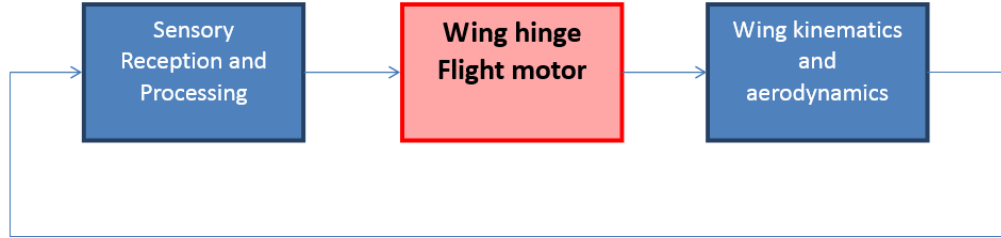


Figure 4.1: Block diagram of generalized flight control system. Sensing, neural signal processing and multisensory integration may be investigated using electrophysiological recordings. Wing kinematics and flight behaviour can be tracked and analysed using high-speed cameras. Complementary *in vivo* imaging is a promising method in the study of the dynamic properties of motor systems.

Blowflies reach wingbeat frequencies in excess of 150 Hz (Dickinson [1990]). In order to observe the contractions of muscles and the movement of sclerites in flying insects, a temporal resolution an order of magnitude faster than the wingbeat period is needed. At the same time, the imaging method should be non-destructive, generate 3D data at a spatial resolution in the micrometre range, and result in sufficient contrast between tissue and background for automatic segmentation. To this end, synchrotron facilities provide x-rays with promising application-specific, appropriate properties which are described below (Willmott [2011]).

4.1.1 Synchrotron-based phase-contrast imaging

The following paragraphs provide a brief overview over the application of synchrotron-generated x-rays to coherent imaging. Individual elements of the experimental setup are only discussed as far as they relate to *in vivo* experiments presented in this chapter.

Synchrotron light sources are ring-shaped particle accelerators that store relativistic electrons (i.e. electrons with velocities near the speed of light) to provide, amongst other probes, hard x-ray radiation of energies higher than 10 keV (Willmott [2011]). As the electron current in the storage ring passes through bending magnets, their trajectory is deviated and radiation is generated. The energy spec-

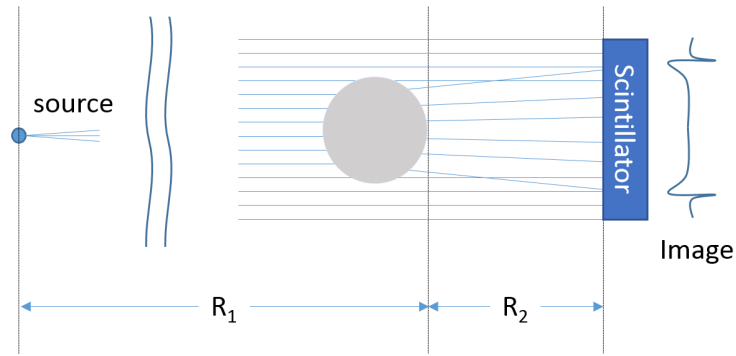


Figure 4.2: Propagation-based phase contrast. At a long distance R_1 from the source, the x-ray beam has fanned out sufficiently for it to be considered of parallel beam geometry. The part of the beam that passes through an object does not only undergo attenuation by means of scattering and absorption, the passage through the object also results in a phase shift compared to the freely-propagating beam. Behind the object, the x-rays interact and form diffraction patterns. At a propagation distance R_2 behind the sample, this results in simple edge-enhancement (single fringes around the edges of the object) if R_2 is in the near field, i.e. if $R_2 < 2\lambda/d$, where d is the size of the smallest distinguishable structure in the object and λ is the wavelength of the x-ray radiation. (Figure adapted from Onuki and Elleaume [2003]).

trum depends, among other factors, on the field strength of the bending magnets and the velocity of the electrons. Using so-called superbending magnets with a high field strength (or other insertion devices such as undulators or wigglers (Onuki and Elleaume [2003]) that induce periodic motion of the electrons and thus emit radiation along straight sections), radiation with high energy, flux and spatial coherence can be generated (Fig. 4.2). Together, flux and coherence define the brilliance of the beam, which is many orders of magnitude higher at dedicated imaging beamlines of synchrotron light sources than that supplied by commercial microCT imaging systems (e.g. Westneat et al. [2008]). Furthermore, if the beam is sufficiently collimated and travels a long distance from the source, the x-rays that are incident on the sample and detector are nearly parallel, which means simpler reconstruction methods can be applied than for fan-shaped beams generated by microfocus x-ray sources (Slaney and Kak [1988]).

In chapter 3, iodine was introduced as a contrast agent that generates absorption contrast between soft tissues and the surrounding ethanol in fixed specimens. The long coherence length of synchrotron-based x-rays lends itself to use a material property that is highly sensitive to differences in biological tissues as a contrast mechanism: X-rays do not only undergo a change in intensity (absorption), but a phase shift (refraction) is introduced as well. This phase change can be measured and used as a contrast mechanism in different ways (Momose et al. [1996]). While absorption contrast increases with the ratio of absorbed photons, and therefore radiation dose, phase contrast makes use of information contained in transmitted x-rays. This means that the dose can be greatly reduced by employing higher x-ray energies, and therefore decreasing attenuation (Momose et al. [1996]; Zhou and Brahme [2008]).

4.1.2 Synchrotron imaging of invertebrates

These extraordinary properties of synchrotron x-ray sources have been used in a number of studies involving insects. Their high flux is well-suited to the investigation of unstained fixed or dried tissue (Betz et al. [2008]; Friedrich and Beutel [2008]; Socha and DeCarlo [2008]). Using hard x-ray tomography, van de Kamp et al. [2011], for instance, discovered a new type of joint in the leg of the bee-

tle *Trigonopterus oblongus weevil*, which resembles the man-made nut-and-screw mechanism. This joint restricts the movement of the coxa, the most proximal segment of the leg, around a single axis.

In other cases the high brilliance of modern imaging beamlines was exploited to acquire radiographs to be acquired using very short exposure times. 2D *in vivo* imaging of behaving insects led to the discovery of a new mode of respiration in insects (Westneat et al. [2008]) as well as a pattern of visceral-locomotory piston-ing in crawling caterpillars (Simon et al. [2010]), where radiographic timeseries clearly show phase-shifted movements of the intestines compared to the rest of the animal.

The amount of physiological information that can be obtained from individual radiographs is limited due to the transmission nature of x-ray imaging: At any given projection angle, a radiograph is a planar projection that only shows the integrated absorption of all tissues that a ray passes through before hitting the scintillator. Their interpretation therefore requires substantial a priori knowledge of the anatomy of the specimen. Only in rare cases, quantitative, volumetric data could be retrieved directly from individual radiographs (Sinclair et al. [2009]; Westneat et al. [2003]).

4.1.3 Gated *in vivo* imaging

The reconstruction of three-dimensional volume data from a set of projection radiographs was described in chapter 3. The acquisition of the full set of projections required for reconstruction is time-consuming. For periodic movements, such an acquisition can be spread out across multiple movement cycles (Desjardins and Kazerooni [2004]). In so-called *gated* imaging paradigms, only radiographs from a single projection angle, or a limited set of projection angles, are obtained during each behaviour cycle. It is important to note that the resulting data set does not describe a single period of motion, but rather a complex reconstruction of the cycle based on multiple periods.

Gated imaging paradigms have been applied to respiratory and cardiac mechanics in rodents with heartrates in excess of 10 Hz. Using clinical systems, a temporal resolution in the order of 10 ms and voxel sizes of $(100\text{ }\mu\text{m})^3$ have been

reported (Drangova et al. [2007]). At the Japanese Synchrotron facility SPring-8 tomography was performed in breathing mice, with 30 μm spatial and 10 ms temporal resolution (Dubsky et al. [2012]). In these experiments, the acquisition was gated according to an external respirometry signal indicating the phase of the breathing cycle. Similar paradigms are in regular use in clinical practice (ECG-gated cardiac imaging), but only at the slower speeds of human heartbeat and more stringent radiation limits (Desjardins and Kazerooni [2004]).

