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CONTRIBUTIONS TOWARDS UNDERSTANDING THE HEART BEAT AND CARDIAC MYOCYTE ACTION POTENTIAL OF THE FRUIT FLY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF SUSSEX IN APPLICATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

March 2021
“IT ALWAYS SEEMS IMPOSSIBLE UNTIL IT'S DONE”.

NELSON MANDELA
DECLARATION

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration with others except where specifically indicated in the text. No part of this dissertation has been submitted to any other university in application for a higher degree.

31/March/2021
ABSTRACT

Studying the heart during normal function and disease is crucial to our understanding of human health and to aspects of animal biology from energy consumption and metabolism to lifespan. The heart of the fruit fly, *Drosophila melanogaster*, has emerged as a model system for studying heart function and particularly the linkage between genes, function, and physiology. Yet many aspects of *D. melanogaster* adult heart physiology remain unclear.

Many of the insights from study of the *D. melanogaster* adult heart are obtained using a semi-intact preparation with artificial haemolymph, in which the beating heart tube is exposed by removing a fly’s head and thorax, and ventral abdomen. We used both in vivo electrophysiology and videography to investigate the heart-beat and cardiac myocyte action potential (AP) of the beating heart in the semi-intact preparation. This allowed the quantification of the heart-beat and AP, showing that there is considerable variation of both even in wild type flies. We show that variation in the heart-beat’s rate is, in part, due to transitions between the different types of heart-beat that the adult heart can generate and, in part, an artefact of the dissection itself. We also identify and describe at least two different patterns of cardiac myocyte AP in wild type flies: a single and a double-action potential. Together, these reveal an unappreciated variability in the adult heart and cardiac myocyte AP that has not previously been recognised or quantified, with implications of the uncritical use of the semi-intact preparation.
At the commencement of the work towards this thesis, the major ion channels supporting the cardiac myocyte AP were unknown, though some channel-types had been identified. Using the semi-intact preparation, we investigated the voltage-gated ion channels and resulting currents that produce the cardiac myocyte AP using a range of Ca\(^{2+}\) and Na\(^{+}\) voltage-gated ion channel mutants, including classical mutations and RNAi lines. Using both videography and electrophysiology, we identify the voltage-gated α1 subunits Ca\(^{2+}\) encoded by the *cacophony* (*cac*) gene. Our evidence comes from changes in the frequency of the heart-beat in RNAi lines coupled with pharmacological evidence from application of the α1 subunits voltage-gated Ca\(^{2+}\) channel blocker, verapamil. We also show that forskolin, which upregulates the protein kinase A pathway, moderately increases heart-beat’s frequency. Despite the effects of *cacophony* RNAi on the heart beat’s frequency, cardiac myocyte APs were unaffected. We discuss how these results can be reconciled.

Finally, we investigated the communication between cardiac myocytes through gap junctions, which is essential for propagation of the cardiac myocyte AP throughout the heart. We used carbenoxolone, a known connexin gap junction blocker, to block *D. melanogaster* cardiac myocyte gap junctions. This block was concentration dependent with an EC\(_{50}\) of 0.071 mM. Using intracellular recordings, we show that carbenoxolone is capable of increasing cardiac myocyte input resistance, consistent with blocking gap junctions. We also identify subthreshold electrical events in cardiac myocytes that are consistent with gap-junctional inputs from neighbouring cells. This emphasises the key role of gap junctions in the adult *D. melanogaster* heart and provides some of the first evidence that carbenoxolone is a blocker of innexin gap junctions.
Taken together, the work in this thesis provides key insights into both the molecular and physiological mechanisms underpinning the adult *D. melanogaster* heart-beat, and the tools used by researchers to study these mechanisms.
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And thanks to me and to my stubbornness that push me to start new scary and sometimes amazing adventures.
THESIS CONTRIBUTION

All the experiments in Chapter 2, Chapter 3 and Chapter 4 were performed by Laura Corona. The Shiny app for analysis of heart-beat’s rates from videos was developed by Mr Mikkel Roald-Arbol. Statistical modelling was performed by Dr A. Sofia David Fernandes.
COVID-19 STATEMENT

The global pandemic has had a significant effect on the work in this thesis. This has been due to several factors. Firstly, the closure of laboratories during the first wave of the pandemic interrupted electrophysiology experiments for many months. These experiments were at a crucial stage because developing the electrophysiological skills needed to perform recordings in vivo from the beating heart of the fruit fly, *Drosophila melanogaster*, is extremely challenging. Secondly, numerous fly stocks that were en route to the laboratory or that were in quarantine were lost because only a limited number of stocks could be maintained during the lockdown due to restricted access to the buildings. Additionally, all crosses that were established and ready at the time of the lockdown were lost. Once the laboratory reopened fly stocks had to be reordered and crosses re-established. Thirdly, limited access to the laboratory even after it reopened due to strict limits on the numbers of individuals within the space meant that the number of electrophysiology experiments that could be performed was severely curtailed. Even when laboratory space could be accessed, the working hours were limited, limiting the number of experiments that could be performed. Finally, almost all supervision had to transfer to online only. These factors caused considerable delays and difficulties to the project that have severely affected the number and quality of the experiments presented here.
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CHAPTER ONE

1 GENERAL INTRODUCTION

1.1 HUMAN HEART DISEASE

The heart is a core component of the cardiovascular system responsible for pumping blood to ensure that tissues receive the oxygenated blood needed to support aerobic respiration. Any impairment in the heart’s performance affecting its ability to pump blood can affect human health with potentially fatal consequences. Several types of heart disease are recognised: arrhythmia, an abnormality in the heart beat rhythm; atherosclerosis, a hardening of the arteries; cardiomyopathy, a weakening or hardening of the heart muscle; congenital heart defects, inherited heart abnormalities; infections, irregularities caused by infection; and coronary artery disease, a restriction of blood flow through the arteries. Numerous factors can affect the heart’s performance from congenital heart defects that develop before birth to cardiac arrhythmias that can affect the heart throughout life to coronary heart disease that typically affects adults (an estimated 2.3 million in the UK) and may lead to heart attacks and heart failure (BHF, 2021). Consequently, the study of heart function and disfunction is essential because insights gained into heart function at the molecular, cellular, and organ/system levels can lead to the development and discovery of new treatments, such as new drugs,
thereby improving human health. Heart disease is not a new phenomenon but has been found even in ancient Egyptians including Pharaoh Merenptah (Allam et al., 2009).

Many diseases of the human heart involve changes in the rhythm of the heart, and the electrical events that generate the heart-beat. These well-studied set of diseases may involve alteration in voltage-gated ion channels and gap junctions that are important to the production and transmission of the cardiac action potential (reviewed in Grant, 2009). Indeed, approximately 50% of heart failure is a consequence of impaired electrical and contractile function producing ventricular arrhythmia. Heart failure is often characterised by a remodelling of ion channels, gap junctions and intracellular calcium. The voltage-gated ion channels expressed by each single cardiac myocyte contributes to the generation of the cardiac action potential. These electrical events are the basis for excitation-contraction coupling through calcium-induced and calcium release and mechanical contraction. Modifications of ion channels, called channelopathies, are responsible for action potential defects that alter the electrical and mechanical heart function (Garcia-Elias and Benito, 2018). Channelopathies predispose the subject to arrhythmias such as Long QT Syndrome (LQTS), Short QT Syndrome (SQTS) and Brugada Syndrome (BrS). The mutation in these diseases is located in specific ion channels’ genes, other occur in genes encoding a protein with an ion channel.

Long QT Syndrome is characterised by a prolongation of the QT-interval in the surface electrocardiogram (ECG) that causes ventricular arrhythmias. The prolongation of the QT interval is correlated with a decrease or increase in depolarization currents late in the cardiac cycle (Moss and Kass, 2005). The mutations associated with Long QT
syndrome arise within three specific ion channels genes KCNQ1 in LQT1, KCNH2 in LQT2 and SCN5A in LQT3 account for 75% of all cases (Ackerman et al., 2011). The KCNH2 gene encodes the voltage-gated K\(^+\) channel involved in the rapid component of the delayed rectifier current. The SCN5A gene encodes the \(\alpha\) subunit of the sodium channel (George et al., 1995). Mutations in SCN5A cause functional alterations in the voltage-gated Na\(^+\) channel. Short QT Syndrome is associated with a loss of function in the L-type Ca\(^{2+}\) channel (Antzelevitch et al., 2007), and mutation in the voltage-gated K\(^+\) channel genes KCNQ1 (Bellocq et al., 2004), KCNH2 (Brugada R. et al., 2004) and KCNJ2 (Priori, 2004). These mutations shorten the effective refractory period (Rahm et al., 2018). Brugada syndrome characterised by an increased risk of developing ventricular tachyarrhythmias and sudden cardiac death (Brugada and Brugada, 1992). Mutations of the SCN5A gene are present in the majority of Brugada syndrome cases.

Gap junctions can also be the cause of heart disease because within the vertebrate heart, gap junctions play a key role in the propagation of the cardiac action potential (reviewed in Rohr, 2004). Early transmission electron microscope studies of the vertebrate heart showed that it was composed of individual muscle cells, and was not a syncytium (Sjöstrand and Andersson, 1954). Subsequent evidence of cable properties, the spread of radioactive K\(^+\) ions in heart tissue, and the presence of low resistance pathways all led to the conclusion that there were gap junctions between cardiomyocytes (Weidmann, 1970; Weidmann and Hodgkin, 1966). Many years of subsequent experimental and computational modelling studies have concluded that gap junctions are the major pathway for signal propagation across the vertebrate heart (reviewed in Rohr, 2004). The disruption of gap junctions in the heart is associated with
heart disease, including arrhythmia of the atria produced by atrial fibrillation in which gap junctions are implicated both in the initiation of the condition and its persistence (Allessie, 2002; Polontchouk et al., 2001; Severs, 2004; Wijffels et al., 1995).

The importance of the heart to human health means that each year many studies are conducted into heart function and disfunction using vertebrate experimental models, particularly mice. For example, in the United Kingdom over 135,000 procedures were performed for cardiovascular research mainly on mice, rats and fish (Statistics of scientific procedures on living animals, 2014). Despite its value to human health, a drawback of this research is the large numbers of vertebrates used. This also limits the types of experiments that can be performed because of the regulations surrounding in vivo work in vertebrates (Animals (Scientific Procedures) Act, 1986). Many studies use isolated cardiac myocytes derived from heart tissue to study the molecular mechanisms underlying heart function (e.g. O’Connell et al., 2007) although it may be difficult to relate findings from the study of isolated cells to function at the organ/system level.

Much of the biology of cardiac myocytes, is common to both vertebrates and invertebrates. Consequently, invertebrate heart preparations offer the possibility of studying the molecular basis of cardiac myocyte function in vivo as well as in isolated cells producing findings with relevance to human health (Piazza and Wessells, 2011; Wolf and Rockman, 2011). The fruit fly (Drosophila melanogaster) heart offers the possibility of combining a well-established and extensive set of molecular and genetic tools with high-quality electrophysiology to reduce/replace the use of vertebrate heart tissue in some aspects of heart research.
1.2 *Drosophila melanogaster* as a Model System

The fruit fly, *Drosophila melanogaster*, has become a model for investigating the molecular mechanisms and genetic basis of numerous aspects of animal biology. The taxonomic classification and phylogeny of the fruit fly are well established (e.g. O’Grady and DeSalle, 2018), with the following classification:

Domain: *Eukarya*

Kingdom: *Animalia*

Phylum: *Arthropoda*

Class: *Insecta*

Order: *Diptera*

Family: *Drosophilidae*

Genus: *Drosophila* ("dew lover")

Species: *melanogaster* ("dark gut")

*D. melanogaster* has been used since the work of Woodsworth, Castle and Morgan in the early 1900s to study inheritance, and the genetic basis of morphological and physiological traits such as the white-eyed mutation used to study sex limited inheritance (reviewed in Beller and Oliver, 2006; Morgan, 1910). Its small size and short generation time combined with an array of techniques for genetic and cellular manipulation (e.g. Brand and Perrimon, 1993; Kennerdell and Carthew, 1998; Yeh et al.,
continue to make it a major model organism in which to study biological mechanisms at the molecular and cellular levels. In particular, the Gal4-UAS system has been a major tool for visualising and manipulating gene expression (Brand and Perrimon, 1993). Also, the temperature sensitivity of Gal4-UAS system coupled with interference RNA constructs have allowed conditional knockdowns of gene expression, allowing interrogation of gene expression (e.g. Bellés, 2009; Heigwer et al., 2018; McGuire et al., 2004; Suzuki, 1970). Moreover, the similarities between the human (Homo sapiens) and D. melanogaster genomes (Adams et al., 2000; Lander et al. 2001; Venter et al. 2001) and the conserved genetic basis of traits make fruit flies an powerful model organism.

1.3 LIFE CYCLE OF DROSOPHILA MELANOGASTER

The cycle life of Drosophila melanogaster is typically considered optimal for laboratory genetics when flies are bred at 25°C (Ashburner, 1989). The fly shows complete metamorphosis, which distinguishes holometabolous insects, going through discrete stages from the egg, to the larval form, to the pupa, and then emerging as an adult fly (Greenspan, 2004). Once that female is fertilized the sperm is stored in a specific structure called receptaculum seminis where it fertilizes hundreds of eggs to be laid over several days. At the optimal temperature, the embryo develops in approximately 21 hours, after which the larva hatches and begins to feed. In the 1st instar (L1) feeding only occurs at the surface of the medium but after moulting the 2nd instar (L2) begins to burrow. During the L2 and L3 (3rd instar) the larvae continue to burrow into the food leaving only the posterior spiracle exposed (Sokal et al., 1960). During larval
development the larval activity changes the pH of the change and microflora (Bridges and Darby, 1933; Gordon et al., 1941).

After egg laying the feeding continues until the wandering stage begins after the cessation of feeding. The larva leaves the food and moves off to search for a suitable site for pupariation. Many organs degenerate or are extensively remodelled during the pupal stages and become adult organs (metamorphosis). The adult fly emerges from the pupal case with unexpanded wings and long, thin, and unpigmented body. The wings expand around 1 hours after eclosion, and the full pigmentation appears within 2-3 hours.

1.4 THE ANATOMY OF THE DROSOPHILA MELANOGASTER HEART AND ACCESSORY STRUCTURES

The D. melanogaster circulatory system consists of a dorsal vessel (heart), a haemocoel or body cavity and different accessory organs and septa, though other cell types and structures are also associated including nephrocytes, the fat body, and the corpora allata (reviewed in Chapman et al., 2013). The heart is a peristaltic pump that circulates the haemolymph through the haemocoel (reviewed in Miller, 1997) but is not involved in the supply of oxygen to the organs and tissues or removal of CO₂, which is done by the tracheal system (reviewed in Chapman et al., 2013). The dorsal vessel is composed of an anterior part, the aorta, that passes through the thorax and ends in the head, and a posterior part, the pulsatile heart. The dorsal vessel is formed from a single layer of muscle cells with striated fibres and scattered lateral nuclei (Rotstein and Paululat, 2016)(Figure 1.1). The aorta, which extends from the anterior heart, has a thin
membranous wall and lacks the striations associated with muscle. The posterior part of the heart is closed and is attached both sixth tergite and the rectal sac.

The *D. melanogaster* heart is also divided into four chambers and extends from along the dorsal body wall of the abdomen. There are three intracardiac valves within the heart that divide it into distinct chambers and block the backflow of haemolymph (Lehmacher *et al.*, 2012; Tang *et al.*, 2014; Zeitouni *et al.*, 2007). The first chamber (conical chamber) is funnelform and occupies the first and second abdominal segments. It joins with the aorta at the anterior end. The second and the third chambers are subcylindrical and occupy the third and fourth segments, respectively. The fourth chamber occupies the fifth and (part of) the sixth segment. Within each chamber are paired ostia, that are thought to act as entrances and passive valves to control the passage of the haemolymph (Miller, 1997; Rizki, 1978; Rotstein and Paululat, 2016). In the adult flies there are five pairs of ostia (Figure 1.1).

The heart is held in place by suspensory fibrils, a rudimentary dorsal diaphragm and several pairs of alary muscles (Miller, 1997; Wigglesworth, 1974). In adult flies there are only four pairs of alary muscles, which originate from single myoblasts (Boukhatmi *et al.*, 2012; Schaub *et al.*, 2015, 2012). Pericardial cells, also called nephrocytes, are located close to the heart tube to filtrate the bypassing haemolymph before it enters the heart and is pumped around the haemocoel (Ivy *et al.*, 2015; Tutor *et al.*, 2014; Weavers *et al.*, 2009; Zhang *et al.*, 2013; Zhuang *et al.*, 2009). The cardiac matrix contributes to holding the pericardial cells in position and to filtration. There are also ventral longitudinal muscles located beneath the heart. These muscles are absent in larvae (Rotstein and
Paululat, 2016; Schaub et al., 2015; Shah et al., 2011), and contribute to the dorsal diaphragm (along with the cardiac matrix) that divides the pericardial sinus (where the heart tube is located) from the abdominal body cavity to improve haemolymph flow (Rotstein and Paululat, 2016). The corpora allata (singular: corpus allatum) is formed by a single spherical tissue mass situated near the dorsal wall of the aorta (Pfeiffer 1939). The corpora allata have a range of endocrine functions including the production of juvenile hormone, which plays key roles during development and in maturation in adult insects. Adipose tissue, known as the fat body, is a mass or sheet of fat cells that fills much of the space within the body not occupied by other organs. The abdomen contains the largest amount of fat body and in general the adipose tissue occurs in area where no muscle attachments are presents (Miller, 1950)(Figure 1.1).

Accessory pulsatile organs are situated in the lateral angles of the scutellum and in the front of the head between the base of the antennae. A small band of muscle fibres forms the two scutellar organs and extends across the angle where the scutellum is connected within the ridge that runs downs the base of the wings. Their likely function is drawing the blood into the hemocoel of the scutellum from the region at the base of the wings (Thomsen 1938).

The nephrocytes appear as discrete large with an ovoid shape and colourless, they can be uni- or binucleate and are located near the dorsal diaphragm in the abdomen and the anterior end of the ventriculus in the thorax. The nephrocytes in the abdomen, also called pericardial cells, appear from the first to the sixth segment as a row of twenty to twenty-five ovoid cells on each side of the heart. The abdominal pericardial cells have a
uninucleate and are larger than their thoracic counterparts. They are connected to the heart and each other by being attached to the lower side of the membranous dorsal diaphragm. Approximately 10 thoracic nephrocytes are found on the anterolateral sides of the cardia and are connected within the lower wall of the trachea connected with the first spiracle and the muscle fibre. The thoracic cells appear as a finely reticulate or spongy cytoplasm with a birefringent peripheral membrane and two spherical nuclei in subcentral position (Miller, 1950). The thoracic cells are incorporated in a delicate membrane that has the function to take them in place. The rule of this cells is not clear but they have an intermediary function in metabolism and excretion. In others insect have been observed that they rale up colloidal particles from the blood (Wigglesworth, 1939)(Figure 1-1).
1.5 **Human and *Drosophila melanogaster* Heart**

In humans the heart pumps the blood through a closed circulatory system composed of blood vessels with distinct structures; arteries, arterioles, veins, venules and capillaries. The four-chambered heart containing the two atria and the two ventricles is the major
pump, separating the oxygenated and deoxygenated blood supplies. The functional contractile unit of the heart is called a sarcomere, which is the structure between two Z lines. Within the sarcomere is possible distinguish the I Band, which is formed solely by actin filaments. Internal to the I Band I is the A Band, which appears darker in colour and is formed by overlapping actin and myosin filaments. Inside this area is collocated the H Band. The H Band is a small area that appear lighter than the A Band and contains only myosin filaments. At the centre of the H Band is the M Line formed by proteins that interconnect the myosin filaments. Myosin is a bipolar filament and interact in a specific area called head of myosin that bins to the actin protein. The interaction between these proteins reduce the length of the sarcomere causing the muscular contractions. Myosin is composed of two heavy chains of 200 KDa and four light chains of 20 KDa. Myosin is a bipolar filament and interact in a specific area called head of myosin that bins to the actin protein. The interaction between these proteins reduces the sarcomere length causing the muscular contractions. During heart contraction the A Band remains a constant length but the I Band reduces in length, reducing the distance between the Z lines (reviewed in Martonosi, 2000).

The contraction of muscles in insects is broadly thought to operate through identical mechanisms to those governing the contraction of vertebrate muscles. Insect muscle is composed by a number of long fibres with multinucleate cells. As in vertebrates, the unit of contraction is called sarcomere and the major proteins are myosin and actin. The myofibrils are embedded in the sarcomere and extend from the end of one fibre to the other. The insect circulatory system incorporates a simple tubular with a contractile myocardial cells where the cells are mononucleate and striated longitudinal and circular
myofibrils. Like the vertebrate heart, this system is activated myogenically without the neuronal input but it can be influenced by nerves that innervate the heart (Chapman and Chapman, 1998). The contraction of insect muscles has been studied in *Drosophila* and other insects (e.g. *Lethocerus*). Muscle contraction in insects is broadly thought to operate via an identical mechanism to those governing the contraction of vertebrate muscle.