This chapter describes how I combined the unique phase-contrast capabilities of a synchrotron beamline dedicated to high-speed imaging experiments with retrospectively gated tomography paradigms to extend the current limits in both the spatial and temporal domains. To the best of my knowledge, this represents the first application of *in vivo* x-ray microtomography to insect wingbeat at the millisecond timescale and on a spatial scale of around 10 micrometres. In contrast to previous *in vivo* work, the entire acquisition was typically as short as 0.5-3 seconds and therefore well suited to capture biological systems under stable conditions. The proposed retrospectively gated reconstruction of volumetric time series recovers gating signals from the projection sequence itself to enable grouping (or sorting) of the projection data after acquisition rather than gating at the time of acquisition. Gating potentially increases the perceived temporal resolution by two to three orders of magnitude.

4.2 Methods and Material

As synchrotron beamlines are run on a 24 hour-basis (100% duty cycle), experiments were conducted with the help of Rajmund Mokso, Martina Wicklein, Simon Walker, Michael Doube, Tonya Mueller and Graham Taylor. The beamline and the ultra-fast end station were the property of the TOMCAT group at the Paul Scherrer Institute, Villigen, Switzerland.

4.2.1 Animals and preparation

Blowfly pupae (*Calliphora vicina*) from the laboratory stock at the Department of Bioengineering, Imperial College London, were relocated to Switzerland where

they were kept on a 12h:12h day/night light cycle after eclosion until the beginning of the experimental shifts.



Figure 4.3: Blowfly attached to a wooden holder using a mixture of beeswax and Collophonium.

Flies were briefly anesthetized by cooling to 4 ° C in ice water and mounted dorsally to a wooden holder. They were given at least 30 minutes of rest to recover before their ability to perform sustained flight was tested prior to use in experiments. The holder was attached to the rotation stage of the beamlines end-station using a custom-made device designed in SolidWorks (Dassault Systemes) and rapid-prototyped from Fullcure-720. The flies were mounted facing upwards such that their anteroposterior axis was approximately aligned with the vertical rotation axis of the sample stage (Fig. 4.3).

4.2.2 Experimental setup

All *in vivo* imaging experiments were conducted using the ultra-fast setup (Mokso et al. [2010]) at the Tomographic and Coherent Radiology Experiments Beamline at port X02DA of the Swiss Light Source, a third-generation synchrotron at the Paul Scherrer Institute, Villigen, Switzerland. The properties of the x-rays supplied by this beamline are reported elsewhere (Stampanoni et al. [2007]).

Imaging was performed using two distinctly different energy spectra. Polychromatic x-rays of 35 keV mean energy were provided using a filtered whitebeam configuration. Monochromatic x-rays were generated by insertion of a double-crystal multilayer monochromator (Fig. 4.4, Mokso et al. [2010]). This reduced the bandwidth of the x-ray energy spectrum to 2% around 18 keV, but also reduced the photon flux by two orders of magnitude.

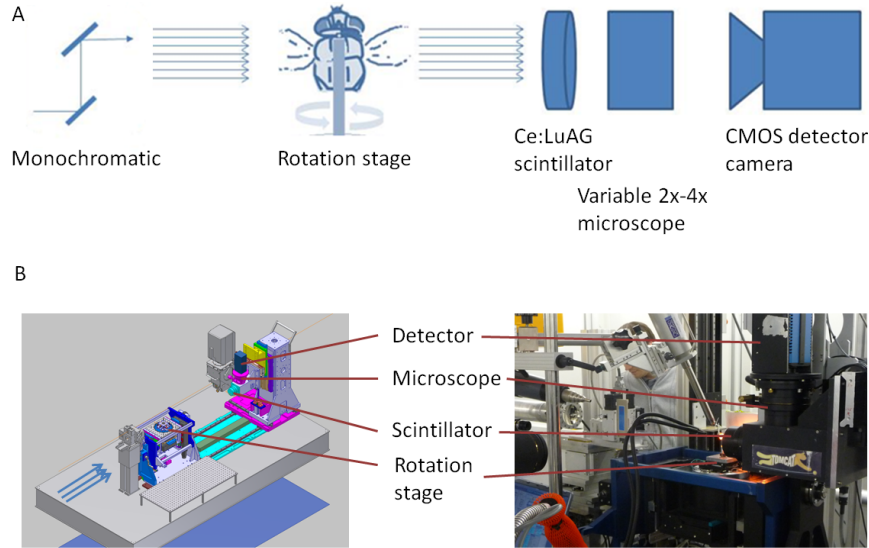


Figure 4.4: (A) Schematic and (B) CAD drawing and photograph of endstation setup. The x-ray beam path is indicated by a group of arrows. CAD drawing courtesy of G. Mikuljan, PSI.

At a free-space propagation distance (source detector distance, SDD) behind the fly, the beam was incident upon a ceramics-doped LuAG scintillator that converted the x-rays into visible light with a high efficiency and fast decay rates (Kučera et al. [2010], Fig. 4.4 B). A high numerical-aperture microscope (Elya solutions s.r.o) was focussed on the scintillator and provided variable two-fold to four-fold magnification. The detector was a pco.dimax (Pco corporation) 12-bit CMOS high-speed camera with a pixel size of $11\ \mu m$. The field-of-view encompassed up to 2048×2048 pixels, but was adjusted to fit the size of the animal in individual trials.

During experiments, tethered flight was initiated by retracting a lever from the legs of the fly and supplying air flow by means of a fan. A metal shutter was opened after a short period of sustained flight and image acquisition was performed at a constant frame rate according to Fig. 4.5. Each exposure lasted between 200 and $300\ \mu s$ and the subsequent readout of the detector lasted between $100\ \mu s$ and $300\ \mu s$, depending on the size of field of view.

Scans of two varieties were performed in June 2011 using whitebeam radiation:

Scan Type	A	B		C	D
X-ray spectrum	Filtered whitebeam (~35 keV)	Filtered whitebeam (~35 keV)		Filtered whitebeam (~35 keV)	Monochromatic beam (~18 keV)
Ring current	400 μ A	400 μ A		350 μ A	350 μ A
Pixel size	2.8 μ m	2.8 μ m		3.3 μ m	3.3 μ m
Exposure	300 μ s	300 μ s		300 μ s	200 μ s
Frame rate	2.5 kHz	2.5 kHz		2.5 kHz	2 kHz
Acquisition Paradigm	Single slow scan (SSS)	Single fast scan (SFS)	Multiple fast scans (MFS)	Multiple fast scans (MFS)	Multiple fast scans (MFS)
Number of half-rotations (scans)	1	1	6	7	7
Projections per scan	8050	1250	1250	1300	1000
Projections per reconstructed wingbeat phase	480	86, 84	513	589	595

Figure 4.5: Parameter sets used in different scans. Three reconstructions were generated from the scan acquired with parameter set B: The first and last half rotations were reconstructed separately, and in a third reconstruction projections acquired across all six scans were used. Note that scans A and B were performed at a different time than scans C and D and needed to be investigated in isolation.

A long, slow scan (parameter set A) and multiple fast scans (parameter set B). In order to test two different acquisition paradigms, the rotation rate of the sample stage was set to either 0.15 Hz or 1 Hz, allowing for varying numbers of projections to be recorded during each rotation. The different rotation speeds also influenced the behaviour of the animal, as fast roll speeds elicited strong equilibrium reflexes in the fly. In February 2012, we performed multiple fast scans in whitebeam mode (parameter set C) and polychromatic configuration (parameter set D).

The ring current of the synchrotron for the second set of experiments was reduced from 400 mA to 350 mA due to a defect in the storage ring. The flux of the beam at the endstation was reduced accordingly. This decrease in flux, combined with the constant evolution of the TOMCAT beamline influences the comparability of data and results generated at different times. For different imaging setups (e.g. changes in x-ray spectra), a host of other parameters including exposure and field-of-view had to be adjusted accordingly.

4.2.3 Processing workflow

4.2.3.1 Pre-processing

The radiographs were processed as follows (Fig. 4.6): Spatial deviations in beam profile were corrected for by the average of between 200 and 300 acquisitions without sample (flatfield images), and between 100 and 120 acquisitions without beam (dark images) acquired at the end of the experiment. Bright pixels caused by x-rays, rather than visible light, incident on the detector were removed by a 3x3 median filter applied to the corrected projections. This process is referred to as speckle removal.