### 1.6 The *Drosophila melanogaster* Heart-Beat

The first studies of the insect heart beat were begun in the second part of the 1900s, but these studies focussed on the movements of the heart and the impact of pharmacology (reviewed by Miller, 1997). Studies of the *D. melanogaster* heart in the larval, pupal, and adult stages have more recently allowed exploration of the molecular details of the heart-beat. Semi-intact preparations in which large portions of the fly are removed to expose the heart coupled with videography techniques have been crucial in these developments (e.g. Dulcis and Levine, 2003; Ocorr et al., 2007a). The cardiac heart-beat in adult flies is formed by two different anterograde and retrograde beats, with potentially different contraction rates (Sláma, 2012; Wasserthal, 2007). These two different heart-beats are generated by two different type pacemakers located in the conical chamber for the retrograde beat and in the terminal chamber for the anterograde beat (Dowse et al., 1995; Dulcis and Levine, 2005; Johnson *et al.*, 2002; Rizki, 1978). The phenomenon of two different pacemakers is known as cardiac reversal in other open circulatory system and it produces a change in the direction of the blood circulation.
It is possible to recognize two different types of synchronous anterograde beat called anterograde 1, in which there is a contraction of the conical chamber, and anterograde 2, in which there is a dilatation of the conical chamber (Sláma, 2012). The anterograde and retrograde heart beats may switch rapidly with an interval of several seconds (Wasserthal, 2007). The switching between the anterograde and retrograde heart beats is thought to be under the control of neural signals to the heart, which can initiate the retrograde heart beat (Dulcis and Levine, 2005).

1.7 THE DROSOPHILA MELANOGASTER HEART AS A MODEL SYSTEM

The general advantages of D. melanogaster as a model system also apply more specifically to the study of the heart. The ease with which flies can be maintained in a laboratory, their short generation time, and the ability to manipulate gene expression through a variety of tools to elucidate molecular and cellular mechanisms all contribute to the value of D. melanogaster as a model system to study the heart. There are several pronounced differences between the mammalian heart and that of the fruit fly including the position of the heart, which is dorsal in contrast to the ventral position of the mammalian heart, and the structure, which is tubular with a single chamber in contrast to the four chambered structure of the mammalian heart. Despite the differences in the size and structure of the heart, numerous similarities have been discovered between fly hearts and human hearts, especially in terms of genetics and development (e.g. Azpiazu and Frasch, 1993; Bodmer et al., 2005, 1990; Bodmer and Frasch, 1999; Bodmer and Venkatesh, 1998; Cripps and Olson, 2002; Harvey, 1996; Zaffran and Frasch, 2002). The
*D. melanogaster* heart expresses a similar set of genes to the human heart (Akasaka *et al.*, 2006; Bodmer *et al.*, 2005; Bodmer and Frasch, 1999; Bodmer and Venkatesh, 1998) and both produce co-ordinated contraction of the cardiac myocytes through the spread of an action potential that leads to contraction of cardiac myocytes (Fleckenstein, 1983; Reuter, 1967). The *D. melanogaster* heart has been extensively studied in terms of development and morphology using a variety of techniques including scanning and transmission electron microscopy, expression of genetically encoded fluorescent proteins, analysis of gene expression, and direct manipulation of gene expression (*e.g.* White *et al.* 1992; Curtis *et al.* 1999; Lehmacher *et al.* 2012; Rotstein and Paululat 2016).

One major breakthrough in understanding both the development and function of the heart was the discovery of *Tinman* in *D. melanogaster*, which was shown to be a key gene in the developmental formation of the heart (Bodmer, 1993). Subsequently, the homologous gene, Nkx2.5, was discovered in vertebrates and shown to specify key stages of heart formation. Null mutations of both *Tinman* and Nkx2.5 prevent the formation of a functioning heart (Alsan and Schultheiss, 2002; Azpiazu and Frasch, 1993; Bodmer, 1993; Lee and Frasch, 2005; Reifers *et al.*, 2000; Schott, 1998; Shishido *et al.*, 1997). Both genes are homeobox transcription factors important in the differentiation of the mesoderm into cardiac tissue, and the differentiation and proliferation of cardiac precursor cells. Subsequently, these genes have also been shown to play key roles in the function of the heart in adults, and leads to congenital heart defects including heart beat’s rate and variability (Harrington *et al.*, 2017; Li *et al.*, 2015).
Another key point of similarity is the progressive reduction in the heart’s performance with age in both fruit flies and humans, which can be a source of heart disease such as cardiac arrhythmia and can lead to increased mortality (Piazza and Wessells, 2011). Indeed, several studies have demonstrated the potential of studies on the *D. melanogaster* aging adult heart to investigate the cardiac aging more generally (e.g. Nishimura et al., 2011; Ocorr et al., 2007a). Some genes are implicated in heart function that with aging or mutation can cause cardiac disfunction such as heart arrythmia. For example, the KCNQ gene encodes a low-threshold voltage-gated $K^+$ channel that encodes an outward current (Brown and Adams, 1980). Mutations in these channels cause loss or decrease of channel function, reduced cardiac repolarization and prolonged action potential that causes an increase of the risk of the early after-depolarization (Jentsch 2000; Robbins 2001; Ocorr et al. 2007). Mutations in one KCNQ gene (KCNQ1) that is expressed in the mammalian heart are a major cause of long QT syndrome (Wang et al., 1996), which produces changes in the electrocardiogram that cause arrhythmia and can lead to sudden death (reviewed in Shah et al., 2019). Mutations in *D. melanogaster KCNQ* also cause age-dependent arrhythmia in the heart beat (Ocorr et al., 2007b).
1.8 **THE ACTION POTENTIAL OF CARDIAC MYOCYTES IN THE *DROSOPHILA MELANOGASTER* HEART**

A key part of understanding the *D. melanogaster* heart-beat and the development of the heart as a model system is being able to study the cardiac myocyte action potential, which allows electrical events to be linked to gene expression and molecular events. In some species the cardiac action potential has been studied electrophysiologically. McCann (1963) used a highly reduced *in situ* preparation of adult *Telea polyphemus* moths to study the cardiac action potential from the exposed heart using microelectrodes. He observed two different types of action potentials. One, typically found in the caudal region, had a unitary spike but the second, recorded in the cephalic region, showed a double spike (peak) (McCann 1963). A double peak in the action potential has been described in the nerves and muscles of other insect species. For example, McCann and Boettiger (1961) used electrophysiology to study the muscles of several different species: *Sacophada bullata*, a flesh-fly; *Vespula diabolique*, a wasp; *Pissodes strobi*, a pine weevil; *Tenebrio molitor*, a meal-worm; and *Nazara viridula*, a stink-bug. An *in situ* electrode inserted directly into the flight muscle of the insect was used. Recordings in beetle flight muscle show double peaks in the action potentials occasionally, though they also described a multiple peaks in some cases caused by the stimulus intensity (McCann and Boettiger, 1961). Ikeda (1964) described the electrical activity of the fibrillar muscle of the bumblebees *Bombus pennsylvanious, Bombus impatiens* and *Bombus terrarius*. Using an electrode inserted in the thoracic sternum he studied the electrical response in nerves and in the longitudinal muscle. In some
recordings, double peaks appeared after the application of repetitive stimuli (Ikeda and Boettiger 1965). A study in house flies (Musca domestica) using in vivo and in vitro experimental approaches application of pyrethroids or DDT evoked double peaks in the action potential (Adams and Miller 1980).

A first step towards the study of the cardiac myocyte action potential was the development by Papaefthmious and Theophilidis (2001) of a semi-intact preparation in which the electrical events of the heart can be recorded, and pharmacological agents can be applied directly to the heart. The semi-intact preparation is highly reduced, lacking the head, ventral thorax and ventral abdomen, which removes the entire brain and ventral nerve cord. Although Papaefthmious and Theophilidis (2001) only performed extracellular recordings, subsequently several authors have performed intracellular recordings from single cardiac myocytes including Dulcis and Levine (2005), Ocorr et al. (2007, 2017) and Magny et al. (2013). These studies and others reported a wide range of action potential amplitudes from ~15 mV (Dulcis and Levine, 2005; Lalevee et al., 2006), to ~50 mV (K. Ocorr et al., 2017). However, to our knowledge, there has been no systematic investigation of the semi-intact preparation and the extent to which it affects the electrical events being recorded.

Several studies have now explored aspects of the electrical event that underpin the cardiac myocyte action potential. These studies have tended to identify genes whose products are involved in the cardiac myocyte action potential based upon their impact on the heart-beat’s frequency or regularity, and only investigating the electrical activity of single cardiac myocytes once an effect on the heart-beat has been established. In
almost all cases, the impact upon the electrical phenotype of the cardiac myocytes is assessed through changes in the size and shape of the action potential (e.g. Dulcis and Levine, 2005; Lalevée et al., 2006; Magny et al., 2013; Ocorr et al., 2017; Ocorr et al., 2007b). For example, Lalevée et al. (2006) investigated the impact of a mutation in the Ork1 gene, which encodes the ORK1 outwardly-rectifying two pore domain K+ channel. Such channels are implicated in setting baseline excitability, and may contribute to leak currents in electrically excitable cells (O’Connell et al., 2002; Goldstein et al., 2001). Cardiac specific RNAi knockdown of Ork1 increased the heart-beat’s frequency, attributed to changing the duration of the action potential. Overexpression of Ork1 silenced the heart abolishing action potentials entirely. However, fully understanding the function of ORK1 channels within an electrical system requires knowledge of many of the electrical components that together produce the cardiac myocyte action potential. Another notable exception is the effect of mutations in KCNQ channels that cause cardiac arrhythmia in aging D. melanogaster, which has been demonstrated not only through changes in the heart-beat’s rate but also in the cardiac myocyte action potential (Ocorr et al., 2007b). Such comprehensive approaches are also becoming more common, with recent studies combining both the behaviour of the heart through videography and electrophysiological techniques (e.g. Ocorr et al., 2017).

One major limitation to the utility of the D. melanogaster heart, however, is that the complete set of ion channels and the genes that encode them that produce currents that are involved in the cardiac myocyte action potential have not yet been fully identified (Zhu et al., 2017). At the outset of this thesis is the voltage-gated ion channel that sustains the major inward current that generates the cardiac myocyte action
potential had not been identified, limiting the interpretation of the contributions of all other gene products to the electrical phenotype of the cardiac myocytes. Pharmacological evidence from the larval *D. melanogaster* heart (Gu and Singh, 1995) suggested that the major inward current of the cardiac myocyte action potential is likely to be an L-type voltage-gated Ca\(^{2+}\) channel, and unlikely to be voltage-gated Na\(^+\) channel due to the slowing and stopping of the heart-beat by verapamil and the lack of an effect of tetrodotoxin, respectively. However, the larval heart is extensively remodelled during pupation, and this may include changes in the expression of voltage-gated ion channels (reviewed in Medioni *et al*., 2009). During our research, a study by Limpitikul *et al.* (2018) was published that showed the major inward current generating the cardiac myocyte action potential is the Ca-\(\alpha_{1D}\), which encodes an L-type voltage-gated Ca\(^{2+}\) channel. They used a variety of techniques including fluorescent *in situ* hybridisation (FISH), recording of inward currents in isolated cardiac myocytes, and analysis of the heart-beat’s rate. This clearly fills a major gap within the understanding of the adult *D. melanogaster* heart. Consequently, we had to alter our approach and re-orientate one chapter of the thesis (Chapter 3) to accommodate the findings of Limpitikul *et al.*, (2018).

The understanding of the contributions of voltage-gated ion channels to the electrical membrane properties of the cardiac myocytes requires not just understanding the contributions to the action potential shape and frequency but also the basic biophysical properties of these channels. This is challenging in a tonically spiking cardiac myocyte. Limpitikul *et al.* (2018) isolated single cardiac myocytes to record the properties of the Ca-\(\alpha_{1D}\) channels. They saw substantial changes in the structure of the
isolated cardiac myocytes but were able to record currents. However, there is still a need for a preparation that allows the electrical properties of cardiac myocyte to be recorded without the potentially damaging processes of dissociation and isolation.

1.9 Thesis Overview

The importance of the *Drosophila melanogaster* adult heart as a model system for understanding the function and disease of the human heart is limited by incomplete understanding of the electrical events that produce the heart-beat. The aim of this thesis is to investigate the heart-beat and the electrical events that underpin it using a combination of approaches including videography, electrophysiology, genetics, and statistical modelling. The impact of pharmacological agents, genetic mutants and gene knockdowns, and individual differences will be considered.

In Chapter 2, the semi-intact preparation, so crucial for investigating the heart-beat in *D. melanogaster*, will be investigated. Numerous genetic and developments’ similarities are presents in both fly and human hearts such as ion channels and contractile proteins. The *D. melanogaster* heart expresses similar genes to the human heart and produces the heart-beat through what is assumed to be a similar mechanism. However, major gaps remain in our understanding of the membrane excitably of cardiac myocytes in *D. melanogaster*. Additionally, individual differences in the functioning of the heart will be investigated. Indeed, although *D. melanogaster* has been a model system for investigating biological mechanisms at the genetic, molecular and cellular levels since the early 1900s. Individual differences have received little attention despite
their importance to human health and disease. Here we describe the heart-beat and cardiac myocyte action potential of *D. melanogaster* using the semi-intact preparation. We studied both the consistency and the variability of the heart-beat, measuring the average frequency and coefficient of variation in short- and longer-term experiments. The experiments suggest that heart-beat’s frequency and variability is consistent, indicating that the semi-intact preparation permits stable recordings of the heart-beat and electrophysiological recordings of the cardiac myocyte action potential. Importantly, both the average heart-beat’s frequency and variability are consistent in each individual, pointing to individual differences. Temperature influences the heart-beat, a decreasing or increasing in temperature causes a decrease or increase in the average heart-beat’s frequency that is specific to individual flies. Using intracellular recordings, we describe the size, shape, and frequency of the action potentials in a single cardiac myocyte. Our experiments reveal both single spikes, and some double peaked action potentials that have not previously been documented in *D. melanogaster* cardiac myocytes. These results demonstrate that the semi-intact preparation is suitable for analysis of the heart-beat and cardiac myocyte action potential of *D. melanogaster* and emphasise the importance of considering individual and cellular differences.

In Chapter 3, the voltage-gated ion channels that are responsible for the major inward current that generates the cardiac myocyte action potential are investigated using a variety of pharmacological and genetic approaches. Many heart diseases in humans are linked (causally or through correlation) with the function of voltage-gated ion channels. One difficulty of studying these diseases is the number of components within the mammalian cardiac myocyte action potential. *D. melanogaster* has the
potential to be a cheaper and easier model in which to study the cardiac myocyte action potential, and to understand the mechanisms of disease because the *D. melanogaster* heart has many similarities in terms of development and physiology with the mammalian heart. In this chapter, we attempt to identify the voltage-gated ion channel type that is responsible for the major inward current that generates the cardiac myocyte action potential in *D. melanogaster*, showing it to be an L-type Ca\(^{2+}\) current. We used substances such as barium chloride, verapamil and forskolin to downregulate and upregulate the heart-beat’s frequency. We also combined these substances with mutations in genes known to produce inward currents, and interference RNA knockdowns of other genes. The substitution of Ba\(^{2+}\) ions for Ca\(^{2+}\) ions in the artificial haemolymph caused a reduction of the heart-beat. The application of different concentrations of verapamil, an L-type Ca\(^{2+}\) channel blocker, demonstrated a correlation between dose and response. At low concentrations, the heart-beat slows whereas at high concentrations a full stop of the heart-beat is produced. Mutations in two specific genes *Cacophony* and *Paralytic* that produce voltage-gated ion channels that support inward currents but that do not encode L-type Ca\(^{2+}\) channels have no effect on the heart-beat’s frequency and little effect on the cardiac myocyte action potential. We used RNAi knockdown of two genes that had been implicated in producing the inward current of the cardiac myocyte action potential, *Caα1-D* and *Caα1-T*, both to verify previous work and to extend it by assessing developmental effects. However, this experiment failed to reproduce the effect of RNAi knockdown of *Ca-α1D* or *Ca-α1T* at 25°C, though the reason for this is unclear. The application of forskolin, which is known to affect molecular pathways capable of
modifying L-type Ca\textsuperscript{2+} channels, caused a substantial increase in heart-beat’s frequency in contrast to previously published results.

The focus of Chapter 4 is on gap junctions between cardiac myocytes in the \textit{D. melanogaster} heart. The chapter investigates gap junctions both to understand their role in the electrical events that generate the heart-beat, and to provide a tool for studying the cardiac myocyte electrical membrane in the intact heart. Intercellular transmission of electrical signals through gap junctions is thought to be important for spreading electrical activity within tissues such as the heart, ensuring co-ordinated cardiac myocyte contraction. Pharmacological blockers are known for vertebrate gap junctions formed from connexins, permitting their study in the vertebrate heart. However, insect gap junctions are formed from innexins, meaning that the efficacy of pharmacological blockers of vertebrate gap junctions is unclear. We determine the effect of carbenoxolone, a vertebrate gap junctional blocker, on the adult heart of the fruit fly, \textit{D. melanogaster}. Intracellular electrophysiological recordings from cardiac myocytes show depolarising inputs capable of initiating action potentials, whilst paired recordings show synchronised action potentials in neighbouring myocytes. Both features are consistent with gap junctional communication between myocytes. Carbenoxolone has a concentration-dependent effect, reducing the heart-beat’s frequency at low concentrations and stopping the heart completely at higher concentrations. The EC_{50} of carbenoxolone is 0.08 mM. Intracellular electrophysiological recordings show that carbenoxolone abolishes spontaneous cardiac myocyte action potentials. Carbenoxolone also increases the input resistance of the cardiac myocytes. Based upon this evidence, we argue that carbenoxolone is a blocker of gap junctions in the adult \textit{D.}
melanogaster heart and may function more broadly as a blocker of invertebrate gap junctions formed from innexins. This will open new avenues of research on invertebrate gap junctions, and on the properties of the cardiac myocyte electrical membrane in the intact heart.
CHAPTER TWO

2 IMPLICATIONS OF A REDUCED PREPARATION FOR STUDYING THE DROSOPHILA MELANOGASTER HEART BEAT AND ACTION POTENTIAL

2.1 INTRODUCTION

Over the last approximately 30 years the fruit fly, Drosophila melanogaster, has become a model for investigating the mechanisms and genetic basis of heart-beat generation and the cardiac myocyte action potential. D. melanogaster has been used since the work of Charles W. Woodsworth, William E. Castle and Thomas Hunt Morgan in the early 1900s to study inheritance, and the genetic basis of morphological and physiological traits such as the white-eyed mutation used to study sex limited inheritance (reviewed in Beller and Oliver, 2006; Morgan, 1910). It’s small size and short generation time combined with an array of techniques for genetic and cellular manipulation (e.g. Brand and Perrimon, 1993; Kennerdell and Carthew, 1998; Yeh et al., 1995) continue to make it a major model organism in which to study biological mechanisms at the molecular and cellular levels. Moreover, the similarities between the human (Homo sapiens) and D. melanogaster genomes (Lander et al., 2001; Adams et al., 2000; Venter et al., 2001) and the conserved genetic basis of traits make fruit flies a powerful model organism.
The general advantages of *D. melanogaster* as a model system also apply more specifically to the study of the heart. For example, numerous similarities have been discovered between fly and human hearts in terms of genetics and development, as well as in terms of physiological components such as ion channels and contractile proteins (e.g. Azpiazu and Frasch, 1993; Bodmer *et al.*, 2005, 1990; Bodmer and Frasch, 1999; Bodmer and Venkatesh, 1998; Cripps and Olson, 2002; Harvey, 1996; Zaffran and Frasch, 2002). The *D. melanogaster* heart expresses a similar set of genes to the human heart (Akasaka *et al.*, 2006; Bodmer *et al.*, 2005; Bodmer and Frasch, 1999; Bodmer and Venkatesh, 1998; Sláma, 2012), and both produce co-ordinated contraction of the cardiac myocytes through a similar mechanism - the depolarization of myocardial cells - and both are affected by the same cardioactive drugs verapamil, 1,4-dihydropyridine and nifedipine (Fleckenstein, 1983; Reuter, 1967). The heart has been studied at each of the post-embryonic developmental stages of the fruit fly life cycle - larval, pupal, early adult and late adult – both in terms of the morphology and physiology using a variety of techniques including scanning and transmission electron microscopy, expression of genetically encoded fluorescent proteins, optical recordings and electrocardiogram (ECG) recordings (Curtis *et al.*, 1999; Lehmacher *et al.*, 2012; Rotstein and Paululat, 2016; White *et al.*, 1992). Optical recording of heart-beat in the pupal heart was performed using intact animals because the larva is nearly transparent that permits the heart to be visible. The beating was recorded, amplified and digitalized with an optical data acquisition system (Johnson *et al.*, 2001). The electrocardiogram (ECG) was used for the first time by Rizki for recording the heart-beat in the pupa using a sharp tungsten electrode inserted laterally into the abdomen near the caudal end of the heart, which is
the pacemaker site, and near the anterior end of the vassal vessel (Rizki, 1978). The performance and rhythmicity of the heart has been studied using both optical recording and ECG in pupae and adult flies (Curtis et al., 1999; Kuo et al., 2014; Papaefthmiou and Theophilidis, 2001; Rizki, 1978; Wessells and Bodmer, 2004). Other studies investigated electrical heart function using intracellular recordings inserted into the contractile posterior heart in larvae (Lavelée et al., 2006). In semi-intact adult flies microelectrodes have been inserted into cardiac myocytes in the heart tube and electrical signals (action potentials) were recorded and amplified (Papaefthmiou and Theophilidis, 2001).