4.2.3.2 Phase-retrieval

A variety of quantitative methods is available to measure the phase contrast of a sample (Pfeiffer et al. [2006]), many of which rely on multiple projections of the specimen from the same angle while varying a physical parameter, such as the distance between sample and detector. Due to time constraints, these methods are generally not suited to the *in vivo* applications targeted here. I have used the

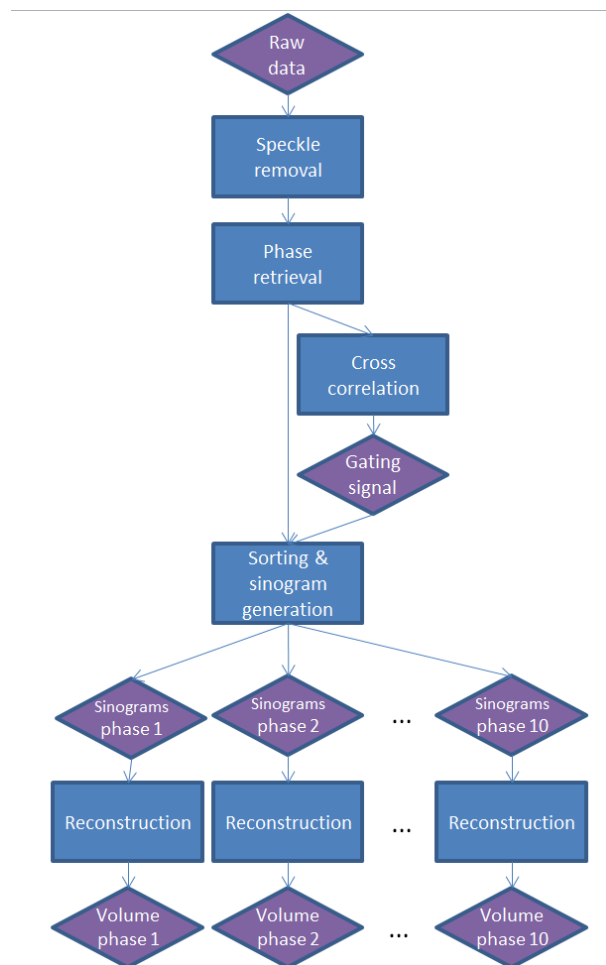


Figure 4.6: Image processing and reconstruction workflow. Note that no external gating signal is provided as input.

well-known single-image inline phase retrieval algorithm proposed by (Paganin et al. [2002]), which is applied to individual projections prior to reconstruction. Strictly speaking, this technique is only applicable if (i) the propagation distance is in the near field, (ii) the object consists of one homogenous material, and (iii) a narrow energy spectrum is used. The results will show that the algorithm is robust enough to the imaging parameters used here. Phase retrieval was performed using the ANKAPhase implementation (Weitkamp et al. [2011]) of this method. The basic operation of this algorithm is the combination of a low-pass filtering operation followed by logarithmic compression:

$$p = -\frac{1}{\mu} \ln \left(\mathcal{F}^{-1} \left[\frac{\mu \mathcal{F}[I]}{\mu + z\delta(u^2 + v^2)} \right] \right) \quad (4.1)$$

where p is the phase shift caused by the tissue projected. I is the two-dimensional projection, μ is proportional to the ratio of absorption coefficient and x-ray wavelength and z is the sample-detector-distance. $\mathcal{F}$ and $\mathcal{F}^{-1}$ are the two-dimensional Fourier transform and inverse Fourier transform, respectively, and u and v are the conjugate coordinates in Fourier space. Lastly, δ is an optical constant of the material, namely the decrement of the refractive index. δ is energy-dependent and strongly influences the result of the phase-retrieval operation.

The linear attenuation coefficient and the refractive index of water were chosen as an initial estimate of the average optical properties of the different tissues present in the animal. Fig. 4.7 illustrates the effect of the assumed refractive index of the material on phase retrieval and reconstruction quality.

4.2.3.3 Gating and sinogram generation

Gating of images was performed retrospectively, i.e. after acquisition at a constant frame rate, and based on a signal derived from the phase contrast radiographs themselves (Fig. 4.8). The movement of the sample along the vertical axis was determined by means of a spatial cross-correlation between frames, confined to the lower thoracic area of the fly. The peaks of this signal were assumed to indicate the beginning of a new wingbeat cycle. The radiographs were sorted into

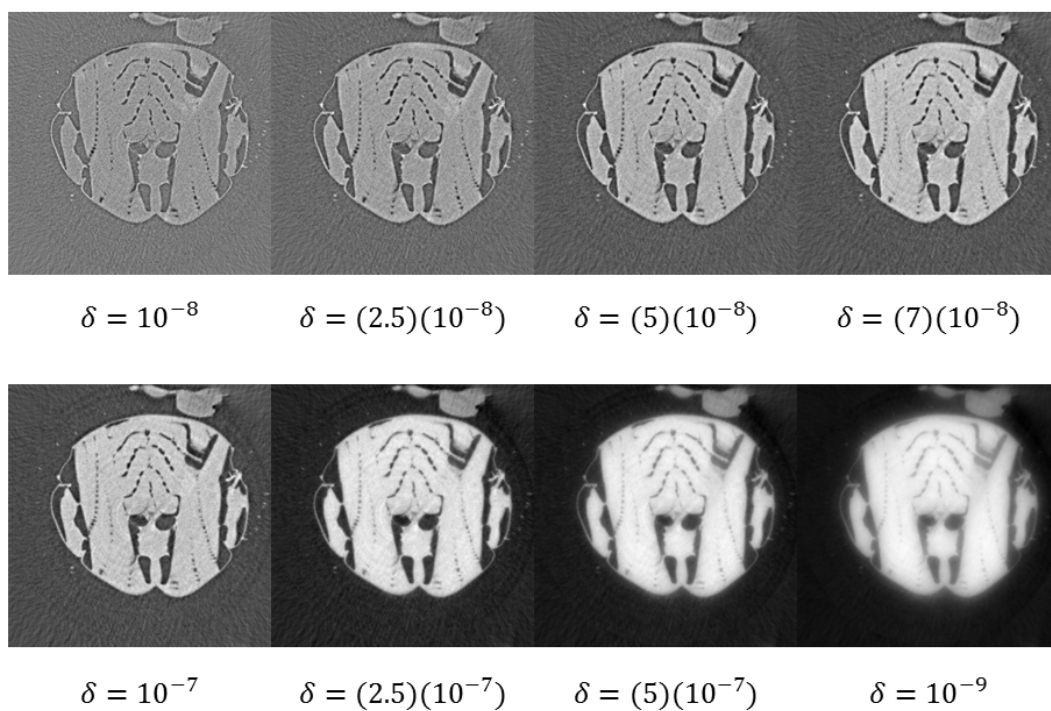


Figure 4.7: Reconstructions of a transverse slice through the thorax in dependence of the decrement of the refractive index δ . Phase retrieval was performed with different optical constants. The resulting reconstructions show the evolution from little contrast and strong edge-enhancement (upper left) to high contrast but strong blurring of structures (lower right).