Another key point of similarity is the progressive reduction in the heart’s performance with age in both fruit flies and humans, which can be a source of heart disease such as cardiac arrhythmia and can lead to increased mortality (reviewed Piazza and Wessells 2011). Indeed, several studies have demonstrated the potential of studies on the D. melanogaster aging adult heart to investigate the cardiac aging more generally (e.g. Nishimura et al., 2011; Ocorr et al., 2007b). Some genes are implicated in heart function that with aging or mutation can cause cardiac disfunction such as heart arrhythmia. The KCNQ1 gene encodes for a specific subunit of a K⁺ channel that regulates the slower repolarizing current (I_{Ks}). Mutations in this gene causes loss or decrease of channel function, reduced cardiac repolarization and prolonged action potential that causes an increase of the risk of the early after-depolarization (Ocorr et al., 2007b; Robbins, 2001; Jentsch, 2000). The hERG gene forms heteromultimeric K⁺ channels, mutations in this gene cause heart repolarization disorders and arrhythmia (Sanguinetti and Tristani-Firouzi, 2006). The Tinman gene is important during the heart development and because it can control the regulation of dSUR, a subunit of the ATP sensitive K⁺ (K_{ATP})
channel complex that protects the heart from ischemia and hypoxia, and with aging a loss expression causes increased failures of the heart-beat (Akasaka et al., 2006). These studies along with several others demonstrates the complex genetic mechanisms that underpin the impact of aging on the performance of the heart-beat (Nishimura et al., 2011).

The first studies on the insect heart-beat were done in the second part of the 1900s (reviewed by Miller, 1997), but all the knowledge was general and only recently have we started to understand more of the physiological and molecular details of the heart-beat. One of the major advances has been the use of video to record the movements of the heart. For example, Dulcis and Levine (2005) used high speed video to record the heart-beat in a semi-intact preparation. They, along with others, showed that the cardiac heart-beat in adult flies is formed by two different and alternative phases call anterograde and retrograde beats, anterograde and retrograde beats show different contraction rate. Moreover, they described periodical changes described as systole and diastole of the heart. Also, they observed that forward-oriented anterograde heart-beat was faster than the retrograde heart-beat (Dulcis and Levine, 2005). In addition, hey described the heart-beat at all stages of the fly, and demonstrated that the heart-beat changes in pupa were faster than in larvae and adult stages (Dulcis and Levine, 2005). In Drosophila, two different pacemakers are recognised, one located in the conical chamber which produces a anterograde heart-beat, and the second is located in the terminal chamber of the heart tube producing the retrograde (Dowse et al., 1995; Dulcis and Levine, 2003; Rizki, 1978; Johnson et al., 2002) The phenomenon of two different pacemaker is known as cardiac reversal in other open circulatory systems,
and it produces a change in the blood circulation direction (Jones, 1977). The anterograde and the retrograde heart-beats switch in an interval of several seconds (Wasserthal, 2007). Recently, Sláma (2012) studied the heart-beat in immobile and decapitate flies comparing the optoelectronic recordings with other electrocardiographic methods such as thermography and strange-gauge posimetric recording, demonstrating the rules and function of both anterograde to and retrograde heart-beat. It is possible to recognize two different types of synchronous anterograde-beat called anterograde 1, in which there is a contraction of the conical chamber, and anterograde 2, in which there is a dilatation of the conical chamber (Sláma, 2012). At the moment, relatively little is known about membrane excitably of cardiac myocytes in *Drosophila*. The study of the action potential has been performed in adult flies where the abdomen was exposed and all the internal organs and fat were removed (Papaefthmiou and Theophilidis, 2001). Using borosilicate glass microelectrodes inserted into the conical chamber action potentials were recorded with amplitudes between 50 and 100 mV (Papaefthmiou and Theophilidis, 2001). More recent intracellular recordings in semi-intact adult flies using microelectrodes have showed that the action potential amplitude is between 15 and 50 mV (Dulcis and Levine, 2005; Ocorr *et al.*, 2007b). A range of studies have used genetic mutations of genes encoding ion channels to identify components of the cardiac myocyte action potential but these have tended to focus on the rate of action potentials (*e.g.* Ocorr *et al.*, 2007b), and the currents these channels produce are largely unknown (but see Limpitikul *et al.*, 2018).

One of the key factors influencing the heart-beat is temperature. The influence of the temperature has been studied in larvae, pupae and adult flies (Andersen *et al.*, 2001).
All of these studies confirmed that the heart-beat increases at higher temperatures and decreases at lower temperatures. Zhu et al. (2016) studied the impact of temperature on the larval *D. melanogaster* heart by expressing a channel rhodopsin, a light-sensitive protein, enabling them to use light to activate the heart. This showed a clear correlation in third instar larvae between temperature, the Ca^{2+} concentration within the cardiac myocytes, and the heart-beat (Zhu et al., 2016). The impact of temperature on the pupal heart-beat has also been studied by Jennings et al. (2009), who showed that across a range from 20°C to 37°C the heart rate increases with temperature. In adults, decreasing temperature in a range between 20°C to 0.2°C lowers the heart rate. At 20°C the heart-beat is ~150 beats per minute but it drops to approximately 10 heart-beats per minute at 0.2°C. Anderson et al. (2015) used two different preparations one in which the flies’ heads were removed, and another where the abdomen was cut away from the head and the thorax (Andersen et al., 2015). They demonstrated a clear change in the relationship between heart-beat’s rate and temperature at 10°C, with the heart-beat’s rate reduction being more pronounced. They also showed that the heart-beat’s rate is slower in more reduced preparations. Here, we study the heart-beat and cardiac myocyte action potential of *D. melanogaster* using a semi-intact preparation. The female flies are anaesthetized, all the internal organs, the fat, the head and the abdomen are removed, leaving the heart tube intact and pulsing and the haemolymph was substituted by artificial haemolymph saline solution to permit recording the heart-beat and myocyte action potentials. After the dissection, all the samples were living in artificial saline solution. The reduced preparation allows the heart to beat for a long time, more than 1 hour, and to remove
neural inputs into the heart (Dulcis and Levine, 2005), nevertheless there may be problems with the heart-beat due to the loss of neural inputs and release of neuromodulators.

The scope of this work is to investigate the variation in the D. melanogaster heart-beat and the cardiac myocyte action potential using a semi-intact preparation. The reduced preparation showed a consistent heart-beat but also the heart is exposed, and this likely causes additional variability in the heart-beat and additional pull from abdominal muscles. This variation in the heart-beat has an impact on the cardiac myocyte action potentials. The experiments demonstrated clearly that it is possible to record single and double action potentials, however, there are also mixtures of both types of action potentials that may not be found in the in vivo beating heart.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Experiments were performed using adult female Drosophila melanogaster (Meigen, 1830) Wild Type (WT) Oregon Red flies, which were obtained from the laboratory of Prof. Claudio Alonso at the School of Life Sciences, University of Sussex, UK. Larvae were incubated for 1 to 2 weeks at 25°C, and adults were collected for experiments 1 to 2 weeks post-eclosion. Flies were fed once a week on a diet consisting of yeast and molasses.
2.2.2 Semi-Intact Preparation

The female flies, 1-2 weeks old, were anaesthetized with Flynap® anaesthetic kit (Carolina Biological Supply Company, Burlington, USA) for ~10 seconds. Individual flies were immobilised in a thin layer of petroleum jelly on a Sylgard® 184 (Dow Corning, Midland, MI, USA) coated petri dish with their dorsal surface down (Papaefthmiou and Theophilidis, 2001). The head, ventral thorax and the ventral abdominal cuticle were removed with Vannas Spring-curved 2.5 mm scissors (Fine Science Tools, Heidelberg, Germany), the still-beating heart was exposed by removing the ventral surface of the abdomen. The gut and reproductive organs were removed, as was the fat around the heart tube (Vogler and Ocorr, 2009). The haemolymph was substituted by an artificial haemolymph 1x AH containing: 10x AHL (1.08 M NaCl, 0.08 M MgCl2, 1.05 M KCl, 0.02 M CaCl2 dehydrate, 0.01 M NaH2PO4), 0.01 M sucrose, 0.1 M trehalose dehydrate, 250 mM NaHCO3 and 120 μM cytochalasin D (Sigma Aldrich, Gillingham, UK) (Magny et al., 2013).

2.2.3 Temperature Control

Temperature control experiments were performed using artificial haemolymph (1xAH) over a range of temperatures between 5°C and 30°C. The temperature of the haemolymph was controlled by a CL-100 bipolar Liquid Cooling System (LCS-a; Warner Instruments LLC, Holliston, MA, USA) and a dual in-line heater/cooler (Model SC-20; Warner Instruments LLC, Holliston, MA, USA). The haemolymph was continually measured throughout the measurements using a thermoprobe attached to the SC-20
heater/cooler. Recordings at a given temperature lasted 40 seconds at each temperature. In each experiment the heart-beat’s rate was recorded at room temperature and then at two other temperatures from 5.5, 11, 15.5, 21, 25.5, and 29°C before a second recording at room temperature.

2.2.4 Video Acquisition

The in vivo beating heart was filmed using an Retiga Electro CCD camera (Digital Imaging Systems, Bourne, UK) attached to a Zeiss Discovery V8 Stereo Achromat S 1.25X FWD 50 mm, lens 8X (Carl Zeiss Limited, Cambridge, UK). A region of interest (164 x 441 pixels) was centred over the conical chamber and the proximal region of the heart tube using Ocular Scientific Imagine Acquisition Software Advanced 2.0 (Teledyne Photometrics, Tucson, Arizona, USA). Videos of six minutes in duration were captured at 20-30 frames per second.

2.2.5 Video Analysis

Each six-minute video was divided into six videos, one minute each, and analysed using Fiji Image J software (http://fiji.sc/). The mean luminosity of the image was used to identify single heart-beats. To ensure reproducibility, the region of interest for each video was recorded. We used batch processing because the images were TIFF stacks. The resulting data files were analysed using a graphical user interface within an R Shiny-app (Chang et al., 2020). Within the app, the user specifies the data files to be processed.
and the parameters to be extracted. The data was low-passed using a Butterworth filter and smoothed using spline interpolation using smooth.spline package. A custom algorithm was then used to identify local maxima. To ensure that the peaks identified represent heart-beats, only peaks with a maximum slope exceeding two standard deviations beyond the baseline were included. Additionally, duplicates were removed by identifying adjacent shared troughs. Peaks that remained unresolved through this process were inspected, the original video assessed, and manually edited if necessary. Once all the heart-beats were identified, the heart-beat’s rate was calculated, and the data exported for each individual heart.

2.2.6 Electrophysiology Recording

Artificial haemolymph for electrophysiological recordings was the same as that for video recordings except for the addition of 120 µM cytochalasin D (Sigma Aldrich, Gillingham, UK) (Magny et al., 2013). In vivo intracellular microelectrode recordings from D. melanogaster cardiomyocytes were obtained using a borosilicate microelectrodes. A P97 puller (Sutter Instruments, Novato, USA) was used to pull microelectrodes with resistances between 80 and 120 MΩ from 10 cm borosilicate glass capillaries 1.0 mm (outer diameter) x 0.58 mm (inner diameter) (Harvard Apparatus, Cambourne, Cambridge, UK). Electrodes were filled with 3 M potassium chloride (KCl) and mounted in a custom-made holder attached to a Leitz micromanipulator for penetration of single cardiac myocytes. Electrical signals were amplified and filtered with using a npi SEC-05X amplifier (npi Electronic, Tamm, Germany; www.npielectonic.com) or a npi BA-01X
amplifier (npi Electronic, Tamm, Germany; www.npielectric.com). In some cases, paired recordings were made from two different myocytes: one in the conical chamber and the other from the proximal segment of the heart tube. Action potential recordings were digitalised at 5 kHz using a CED micro1401 mark II 500 KHZ 16-bit ADC (Cambridge Electronic Design Ltd, Cambridge, UK) analogue-to-digital conversion interface and Spike2 software for subsequent off-line analysis. All recordings were performed at room temperature (20-24°C).

2.2.7 Electrophysiological Analysis

Electrophysiological recordings were analysed offline using Spike 2 software. Only those recordings in which the action potentials exceeded 30 mV in amplitude, and the minimum potential between action potentials was below -30 mV were analysed further. Three measures were taken from each recording: the height, width, and frequency of the action potentials. The height was measured between the peak of and the minimum potential within the 10 ms preceding the action potential. The width was measured at half the full height of the action potential and is referred to as the half-width. The average action potential frequency was calculated as the number of action potentials within one minute.
2.2.8 Statistical Analysis

Statistical analysis was performed using R (version 3.5.1). For analysing how different pulses differed from one another and how were they influenced by other movements present during the recording and by time, we used data acquired from videos (48 flies, 6 recordings each). Mean heart-beat’s rate was analysed using linear mixed models and coefficient of variation (CV) was analysed using generalized linear mixed models, with family Gamma (link=log). For both cases, the package ‘lmer’ was used and the type of pulse and/or other movements present and/or time were taken as fixed factors, while the flies’ ID was taken as a random factor. Repeatability of heart-beat’s rate and CV were analysed using the package ‘rptR’. Furthermore, we analysed if the mean heart-beat’s rate and CV on the first minute could predict the heart-beat’s rate and CV on the last (sixth) minute, using linear or generalized regression (family Gamma, link=log) respectively. For analysing how temperature influenced heart-beat’s rate, a linear mixed model (‘lmer’ package) was used, and the model including Temperature as a fixed factor and (Temperature|ID) as a random factor was considered the best model (lowest AIC). The temperature coefficient (Q10) was calculated per pair of recordings, within the same individual, at different temperatures, as \((R2/R1)^{(10/(T2-T1)},\) where R’s correspond to the heart-beat’s rates in each recording and T’s the corresponding temperature. The reaction norm (how heart beat ratio changes with the difference in temperature) was analysed using a generalized mixed effects model, with (Temperature_difference|ID) as a random factor and with Gamma (link=log) distribution. Lastly, action potential
height and width, from electrophysiology data, were analysed using linear mixed effects models.

2.3 RESULTS

2.3.1 The Adult *Drosophila Melanogaster* Heart Produces Two Distinct Heart Beats

We filmed the beat of the adult *D. melanogaster* heart in the semi-intact preparation over a six minute period (N = 48). Each video was divided into six, 30 second sections each separated by 30 seconds for analysis. The adult heart is known to produce three distinct heart-beats: two types of anterograde heart-beat, and a retrograde heart-beat (Sláma, 2010). Both anterograde heart-beats are synchronic but differ in whether haemolymph is pumped into the head and thorax (anterograde 1), or the abdomen (anterograde 2). We analysed the videos to determine the type of heart-beat produced by the heart in the semi-intact preparation. Both anterograde heart-beats were visible in videos, distinguished by the movements of the conical chamber (Figure 2-1). Anterograde 1 heart-beats were the most common being observed in 64.58% (186 of 288) 30 second videos (Figure 2-1A), whereas Anterograde 2 heart-beats were observed in 35.42% (102 of 288) of videos (Figure 2-1B). No retrograde heart-beats were observed. Only one pulse type was visible during the 30 second videos in 78.13% (225 of 288; 156 anterograde 1, 69 anterograde 2) of the videos. In all cases, only one type of anterograde pulse was observed from a single individual. Anterograde 1 pulses were observed in 31
individuals, whereas anterograde 2 pulses were observed in 17 individuals. Accompanying the anterograde 1 or 2 heart-beats, some videos showed pulses in a region of the heart posterior to the conical chamber that were not synchronised with heart-beat visible in the conical chamber (Figure 2-1C). Such unsynchronised posterior pulses were present in 16.13% of hearts producing anterograde 1 pulses (30 of 186), and 32.35% of hearts producing anterograde 2 pulses (33 of 102). In addition, a small number of recordings showed lateral movements of the conical chamber walls, likely due to the contraction of abdominal wall muscles. These movements were more often observed in hearts producing anterograde 1 pulses (65 of 186) than those producing anterograde 2 pulses (26 of 102) (Figure 2-1D).
Figure 2-1 The semi-intact D. melanogaster adult heart produces two distinct heart beats.

Example frames and traces showing each type of movement: (A) anterograde 1 pulses; (B) anterograde 2 pulses; (C) anterograde 1 pulse with distinct posterior pulses; and (D) anterograde 1 pulses with lateral drag. Arrows indicate movements of the heart. Heart-beats contractions are indicated with dashed lines.
2.3.2 Hearts Producing the Anterior 1 and Anterior 2 Heart-Beats do not Differ in Frequency

The anterograde 1 and anterograde 2 heart-beat may differ in their rate and variability. Semi-intact hearts showed a considerable range of heart-beat’s frequencies within the 30 second videos from 0.29 to 1.71 beats per second for anterograde 1 pulses, and from 0.44 to 1.99 beats per second for anterograde 2 pulses. The mean heart-beat’s frequency of anterograde 1 pulses was 1.00 (± 0.30; mean ± standard deviation unless otherwise stated) beats per second, similar to the mean heart-beat’s frequency of anterograde 2 pulses (1.06 ± 0.32 beats per second) (Figure 2-2A). The distribution of heart-beat’s frequencies was not significantly different between the anterograde 1 and 2 pulse types, when accounting for the repeated measures from single individuals (Linear Mixed Model; lmer (heart-beat_frequency ~ pulse_type + (1|individual)); t = 0.70, p = 0.48).

The variability in the heart-beats measured as the coefficient of variation (CV) produced by the anterograde 1 and 2 pulse types had a gamma distribution (Figure 2-2B). The CV of the anterograde 1 pulses was 0.25 ± 0.20, similar to that of anterograde 2 pulses (0.23 ± 0.13). The distribution of heart-beat CV was not significantly different between the anterograde 1 and 2 pulse types, when accounting for the repeated measures from single individuals (Generalized Linear Mixed Model; glmer (heart-beat_CV ~ pulses_type + (1|individual), family = gamma(link=log)); t = 0.04, p = 0.97).
Figure 2-2. The average heart beat’s rate and variability of anterograde 1 and anterograde 2 pulses.

(A) The distribution of the mean heart-beat’s rate for anterograde 1 (green) and anterograde 2 (beige) pulse types. (B) The distribution of the coefficient of variation of heart-beats for anterograde 1 (green) and anterograde 2 (beige) pulse types. The mean values of anterograde 1 and anterograde 2 pulses are indicated by pale green and pale brown dashed lines, respectively.
2.3.3 Unsynchronised Posterior Movements do not Affect the Heart-Beat’s Frequency

Movements of the heart posterior to and not necessarily synchronised to those of the conical chamber could influence the frequency of the anterograde 1 and anterograde 2 pulses and may do so differently. There was no differential effect of the posterior movements on the heart-beat’s frequency produced by the two different anterograde pulses (Linear Mixed Model; lmer (heart-beat_frequency ~ pulse_type*posterior_pulses + (1|individual)); t = 0.68, p = 0.50) (Figure 2-3A). There was also no differential effect upon CV of the anterograde 1 and anterograde 2 pulses (Generalised Linear Mixed Model; glmer (heart-beat_CV ~ pulse_type*posterior_pulses + (1|individual), family = gamma(link=log)); t = 0.43, p = 0.66) (Figure 2-3B). Moreover, there was no overall effect of the presence of posterior pulses on the heart-beat’s frequency (Linear Regression; lmer (heart-beat_frequency ~ posterior_pulses + (1|individual)); t = -0.75, p = 0.46) (Figure 2-3A). There was also no overall effect of the presence of posterior pulses on the CV of the heart-beat (Generalised Linear Mixed Model; glmer (heart beat_CV ~ posterior_pulses + (1|individual), family = gamma(link=log)); t = -0.06, p = 0.95) (Figure 2-3B).
Figure 2.3. The heart-beat’s frequency and coefficient of variation (CV) of anterograde 1 and anterograde 2 pulses are unaffected by the presence of posterior movements.

(A) The heart-beat’s frequency of anterograde 1 (dark green) and anterograde 2 pulses (dark brown) are unaffected by the presence of posterior movements (pale green and pale brown, respectively). (B) The CV of the heart-beats of anterograde 1 (dark green) and anterograde 2 pulses (dark brown) are unaffected by the presence of posterior movements (pale green and pale brown, respectively). Bars show the mean, error bars show the standard error of the mean (SEM). For details of the model see the main text.
2.3.4 Lateral Movement of the Conical Chamber does not Affect the Average Frequency of Anterior 1 and Anterior 2 Heart-Beats but does Differentially Affect the Variability

The lateral movements of conical chamber could also influence the frequency of the anterograde 1 and anterograde 2 pulses and may do so differently. However, there was no differential effect of the lateral movements on the heart-beat’s frequency produced by the two different anterograde pulses (Linear Mixed Model; lmer(heart-beat_frequecy ~ pulse_type*lateral_movement + (1|individual)); t = 1.87, p = 0.06) (Figure 2-4A). There was, however, a differential effect upon CV of the anterograde 1 and anterograde 2 pulses (Generalised Linear Mixed Model; glmer(heart beat_CV ~ lateral_movement + (1|individual), family = gamma(link=log)); t = 2.37, p = 0.02) (Figure 2-4B). There was no overall effect of the presence of lateral movements on the heart-beat’s frequency (Linear Regression; lmer(heart-beat_frequency ~ lateral_movement + (1|individual)); t =-1.34, p = 0.18) (Figure 2-4A). There was, however, no overall effect of the presence of lateral movements on the CV of the heart-beat (Generalised Linear Mixed Model; glmer(heart-beat_CV ~ lateral_movement + (1|individual), family = gamma(link=log)); t = 5.80, p = 0.99) (Figure 2-4B).
Figure 2-4. The effect of lateral drag on the heart-beat’s frequency and coefficient of variation (CV) of anterograde 1 and anterograde 2 pulses.