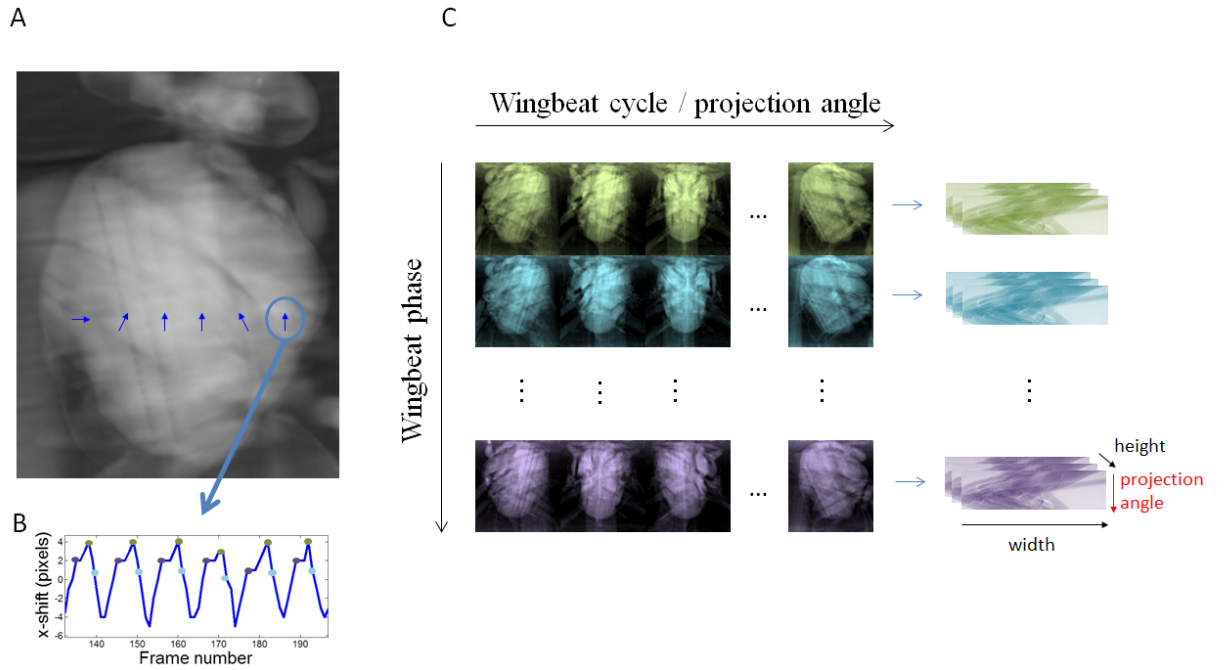


Figure 4.8: Retrospective gating of radiographic timeseries. (A) The windowed, spatial cross-correlation between normalized patches in the lower thoracic region of subsequent frames is calculated. (B) The vertical component of the location of the maximum of this cross correlation is used as gating signal. (C) Radiographs are sorted into 10 groups based on wingbeat phase. Stacks of sinograms are prepared for each wingbeat phase.

groups according to 10 equidistant wingbeat phases. The 10 sets each contained radiographs acquired at a single wing beat phase, but from a range of projection angles.

4.2.3.4 Reconstruction

Sinograms containing all angular projections of a single slice at a single wingbeat phase were generated using a custom-written MATLAB function. Reconstruction was performed using `gridrec`, an implementation of the Fourier slice theorem (Marone and Hintermuller [2008]). The Fourier slice theorem allows fast tomographic reconstruction equivalent to the filtered backprojection algorithm briefly discussed in section 3.2.

4.2.3.5 Renderings

Renderings displayed in this thesis were performed from downsampled data sets using `Drishti`, an open-source scientific visualisation engine (Limaye [2006]). The transfer functions from voxel intensities and gradients to colour and opacity values used in the renderings were chosen and optimized manually.

4.2.3.6 Analysis of Image Quality

Two metrics were calculated to compare the quality of image data obtained with different acquisition parameters, the contrast-to-noise ratio (CNR) and the resolution. For the purposes of this chapter, the CNR is defined as

$$CNR = \frac{\mu_o - \mu_b}{\sqrt{\sigma_o^2 + \sigma_b^2}}, \quad (4.2)$$

where μ_o and μ_b are the mean intensity of patches of 12x12 object and background pixels, respectively. σ_o and σ_b are the standard deviation of pixel intensity in the same object and background patch. Twelve pairs of patches were chosen pseudo-randomly across the field of view in each of four slices.

The spatial resolution was quantified using the edge response function of naturally occurring sharp edges in the tomographic slices of the object. Eight such

edges in the dorsal region, and at the most ventral unit of the dorsal longitudinal flight muscle were chosen manually. I fitted an error function to a line profile two pixels wide, and identified the resulting edge response function with an error function as described in (Bentzen [1983]). This allowed for the derivation of the full width at half maximum (FWHM) of the estimated point spread function (PSF) as a measure of spatial resolution.

4.3 Results

4.3.1 Phase-retrieval results in high tissue-to-air contrast

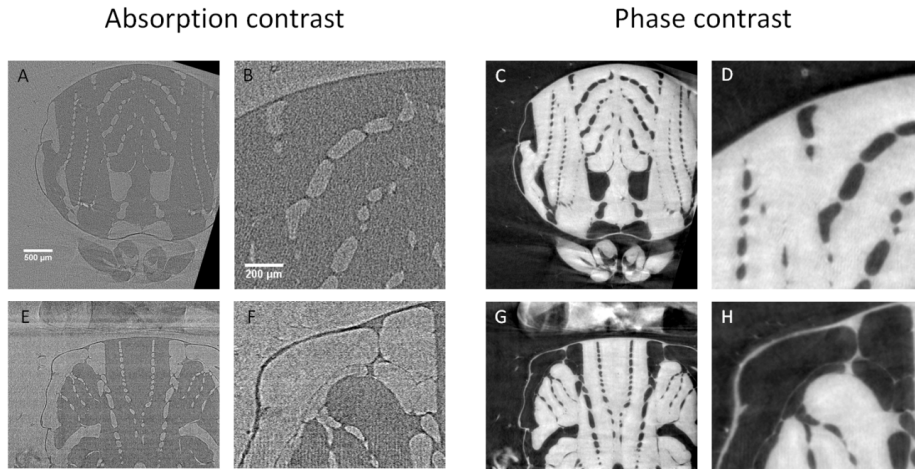


Figure 4.9: Absorption contrast and phase contrast thoracic cross sections, transverse (A-D) and frontal (E-H) planes. (A,B,E,F) Without phase retrieval, edge-enhancement is clearly visible and contrast is poor. (C,D,G,H) Phase contrast provides high signal-to-noise ratio, but no differential tissue contrast (Mokso, Schwyn et al., in preparation). See Movie 4.1 on accompanying CD for animated slices.

The radiographs for the single longs scan (parameter set A) were sorted according to ten equidistant wingbeat phases and reconstructed with and without phase retrieval (ventral stroke reversal: Fig. 4.9). Reconstructions of transverse and frontal sections show a marked difference in contrast. The borders of tissues appear very sharp, no motion blur is visible in the thoracic region, however, the

reconstruction of the head suffers from major motion artefacts (Fig. 4.9 E and G, upper quarter of the image). Note also the sizes and shapes of tissues compared to the microCT slices in chapter 3 (e.g. Fig. 3.4).

4.3.2 Dependence of image quality on acquisition paradigm

The rotation stage used at the TOMCAT beamline allows faster rotation speeds than that used in the single slow scan above. Faster scans mean the animals behaviour is sampled across fewer wingbeats, but reconstructions suffer from streaking artefacts, radial streaks that result from excessive undersampling.