(A) The heart-beat’s frequency of anterograde 1 (dark green) and anterograde 2 pulses (dark brown) are unaffected by the presence of lateral drag (pale green and pale brown, respectively). (B) The CV of the heart-beats of anterograde 1 (dark green) and anterograde 2 pulses (dark brown) are unaffected by the presence of lateral drag (pale green and pale brown, respectively). Bars show the mean, error bars show the standard error of the mean (SEM). For details of the model see the main text.
2.3.5 Stability of the Average Heart-Beat’s Frequency

Although the frequency of the heart-beat is unaffected by the type (anterograde 1 or 2), or the presence of posterior or lateral movement, the heart-beat may change over time. To test for this possibility, we compared the average heart-beat’s frequency over the six minutes of our recordings. The average heart-beat’s frequency did not change during the six minutes (Linear Mixed Model; lmer (heart-beat_frequency ~ time + (1|individual)); t = -1.04, p = 0.29) (Figure 2-5A). We also tested whether the coefficient of variation (CV) changed during the six minutes. There was a significant change in the CV over the six minutes of our recordings (Generalised Linear Mixed Model; glmer (CV ~ time + (1|individual), family = gamma (link = log)); t = -11.78, p = 0.048) (Figure 2-5B). To test whether the heart-beat’s frequency changed over a longer time, we extended our recordings to 18 minutes in 10 hearts. Over the 18 minute period, there was no significant change in the heart-beat’s frequency (Linear Mixed Model; lmer (heart-beat_frequency ~ time + (1|individual)); t = -1.45, p = 0.149) (Figure 2-5C). There was also no significant change in the heart-beat CV during the 18 minute recordings (Generalised Linear Mixed Model; glmer (CV ~ time + (1|individual), family = gamma (link = log)); t = -7.42, p = 0.79) (Figure 2-5D).
2.3.6 Individual Hearts have Repeatable Heart-Beat’s Frequencies and Variability

The steady heart-beat’s frequency coupled with a single heart-beat type in each individual raised the possibility that individuals may have a characteristic heart-beat’s frequency that is consistent over time. To test this possibility, we calculated the repeatability of an individual’s heart-beat’s frequency over the six minute recording period (Figure 2-6). The heart-beat’s frequency of individual flies was repeatable over the six minutes ($R = 0.83$ (Confidence Interval: 0.74 to 0.88), (Heart beat_frequency ~}
This repeatability also occurred over 18 minutes ($R = 0.66$ (Confidence Interval: 0.34 to 0.81), (Heart-beat_frequency ~ (1|individual)), $p < 0.00001$). This repeatability also extended to the CV of the heart-beat over six minutes ($R = 0.69$ (Confidence Interval: 0.57 to 0.78), (Heart-beat_CV ~ (1|individual)), $p < 0.00001$) (Figure 2-6B), and over 18 minutes ($R = 0.80$ (Confidence Interval: 0.55 to 0.90), (Heart-beat_CV ~ (1|individual)), $p < 0.00001$). Additionally, the heart-beat’s frequency of an individual fly in the final minute of the recording could be predicted by the heart-beat’s frequency during the first minute of the recording (Linear Regression; $\text{lm (heart-beat_frequency_minute6 ~ heart-beat_frequency_minute1); } t = 7.00, p < 0.00001$). Likewise, the heart-beat CV of an individual fly in the final minute of the recording could be predicted by the heart-beat CV during the first minute of the recording (Generalised Linear Regression; $\text{glm (heart-beat_CV_minute6 ~ heart-beat_CV_minute1); } t = 5.37, p < 0.00001$).
Figure 2-6 The heart-beat’s frequency and coefficient of variation (CV) are predictable.

(A) The heart-beat’s frequency during the sixth minute of recordings is predictable from the heart-beat’s frequency during the first minute. (B) The heart-beat CV during the sixth minute of recordings is predictable from the heart-beat’s frequency during the first minute. Black line indicates the model fit to the data; the grey region indicates the 95% confidence interval.

2.3.7 The Effect of Temperature on the Heart-Beat’s Rate Differs Among Individuals

The differences in the heart-beat’s rate of individual adult *D. melanogaster* may also extend to their responses to changes in temperature. To determine whether individuals differ in their responses to temperature, we recorded the heart-beat’s frequency of hearts at a range of temperatures from 6 °C to 29 °C. The heart-beat’s rate of 29 individuals were recorded with 3-6 observations per individual to produce 123 observations in total. There was a significant linear relationship between heart-beat’s rate (beats per minute) and temperature; heart-beat’s rate increasing at higher
temperatures (Figure 2-7). The best fit model selected using the AIC (Akaike Information Criterion) incorporated both temperature and the individual response to temperature (Linear Mixed Model; lmer (Heart-beat_rate ~ Temperature + (Temperature | individual)). Models that did not incorporate the individual response to temperature had a higher AIC showing that individual hearts differ in their response to temperature.

We calculated the Q10 of the heart-beat’s rate for each pair of temperatures from each individual. The Q10 value quantifies the change heart-beat’s rate for each 10 °C increase in temperature. There was a broad range Q10 values for the heart-beat’s rates of individuals (Figure 2-8), though the majority of values were close to 2 (2.22 ± 1.94, median ± interquartile range) producing a highly skewed distribution. There were significant outliers beyond the 95th percentile (9.82). The ratio of the heart-beat’s rate at the higher and lower temperatures was larger the greater the temperature difference over which it was calculated (Figure 2-9). However, individuals responded to changes in temperature significantly differently (Generalised Linear Mixed Model; glmer (Heart-beat_rate ~ Temperature_difference + (Temperature_difference | individual), family = gamma (link = log)). Models that did not incorporate the individual response to temperature had a higher AIC showing that individual hearts differ in their response to temperature. The outliers with Q10 values greater than the 95th percentile were all derived from just three individuals.
Figure 2-7. The effect of temperature on the heart-beat’s frequency of the adult D. melanogaster heart depends on the individual.

A linear regression (black line) is fitted to the relationship between temperature and heart-beat’s frequency for 29 individuals (123 observations, 3-6 observations per individual). The 95% confidence interval is shown in grey. Individual measurements are indicated with dots. For details of the model see the main text.
Figure 2-8 *The Q10 of heart-beat’s frequency of the adult D. melanogaster heart depends on the individual.*

Each Q10 value is calculated from the difference in heart-beat’s frequency measured at different temperatures (29 individuals, 123 observations, 3-6 observations per individual). Grey bars indicate the number of observations. The median heart-beat’s frequency is shown as a dashed pink line, the 95th percentile is shown as a dashed blue line.
Figure 2-9 The temperature dependency of heart-beat’s frequency differs among individuals.

As temperature increases, the ratio between heart-beat at the higher and lower temperatures increases significantly. A generalised linear model (black line) is fitted to the relationship between difference in temperature and ratio of heart-beat’s frequencies for 29 individuals (123 observations, 3-6 observations per individual). The 95% confidence interval is shown in grey. Individual measurements are indicated with black dots, outliers are shown in blue dots, and the single pink dot indicates the ratio at a temperature of 10 degrees. Outliers are all derived from three individuals. For details of the model see the main text.
2.3.8 The Cardiac Myocyte Action Potential of the *D. melanogaster* Adult Heart

Individual differences in the heart-beat may be produced, at least in part, by differences in the electric events triggering these movements. To measure these electrical events, we made sharp intracellular recordings from single cardiac myocytes in a beating heart using the same semi-intact preparation that was used for video recordings of the heart. Single electrode recordings could last up to 30 minutes but typically were less than 10 minutes (Figure 2-10A). We measured the frequency, height, and half width of 10 successive action potentials from single cardiac myocytes from 20 different wild type flies. Action potentials typically occurred at 5.01 ± 1.99 Hz (mean ± standard deviation) and were 42.00 ± 9.13 mV in amplitude with a half width of 58.58 ± 0.37 ms. The minimum potential between action potentials was -34.98 mV.

In some recordings, double-peaked action potentials in which two distinct peaks were clearly visible on each action potential (Figure 2-10B). These double-peaked action potentials were less common than single action potentials. We measured the frequency, height, and half width of 10 successive action potentials from single cardiac myocytes from 12 different wild type flies. These double action potentials occurred at a frequency of 5.38 ± 1.85 Hz. The first spike was 38.52 ± 13.64 mV with a half width of 64.11 ± 0.48 ms. The second spike was 30.13 ± 11.72 mV with a half width of 38.43 ± 0.24 ms. The minimum potential between the double action potentials was -33.15 mV, whereas the minimum potential between the two spikes of the double action potential was -24.20 mV.
The electrophysiology recordings show that the first spikes of double action potentials are significantly larger and longer than the second spikes, but they appear smaller than the single spike. The mean height of the first double action potential is higher than the mean height of the second double spike (Paired T-test; \( t = 10.56, p < 0.0001; \) Figure 2-11A). The mean half width of the first double action potential spike is significantly higher than the mean of the half width of the second action potential spike (Paired T-test; \( t = 8.58, p < 0.0001; \) Figure 2-11B). The mean height of first double action potential spike appears smaller than mean height of single action potential spike (ANOVA; \( F = 7.01, p = 0.009; \) Figure 2-11C). The mean half width of the first double action potential compared with the mean of the half width of single action potential spike showed not different (ANOVA; \( F= 3.01, p = 0.084; \) Figure 2-11D).
Figure 2-10 The cardiac myocyte action potential of the adult D. melanogaster heart.

(A) Left. A sequence of action potentials from a sharp electrode recording of a single cardiac myocyte. Right. A single cardiac myocyte action potential showing the measurement of the height and half width.

(B) Left. A sequence of double-peaked action potentials showing the measurement of the height and half width. Right. A double-peaked cardiac myocyte action potential showing the measurement of the height and half width.
Figure 2-11 Mean height and half width in first and second in double action potentials and mean height and half width in single action potentials.

(A) Mean height (pink) of first action potential spike and mean height of second action potential (dark pink). (B) Mean half width of first double action potential spike (blue) and mean half width of second action potential (dark blue). (B) Mean height of first double action potentials (pink) and mean height of single action potential (pale pink). (D) Mean half width of first double action potential spike (blue) and mean half width of single action potential spike (pale blue). Bars indicate mean ± standard error of the mean.
2.3.9 **Current Injection Alters the Shape and Frequency of the Cardiac Myocyte Action Potential**

Within single cardiac myocyte recordings, the action potential frequency was consistent, however, the substantial differences between cardiac myocytes even within the same fly suggests that this can be modified. We tested this by injecting depolarising or hyperpolarising currents into single cardiac myocytes. Depolarising currents generally increased the frequency of action potentials and reduced their height (Figure 2-12A, B, and D). Hyperpolarising currents decreased the action potential frequency, but typically without a (or with only a small) change in height (Figure 2-12A, C, and D). The rise of the action potential was also slowed by depolarising current injection (Figure 2-12E).

The injection of negative current into the cardiac myocytes reduced the frequency of the action potential (Figure 2-12B). However, the action potential spike height did not show a consistent change in amplitude across all recordings. The width of the action potential also showed no consistent change in duration across all recordings (Figure 2-12D, E). The injection of positive current depolarised the cardiac myocyte reducing the height of the action potential and increasing the half width (Figure 2-12D, E).
Figure 2-12 Tonic shifts in membrane polarisation cause changes in the frequency and shape cardiac myocyte action potential.

(A) Left. A sequence of action potentials from a sharp electrode recording of a single cardiac myocyte. Right. A single cardiac myocyte action potential from the sequence shown in ‘A’. (B) Left. A sequence of action potentials from the same cardiac myocyte as in ‘A’. A tonic depolarising current is injected into the myocyte. Right. A single cardiac myocyte action potential from the sequence shown in ‘B’. (C) Left. A sequence of action potentials from the same cardiac myocyte as in ‘A’. A tonic hyperpolarising current is injected into the myocyte. Right. A single cardiac myocyte action potential from the sequence shown in ‘C’. (D) Overlaid single action potentials from the same cardiac myocyte as in ‘A-C’. (E) Overlaid single action potentials from the same cardiac myocyte as in ‘A-C’ normalised for action potential height and inflection.
2.4 Discussion

We aimed to understand the consistency and variability of the adult *Drosophila melanogaster* heart-beat in a semi-intact preparation, that allows access to the heart by glass microelectrodes permitting electrophysiological recordings. We examined the consistency and variability by measuring the average heart-beat’s frequency and coefficient of variation (CV) over the short-term and the longer-term (6 and 18 minutes, respectively). We found that the average heart-beat’s frequency and CV were stable over time, indicating a consistency over time that suggests the semi-intact preparation is suitable for performing stable electrophysiological recordings. We also found substantial evidence that individual flies have a stable average heart-beat’s frequency and CV. This has not previously been documented and was supported by logged changes in temperature. Increasing the temperature increased the average heart-beat’s frequency but did so in a way specific to each individual. The consistency and individuality of the adult heart-beat was confirmed by analysing the repeatability of the average heart-beat’s frequency and CV over 6 and 18 minutes. Together, these results demonstrate a key overlooked feature of the *D. melanogaster* heart-beat, that individuals have an average frequency and CV that is stable over time in the semi-intact preparation.
2.4.1 The Semi-intact Preparation for the Measurement of the Adult *D. melanogaster* Heart-Beat

The semi-intact preparation involves the removal of the head, the ventral and anterior thorax, and the ventral abdomen (Magny *et al.*, 2013). The semi-intact preparation is widely used for the study of the adult *D. melanogaster* heart-beat, allowing direct access to the dorsal heart tube (*e.g.* Vogler and Ocorr, 2009), though other non-invasive methods are used in some studies permitting extended recordings of the heart-beat in excess of one hour (*e.g.* Sláma and Farkaš, 2005; Wasserthal, 2007; Sláma, 2010). The semi-intact preparation is particularly important for the study of the cellular and molecular mechanisms that underpin the adult *D. melanogaster* heart-beat (*e.g.* Dulcis and Levine, 2005; Ocorr *et al*., 2007; Santalla *et al*., 2014; Balcazar *et al*., 2018). In particular, the study of electrical events within the cardiac myocytes necessitates the use of the semi-intact preparation to permit access to the heart tube with glass microelectrodes (*e.g.* Dulcis and Levine, 2005; Ocorr *et al*., 2007).

This preparation removes the entire brain and ventral nerve cord, which in turn eliminates neural control of the heart-beat (Vogler and Ocorr, 2009). This neural control is known to be capable of reversing the direction of the heart-beat’s from an anterograde movement that pushes the haemolymph anteriorly to a retrograde movement that pushes the haemolymph posteriorly (Dulcis and Levine, 2005). Two distinct anterograde heart-beats have also been discerned, an anterograde heart-beat that moves haemolymph to the head and thorax, termed anterograde 1, and a distinct anterograde heart-beat that moves haemolymph within the abdomen, termed anterograde 2.
(Sláma, 2010). Our analysis shows that both anterograde 1 and 2 heart-beats are found in the semi-intact preparation. We did not observe the retrograde heart-beat in any of the 48 adult fly hearts we recorded suggesting that the retrograde heart-beat is either rare or absent in the semi-intact preparation.

Although both the anterograde 1 and 2 heart-beats are present in semi-intact preparations, our recordings suggest that in the short-term, at least, there is no switching between them. Consequently, a single heart produces a single heart-beat type throughout the recording period. This contrasts with Wasserthal (2007) and Sláma (2010), who observed regular switches between the anterograde and retrograde heart-beats in intact (or nearly intact) flies. This suggests that the absence of switching may be a consequence of the loss of neural control, though Sláma (2006) shows that in another insect (*Manduca sexta*) the removal of the nervous system does not influence switching between anterograde and retrograde heart-beats.

The anterograde 1 and 2 heart-beats did not differ in their average frequency or variability for up to 18 minutes, suggesting that these are stable in the semi-intact preparation. This is consistent with the findings of Wasserthal (2007) and Sláma (2010), who also found no difference in the heart-beat’s frequency of the anterograde 1 and 2 rhythms. Neither study investigated the variability of the heart-beat’s frequency. Indeed, the variability of the adult heart-beat is largely ignored (but see Ocorr et al., 2007b). However, there was considerable variability in the average frequency between preparations. And this variability was consistent throughout the recording period.
Whether such variability is a consequence of the semi-intact preparation or represents natural variation is unclear.

2.4.2 The Temperature Dependency of the Adult *D. melanogaster* Heart Beat

Our recordings are suggesting a strong dependence of the average heart-beat’s frequency of the adult *D. melanogaster* heart-beat upon temperature; increasing the temperature increases the heart-beat’s rate from close to 10 heart-beats per minute at 6°C to more than 100 heart-beats per minute at 29°C. A similar rise in average heart-beat’s frequency of the *D. melanogaster* adult heart with temperature has been observed in other studies (Andersen et al., 2015). Further studies have focussed on the impact of temperature on the larval heart (e.g. Jennings et al., 2009; White et al., 1992), however, comparison with adult heart is difficult because of their very different life history stages. Andersen et al. (2015) showed that below approximately 11°C there is a distinct ‘breakpoint’ in the relationship between temperature and average heart-beat’s frequency; the slope of the relationship becomes steeper so that small reductions in temperature produce greater reductions in average heart-beat’s rate. This change in slope was also found in other species from the genus *Drosophila*, the temperature at which the slope’s shift occurs, depends on the ambient temperature of the species’ habitat (Andersen et al., 2015). However, we found no evidence for a breakpoint in the semi-intact preparation. One possible explanation for this is the removal of the brain and ventral nerve cord. Andersen et al. (2015) show that removal of the head and thorax shifts the breakpoint to lower temperatures and reduces the average heart-beat’s
frequency. Although they did not directly assess the impact of the semi-intact preparation, it seems likely that this is the explanation for the absence of a breakpoint in our data.

The relationship between temperature and average heart-beat’s rate differs among individuals, some respond strongly to temperature whilst others respond little even to large temperature changes. The individual’s response to temperature is reflected in the Q10, which characterises the response of the heart-beat to temperature. The Q10 differs among individuals, though the majority have a Q10 close to 2, which indicates a doubling of heart-beat’s frequency for each 10°C increase in temperature. This is lower than the Q10 of 3 calculated by Andersen et al. (2015). In part this may be a consequence of differences in the way the temperature was changed during experiments; Andersen et al. (2015) reduced temperature from 20°C, whereas we both decreased and increased the temperature. However, it may also be a consequence of the semi-intact preparation, suggesting that not only is the average heart-beat’s frequency reduced but also the response to temperature increases and decreases. Nevertheless, some hearts in semi-intact preparations respond strongly to temperature change, with the average heart-beat’s frequency of a few individuals having a Q10 in excess of 20. Andersen et al. (2015) did not use statistical models that would permit individual differences to be detected, and so the contribution of such individuals to their Q10 values and breakpoint analysis are unknown. Our data emphasises the importance of such analysis in distinguishing the responses of individuals, particularly those with extremely strong or weak responses to temperature.
2.4.3 The Cardiac Myocyte Action Potential of the Adult *D. melanogaster* Heart-Beat

The semi-intact preparation is essential for recording the electrical activity of the cardiac myocytes that compose the adult *D. melanogaster* heart tube. Although the semi-intact preparation provides easy access to the heart tube, there remain considerable challenges in obtaining intracellular sharp electrode recordings from cardiac myocytes because of their small size and the rhythmic movement of the heart. To this end, we used electrodes with resistances of approximately 120 MΩ and designed a bespoke electrode holder that ‘loosely’ holds the electrodes within a sleeve lined with petroleum jelly. This permitted small movements of the electrode and allowed high-quality recordings from single cardiac myocytes. Our recordings show that wild type cardiac myocytes have action potentials of ~40 mV. This amplitude is considerably larger than reported in earlier studies (*e.g.* Dulcis and Levine, 2005), but is similar to that reported by Ocorr *et al.* (2017). However, their recordings were made from a stationary heart treated with blebbistatin for one hour prior to recording, which prevents the heart from moving but leaves the cardiac myocyte action potential intact. The minimum potential between action potentials was ~60 mV, which again is similar to that reported by Ocorr *et al.* (2017). Although this is often assumed to represent a resting potential, in tonically firing cardiac myocytes, it may be substantially depolarised from the resting potential as in other tonically-firing electrically active neurons (reviewed in Koch, 2004).
Our recordings show that action potentials from single cardiac myocytes are consistent in size and shape but that they differ among individuals. These differences in the size and shape of action potentials are unrelated to their frequency. This suggests that it is not the frequency that this determining the size and shape of the action potential, and that they are not both being solely determined by the depolarisation of the cardiac myocyte. This is emphasised by some cardiac myocytes that produce double-peaked action potentials. The majority of recordings show a consistent action potential size, shape, and frequency during the period of stable recording. However, current injection to depolarise or hyperpolarise the cardiac myocyte is capable of altering the size and shape of the action potential and whether it is a double-peaked action potential. This implies that there are differences between individual cardiac myocytes in terms of electrical membrane properties, even within the same heart. This is also supported by the statistical modelling, which shows that the individual cardiac myocyte is an important factor in the properties (height, half width, frequency) of the action potential that are recorded. This is likely due to the set of voltage-gated ion channels expressed in the cell membrane. Differences between individual neurons in electrical membrane properties, even when comparisons are made between homologous neurons, are well documented in a variety of neurons such as neurons from the stomatogastric ganglion of the crayfish (e.g. Prinz et al., 2004). In the case of the stomatogastric ganglion neurons, the internal concentration of Ca\textsuperscript{2+} ions are thought to be key to the regulation of the expression of voltage-gated ion channels in the cell membrane (e.g. Turrigiano et al., 1994). Similar variability may exist within the cardiac myocytes of the adult D. melanogaster heart.
and may be regulated through intracellular Ca$^{2+}$ ions as in stomatogastric neurons, though this has not been documented previously.