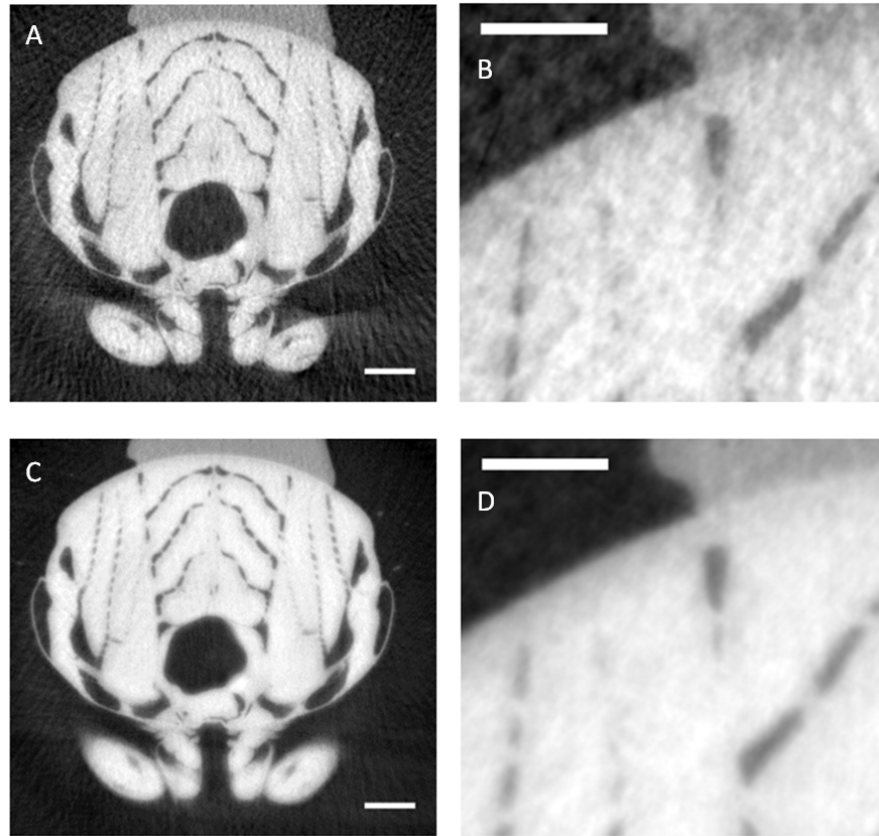


Figure 4.10: Acquisition paradigms involving fast rotations. (A,B) A single fast scan shows strong streaking artefacts. (C,D) Reconstruction across multiple fast scans results in smoother edges due to motion blur, but also higher contrast. Scalebars: A, C: 500 μm . B, D: 200 μm . (Mokso, Schwyn et al., in preparation)

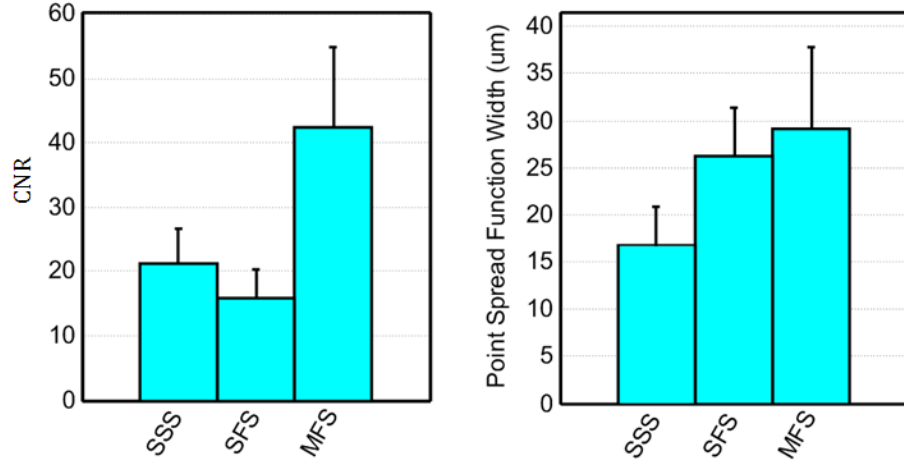


Figure 4.11: Contrast and resolution for acquisition with filtered whitebeam and three acquisition paradigms (mean $\pm$ standard deviation, see also Fig. 4.5).

A single fast scan of only 500 ms duration allowed for acquisition of 86 projections per wingbeat phase (ventral stroke reversal: Fig. 4.10 A and B) and clearly exhibits lower contrast, resolution and more reconstruction artefacts than the single slow scan in Fig. 4.9. A sufficient number of projections can be obtained if multiple fast scans are used in a single reconstruction (Fig. 4.10 C and D). This results in similar signal-to-noise ratios, but inferior spatial resolution. Changes of the animals behaviour during image acquisition, such as leg movements or a change in wing kinematics, result in motion blur that is easily identifiable. The resolution and contrast of the three paradigms are summarised in Fig. 4.11. The contrast-to-noise ratio was highest when multiple fast rotations were used. The spatial resolution of the single-slow-scan was superior to the other paradigms.

4.3.3 Monochromatic and polychromatic acquisitions

We also acquired image data with the monochromatic 18 keV beam. Surprisingly, there was little difference in either image quality or experimental yield. Flies survived in the monochromatic beam approximately as long as in the whitebeam configuration. Cross-sections through volumes acquired in February 2012, when the ring current was limited to 350 mA, are provided for completeness Fig. 4.12.

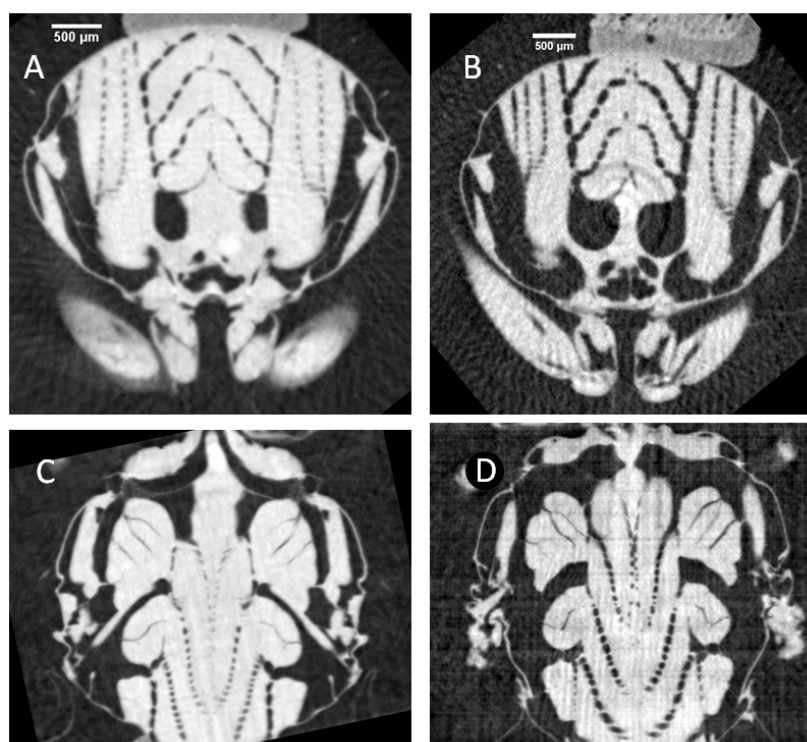


Figure 4.12: Transverse and frontal slices of filtered whitebeam (A, C) and monochromatic beam (B, D).

4.3.4 Potential applications

4.3.4.1 Visualisation of the trachea network

The tracheal network in the power muscle, a branching network of air-filled tubes, exhibits high contrast with respect to the surrounding muscle tissue. Visualisation by means of volume rendering shows the regular structure of these hollow tubes (Fig. 4.13).

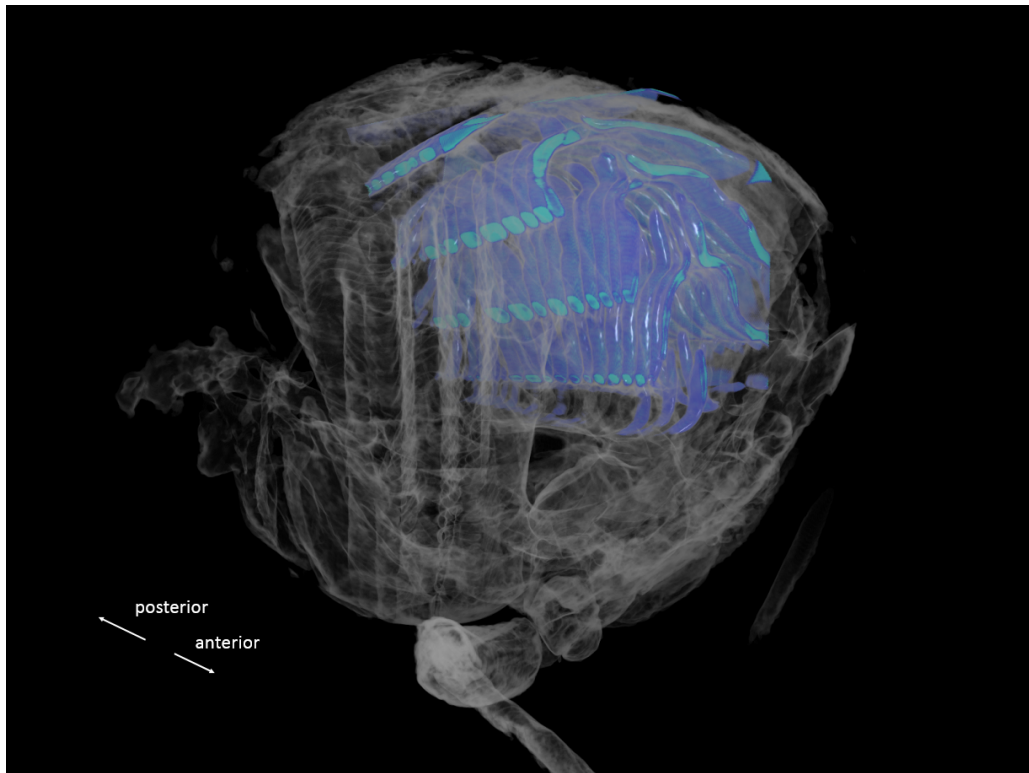


Figure 4.13: Volume rendering of part of the trachea network inside the dorsal longitudinal muscle (DLM). Only the first order of branches can be identified. Scan performed according to parameter set A in Fig. 4.5. Rendering performed using Drishti (ANU).