2.4.4 Individual Differences in the Adult *D. melanogaster* Heart-Beat

Numerous lines of experimental evidence suggest that there are meaningful differences among individuals in terms of the average heart-beat’s frequency, the variance in the heart-beat (CV), the response of the heart-beat’s rate to temperature, and the properties of the cardiac myocyte action potential. When coupled with statistical mixed models that explicitly separate the random effect of individuals, this provides strong evidence for significant individual differences in numerous aspects of the behaviour and physiology of the heart-beat. Moreover, the individual differences in the average heart-beat’s frequency and the variance are repeatable, at least over the short-term. This suggests that individual adult *D. melanogaster* possess differences in the behaviour and physiology of the heart that are repeatable. Such individual differences have not, to our knowledge, previously been reported for the *D. melanogaster* heart or for any insect heart. In fact, the majority of studies on the adult *D. melanogaster* heart assume that individuals should be similar, and that differences arise through random chance rather than through systematic differences between individuals. Such repeatable differences among individuals have been described for other insect behaviours such as walking (e.g. Videlier et al., 2019). Such behaviours (e.g. walking) are the outputs of complex interactions between neural, muscular, and biomechanical systems and the underlying
origin of individual differences are unknown, however, the heart tube offers a simpler system in which the individual cellular and molecular components can be assessed, offering the possibility of identifying mechanisms.
3 Towards Identifying the Voltage-gated Ion Channels that Sustain the Major Inward Current of the Cardiac Myocyte Action Potential in the D. melanogaster Heart

3.1 Introduction

Understanding the functioning of the human heart is crucial to improving human health and longevity. More than 160,000 people in the UK die from heart disease and circulatory issues each year, and more than 900,000 people are living with heart failure in the UK (British Heart Foundation, 2021). Many aspects of heart disease have been linked to the function of voltage-gated ion channels that contribute to the production of the cardiac action potential (reviewed in Grant, 2009). One such example is Brugada Syndrome, in which there is a problem with the major inward current that leads to the production of the action potential (Sarquella-Brugada et al., 2016). Mutations in the SCN5A voltage-gated Na\(^+\) channel cause the cardiac action potential to fail and increase arrhythmia, which can be life threatening (Kapplinger et al., 2010). Several other diseases are also linked to various channelopathies, including Long QT syndrome, short QT syndrome, and atrial fibrillation, which are all associated with mutations in voltage-gated Na\(^+\) or K\(^+\) channel mutations (reviewed in Grant, 2009). Such mutations have a powerful
effect on the heart and, consequently, on human health. This occurs even though other components of the heart, including the contractile machinery, may be intact and unaffected by the mutation.

Despite more than 100 years of research on the mammalian heart (Engelmann, 1875; Lewis et al., 1914), which is used as a model system to understand the human heart, there is still no comprehensive understanding of the cardiac action potential and its relationship with contractile machinery (reviewed Veeraraghavan et al., 2014). One of the difficulties is the number of components that contribute to the heart and the cardiac action potential. Another issue that is important to consider is the number of animals that are consumed in the study of the mammalian heart, and the cost of this research. A range of mammalian models are used, each to study the heart (reviewed in Camacho et al., 2016). One potential means by which the number of mammals used in heart research, and the costs of the research, can be reduced is to use another model organism that shares many features with the mammalian heart but is simpler in terms of the numbers of cells and molecular components.

One such model system is the heart of the fruit fly, *Drosophila melanogaster* (Bier and Bodmer, 2004; Medioni et al., 2009; Piazza and Wessells, 2011). There are several pronounced differences between the mammalian heart and that of the fruit fly including the position of the heart, which is dorsal in contrast to the ventral position of the mammalian heart, and the structure, which tubular with a single chamber in contrast to the four chambered structure of the mammalian heart. However, there are also similarities in the development and physiology of the fly and mammalian hearts.
(reviewed in Bodmer, 1995; Medioni et al., 2008; Tao and Schulz, 2007). For example, the genes *Tinman, Bagpipe, Heartless* are homologous to vertebrate Nkx2.5, NK family and FGF signalling genes respectively, and are expressed in both the insects and vertebrates to specify the origins of the heart (Alsan and Schultheiss, 2002; Azpiazu and Frasch, 1993; Bodmer, 1993; Lee and Frasch, 2005; Marques et al., 2008; Reifers et al., 2000; Schott, 1998; Shishido et al., 1997). Additional similarities include common functions for genes specifying aspects of cardiac physiology. For example, the *KCNQ* gene encodes a low-threshold voltage-gated K⁺ channel that encodes an outward current (Brown and Adams, 1980). Mutations in one *KCNQ* gene (*KCNQ1*) that is expressed in the mammalian heart are a major cause of long QT syndrome (Wang et al., 1996), which produces changes in the electrocardiogram that cause arrhythmia and can lead to sudden death (reviewed in Shah et al., 2019. Mutations in *D. melanogaster* *KCNQ* also cause age-dependent arrhythmia in the heart beat (Ocorr et al., 2007a).

One limitation to the utility of the *D. melanogaster* heart is that the various ion channels and the genes that encode them that produce currents that are involved in the cardiac myocyte action potential have not yet been fully identified (Zhu et al., 2017). Some ion channels that contribute to the action potential have been identified, though in most cases the effects on the cardiac action potential that they generate have not been measured. A notable exception is the effect of mutations in *KCNQ* channels that cause cardiac arrhythmia in aging *D. melanogaster*, which has been demonstrated though changes in the heart-beat’s rate but also in cardiac myocyte action potential (Ocorr et al., 2007b). However, such comprehensive approaches have become more
common, with recent studies combining both the behaviour of the heart through videography and electrophysiological techniques (e.g. Ocorr et al., 2007a).

We determined to identify the main inward current that contributes to the generation of the action potential. Genes that encoded voltage-gated ion channels capable of producing inward currents had been identified including Cacophony, which encodes a voltage-gated Ca\textsuperscript{2+} channel (Astorga et al., 2012; Peng and Wu, 2007; Ryglewski et al., 2012; Smith et al., 1996), and Paralytic, which encodes a voltage-gated Na\textsuperscript{+} channel (e.g. Usherwood et al., 2005). However, these genes had not been shown to encode the major inward current of the cardiac myocyte action potential. Pharmacological evidence from the larval D. melanogaster heart (Gu and Singh, 1995) had already suggested that the major inward current of the cardiac myocyte action potential is likely to be an L-type voltage-gated Ca\textsuperscript{2+} channel, and unlikely to be voltage-gated Na\textsuperscript{+} channel due to the slowing and stopping of the heart-beat by verapamil and the lack off an effect of tetrodotoxin, respectively. However, the larval heart is extensively remodelled during pupation, and this may include changes in the expression of voltage-gated ion channels (reviewed in Medioni et al., 2009). We began by assessing the effect of the Cacophony and Paralytic mutations and the L-type voltage-gated Ca\textsuperscript{2+} channel blocker verapamil.

During our research, a study by Limpitikul et al. (2018) was published that showed the major inward current generating the cardiac myocyte action potential is the Ca-\alpha1D, which encodes an L-type voltage-gated Ca\textsuperscript{2+} channel. They used a variety of techniques to support their findings including fluorescent in situ hybridisation (FISH), recording
of inward currents in isolated cardiac myocytes, and analysis of the heart-beat’s rate. We decided to replicate the major finding of Limpitikul et al. (2018) and extend this to compare the effect of reducing Ca-α1D throughout development with an acute reduction during adulthood.

3.1.1 Materials and Methods

3.1.1.1 Animals

The Drosophila melanogaster (D. melanogaster) stocks were raised at 25°C on standard molasses medium. The wild type Oregon Red (Ore-R; control) TinC and yellow white (YW; control) stocks have been provided by Claudio Alonso lab. The w1118 control stock obtained from the Bloomington Stock Center. Flies between 1 and 8 weeks old were used in the experiments. The mutants and control stocks were incubated and maintained at 25°C for their entire life unless otherwise stated. The vials in which they were bred and maintained were changed once per week.

3.1.1.2 Fly Stocks and Crosses

A range of stocks were used to determine the potential genetic basis for the main depolarising current of the action potential (Table 3-1). In some cases, mutations were expressed throughout the entire fly (e.g. cacH18). However, for all RNAi lines expression of the RNAi was under the control of the GAL4-UAS system (Brand and Perrimon, 1993) and restricted to the heart (Table 3-1). We bred flies at both 25°C and 18°C to make use
of the mild sensitivity of the GAL4-UAS system in *D. melanogaster* (Brand and Perrimon, 1993; McGuire *et al.*, 2004) to reduce the expression of RNAi constructs. Stocks were divided into two different groups. The first group was raised at 25°C throughout their lives. The second group was raised at 18°C until the adult eclosion and then switched to 25°C; the control line for five days and the RNAi mutant lines for 15 days. We used *W*1118 flies as a control for all of the RNAi lines.

*Table 3-1. Fly stocks.*

<table>
<thead>
<tr>
<th>ABBREVIATED NAME</th>
<th>GENOTYPE</th>
<th>ORIGINAL SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Oregon-R</td>
<td>Abarna Laboratory, University of Sussex</td>
</tr>
<tr>
<td><em>W</em>1118</td>
<td><em>W</em>1118</td>
<td>Ashburner Laboratory, University of Cambridge</td>
</tr>
<tr>
<td><em>yw</em></td>
<td><em>y</em>1</td>
<td>Kaufmann Laboratory</td>
</tr>
<tr>
<td>CasDi 200</td>
<td>U[1]HR:200; caspase-3; G418 M/0</td>
<td>Kyoto Urasoebo Center #10646</td>
</tr>
<tr>
<td>CasDi19336</td>
<td>y[1]hap[156M]; y(Diap-GAL4DBD 0)caspaseM03856; T7GDBD.G[1]M7a</td>
<td>Bloomington Drosophila Center #80325</td>
</tr>
<tr>
<td>Cas11B</td>
<td>Cas[11B]</td>
<td>Bloomington Drosophila Center #42245</td>
</tr>
<tr>
<td>Cas-AT RNAi</td>
<td>P[rok(10093)3]VIE-2626</td>
<td>Vienna Drosophila Resource Center #101827</td>
</tr>
<tr>
<td>Cas-upD(1) RNAi</td>
<td><em>W</em>1118, P[GDa1757]y51491</td>
<td>Vienna Drosophila Resource Center #51491</td>
</tr>
<tr>
<td>Cas-upD(2) RNAi</td>
<td><em>W</em>1118, P[GDa1757]y2544/TM3</td>
<td>Vienna Drosophila Resource Center #52544</td>
</tr>
<tr>
<td>Casophilory RNAi</td>
<td>P[rok(101478)3]VIE-2626</td>
<td>Vienna Drosophila Resource Center #104143</td>
</tr>
<tr>
<td>TnT</td>
<td>TnT*GSAle16a</td>
<td>Jose Ignacio Pacito-Mellet</td>
</tr>
<tr>
<td>ParaS1</td>
<td>para[5175C(1)X,y11]11</td>
<td>Kyoto Urasoebo Center #108353</td>
</tr>
<tr>
<td>ParaS776</td>
<td>Para[5776]</td>
<td>Bloomington Drosophila Center #25701</td>
</tr>
</tbody>
</table>
Figure 3-1. Crossing scheme to produce a $W^{1118}$ control for the Caα1D(1) RNAi lines.

Figure 3-2 Crossing scheme to express UAS-Cacophony RNAi in the dorsal heart.
Figure 3-3. Crossing scheme to express UAS-Caa1T RNAi in the dorsal heart.

Figure 3-4. Crossing scheme to express UAS-Caa1D(1) RNAi in the dorsal heart.


Figure 3-5 Crossing scheme to express UAS-Caa1D(2) RNAi in the dorsal heart.

3.1.1.3 Semi-intact Preparation and Artificial Haemolymph

Following previous studies (e.g. Magny et al., 2013; Vogler and Ocorr, 2009), the flies were anaesthetized using a FlyNap© kit (Carolina Biological Supply Company, Burlington, NC, USA). They were immobilised in a Sylgard 184 (Dow Corning, Midland, MI, USA) coated petri dish dorsal surface down and fixed in a thin layer of petroleum jelly. Using fine scissors (Vannas Spring curved 2.5 mm; Fine Science Tools, Heidelberg, Germany), the head, the thorax and abdomen were removed. The gut and the fat were removed to reveal the beating dorsal heart. The haemolymph was substituted by an artificial haemolymph (AH) solution 1X made fresh containing: 10X AHL (1.08 M NaCl, 0.08 M MgCl₂, 1.05 M KCl, 0.02 CaCl₂ dehydrate, 0.01 NaH₂PO₄), 0.01 M sucrose, 0.1 M trehalose dehydrate, 250 mM NaHCO₃ and 120 µM cytochalasin D (Sigma Aldrich, Gillingham, UK). Flies were perfused with artificial haemolymph (AH) for 20 minutes prior to the start of any experiment.
3.1.1.4 Microelectrode Preparation and Electrophysiology

Artificial haemolymph for electrophysiological recordings was the same as that for video recordings except for the addition of 120 µM cytochalasin D (Sigma Aldrich, Gillingham, UK) (Magny et al., 2013). In vivo intracellular microelectrode recordings from *D. melanogaster* cardiomyocytes were obtained using a borosilicate microelectrodes. A P97 puller (Sutter Instruments, Novato, USA) was used to pull microelectrodes with resistances between 80 and 120 MΩ from borosilicate glass capillaries 1.0 mm O.D. x 0.58 mm I.D (Harvard Apparatus, Cambourne, Cambridge, UK). Electrodes were filled with 3 M potassium chloride (KCl) and mounted in a custom-made holder attached to a Leitz micromanipulator for penetration of single cardiac myocytes. Electrical signals were amplified and filtered with using an npi SEC-05X amplifier (npi Electronic, Tamm, Germany; www.npielectonic.com) or an npi BA-01X amplifier (npi Electronic, Tamm, Germany; www.npielectonic.com). In some cases, paired recordings were from two different myocytes: one in the conical chamber and the other from the proximal segment of the heart tube. Action potential recordings were digitalised at 5 kHz using a CED micro1401 mark II 500 KHZ 16-bit ADC (Cambridge Electronic Design Ltd, Cambridge, UK) analogue-to-digital conversion interface and Spike2 software for subsequent off-line analysis. All recordings were performed at room temperature (20-24°C).
3.1.1.5 Pharmacological Treatments

Verapamil (Sigma Aldrich, Gillingham, UK) was added to artificial haemolymph (AH) in a range of concentrations (50 µM, 71.6 µM, 100 µM, 500 µM, 1000 µM, 2000 µM). Next, the artificial haemolymph in which the semi-intact preparation was bathed was replaced with artificial haemolymph containing verapamil at one concentration. After 10 minutes a video of one minute was taken to assess the effects of verapamil upon the heart. In some cases, the artificial haemolymph containing verapamil was subsequently removed, and replaced with artificial haemolymph.

Barium chloride (Sigma Aldrich, Gillingham, UK) was substituted for calcium chloride in artificial haemolymph. A one minute video was made of the heart in artificial haemolymph. Next, the artificial haemolymph in which the semi-intact preparation was bathed was replaced with barium chloride artificial haemolymph. After 10 minutes a video of one minute was taken to assess the effects of barium upon the heart. In some cases, the artificial haemolymph containing barium chloride was subsequently removed, and replaced with artificial haemolymph.

Forskolin (Sigma Aldrich, Gillingham, UK) was dissolved in artificial haemolymph to produce final concentrations of 10 µM, 50 µM, 100 µM or 500 µM. Following preliminary experiments, a concentration of 10 µM was used for subsequent experiments. A one minute video was made of the heart in artificial haemolymph. Next, the artificial haemolymph in which the semi-intact preparation was bathed has been replaced with artificial haemolymph containing forskolin at one concentration. After 10 minutes a video of one minute was taken to assess the effects of forskolin upon the
heart. In some cases, the artificial haemolymph containing forskolin was subsequently removed, and replaced with artificial haemolymph.

### 3.1.1.6 Electrophysiological Analysis

Electrophysiological recordings were analysed offline using Spike 2 software. Only those recordings in which the action potentials exceeded 30 mV in amplitude, and the minimum potential between action potentials was below -40 mV were analysed further. Three measures were taken from each recording: the height, width, and frequency of the action potentials. The height was measured between the peak of and the minimum potential within the 10 ms preceding the action potential. The width was measured at half the full height of the action potential and is referred to as the half-width. The average action potential’s frequency was calculated as the number of action potentials within one minute.

### 3.1.1.7 Video Acquisition

The heart beating of the flies was filmed using an Electro Retiga camera (Teledyne Photometrics, Tucson, AZ, USA) attached to a Zeiss Discovery V8 Stereo Achromat microscope (Zeiss House, Cambourne, Cambridge, UK). Videos were acquired using Ocular Scientific Imagine Acquisition Software Advanced 2.0 (Teledyne Photometrics, Tucson, AZ, USA). The region of interest was approximately 164 x 441 pixels and covered
the conical chamber and the proximal part of the heart tube. Videos had frame rates between 20 and 30 frames per second and were up to six minutes in duration.

**Video Analysis**

Each six minute video was divided into six videos of one minute each and analysed using Fiji Image J software (http://fiji.sc/). The mean luminosity of the image was used to identify heart-beats. To ensure reproducibility, the region of interest for each video was recorded. We used batch processing because the images were TIFF stacks. The resulting data files were analysed using a graphical user interface within an R Shiny-app (Chang et al., 2020). Within the app, the user specifies the data files to be processed and the parameters to be extracted. The data was low-passed using a Butterworth filter and smoothed using spline interpolation using smooth. Spline package. A custom algorithm was then used to identify local maxima. To ensure that the peaks identified represent heart-beats, only peaks with a maximum slope exceeding two standard deviations beyond the baseline were included. Additionally, duplicates were removed by identifying adjacent shared troughs. Peaks that remained unresolved through this process were inspected, the original video assessed, and manually edited if necessary. Once all the heart beats were identified, the heart rate was calculated, and the data exported for each individual heart.
3.1.1.8 **Statistical Analysis**

Statistical analysis was performed using R (version 3.5.1). The heart-beat’s rate of flies extracted from the video analysis before, during and after drug exposure (barium, forskolin or verapamil) were compared using ANOVAs with repeated measures. Where applicable, post-hoc tests were performed using paired t-tests. ANOVAs were also performed, without repeated measures, for comparing the heart-beat’s rate, extracted from the video analysis, between different groups of flies (WT, Cacophony, Paralytic, Ca-α1D1, Ca-α1D2, Ca-α1T and W1118) and for comparing action potential height, half-width and frequency, from electrophysiology data, between different groups. The drug response curve of verapamil and EC50 were obtained using the package ‘drm’.
3.2 RESULTS

3.2.1 Barium Reduces the Adult *D. melanogaster* Heart-Beat’s Rate

Barium (Ba\(^{2+}\)) is commonly used to study the involvement of Ca\(^{2+}\) ions in physiological studies because it alters the responses of proteins that bind Ca\(^{2+}\) ions (*e.g.* Ferreira *et al.*, 1997; Kreye *et al.*, 1986; Satoh *et al.*, 1987). To assess whether Ca\(^{2+}\) ions were involved in the adult *D. melanogaster* heart-beat, we compared the heart-beat’s rate in artificial haemolymph (AH) containing Ca\(^{2+}\) ions or with artificial haemolymph in which CaCl\(_2\) was replaced with BaCl\(_2\) (hereafter barium AH). Exposure to barium caused a significant difference in the heart-beat’s rate (ANOVA; \(F = 7.26, p = 0.007, N = 10\)). The heart-beat’s rate of wild type adult hearts recorded in the semi-intact preparation was 1.318 ± 0.068 Hz (mean ± standard error of the mean (SEM)) (Figure 3-6). After exposure to barium AH for 10 minutes, the heart-beat’s rate had dropped to 0.368 ± 0.053 Hz (Figure 3-6). This reduction in heart-beat’s rate was significant (Paired T-test; \(T = 3.55, p = 0.0188, N = 10\)). The removal of the barium AH and replacement with AH (wash out) caused an increase in the heart-beat’s rate to 1.417 ± 0.092 Hz (Figure 3-6). This was a significant increase in heart-beat’s rate (Paired T-test; \(T = 3.52, p = 0.05, N = 6\)). There was no significant difference between the heart-beat’s rate before and after exposure to barium AH.
(Paired T-test; $T = 0.26$, $p = 0.26$, $N = 6$). This shows that $Ca^{2+}$ plays an important role in one or more aspects of the heart-beat.

Figure 3-6 Barium reduces the heart-beat’s frequency of the adult Drosophila melanogaster heart.

The mean heart-beat’s rate of wild type flies in the semi-intact preparation: with artificial haemolymph (AH) (blue, left); following replacement of AH with barium AH (dark blue, centre); following wash-out of barium AH (blue, right). Bars indicate mean ± standard error of the mean.
3.2.2 The *Cacophony* Ca$^{2+}$ Channel Mutation does not Affect the Heart-Beat’s Rate of Adult *D. melanogaster*

Our barium experiments suggested that a voltage-gated Ca$^{2+}$ channel may be involved in the generation of the heart-beat. The *Cacophony* gene encodes a voltage-gated Ca$^{2+}$ channel that is expressed in many excitable tissues (Astorga et al., 2012; Peng and Wu, 2007; Ryglewski et al., 2012; Smith et al., 1996). The heart-beat of the *Cacophony* mutant flies did not differ from that of wild type flies in AH or in barium AH (ANOVA; $F = 0.85$, $p = 0.44$, $N = 20$). Exposure to barium caused a significant difference in the heart-beat’s rate (ANOVA; $F = 37.81$, $p < 0.0001$, $N = 10$). The heart-beat’s rate of Cacophony adult hearts recorded in the semi-intact preparation was $0.827 \pm 0.035$ Hz (mean ± standard error of the mean (SEM)) (Figure 3-7). After exposure to barium AH for 10 minutes, the heart-beat’s rate had dropped to $0.057 \pm 0.01$ Hz (Figure 3-2). This reduction in heart-beat’s rate was significant (Paired T-test; $T = 7.36$, $p = 0.0001$, $N = 100$). The removal of the barium AH and replacement with AH (wash out) caused an increase in the heart-beat’s rate to $1.289 \pm 0.051$ Hz (Figure 3-7). This was a significant increase in heart-beat’s rate (Paired T-test; $T = 11.23$, $p = 0.0003$, $N = 6$). There was no significant difference between the heart-beat’s rate before and after exposure to barium AH (Paired T-test; $T = 1.69$, $p = 0.45$, $N = 6$). This shows that Ca$^{2+}$ plays an important role in determining the heart-beat’s rate but that this does not involve Cacophony Ca$^{2+}$ ion channel.
The heart-beat’s rate does not necessarily reflect changes in the cardiac myocyte action potential that generates the heart-beat. To assess the effect of the Cacophony mutation on the cardiac myocyte action potential, we performed sharp intracellular electrophysiological recordings from single cardiac myocytes in the intact beating heart, and measured the action potential frequency, height, and half width from single cardiac myocytes, comparing the Cacophony mutant flies with wild type flies (Figure 3-8A-C).
The action potential height of *Cacophony* mutants was 35.986 ± 0.055 mV (N = 11), which was significantly shorter than the action potential height of wild type flies (43.996 ± 0.040 mV, N = 20) (ANOVA, F = 96.84, p < 2e-16, N = 31). The action potential half width of *Cacophony* mutants was 0.048 ± 0.0001 ms, which was significantly shorter than the action potential height of wild type flies (0.060 ± 0.0001 ms) (ANOVA, F = 23.88, p = 1.81e-06, N = 31). The action potential frequency of *Cacophony* mutants was 6.217 ± 0.134 Hz, which did not differ from that of wild type flies (5.016 ± 0.097 Hz) (ANOVA, F = 3.197, p = 0.084, N = 31). This suggests that the *Cacophony* mutation affects the size and shape of the cardiac myocyte action potential.
Figure 3-8 Cacophony affects the size and shape of the cardiac myocyte action potential.

(A). The height of the cardiac myocyte action potential is shorter in Cacophony mutant (red) than wild type (grey) flies. (B). The half width of the cardiac myocyte action potential is greater in Cacophony mutant than wild type flies. (C). The frequency of cardiac myocyte action potentials does not differ between Cacophony mutant and wild type flies. Bars indicate mean ± standard error of the mean.
3.2.3 The Paralytic Na⁺ Channel Mutation does not Affect the Heart-Beat’s Rate of Adult D. melanogaster

The Paralytic gene encodes a voltage-gated Na⁺ channel that is expressed in many excitable tissues and the main inward current in neural action potentials (e.g. Usherwood et al., 2005). The heart-beat’s rate of Paralytic adult hearts recorded in the semi-intact preparation was 0.898 ± 0.033 Hz (mean ± standard error of the mean (SEM); N = 10) (Figure 3-9). This did not differ significantly from the heart-beat’s rate of wild type flies (1.318 ± 0.068 Hz; ANOVA; F = 3.098, p = 0.0954, N = 20). This shows that Na⁺ does not play an important role in determining the heart-beat’s frequency. We calculated the height, the half width, and the frequency of the myocardial action potentials in both WT and Paralytic flies (Figure 3-10). The height of the Paralytic action potential was 35.555 ± 0.032 mV (mean ± standard error of the mean (SEM); N =15) shorter than the control (Figure 3-10A), though the half width did not differ between strains (Paralytic: 0.056±0.0002 ms; WT: 0.06 ± 0.0001 ms ANOVA, F =1.918, p = 0.167, N =33; Figure 3-10B). The frequency of the Paralytic cardiac myocyte action potentials did not show any difference compared to the control (Paralytic: 4.8 ± 0.127 Hz, WT: 4.861 ± 0.109 Hz ANOVA, F = 0.008, p = 0.929, N = 33; Figure 3-10 C). This suggests that the Paralytic mutation affects the size and shape of the cardiac myocyte action potential.
Figure 3-9. **Paralytic does not alter the response of the adult D. melanogaster heart-beat’s rate.**

The heart-beat’s rate of Paralytic flies in the semi-intact preparation: (brown, left) in comparison to the heart-beat’s rate of wild type flies (grey, right). Bars indicate mean ± standard error of the mean.
Figure 3-10 *Paralytic affects the size and shape of the cardiac myocyte action potential.*

(A). The height of the cardiac myocyte action potential is shorter in Paralytic mutant (brown) than wild type (grey) flies. (B). The half width of the cardiac myocyte action potential does not differ between Paralytic mutant and wild type flies. (C). The frequency of cardiac myocyte action potentials does not differ between Paralytic mutant and wild type flies. Bars indicate mean ± standard error of the mean.
3.2.4 Verapamil has a Concentration Dependent Effect Upon the Heart-Beat of Wild Type Flies

To specify the inward voltage-gated current that generates the cardiac myocyte action potential more precisely, we applied verapamil an L-type Ca\textsuperscript{2+} channel to the adult \textit{D. melanogaster} heart (reviewed in Striessnig \textit{et al.}, 2015.). We applied AH or AH + verapamil at one of seven concentrations (0.05, 0.1, 0.15, 0.5, 1, 1.5 and 2 mM) and recorded the heart-beat’s rate. At low concentrations, verapamil caused a reduction in the heart-beat’s rate, but at high concentrations the heart stopped completely (Figure 3-11). We fitted the Hill equation to the heart-beat’s rate– concentration data, which showed that the half maximum effective concentration (EC\textsubscript{50}) of 0.7 (± 0.82) mM, and the EC\textsubscript{90} was 0.21 ± 0.12 mM. This suggests that the inward current of the cardiac myocyte action potential is produced by an L-type Ca\textsuperscript{2+} current.

![Figure 3-11](image)

\textbf{Figure 3-11. Verapamil reduces the heart-beat’s rate (HR) of adult Drosophila melanogaster heart and stops the heart at high concentrations.}

\textit{A dose-response curve for the effect of verapamil on the heart-beat’s rate of the adult D. melanogaster heart. The line shows a fit of the Hill equation, the open circles indicate the mean heart-beat’s rate at a given 0.05 (N=6), 0.1 (N=6), 0.15 (N=6), 0.5 (N=6), 1 (N=6), 1.5 (N=6), 2 (N=4), control (N=5).}
Due to the effects of the *Cacophony* or *Paralytic* mutations on the cardiac myocyte action potential, we assessed the effect of verapamil on the heart-beat’s rate of mutants. The EC$_{50}$ for verapamil applied to wild type flies is 0.21 mM. We applied this concentration to the hearts of *Cacophony* and *Paralytic* flies. We expected the heart-beat’s frequency of *Cacophony* flies to be 0.415 Hz if verapamil had a similar effect as in wild type flies (Figure 3-12). We expected the heart-beat’s frequency of *Paralytic* flies to be 0.449 Hz if verapamil had a similar effect as in wild type flies (Figure 3-12). The values for *Cacophony* and *Paralytic* flies were within the standard deviation for wild type flies (Figure 3-6), suggesting that there is no difference in response to verapamil. The heart-beat’s rate of *Cacophony* flies was 0.83 ± 0.058 Hz in AH but when exposed to AH + verapamil (0.21 mM) this dropped to 0.287 ± 0.049 Hz, a significant reduction (Paired t-test; $t = 3.076$, $p = 0.040$, $N = 10$). Removal of the verapamil allowed the heart-beat’s rate to recover to 0.728 ± 0.080 Hz, a significant increase (Paired t-test; $t = 3.912$, $p = 0.033$, $N = 6$). There was no difference in heart-beat’s rate before and after verapamil exposure (Paired t-test; $t = 0.786$, $p = 0.999$, $N = 6$). The heart-beat’s rate of *Paralytic* flies was 0.898 ± 0.033 Hz in AH but when exposed to AH + verapamil (0.21 mM) this dropped to 0.478 ± 0.017 Hz, a significant reduction (Paired t-test; $t = 6.073$, $p = 0.0006$, $N = 10$). Removal of the verapamil allowed the heart-beat’s rate to recover to 0.794 ± 0.056 Hz, a significant increase (Paired t-test; $t = 2.033$, $p = 0.293$, $N = 6$). There was no
difference in heart-beat’s rate before and after verapamil exposure (Paired t-test; \( t = 1.297, p = 0.754, N = 6 \)).

Figure 3-12 Verapamil has a similar effect on wild type, Cacophony and Paralytic flies.

(A) The heart beat’s rate of Cacophony flies before (green, left), during (dark green, centre) and after (green, right) exposure to verapamil. (B). The heart-beat’s rate of Cacophony flies before (brown, left), during (dark brown, centre) and after (brown, right) exposure to verapamil. The horizontal dashed line indicates a 50% decrease heart beat’s rate as expected from wild type flies treated with the same verapamil concentration. Bars indicate mean ± standard error of the mean.
3.2.6 Interference RNA (RNAi) Knockdown of Ca-\(\alpha_{1D}\), Ca-\(\alpha_{1T}\) and Cacophony does not Alter Heart-Beat’s Rate

Limpitikul et al. (2018) showed that two genes that encode the L-type and T-type voltage-gated Ca\(^{2+}\) channels in cardiac myocyte action potentials were identified as Ca-\(\alpha_{1D}\) and Ca-\(\alpha_{1T}\), respectively. We attempted to reproduce and extend the findings of Limpitikul et al. (2018) by using the same interference RNA (RNAi) knockdowns. We compared the heart-beat’s rate of two knockdowns of the Ca-\(\alpha_{1D}\) gene (Ca-\(\alpha_{1D}(1)\) and Ca-\(\alpha_{1D}(2)\)), with RNAi knockdown of Ca-\(\alpha_{1T}\) and Cacophony, and controls. We bred flies at 25°C, which produces strong expression of the GAL4-UAS system, and at 18°C, which produces much weaker expression (Brand and Perrimon, 1993). All flies were transferred to 25°C post-pupation, allowing us to compare the effects of a developmental RNAi knockdown with RNAi knockdown only in adult flies, allowing us to distinguish the acute and chronic effects of RNAi knockdown.

The heart-beat’s rate of RNAi knockdowns differed among the fly lines, both we tested at 18°C and 25°C but they also showed considerable variability (Table 3-2; Figure 3-13, 3-14). However, there was no significant difference in heart-beat’s rate among the fly lines that developed at 18°C (ANOVA; \(F = 1.44, p = 0.24, N = 53\)). Likewise, there was no significant difference in heart-beat’s rate among the fly lines that developed at 25°C (ANOVA; \(F = 2.32, p = 0.068, N = 59\)). There was also no significant difference among the fly lines that developed at 18°C and 25°C (ANOVA; \(F = 0.63, p = 0.44, N = 102\)). We also tested the effect of two concentrations of verapamil (0.21 and 2 mM) upon each of the fly lines at 18°C and 25°C. Exposure to 0.21 mM produced a significant reduction in
the Heart-beat’s rate across all lines at both temperatures (ANOVA; F = 110.69, p < 0.0001, N = 102). However, there was no significant difference between any of the fly lines in their response to 0.21 mM verapamil (ANOVA; F = 0.19, p = 0.95, N = 102). At the higher concentration of 2 mM all of the hearts stopped.

Table 3.2. The mean and standard error of the mean of the heart-beat’s rate for each of the RNAi lines (Cacophony (Cac), Ca-α1D (D1), Ca-α1D (D2), Ca-α1T (T); W1118).

<table>
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<th>Line</th>
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<th>Mean heart-beat’s rate (Hz)</th>
<th>SEM heart-beat’s rate (Hz)</th>
<th>Number of Individuals</th>
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Figure 3-13. Expression of RNAi constructs that knockdown the activity of Ca-α1D(1), Ca-α1D(2), or Ca-α1T do not alter the heart-beat’s rate in comparison to control or Cacophony mutant flies.

(A). The heart-beat’s rate of Ca-α1D(1) (green), Ca-α1D(2) (green), Ca-α1T (pale blue), Cacophony (pale blue) and control (w1118; pale blue) flies bred at 18°C. (B). The heart-beat’s rate of Ca-α1D(1) (dark green), Ca-α1D(2) (dark green), Ca-α1T (grey), Cacophony (grey) and control (w1118; grey) flies bred at 25°C. All bars show mean ± standard error of the mean.
Figure 3-14. Expression of RNAi constructs that knockdown the activity of Ca-α1D(1), Ca- α1D(2), or Ca- α1T does not alter the effect of verapamil on the heart-beat’s rate in comparison to control or Cacophony mutant flies.

(A). The heart-beat’s rate of Ca-α1D(1) (green), Ca-α1D(2) (green), Ca-α1T (pale blue), Cacophony (pale blue) and control (w1118; pale blue) flies bred at 18°C exposed to 0.21 mM verapamil. (B). The heart-beat’s rate of Ca-α1D(1) (dark green), Ca-α1D(2) (dark green), Ca-α1T (grey), Cacophony (grey) and control (w1118; grey) flies bred at 25°C exposed to 0.21 mM verapamil. All bars show mean ± standard error of the mean.
3.2.7 Forskolin Increases the Heart-Beat’s Frequency of the Adult *D. melanogaster* Heart

Forskolin is an activator of adenyl cyclase (Insel and Ostrom, 2003), capable of augmenting the inward voltage-gated Ca\(^{2+}\) current in isolated *D. melanogaster* cardiac myocytes. To determine whether forskolin affects the heart-beat’s frequency of an intact heart *in situ*, we recorded the heart-beat of wild type flies and substituted the artificial haemolymph (AH) with AH + forskolin (10 mM) (Figure 3-15). With AH the mean heart-beat’s rate was 1.109 ± 0.053 Hz, which increased to 2.392 ± 0.061 Hz following 10 minutes of exposure to forskolin. This was a significant increase in the heart-beat’s rate (Repeated Measures ANOVA; \(F = 60.32\), \(p = 0.000015\), \(N = 11\)). Thus, forskolin increases the adult *D. melanogaster* heart-beat’s rate.
Figure 3-15. Forskolin increases the heart beat’s frequency of the adult Drosophila melanogaster heart.

The mean heart-beat’s rate (beats per second) of wild type flies in the semi-intact preparation: with artificial haemolymph (AH) (blue, left); following replacement of AH with AH + Forskolin (dark blue, right). Error bars represent mean ± standard error of the mean.
3.3 DISCUSSION

We aimed to find the gene or genes that encode the inward voltage-gated conductances that generate the cardiac myocyte action potential of the adult *Drosophila melanogaster* heart. To this end, we first attempted to confirm existing evidence that the major inward voltage-gated conductance is carried by Ca\(^{2+}\) ions. We used several experiments to show that the inward voltage-gated conductance in indeed carried by Ca\(^{2+}\) ions through L-type voltage-gated Ca\(^{2+}\) channels. We were, however, unable to verify another study published after our experiments had begun (Limpitikul et al., 2018) that these L-type voltage-gated Ca\(^{2+}\) channels are encoded by the *Ca-α1D* and *Ca-α1T* genes. Below, we discuss our positive results and our inability to verify the findings of Limpitikul et al. (2018).

3.3.1 Evidence for an L-type Voltage-gated Ca\(^{2+}\) Conductance Generating the Cardiac Myocyte Action Potential

Several distinct lines of evidence point to the main inward voltage-gated current generating the cardiac myocyte action potential in the adult *D. melanogaster* heart being an L-type Ca\(^{2+}\) current: (1) the effect of barium chloride on the heart-beat’s frequency; (2) the concentration dependent reduction in the heart-beat’s frequency by verapamil; (3) the lack of an effect of the *Cacophony* or *Paralytic* mutations on the heart-beat’s frequency; (4) the lack of a consistent effect of the *Cacophony* or *Paralytic*
mutations on the cardiac myocyte action potential; (5) the lack of an effect of Cacophony or Paralytic mutations on the response of the heart-beat’s frequency to verapamil.

Barium (Ba\(^{2+}\)) has long been used in physiological studies because it is a smaller ion than Ca\(^{2+}\) but carries a similar charge, can move through voltage-gated Ca\(^{2+}\) channels, can bind with Ca\(^{2+}\) sensitive proteins like calmodulin, and often interferes with specific properties of proteins such as inactivation (e.g. Ferreira et al., 1997; Kreye et al., 1986; Satoh et al., 1987). The substitution of Ba\(^{2+}\) ions for Ca\(^{2+}\) ions in the artificial haemolymph of the semi-intact preparation caused a substantial reduction in the heart-beat’s frequency. A similar reduction also occurred in Cacophony mutant flies. Although this suggests that Ca\(^{2+}\) ions play an important role in the heart-beat, it is not possible to identify the physiological process that is affected by Ba\(^{2+}\) ions, or whether only a single physiological process is affected; many physiological processes depend on Ca\(^{2+}\) ions including muscle contraction (Lehman et al., 1994).

To provide greater insight into the inward voltage-gated current that generates the cardiac myocyte action potential, we used the phenylalkylamine L-type Ca\(^{2+}\) channel blocker verapamil (reviewed in Grossman and Messerli, 2004; Striessnig et al., 2015). In the larval D. melanogaster heart, verapamil slows the heart in a concentration dependent manner, stopping the heart at high concentrations (Gu and Singh, 1995). We showed for the first time that verapamil has a similar effect in the adult D. melanogaster heart, slowing the heart-beat at low concentrations and stopping the heart at high concentrations. Although this was not surprising, but it was important to confirm because the regulation of Ca\(^{2+}\) within muscle cells can change during pupation (Magny...
et al., 2013). The effect of verapamil on the heart strongly implicates L-type Ca\(^{2+}\) channels in the cardiac myocyte action potential, however, verapamil is known to have other targets including some K\(^+\) channels (e.g. Xu et al., 2008; Zhang et al., 1999). Such ‘off-target’ effects are common in pharmacological agonists and antagonists (Ritter et al., 2019).

To confirm that the major inward voltage-gated current was an L-type voltage-gated Ca\(^{2+}\) channel, we used mutations of two genes that encode inward currents, *Cacophony* and *Paralytic*, that do not encode an L-type channel and are not expressed in the heart, but which have been extensively studied and have known functions. The *Cacophony* gene encodes a voltage-gated Ca\(^{2+}\) channel that is homologous to N-, P/Q- and R-type voltage-gated Ca\(^{2+}\) channels in vertebrates but not to L-type voltage-gated Ca\(^{2+}\) channels (Astorga et al., 2012; Feng Peng and Wu, 2007; Ryglewski et al., 2012; Smith et al., 1996). Our data shows that *Cacophony* mutant flies do not have any obvious defect in their heart-beat’s frequency. There are also no major differences in the height, width, or frequency of the cardiac myocyte action potential between *Cacophony* flies and their wild type counterparts. *Paralytic* encodes a voltage-gated Na\(^+\) channel (Usherwood et al., 2005) that is homologous to the vertebrate voltage-gated Na\(^+\) channel. Again, our data shows that *Paralytic* flies have no defects in the heart-beat’s frequency and cardiac myocyte action potential. We confirmed that there was no impact of either *Cacophony* or *Paralytic* on the adult *D. melanogaster* heart-beat by comparing the response of the heart-beat in these mutants and wild type flies to verapamil. There was no change in the reduction of the heart-beat in the mutants and the wild type flies, which is consistent with an L-type voltage-gated Ca\(^{2+}\) channel but is not a direct proof.
3.3.2 Failure to Reproduce the Findings of Limpitikul et al. (2018)

The publication of a paper by Limpitikul et al. (2018) during our experiments meant that the genes that encode the L-type voltage-gated Ca$^{2+}$ channels were identified as $Ca$-$\alpha$1D and $Ca$-$\alpha$1T. This caused us to abandon our intended experimental work, and to build upon the published work. Limpitikul et al. (2018) used several complementary lines of evidence to verify the contribution of these genes to the heart-beat of adult $D$. melanogaster including fluorescent in situ hybridisation (FISH) to examine the abundance of $Ca$-$\alpha$1D and $Ca$-$\alpha$1T mRNA within the adult heart, and RNA interference (RNAi) to knockdown the expression of $Ca$-$\alpha$1D or $Ca$-$\alpha$1T genes coupled with assessment of the heart-beat’s frequency. This experimental evidence, coupled with whole cell patch clamp of isolated cardiac myocytes to show the presence of an L-type voltage-gated Ca$^{2+}$ current, linked the $Ca$-$\alpha$1D and $Ca$-$\alpha$1T to the major inward conductance of the cardiac myocyte action potential.