4.3.4.2 Length oscillations of the dorso-ventral muscle

The 4D data sets clearly show the contractions of dorso-ventral and dorsal longitudinal flight muscles, and indeed the deformations of the whole thorax. I selected

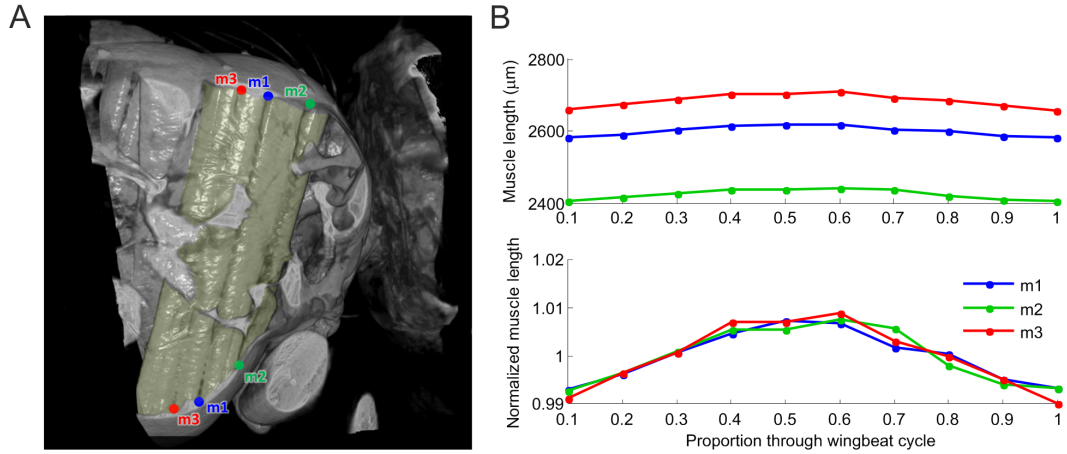


Figure 4.14: Muscle contraction cycle measured using virtual markers (scan parameters: A). Normalised muscle length is shown for comparative purposes only - in order to perform accurate strain calculations resting lengths of muscles would need to be known. See Movie 4.2 on CD for a time series of the muscle contraction. (Mokso, Schwyn, et al., in preparation)

virtual markers at the dorsal and ventral attachment sites of three individual subunits of DVM I (Fig. 4.14) and used a straightforward template matching routine (3D cross-correlation maximization) to find the displacements of markers for all time steps (compared to the first time step). The length oscillations allowed estimation of strain at different points in the wingbeat cycle. The strain cycle is surprisingly uniform considering the differences in average length between marker pairs and the fact that the individual attachment sites are rigidly connected by the exoskeleton.

4.3.4.3 Movement of the I.1 steering muscle in the wing hinge

The wing hinge was visualised using x-ray microCT in chapter 3 and individual steering muscles were clearly identifiable. Volume renderings at dorsal and ventral stroke reversal suggest that the image quality of the synchrotron *in vivo* time series is sufficient to investigate the movements of a subset of control muscles during wingbeat (Fig. 4.15). The I.1 steering muscle and its tendon (see also Fig. 3.8) are clearly visible at both timesteps.

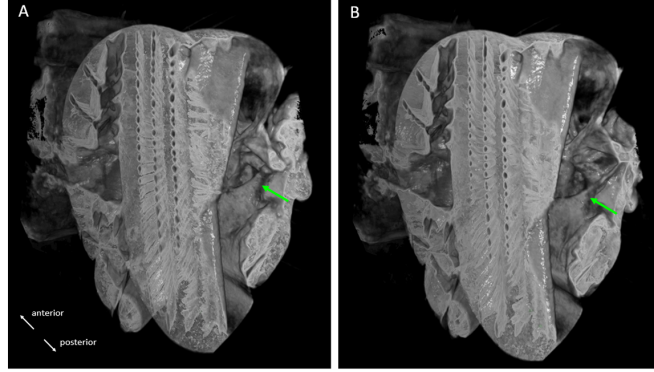


Figure 4.15: Visualisation of the wing hinge (partly covered by the first unit of the DVM). The I.1 pterale muscle (arrows) is clearly visible and seems to move between the two stroke reversal points (A: ventral, B: dorsal).

4.4 Conclusion

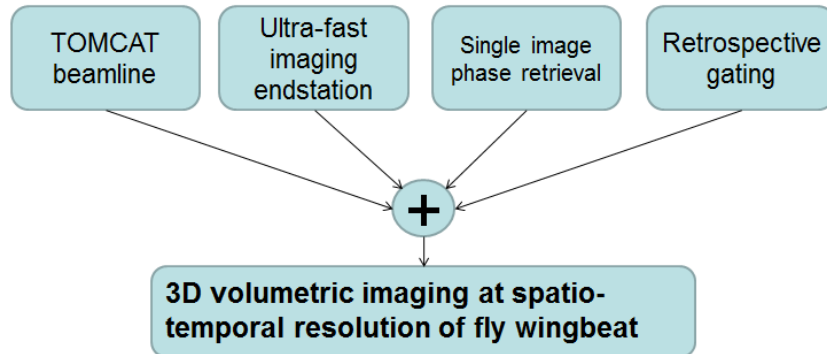


Figure 4.16: Relevant components for ultra-fast x-ray tomography at the temporal scale of fly wingbeat.

The high speed of image acquisition demonstrated in this chapter was achieved using a combination of four elements (Fig. 4.16). A synchrotron beamline provided an x-ray beam with high flux and coherent radiation. A dedicated ultra-fast end station consisted of a fast and accurate rotation stage, efficient x-ray conversion and a fast high speed camera as detector. A single-image phase retrieval algorithm enabled the exploitation of a contrast mechanism that is very sensitive

for many biological tissues. Lastly, data sets were acquired continuously across multiple wing beats using a retrospective gating paradigm.

4.4.1 Requirements for fast *in vivo* imaging

On the one hand, the data presented here offers a first, direct observation of the biomechanics of the fly wing hinge during tethered flight. On the other, the methods and results in this chapter serve as a proof-of-concept that gated imaging using hard, coherent x-rays is well-suited to *in vivo* studies of fast, quasi-periodic behaviour. The spatiotemporal resolutions of all scans in this chapter are far in excess of those of mammalian cardiac and respiratory imaging (Drangova et al. [2007]; Dubsky et al. [2012]), and a previous invertebrate imaging study: Momose et al. [2011] imaged a living moth larva (*nokona regalis*) using subsequent phase contrast scans of only 500 ms each (without gating) and superior soft-tissue contrast. Their camera frame rate, however, was only on the order of 500 Hz and the voxel edge lengths of the imaging system were 12.7 μm .

The retrospective gating method used in the first set of experiments has a range of distinct advantages over the generation of a gating signal by means of wingbeat tracking. Firstly, the cross-correlation algorithm means no cameras have to be placed in the hutch, facilitating experiments where the fly is not always in full view during the rotation. This may enable the fitting of specialised visual stimulators (e.g. Reiser and Dickinson [2008]; Taylor et al. [2008]) or the use of electrophysiology equipment. Secondly, where wingbeat tracking often requires more time and supervision, cross-correlation gating takes less than 10 minutes for sequence of 1000 frames, meaning gated reconstructions can be available within the hour after an experiment.