We decided to build upon Limpitikul et al. (2018) by comparing the effect of RNAi knockdown of $Ca$-$\alpha$1D or $Ca$-$\alpha$1T expression in the heart throughout the entire lifetime of the fly or just in the adult flies. We used the temperature dependency of the GAL4-UAS expression system in $D$. melanogaster, which is weaker at lower temperatures below 25°C (Brand and Perrimon, 1993) to reduce RNA interference in flies bred at 18°C. Our experiment involved a direct comparison between flies with RNAi knockdown of $Ca$-$\alpha$1D or $Ca$-$\alpha$1T at 25°C and 18°C with two controls (an RNAi knockdown of $Cac$, and $w^{1118}$). However, this experiment failed to reproduce the effect of RNAi knockdown of $Ca$-$\alpha$1D or $Ca$-$\alpha$1T at 25°C demonstrated by Limpitikul et al. (2018). Given that this
effect was not reproduced in three separate crosses (two Ca-α1D crosses and a Ca-α1T cross) this suggests that there is a problem with the GAL4 expression in the heart (see Methods). An obvious approach would be to perform new crosses with a new driver line; however, this was not possible within the time available (see Covid-19 Statement).

3.3.3 Forskolin Increases the Frequency of the Adult *D. melanogaster* Heart

Forskolin is an activator of adenyl cyclase (Insel and Ostrom, 2003), a membrane protein that catalyses the conversion of ATP to cAMP (Zhang *et al.*, 1997). Limpitikul *et al.* (2018) showed that forskolin is capable of augmenting the inward voltage-gated Ca\(^{2+}\) current in isolated cardiac myocytes from the adult *D. melanogaster* heart. However, this was not accompanied by any proof that forskolin affects the heart-beat’s frequency in an intact heart *in situ*. Moreover, Johnson *et al.* (2002) demonstrates that forskolin does not produce a significant change in heart-beat’s frequency of the pupal heart. We showed that forskolin is capable of producing a substantial increase in the frequency of the adult *D. melanogaster* heart in the semi-intact preparation. The role of such an upregulation of the heart-beat’s frequency is unknown but cAMP is a key second messenger that is involved in multiple signalling cascades (reviewed in Sassone- Corsi, 2012). Limpitikul *et al.* (2018) argue that adenyl cyclase augments the inward voltage-gated Ca\(^{2+}\) current in *D. melanogaster* cardiac myocytes through protein kinase A phosphorylation of the L-type Ca\(^{2+}\) channels by analogy with modulation of Cav1.2 channels in mammalian cardiac myocytes (reviewed in Weiss *et al.*, 2013). Indeed, phosphorylation of L-type
Ca\textsuperscript{2+} channels by cAMP-dependent protein kinases has been reported extensively (e.g. Sculptoreanu and Scheuer, 1993). Although there may be alternative pathways through which activation of adenyl cyclase by forskolin could augment the inward L-type Ca\textsuperscript{2+} current, a strong candidate is the protein kinase A signalling cascade. All of the components of the protein kinase A signalling cascade are present in \textit{D. melanogaster}, including phosphodiesterase (Pavot \textit{et al.}, 2015) and protein kinase A (Li \textit{et al.}, 1996; Taylor and Yonemoto, 1990). The signalling cascade may be activated \textit{in vivo} by octopamine as part of the β-adrenergic signalling cascade in \textit{D. melanogaster}. Octopamine has also been shown to increase the heart-beat’s frequency in the \textit{D. melanogaster} pupal heart (Johnson \textit{et al.}, 1997). However, additional experiments will be needed to confirm that this is the signalling cascade because numerous modulators are capable of increasing the heart-beat’s frequency in \textit{D. melanogaster} (Johnson \textit{et al.}, 1997). Further experiments will also be needed to determine the biological function of the increase in heart-beat’s frequency through adenyl cyclase activation.
4.1 INTRODUCTION

Gap junctions are essential for intercellular communication within almost all animal tissues and organs (Sáez et al., 2003; Güiza et al., 2018; Panchin, 2005). They have been identified in organs such as heart, brain and kidney/Malpighian tubules in vertebrates and invertebrates (e.g. Biava and West, 1966; Brightman and Reese, 1969; Connors and Long, 2004; Furshpan and Potter, 1959; Loewenstein et al., 1965; Phelan et al., 2008; Rohr, 2004; Taugner et al., 1978). Within excitable tissues, gap junctions form electrical synapses that allow electrical signals to be propagated between cells (e.g. Connors and Long, 2004; Furshpan and Potter, 1959; Phelan et al., 2008). Gap junctions are important for the co-ordinated activity of cells both during development when the structure and cellular properties of tissues are being determined, and for physiological functioning in growing and adult tissues (reviewed in Panchin, 2005; Phelan et al., 1998; Phelan and Starich, 2001; Sáez et al., 2003 Güiza et al., 2018).
Gap junctions are present in both vertebrates and invertebrates (though more correctly this should be deuterostomes and protostomes, respectively), and perform similar functions in these lineages (Bruzzone et al., 1996; Güiza et al., 2018; Panchin, 2005; Phelan et al., 1998; Phelan and Starich, 2001; Sáez et al., 2003; Skerrett and Williams, 2017; Söhl et al., 2005). In both deuterostome and protostome lineages gap junctions have complex structures composed of many subunits, thought to be a six subunit hemichannel that docks with a similar hemichannel on a neighbouring cell, as determined by both X-ray crystallography and cryo-electron microscopy (Caspar et al., 1977; Maeda et al., 2009; Makowski et al., 1977; Oshima et al., 2016, 2007; Perkins et al., 1997; Unger, 1999). Gap junctions form cytoplasmic connections between neighbouring cells, allowing passage of small molecules (e.g. secondary messengers inositol 1,4,5- triphosphate and cAMP) and ions (K+, Na+ and Ca2+) ions and between these cells (Bruzzone et al., 1996; Chailakhyan, 1990; Lawrence et al., 1978; Sáez et al., 1989; Spray et al., 1982; White and Paul, 1999). Gap junctions in deuterostomes permit the movement of molecules up to 485 Daltons whereas molecules of up to 1100 Daltons can travel through protostome gap junctions (Hu and Dahl, 1999; Loewenstein, 1981). The junctional conductances in vertebrates are 30-300 pS whereas those of invertebrates are larger 270-330 pS (e.g. Bruzzone et al., 1996; Gho, 1994).

Despite their similarities in terms of function, deuterostome and protostome gap junctions are formed of proteins that are coded for by different, unrelated gene families with distinct lineages, though in both cases the proteins span the membrane four times (reviewed in Panchin, 2005). In vertebrates gap junction subunits are the products of connexin genes (reviewed in Kumar and Gilula, 1996), and have been found other
members of the chordates including tunicates and ascidians (e.g. Sasakura et al., 2003). In the protostomes, gap junctions are formed from protein subunits that are the products of innexin genes (reviewed in Phelan and Starich, 2001). No connexin genes have been found in protostomes but vertebrates do possess genes that are homologous to innexins leading to the formation of the pannexin gene family (Bruzzone et al., 2003; Panchin, 2005). Further identification of innexin genes but not connexin genes in the diploblastic animal Hydra, which is thought to represent a lineage that pre-dates the deuterostome-protostome split, has led to a general hypothesis that innexins were present in the earliest animal lineages but were subsequently lost in some deuterostome lineages (Alexopoulos et al., 2004; Panchin, 2005). The Hydra expresses structural gap junctions that are involved in patterning, suggesting that the innexin genes are functional (Fraser et al., 1987; Hand and Gobel, 1972). Several key studies demonstrated that the products of innexin genes from Drosophila melanogaster and Caenorhabditis elegans could form functional gap junctions (e.g. Landesman et al., 1999; Phelan et al., 1998; Starich et al., 1996).

Within the vertebrate heart, gap junctions play a key role in the propagation of the cardiac action potential (reviewed in Rohr, 2004). Early transmission electron microscope studies of the vertebrate heart showed that it was composed of individual muscle cells, and was not a syncytium (Sjöstrand and Andersson, 1954). Subsequent evidence of cable properties, the spread of radioactive K+ ions in heart tissue, and the presence of low resistance pathways all led to the conclusion that there were gap junctions between cardiomyocytes (Weidmann, 1970; Weidmann and Hodgkin, 1966). Many years of subsequent experimental and computational modelling studies have
concluded that gap junctions are the major pathway for signal propagation across the vertebrate heart (reviewed in Rohr, 2004). The disruption of gap junctions in the heart is associated with heart disease, including arrhythmia of the atria produced by atrial fibrillation in which gap junctions are implicated both in the initiation of the condition and its persistence (reviewed in Allessie, 2002; Polontchouk et al., 2001; Severs, 2004; Wijffels et al., 1995). Despite the importance of gap junctions to the function of the vertebrate heart, and the emergence of the *Drosophila melanogaster* heart as a model system for studying the molecular basis of the heart beat and heart disease (reviewed in Bier and Bodmer, 2004; Piazza and Wessells, 2011), to our knowledge no previous study has explored the role of gap junctions on the fly’s heart-beat. However, the proteome of the adult *Drosophila melanogaster* heart shows that both innexin2 and innexin3 are expressed (Cammarato et al., 2011). These two innexins are known to produce oligomeric gap junctions when co-expressed (Stebbings et al., 2000).

Studies have shown broad structural similarities between connexins and innexins. In particular, studied the structure of the innexin-6 gap junction channel using cryo-electron microscopy. This study demonstrated that *Caenorhabditis elegans* innexins-6 gap junction show a high degree of broad structural similarity to connexin-26 in vertebrate. The communication between cells through gap junction channel is important for the homeostasis. In the study Oshima et al. (2016) determined that innexin-6 trans-membrane helices and extracellular loops are very similar to the structure in connexin-26 despite low sequence similarity. The structure of the innexin-6 reveals the N-terminal part funnel consistent with connexin-26 (Oshima et al. 2016). Other studies on the similarity of the connexins and innexins and the effect of
pharmacological drug carbenoxolone are known. For example, Bao et al., (2007) tested whether innexins are similar to vertebrate pannexin. Using whole cell voltage clamp they demonstrated that innexin expressed in *Xenopus* oocytes form functional innexons. Moreover, *Hirudo sp.* innexin has several properties in common with human and mouse pannexin 1 channels. Carbenoxolone closed the channels in a dose-dependent manner (Bao et al. 2007). Luo and Turnbull (2011) showed that vertebrate and invertebrate gap junctions have similar responses to carbenoxolone. They studied two *Lepidopteron* cell lines and haemocytes using flow cytometry and fluorescent microscopy. Carbenoxolone consistently inhibited dye uptake in two cultured lines and haemocytes (Luo and Turnbull 2011).

The effect of carbenoxolone on the vertebrate heart has been studied in the rabbit. De Groot (2003) used an intact heart after a profusion of Tyrode’s solution containing carbenoxolone. Carbenoxolone caused moderate cellular electrical uncoupling and slowing of conduction in intact atrial and ventricular myocardium in a reversible manner (de Groot et al. 2003). Carbenoxolone also causes a decrease of the velocity in atrial and ventricular conduction in guinea pigs by increasing intercellular resistivity but it does not affect action potential parameters (Gray 2005). In human hearts, carbenoxolone slowed both atrial and ventricular wavefront propagation velocities most likely due to gap junctional uncoupling. The effect of carbenoxolone was particularly marked in patients with ischemic heart disease (Kojodjojo et al., 2006).

At least part of the reason gap junctions within the *Drosophila melanogaster* heart have not been studied is that a pharmacological agent proven to block gap junctions
is not, to our knowledge, available. Many pharmacological agents are known to block gap junctions in vertebrates (reviewed in Juszczak and Swiergiel, 2009; Rozental et al., 2001), but these are formed from connexins not the innexin proteins from which insect gap junctions are formed (e.g. Panchin, 2005; Phelan, 2005). Consequently, the identification of a pharmacological gap junction blocker would be an invaluable tool to enable the study of gap junctions in the *Drosophila* heart. Molecules from various classes have been identified for their ability to block vertebrate gap junctions (Juszczak and Swiergiel, 2009; Rozental et al., 2001). Here, we focus on one particular pharmacological agent carbenoxolone (3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate), which is a glycyrrhetinic acid derivative that has a structure similar to molecules found in licorice root (Connors, 2012). Several recent studies of invertebrates have used carbenoxolone but have assumed rather than proved that it blocks innexin gap junctions (e.g. Calkins and Piermarini, 2015; Miriyala et al., 2018; Sangaletti et al., 2014). We show that many effects of carbenoxolone on the *D. melanogaster* heart are consistent with an ability to block gap junctions, though unequivocal proof requires additional experimental evidence. Consequently, we argue that carbenoxolone is a putative blocker of innexin gap junctions, with potential application across a broad range of protostome preparations.
4.2 MATERIALS AND METHODS

4.2.1 Animals

Experiments were performed using adult female *Drosophila melanogaster* (Meigen 1830) Wild Type (WT) Oregon Red flies. Larvae were incubated for 1 to 2 weeks at 25°C, and adults were collected for experiments 1 to 2 weeks post-eclosion. Flies were fed once a week on a diet consisting of yeast and molasses. The Oregon Red flies were provided by Prof. Claudio Alonso from his laboratory at the School of Life Sciences, University of Sussex, UK.

4.2.2 Semi-intact Preparation

The female flies, 1-2 weeks old, were anaesthetized with Flynap® anaesthetic kit (Carolina Biological Supply Company, Burlington, USA) for ~10 seconds. Individual flies were immobilised in a thin layer of petroleum jelly on a Sylgard® 184 (Dow Corning, Midland, MI, USA) coated Petri dish with their dorsal surface down (Magny et al., 2013). The head, the thorax and the abdominal cuticle were removed with Vannas Spring-curved 2.5 mm scissors (Fine Science Tools, Heidelberg, Germany), the still-beating heart was exposed by removing the ventral surface of the abdomen. The gut and reproductive organs were removed, as was the fat around the heart tube (Vogler and Ocorr, 2009). The haemolymph was substituted by a 1x Artificial Haemolymph (AH) containing: 10x AHL (1.08 M NaCl, 0.08 M MgCl₂, 1.05 M KCl, 0.02 M CaCl₂ dehydrate, 0.01 M
NaH₂PO₄), 0.01 M sucrose, 0.1 M trehalose dehydrate, 250 mM NaHCO₃ and 120 µM cytochalasin D (Sigma Aldrich, Gillingham, UK) (Magny et al., 2013). The experiments were carried out at room temperature (17-20°C).

4.2.3 Pharmacological Treatment

Dissected hearts (see Semi-intact Preparation) were exposed to carbenoxolone disodium salt (Sigma Aldrich, Gillingham, UK), a known blocker of vertebrate gap junctions (Calkins and Piermarini, 2015; Miriyala et al., 2018; Sangaletti et al., 2014). Carbenoxolone was dissolved in 1x AH saline solution (see above) at five different concentrations: 0.03 mM, 0.1 mM, 0.3 mM, 0.7 mM and 1.5 mM. Each experiment began with 30 minutes during which the heart was covered with oxygenated saline. The saline was refreshed, and the heart was then filmed for the subsequent six minutes. The saline was then substituted with saline containing carbenoxolone disodium salt at one of five concentrations (0.03, 0.1, 0.3, 0.7 or 1.5 mM) or fresh saline, which acted as a control. In each case, the heart was filmed for the subsequent six minutes. Finally, the saline (both control and that containing carbenoxolone) was substituted with saline to ‘wash-out’ the carbenoxolone. Again, the heart was filmed for the subsequent six minutes. Experiments were repeated between five and ten times for each concentration and the control.
4.2.4 Video Acquisition

The *in vivo* beating heart was filmed using an Retiga Electro CCD camera (Digital Imaging Systems, Bourne, UK) attached to a Zeiss Discovery V8 Stereo Achromat S 1.25X FWD 50 mm, lens 8X (Carl Zeiss Limited, Cambridge, UK). The region of interest (164 x 441 pixels) was centred on the conical chamber and posterior region of the heart tube selected using Ocular Scientific Imagine Acquisition Software Advanced 2.0 (Teledyne Photometrics, Tucson, Arizona, USA), and videos of six minutes in duration were captured at 20-30 frames per second.

4.2.5 Video Analysis

Each six minute video was divided into six videos of one minute each and analysed using Fiji Image J software (http://fiji.sc/). The mean luminosity of the image was used to identify heart-beats. To ensure reproducibility, the region of interest for each video was recorded. We used batch processing because the images were TIFF stacks. The resulting data files were analysed using a graphical user interface within an R Shiny-app (Chang *et al.*, 2020). Within the app, the user specifies the data files to be processed and the parameters to be extracted. Any frames not containing values were removed, and the data inverted if necessary to improve detection. The data was low passed using a Butterworth filter and smoothed using spline interpolation using smooth.spline package. A custom algorithm was then used to identify local maxima. To ensure that the
peaks identified represent heart-beats, only peaks with a maximum slope exceeding two standard deviations beyond the baseline were included. Additionally, duplicates were removed by identifying adjacent shared troughs. Peaks that remained unresolved through this process were inspected, the original video assessed, and manually edited if necessary. Once all the heart-beats were identified, the heart rate was calculated and the data exported for each individual heart.

4.2.6 Electrophysiology Recording

Artificial haemolymph for electrophysiological recordings was the same as that for video recordings except for the addition of 120 µM cytochalasin D (Sigma Aldrich, Gillingham, UK) (Magny et al., 2013). In vivo intracellular microelectrode recordings from D. melanogaster cardiomyocytes were obtained using a borosilicate microelectrodes. A P97 puller (Sutter Instruments, Novato, USA) was used to pull microelectrodes with resistances between 80 and 120 MΩ from 10 cm borosilicate glass capillaries 1.0 mm O.D. x 0.58 mm I.D (Harvard Apparatus, Cambourne, Cambridge, UK). Electrodes were filled with 3 M potassium chloride (KCl) and mounted in a custom-made holder attached to a Leitz micromanipulator for penetration of single cardiac myocytes. Electrical signals were amplified and filtered with using an NPI SEC-05X amplifier (npi Electronic, Tamm, Germany; www.npielectonic.com), and filtered using a four-pole Butterworth filter. In some cases, paired recordings were from two different myocytes: one in the conical chamber and the other from the proximal segment of the heart tube. Action potential
recordings were digitalised at 5 kHz using a CED micro1401 mark II 500 KHZ 16-bit ADC (Cambridge Electronic Design Limited, Cambridge, UK) analogue-to-digital conversion interface and Spike2 software for subsequent off-line analysis. All recordings were performed at room temperature (20-24°C).

4.2.7 Statistical Analysis

Statistical analysis was performed using R (version 3.5.1). The concentration dependent effect of carbenoxolone on the number of heart-beats was analysed using a zero-inflated Poisson model with the package glmmTMB. In this model, concentration, and the number of heart-beats prior to carbenoxolone exposure were fixed factors, and the individual fly was included as a random factor. A similar model was used to assess the concentration dependent recovery from carbenoxolone exposure during the wash-out phase. This model incorporated the number of heart-beats during the exposure phase as additional fixed factor. Comparisons of numbers of heart-beats between the three phases of the experiment for each carbenoxolone concentration or between concentrations for each phase were made using Wilcoxon Rank Sum tests for the final two minutes of each experimental phase. All p-values were Bonferroni corrected, the significance being adjusted to 0.025 or 0.01, respectively.

The effect of carbenoxolone concentration on heart-beat’s rate during the last two minutes of the exposure phase was modelled as a typical drug response curve using the package drm. The EC$_{10}$, EC$_{50}$, EC$_{90}$ were also calculated using the same package. This
permitted calculation of the Hill coefficient \(n_H\) and the Hill working range \(WR_H=EC_{10}-EC_{90}\) (Calkins and Piermarini, 2015; Miriyala et al., 2018; Sangaletti et al., 2014).

The time constant of the effect of carbenoxolone on heart-beat’s rate was calculated for each individual as an exponential decay curve using the drm package. The concentration dependent effect of carbenoxolone on the decrease in heart-beat’s rate was modelled using concentration as a fixed factor and the individual fly as a random factor using the glmer package with a Gamma distribution and an identity link.

The effect of carbenoxolone concentration on the likelihood of suffering a full heart-beat stoppage of at least one minute while exposed to carbenoxolone, was analysed using a logistic regression model with the glmer package. The model incorporated concentration as a fixed factor and the individual fly as a random factor. The effect of carbenoxolone concentration on the likelihood of recovering from full stoppage during the wash-out phase (heart-beats present during the final two minutes) was modelled using a logistic regression with the glmer package. The model incorporated concentration as a fixed factor and the individual fly as a random factor.

Heart-beat rhythmicity was quantified using the coefficient of variation (CV), the standard deviation divided by the mean. The effect of carbenoxolone concentration on the CV was modelled with the glmer package (Gamma family, identity link) using the concentration and CV from part 1 as fixed factors, and the individual fly as a random factor. The effect of carbenoxolone concentration on CV during the wash-out phase was modelled with the glmer package (Gamma family, identity link) using concentration, the CV from part 1 and the CV from part 2 as fixed factors, and the individual fly as a random factor.
factor. Only hearts that were beating for at least one minute of the test phase and wash-out phase (i.e. CV>0) were included in the analysis.