4.4.2 Limitations and future improvements

The limitations of the technique presented here are similar to those discussed in the preceding chapter, as x-rays were the probe used in both studies. Rather than adding a contrast agent, inherent differences in refractive index were exploited to achieve sufficient contrast between tissue and air. There is some evidence for soft-tissue differentiation: In some parts of the phase-contrast images acquired with

parameter sets C and D (Fig. 4.5), cuticle appears lighter than muscle or nervous tissue. However, the differences are so faint that automatic segmentation is not possible. As a remedy, contrast enhancement using electron-dense materials, such as iodine or cadmium, has been proposed for *in vivo* synchrotron applications (Westneat et al. [2008]). These would lead to increased absorption contrast. Although the aim of my experiments was to image the fly using phase contrast, reconstructions without phase retrieval (Fig. 4.9) suggest that the fly absorbs a significant proportion of the x-ray flux. The radiation dose absorbed by the fly may damage tissues and lead to a change in behaviour, for example in metabolic or respiratory rate (Socha et al. [2007]). Preliminary investigations indicate that the dose rates absorbed in the whole animal were on the order of between 1 and 5 Gy/s in monochromatic configuration (G. Lovric, personal communication). With the whitebeam setup, similar dose rates were to be expected. These are well below the limits for respiratory anomalies given in (Socha et al. [2007]). Radiation doses nonetheless proved lethal within approximately of 10 seconds. The high contrast-to-noise ratio of all the image data acquired with all of the paradigms and configurations presented here suggests that the flux could be further reduced while maintaining sufficient image quality. Such a reduction of flux could be achieved by introducing further filters to the beam, to reduce lower-energy photons, which contribute above-average to dose, but below-average to image contrast. Another possible improvement is the use of iterative reconstruction algorithms that have been shown to outperform filtered backprojection for undersampled data sets (Andersen and Kak [1984]). Techniques that make use of *a priori* information on the object being imaged, so-called compressive sensing methods, may allow me to reduce scan duration and therefore those without a reduction in image quality (Ritschl et al. [2012]).

Chapter 5

Discussion

The results presented in this thesis provide further insight into the control architecture of fly gaze control, and into the animals neck and flight motor systems. The behavioural experiments described in chapter 2 served to elucidate and analyse the contributions of different sensory pathways to reflex stability and robustness. The two-degree-of-freedom control architecture and the large stability margins of the visually mediated feedback component in the fly gaze stabilization system merit further discussion in the light of previous studies on biological control systems. *In situ* and *in vivo* imaging methods were presented in chapters 3 and 4 of this thesis as promising techniques in the comparative and functional investigation of gaze and flight biomechanics. The second part of this chapter focuses on future applications of the approaches and methods I introduced and how a combination of techniques may advance our studies on reflex control in invertebrates.

5.1 Reflex stability in gaze and flight control

The high marginal stability of head roll responses to forced thorax oscillations is a defining property of the reflex arc as a whole. Assuming all subsystems are linear, a simple low pass filter operation at any stage of the processing chain would increase the marginal stability. Behavioural experiments on their own are not suited to test how individual processing stages contribute to compensatory head roll. Using a combination of behavioural experiments and electrophysio-

logical recordings from a lobula plate tangential cell sensitive to yaw rotations, Warzecha and Egelhaaf [1996] studied the effect of EMD (elementary motion detector) properties, in the early motion vision pathway (Warzecha and Egelhaaf [1989]) on the stability of optomotor behaviour. Warzecha and Egelhaaf [1996] concluded that the nonlinear dependence of EMD responses on retinal slip speed limits the contribution of high frequency components in the feedback loop and thus guarantees stable performance. This result is consistent with large stability margins, as this nonlinear tuning curve limits the bandwidth of the whole sensorimotor pathway, too. Further experiments may be needed to establish the superior performance of EMDs over a simple, linear low-pass filter that could be represented in the motor system, for example. Further evidence may be added based on studies of non-linear contributions at individual processing stages, for example, through the identification of subsystem transfer functions [46].

The trade-off between delay, bandwidth and gain is a generic property of linear feedback control systems and therefore affects flight as well as gaze control. Long response delays and high gain lead to instability if components that are phase shifted by 180 are amplified by the feedback loop. The two-degree-of-freedom control structure is specific to gaze control. In a recent study Elzinga et al. [2012] analysed the sensitivity of the fruit fly yaw rate controller on sensorimotor delays. Force measurements on a robotic model, combined with simulations of the effects of wing forces on yaw rate allowed them to include highly non-linear aerodynamics. Their study reported a similar trade-off between gain and delay as the one I found in the analysis of head roll responses. At the same time it provided evidence for a fundamentally different architecture implemented to control thorax yaw rate than I propose for compensatory head roll (Fig. 5.1). For yaw rate control, the authors proposed a serial combination of visual and mechanosensory inputs where fast haltere signals provide active damping to a slower visual controller.

There is a surprisingly simple explanation for the use of different designs in these two behaviours: In flight control halteres and visual sensors both sense feedback information. Furthermore, while there is a natural null-position for head roll (unless the fly is navigating towards a target or in a tunnel) there is no external reference direction for yaw the controller is a rate controller rather

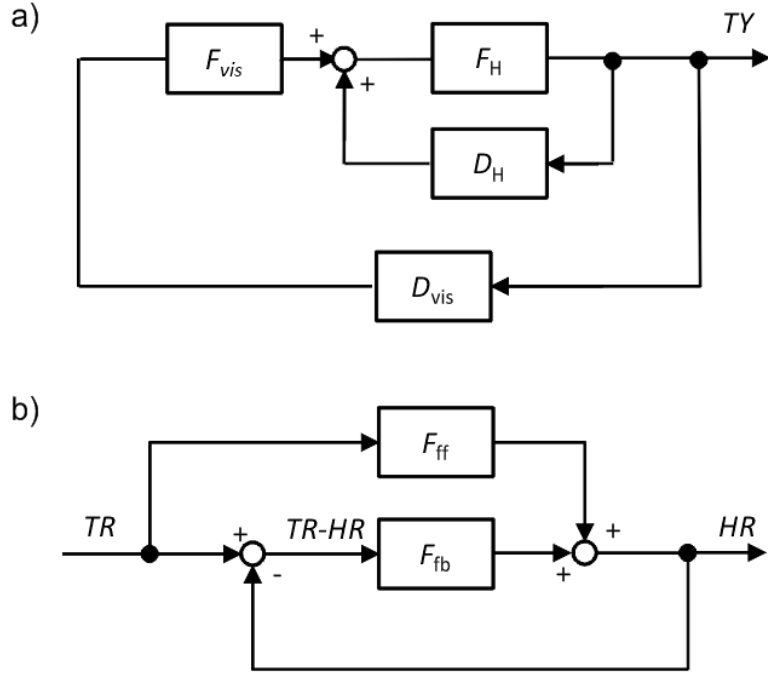


Figure 5.1: Control architectures in fly flight control and gaze control. (a) Thorax yaw rate (TY) equilibrium control is provided by fast active damping. The delay of mechanosensory feedback from the halteres (D_H) is much smaller than the visual feedback delay (D_{vis}). Processing of visual feedback occurs in series with mechanosensory feedback. (b) Compensatory head roll (HR) in response to externally induced thorax roll (TR). The processing and integration of feedback (F_{fb}) and feedforward (F_{ff}) information occurs in a two-degree-of-freedom control architecture. (Fig.5.1(a) adapted and generalised from Elzinga et al. [2012].)

than a controller of an absolute angle. The physical locations of the sensors and the nature of the controlled variable lead to different control architectures. This may shine a new light on questions regarding the principles of multi-sensory integration: The effect of sensor noise and reliability on the weighting of information has been studied in eye movements in mammals (e.g. Green and Angelaki [2010]; Morgan et al. [2008]; Pouget et al. [2002]). However, the weighting may also depend on the different signals carried by these sensors, be it disturbance, reference or error information. As mentioned in the conclusions of chapter 2 (section 2.4), compensatory head roll is only one part of the response of flies undergoing forced thorax oscillations. Aerial disturbances encountered in free flight will elicit equilibrium responses by the flight and the neck motor systems. The behavioural experiments were therefore not truly "closed-loop", as changes in wing kinematics did not result in a change of the visual or inertial stimulus. Vice versa, visual self-motion information employed in flight stabilisation is altered by changes in head position. Head movements have been shown to affect the processing of motion vision in fly lobula plate tangential cells (Kern et al. [2006]). Gaze stabilisation does not only align the principal axes of compound eyes and ocelli with an inertial coordinate system (see also the mode-sensing hypothesis: Krapp et al. [2012]; Taylor and Krapp [2007]), but also reduces the maximal values of rotation-induced optic flow. In technical systems, the design and operation of the controller is often based on a linearized version of the plant around one or multiple reference (equilibrium) states (e.g. Guzzella [2009]; Nelson [1997]). While in technical applications the computations needed to remove the equilibrium offset are almost always performed in silica, stabilising head movements can be seen as a mechanical solution of linearizing a control system.

5.2 Comparison of microCT and synchrotron-based CT

In chapter 3 I argued that laboratory-based micro-focus x-ray scanning is well suited for high throughput comparative studies of anatomical structures (see also

Richards et al. [2012]). I should like to emphasize that such studies are feasible using synchrotron beamlines, too, but at a considerably higher cost. The acquisition of still tomographs using the ultra-fast end station at the Swiss Light Source resulted in a 3D representation of the insect anatomy in situ, without fixation or drying (Schwyn et al., invited, in preparation). Tissue-specific contrast in synchrotron data, however, was low compared to microCT scans of fixed and iodine-stained flies, which may be considered the cost for obtaining naturalistic anatomical representation of an insect free from shrinkage and movement artefacts. Longer scans of unfixed, sacrificed insects using the standard setup endstation at the TOMCAT beamline (Stampanoni et al. [2007]) present a compromise between these two extremes. Compared to the microCT scans presented in chapter 3, tomographic imaging using monochromatic synchrotron radiation at exposure times on the order of 100 ms and scan durations between 1 and 10 minutes enable superior tissue contrast for unstained, untreated samples. Scan duration on such a short timescale significantly mitigates movement artefacts. For studies on arthropod morphology, conventional microCT and modern synchrotron facilities are complementary due to differences in image quality, time required for sample preparation and scan duration, but also cost effectiveness as well as the availability and complexity of the experimental setup.

5.3 A multi-level approach to fly reflex control

While fast *in vivo* imaging methods allow us to gather new insights into the biomechanics of musculo-skeletal systems, many of the open questions in biological flight and gaze control will ultimately be answered by a combination of methods. Two complementary techniques are available to investigate the functional organization of the neck motor system (5.2): (i) Electrophysiological recordings from individual motor neurons (Huston [2008]) are used to study the transformation of sensory stimuli into motor commands. (ii) Electrical stimulation of motor neurons or muscles may be applied to quantify the dynamic relationship between muscular activation patterns and the behavioural responses (Gilbert et al. [1995]). Stimulation parameters such as the waveform of the injected current, its ampli-

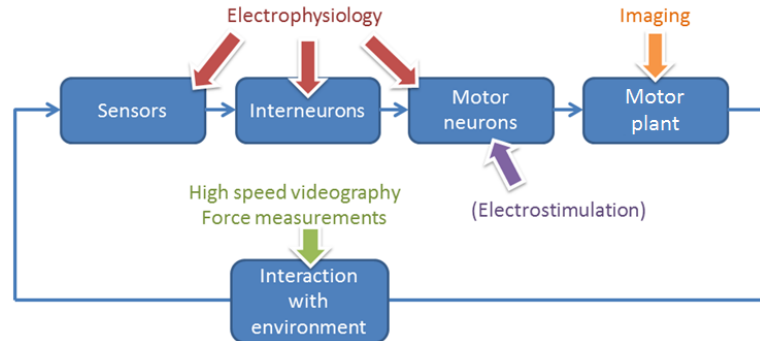


Figure 5.2: Selection of methods available for the investigation of sensorimotor control. Electrophysiology and behaviour experiments have long been pillars of neuroethology research, while imaging techniques are expected to provide information on the biomechanics linking motor commands to behaviour.

tude and frequency may be chosen in a range deduced from intracellular motor neuron recordings. However, electrophysiological methods may only indicate the activation of muscles, not the comprehensive state of the motor system at the time of activation: The pulling planes of individual muscles and the disposition of sclerites modulate the effects of muscle contractions on behaviour, e.g. head movements. The existence of indirect flight and neck muscles indicate that such effects are not only passively present, but actively exploited by flying insects: Indirect muscles fine-tune the configuration of the motor system in subtle ways such as adjusting the pulling planes of other direct muscles or change the stiffness of a structure. The observation of the muscular apparatus *in vivo* using non-invasive imaging techniques may help to formulate and test hypotheses on the specific function of individual muscles in a given motor system. A combination of imaging with simultaneous sensory stimulation and microelectrode recordings would be a powerful approach in sensorimotor control research, in general, and will be attempted in future studies. The use of electrostimulation in combination with gated imaging paradigms could potentially improve the consistency of the behaviour and therefore the quality and accuracy of reconstructed images. However, metal electrodes may cause beam hardening artefacts (Hsieh [2003]) and increase the complexity of the experimental technique not least due to the

challenge of maintaining precise electrode placement for stable recordings.

The imaging techniques established in this thesis are expected to close an important gap in the repertoire of methods available for sensorimotor research: the observation of the displacement of muscles represents the link from the electrical activation of motor neurons and muscles to the resulting behaviour.

5.4 Potential for translational research

Some examples of insect-inspired, translational research projects were presented in chapter 1. The research presented in this thesis, while not directly geared towards technical applications, may be of methodological interest in several areas: *In vivo* phase contrast imaging is moving towards clinical implementation (Stampanoni et al. [2011]) and small-animal imaging will continue to provide challenging proof-of-concept applications. The imaging of insect motor systems is expected to offer inspiration for micro-mechanical systems in an age of continued miniaturization. And a range of general control principles, derived from studies on flies, have already been applied in bio-mimetic and bio-inspired designs, particularly in the development of autonomous robots (Beyeler et al. [2009]; Franceschini et al. [1992]; Hyslop and Humbert [2010]). The two-degree-of-freedom control architecture that underlies fly gaze control is a common configuration for simple, intuitive control systems (e.g. Sugje and Yoshikawa [1986]). Kerhuel et al. [2007] designed an aerial robot that stabilizes both gaze and heading based on the visual fixation reflex (VFR) and on the vestibulo-ocular reflex (VOR) present in mammals, a concept previously envisioned by Yamaguchi and Yamasaki [1994]. The combination of high-bandwidth feedforward compensation and slow, but accurate feedback control, as well as the mechanical conditioning of input signals before electronic processing occurs, may improve the performance in a range of control applications.

5.5 Conclusions

I calculated the frequency responses of sensory-motor pathways for a major component of fly gaze control, the head roll response. Behavioural recordings were used to derive the pathway bandwidths, response delays and stability margins of this reflex under the assumption of linearity. A two-degree-of-freedom control architecture and the reduction of the visual latency through ocellar information enables flies to achieve accurate, robust and stable control in the face of uncertainty, and inevitable processing delays. Despite the findings presented here, a series of fundamental questions regarding biological control design remain open. How does a series of non-linear components give rise to a nearly linear behaviour? In which way do individual sensors and processing stages contribute to stable responses? Answers to these questions require comprehensive studies of individual components of the control systems involved. I have approached specific aspects of this rather complex subject, by advancing the methodology to investigate the functional anatomic structure of fly motor systems. Two imaging techniques hold great promise to study motor systems involved in sensorimotor control: I found that computed tomography with microfocus x-ray tubes enables imaging of multiple samples per hour where sufficient contrast can be achieved using iodine, a contrast agent that is less dangerous and easier to use than many other conventional histology stains. I also advanced synchrotron x-ray microtomography, which is well-suited to 3D imaging of behaving insects. The unique capabilities of this technique were demonstrated using microtomographic timeseries of the thoraces of flies during tethered flight. The spatio-temporal resolution of this technique exceeds that of previously published work by two orders of magnitude and holds promise for a range of imaging applications. A combination of novel imaging techniques, behavioural experiments and electrophysiology is expected to reveal further general principles of biological sensorimotor control.

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