4.3 RESULTS

4.3.1 Carbenoxolone (CBX) Affects the Heart-Beat’s Rate (HbR) in a Concentration-Dependent Manner

We investigated the effect of carbenoxolone (CBX) on the adult *D. melanogaster* heart using a reduced *in vivo* preparation in which the heart was exposed (see Methods). The HbR of each individual was videoed for six periods of one minute each in saline to establish a baseline (Figure 4-1A). As a control, the saline was replaced by new saline and videoed for a further six periods of one minute each (Figure 4-1A). To test the effect of CBX, the saline was replaced by saline containing 0.03, 0.1, 0.3, 0.7 or 1.5 mM CBX and videoed for a further six periods of one minute each (Figure 4-1A). Finally, during the wash-out phase new saline was applied to both control and test hearts to determine the recovery from CBX exposure (Figure 4-1A).

There was no significant difference in the baseline HbR among the different treatments and control (Figure 4-1B). However, upon exposure to concentrations of CBX of 0.1 mM or greater during the test, there was a significant reduction in the HbR in comparison to the baseline, though no such reduction occurred with 0.03 mM CBX or in controls (Figure 4-1B). Exposure to concentrations of CBX of 0.1 mM or greater during the test also caused a significant reduction in the HbR in comparison to the control,
though no such reduction occurred with 0.03 mM CBX (Figure 4-1B). We modelled the concentration-dependent effect of CBX on HbR during the test phase using a dose-response curve (Figure 4-1C; see Methods). From this curve, we estimated the CBX concentration needed to produce a 50% reduction in HbR, the EC$_{50}$, which was 0.083 ± 0.011 (estimate ± SEM) (Figure 4-1C).

The reduction in HbR caused by CBX during the test phase was faster at higher concentrations (Figure 4-2A, B). Indeed, for some flies the highest concentrations of CBX produced a marked reduction in HbR within less than a minute (Figure 4-2B). We fitted an exponential decay to the reduction of HbR during the test phase for each heart at each CBX concentration. The time constant, $\tau$, of the HbR reduction was dependent upon CBX concentration, higher concentrations having significantly shorter time constants (Figure 4-2C).
Figure 4-1. **Carbenoxolone (CBX) produces a concentration-dependent reduction in heart-beat’s rate (HbR).**

(A) The mean (± standard deviation) HbR of adult flies in a semi-intact preparation exposed to CBX. The grey region indicates when CBX was applied at 0 (control), 0.03, 0.1, 0.3, 0.7, 1.5 mM concentrations. Regions either side of the grey region indicate only saline. (B) The mean (± standard deviation) HbR for the three parts of the experiment shown in ‘A’ (light, mid or dark colour) in the control or each of the concentrations shown in ‘A’. (C) A dose-response curve produced by the Hill equation for the effect of CBX upon HbR (black line). The 95% confidence interval for the curve is shown in grey, the dashed vertical line shows the EC50.
4.3.2 The Effect of Carbenoxolone (CBX) on the Heart-Beat’s Rate (HbR) is Concentration-Dependent

The reduction in the number of heart-beats four to six minutes after application of CBX was dependent upon the CBX concentration (Zero-inflated Poisson; estimate = 2.611, \( p < 0.0001 \); Figure 3A), but did not depend on the number of heart-beats prior to CBX application (Zero-inflated Poisson; estimate = 0.018, \( p = 0.124 \)). Four to six minutes after washout, the number of heart-beats remained dependent upon the CBX concentration to which the heart was exposed (Zero-inflated Poisson; estimate = 1.534, \( p < 0.0001 \);
Figure 4-3B), but did not depend on the number of heart-beats prior to CBX application (Zero-inflated Poisson; estimate = 0.012, p = 0.201). However, the number of heart-beats four to six minutes after washout was dependent upon the number of heart-beats four to six minutes after application of CBX (Zero-inflated Poisson; estimate = 0.013, p = 0.039; Figure 4-3C).

Figure 4-3. The number of heart-beats following carbenoxolone (CBX) application is concentration dependent.

(A) The number of heart-beats 4-6 minutes after CBX application decreases with increasing CBX concentration. (B) The number of heart-beats 4-6 minutes after CBX washout decreases with increasing CBX concentration. (C). The number of heart-beats beats 4-6 minutes after CBX washout depends on the number of heart-beats 4-6 minutes after CBX application. Black line indicates the statistical model, grey region indicates the 95% confidence interval. Points indicate a single heart at a single concentration.
4.3.3 Termination of the Heart-Beat’s Rate (HbR) by Carbenoxolone (CBX) is Concentration-Dependent and Affects Recovery

There was considerable variation in the extent of the reduction in the HbR during the test phase caused by CBX at each concentration greater than 0.06 mM. In some cases, particularly at lower concentrations, CBX produced only a small reduction in the HbR (Figure 4-1A). In other cases, particularly at higher concentrations, CBX terminated the Hb (Figure 4-1A, 4-4A, B). The termination of the Hb was significantly predicted by CBX concentration, higher concentrations increasing the probability of termination (logistic regression; $z = 3.601, p < 0.0001$; Figure 4-4C).

Replacement of saline containing CBX with control saline during the wash-out phase produced recovery of the HbR (Figure 4-1A, 4-4A). In fact, 39 of 41 hearts of hearts exposed to CBX during the testing phase (excluding controls) that did not stop fully, recovered during the wash-out phase. In some cases, however, there was no recovery during the wash-out phase (Figure 4-4B). The recovery of the Hb was significantly predicted by CBX concentration, higher concentrations reducing the probability of recovery (logistic regression; $z = 2.80, p < 0.005$; Figure 4-4D). The failure to recover was significantly higher in those hearts in which the HbR was terminated by exposure to CBX (G-test of independence; $G = 15.21, p < 0.0001$).
4.3.4 Gap junctions are important for the initiation and timing of cardiac action potentials

Intracellular *in vivo* recordings from single cardiac myocytes revealed tonic action potentials (Figure 4-5A). Occasionally, action potentials failed to initiate revealing a distinct subthreshold depolarisation that occurred at the moment when an action
potential would have been expected. These events were more clearly revealed by hyperpolarising single cardiac myocytes (Figure 4-5B). Intracellular in vivo recordings from pairs of cardiac myocytes revealed action potentials co-occurring in both cells, with a similar shape (Figure 4-5C). The timing of these action potentials was similar in both cardiac myocytes (Figure 4-5D).

Figure 4-5 Electrophysiological evidence for the role of gap junctions in cardiac myocyte function.

(A) An in vivo intracellular recording from a single cardiac myocyte showing action potentials. (B) Hyperpolarisation of the cardiac myocyte in ‘A’ reveals distinct inputs that trigger action potentials. (C) A paired intracellular recording from two cardiac myocytes showing action potentials. (D) An overlay of the action potentials in the recording shown in ‘C’.
4.3.5 Carbenoxolone (CBX) Terminates Cardiac Action Potentials and Increases Cardiac Myocyte Input Resistance

Intracellular in vivo recordings single cardiac myocytes showed that application of CBX (0.7 mM) to the heart rapidly stopped the action potentials, as expected from the heart-beat. Additionally, after CBX application there were no subthreshold events. Measurement of cardiac myocyte input resistance before (Figure 4-6A) and after CBX application (Figure 4-6B) revealed an increase in input resistance. Indeed, CBX significantly increased cardiac myocyte input resistance (Figure 4-6C).
Figure 4-6. Electrophysiological evidence for the effect of carbenoxolone (CBX) on cardiac myocytes.

(A) Injection of hyperpolarising current pulses (below) produced a negative deflection of the membrane potential (above). (B) Following application of CBX, the negative deflection of the membrane potential (green) to hyperpolarising current pulses was larger. Same cardiac myocyte as in ‘A’. (C) Comparison of the hyperpolarising membrane deflections shown in ‘A’ and ‘B’.
4.4 **Discussion**

We aimed to determine the effect of carbenoxolone (CBX) upon the adult *D. melanogaster* heart. To this end, we used a semi-intact preparation in which the heart remains intact but the ventral abdomen and thorax along with the head is removed (see Chapter 2). This allowed us to record the heart-beat before, during and after exposure to carbenoxolone, as well as to make electrophysiological intracellular recordings from single cardiac myocytes. Evidence from analysis of video recordings shows that carbenoxolone is sufficient to reduce the heart-beat’s rate or even terminate the heart-beat entirely. Electrophysiological evidence shows that carbenoxolone is sufficient to abolish cardiac myocyte action potentials and increase their input resistance. Below, we discuss this evidence, arguing that taken together it suggests that carbenoxolone may block innexin gap junctions in the adult *D. melanogaster* heart.

### 4.4.1 Carbenoxolone (CBX) Slows and Stops the Adult *D. melanogaster* Heart-Beat

The effect of carbenoxolone was concentration dependent. At low concentrations there was no effect on the heart-beat but concentrations of carbenoxolone greater than 0.3 mM slowed the heart-beat, whilst concentrations 0.7 mM or higher were capable of stopping the heart-beat. The rapidity within which carbenoxolone had an effect on the heart-beat was also dependent upon concentration, lower concentrations having a
longer time constant than higher concentrations. Moreover, complete stoppage of the heart-beat, which happened in 80% of experiments with concentrations 0.7 mM or higher, was rapid, occurring within less than a minute of exposure. Both slowing and stoppage of the heart-beat are consistent with carbenoxolone blocking gap junctions between cardiac myocytes.

Slowing may be the result of blocking only a fraction of the gap junctions that allow the spread of an electrical signal between cardiac myocytes. Fewer gap junctions may lead to smaller electrical inputs that slow the heart-beat, possibly due to the need for a large pool of voltage-gated Ca\textsuperscript{2+} channels that are needed to generate the action potential (see Chapter 3). More voltage-gated Ca\textsuperscript{2+} channels released from inactivation may allow smaller or slower inputs to trigger action potentials (Azouz and Gray, 2003, 2000; Fricker \textit{et al.}, 1999; Platkiewicz and Brette, 2010; Wilent and Contreras, 2005). However, this would be expected to increase the variance of the heart-beat, which we did not observe. Instead, the variance in the heart-beat, even after the application of carbenoxolone depends more on the individual fly than the effect of the drug. This emphasises the importance of individual variability, which occurs in wild type flies even in the absence of any pharmacological treatment (see Chapter 2). Stopping the heart-beat completely at high concentrations is also consistent with sufficient block of gap junctions that the spread of the cardiac action potential between neighbouring myocytes is prevented.

At lower concentrations of 0.3 mM or less, removal of carbenoxolone produces a recovery in heart-beat’s rate to levels prior to application. However, at higher
concentrations following complete stoppage of the heart-beat, there is no recovery of
the heart-beat following removal of carbenoxolone. A small proportion of hearts do
restart approximately 5-6 minutes after washout, however, which suggests that a longer
period after removal of carbenoxolone would allow most hearts to restart. Thus, it is
likely that the effect of carbenoxolone on the heart-beat is reversible at all
concentrations.

4.4.2 Is Carbenoxolone (CBX) a Reversible Blocker of Gap Junctions in the
adult D. melanogaster Heart?

Evidence from video analysis of the heart-beat shows that the effect of carbenoxolone
is concentration-dependent; the reduction in the heart-beat’s rate is smaller at low
concentrations than at high concentrations. A dose-response curve calculated from this
data shows that the EC$_{50}$ of the heart-beat’s rate is approximately 0.08 mM. This is a
similar concentration to the EC$_{50}$ of carbenoxolone calculated from other preparations,
though slightly higher than in other preparations (Calkins and Piermarini, 2015; Miriyala
et al., 2018; Sangaletti et al., 2014). Any increase in the EC$_{50}$ may be a consequence of
applying carbenoxolone to the entire semi-intact preparation, and an intact heart tube;
the actual concentration of carbenoxolone within the heart tissue may be lower.

Application of carbenoxolone to the heart abolishes the cardiac myocyte action
potential and the sub threshold inputs to cardiac myocytes. This is consistent with
carbenoxolone blocking gap junctions, which would prevent the spread of electrical
events between cardiac myocytes. In addition, carbenoxolone produces an increase in the input resistance of cardiac myocytes as measured through the injection of negative current pulses. Again, this is consistent with blocking gap junctions, which reduces the cellular input resistance even at negative potentials (e.g. Moreno et al., 1994; Palacios-Prado and Bukauskas, 2009) when most voltage-gated channels are closed (reviewed in Hille, 2001).

Innexin gene products (innexin2 and innexin3), which are known to form oligomeric gap junctions when co-expressed (Stebbings et al., 2000), are expressed in the proteome of the adult *D. melanogaster* heart as determined by mass spectroscopy (Cammarato et al., 2011). This suggests that gap junctions are present in the heart, likely between cardiac myocytes allowing transmission of electrical signals.

This evidence is not, however, sufficient to unequivocally demonstrate that carbenoxolone blocks gap junctions in the adult *D. melanogaster* heart. The slowing and stopping of the heart are produced by a range of pharmacological agents, including the voltage-gated Ca\(^{2+}\) channel blocker verapamil (see Chapter 3). It could be argued that, a voltage-gated Ca\(^{2+}\) channel blocker would also abolish cardiac myocyte action potentials and subthreshold inputs to cardiac myocytes. Moreover, carbenoxolone is known to have several ‘off-target’ effects, including on voltage-gated Ca\(^{2+}\) channels (e.g. Bramley et al., 2011; Buckley et al., 2021; Connors, 2012; Vessey et al., 2004). The increase in the input resistance of cardiac myocytes following carbenoxolone application occurs even in the presence of verapamil, a voltage-gated Ca\(^{2+}\) channel blocker (preliminary observation), suggesting that the impact of carbenoxolone on the adult *D. melanogaster*
heart is not an ‘off-target’ effect on voltage-gated Ca$^{2+}$ channels. Nevertheless additional evidence is needed for an unequivocal proof that carbenoxolone blocks gap junctions in the adult D. melanogaster heart. Two forms of proof are important in this context. Firstly, demonstrating dye coupling between cardiac myocytes suggests that there are gap junctions present, and the prevention of this by carbenoxolone. Secondly, the injection of current into one cardiac myocyte that is detected in a neighbouring cardiac myocyte, and the prevention of this by carbenoxolone. Both dye coupling and current injection have been used to investigate gap junctions in insects (e.g. Dermietzel et al., 1990; Furshpan and Potter, 1959; Levin, 2002; Loewenstein, 1981). Without such proof, carbenoxolone can only be considered a putative blocker of gap junctions within the adult D. melanogaster heart.

4.4.3 Is Carbenoxolone (CBX) a Blocker of Protostome Gap Junctions Formed from Innexins?

Numerous studies have investigated the gap junctions in invertebrates, in a broad range of tissues, and across a range of developmental and life history stages (reviewed in Güiza et al., 2018; Panchin, 2005; Phelan, 2005; Phelan et al., 1998; Phelan and Starich, 2001; Skerrett and Williams, 2017; Söhl et al., 2005). Gap junctions have been studied in cnidarians (Takaku et al., 2014), nematodes (Jang et al., 2017; Starich et al., 1993), and numerous arthropods (e.g. Anava et al., 2009; Giuliani et al., 2013; Phelan et al., 1998; Shruti et al., 2014). Several recent studies have assessed the effects of carbenoxolone on invertebrate gap junctions (e.g. Calkins and Piermarini, 2015; Miriyala et al., 2018;
Sangaletti et al., 2014). These include studies on the nematode worm *Caenorhabditis elegans* (Sangaletti et al., 2014), the yellow fever mosquito *Aedes aegypti* (Calkins and Piermarini, 2015), and the bumblebee *Bombus terrestris* (Miriayala et al., 2018). For example, Calkins and Piermarini (2015) used carbenoxolone on mosquitoes, testing its efficacy as a putative insecticide alongside another putative gap junction blocker, mefloquine. Both carbenoxolone and mefloquine were effective in killing (or preventing flight) in mosquitoes; mefloquine was more effective than carbenoxolone at lower concentrations when applied directly to haemolymph, but carbenoxolone was more effective when applied to the cuticle. There was also a reduction in mosquito excretion when treated with gap junction blockers (Calkins and Piermarini, 2015). These pharmacological effects were also juxtaposed with RNAi knockdown of innexin gene products, but Calkins and Piermarini (2015) present no evidence that carbenoxolone or mefloquine are indeed blockers of mosquito gap junctions. Likewise, Miriyala et al. (2018) applied carbenoxolone to pairs of gustatory receptor neurons to block gap junction-mediated communication between them, but no evidence that carbenoxolone blocks gap junctions in bumblebees.

The effects of carbenoxolone in many invertebrate preparations and assays are consistent with an ability to block gap junctions. However, none of these studies provide unequivocal evidence that carbenoxolone is indeed a blocker of innexin gap junctions. Instead, this function is assumed because of the role of carbenoxolone as a vertebrate gap junction blocker. However, vertebrate gap junctions are formed from connexins, a distinct class of proteins from innexins. Consequently, the evidence that carbenoxolone
acts as a blocker of vertebrate gap junctions formed from connexins (Vessey et al., 2004) is not necessarily applicable to protostome gap junctions formed from innexins.

Our evidence suggests that carbenoxolone is likely to be a reversible blocker of gap junctions within the adult D. melanogaster heart (see above). Together with studies showing that carbenoxolone blocks connexin gap junctions between cells in vertebrate tissues, and the broad range of studies showing effects on a large number of tissues that are known to express innexins that likely form gap junctions between cells, our findings from the adult D. melanogaster heart raise the possibility but do not unequivocally establish that carbenoxolone is capable of blocking protostome innexin gap junctions. Establishing that carbenoxolone is a blocker of innexin gap junctions would provide a major tool for the acute physiological study of the role of gap junctions in a broad range of tissues from a broad phylogenetic range of protostomes, including nematode worms and arthropods (Phelan et al., 1998; Phelan and Starich, 2001; Guiza et al., 2018).
CHAPTER FIVE

5 GENERAL DISCUSSION

5.1 CONTRIBUTIONS OF THIS THESIS

At the start of the work that contributed to this thesis, we sought to understand the semi-intact preparation for the study of the heart-beat and cardiac myocyte action potential of the adult *Drosophila melanogaster* heart. We also sought to make contributions to the understanding of the heart-beat and cardiac myocyte action potential themselves. These are important issues because the heart of adult *D. melanogaster* is increasingly used as a model system in which to study the fundamental molecular and genetic mechanisms (Bier and Bodmer, 2004; Piazza and Wessells, 2011). As a matter of fact, numerous authors have advocated that the *D. melanogaster* heart has the potential to provide vital insights into the functioning of cardiac myocytes because of the molecular and genetic similarities with the mammalian heart, and because of the tools available for reporting and manipulating gene expression.

This thesis makes novel contributions to understanding both the semi-intact preparation for the study of the heart-beat and cardiac myocyte action potential, as well as to the understanding of the heart-beat and cardiac myocyte action potential more generally. Considered as a whole, this thesis provides the first evidence that there are substantial individual differences among adult flies in terms of both their heart-beat
and the response of this heart-beat to either environmental factors (temperature) or pharmacological treatments (carbenoxolone). This is, to our knowledge, the first example of such individual variability within the performance and physiology of the *D. melanogaster* heart or in any insect heart. Importantly, this suggests that even in individuals with identical environments during their development and growth, and similar genomes, the heart can differ substantially. These differences in the heart could arise from the electrical membrane of the cardiac myocytes, the molecular machinery associated with contraction including the structure and composition of the sarcomere, and/or the cells that generate the heart beat in the posterior region of the heart tube.

In this thesis, we have presented some evidence that the main depolarising conductance that generates the action potential is a voltage-gated Ca\(^{2+}\) conductance, though our findings are equivocal about the specific gene/s encoding it. We have also provided evidence that gap junctions between cardiac myocytes are essential for the transmission of the cardiac myocyte action potential throughout the heart. We identify carbenoxolone as a putative gap junction blocker, which has broad implications not just for the study of the *D. melanogaster* heart but more broadly to the study of gap junctions in formed from members of the pannexin gene family.

In Chapter 2, we explore the semi-intact preparation that has been widely used for the study of the adult *D. melanogaster* heart-beat. In line with many other studies (*e.g.* Ocorr *et al.*, 2017), we find that this preparation allows the heart-beat to continue for prolonged periods. Indeed, the heart is often still beating after an hour when perfused with oxygenated saline. We develop a novel software app to permit the quantification of
the heart-beat’s frequency and variability from the videos we capture of the beating adult heart. We find that there are marked individual differences in the heart-beat of wild type flies that cannot be attributed to differences in the preparation or in environmental conditions during development and growth. We find that these differences extend to the response of individuals to changing temperature, with individual hearts showing markedly different responses to increases or decreases in temperature. By developing a novel electrode holder, we show it is possible to obtain high quality recordings from the adult *D. melanogaster* heart while it is beating. We show that there are differences in the cardiac myocyte action potential among individuals and among cells within individuals. We also identify action potentials with double spikes that, to our knowledge, have not previously been reported in any electrophysiological study of the *D. melanogaster* heart. We show that such ‘double’ action potentials can be generated in cardiac myocytes that also generate more typical single action potentials, and that the switch depends on the level of depolarisation experienced by a single cardiac myocyte.

In Chapter 3, we examine the role of voltage-gated Ca\(^{2+}\) channels on the heart-beat of *D. melanogaster* and the cardiac myocyte action potentials. We show that the replacement of calcium with barium in the artificial haemolymph causes a large reduction in the heart-beat’s frequency despite preventing voltage- gated Ca\(^{2+}\) channel inactivation. We also show that verapamil, which is known to block voltage-gated Ca\(^{2+}\) channels, reduces the heart-beat’s frequency at low concentrations and stops the heart at higher concentrations. By comparing the heart-beat’s frequencies of wild type, *Cacophony* and *Paralytic* flies, we show that these mutations do not affect the heart-
beat’s frequency. We further show that wild type, Cacophony and Paralytic hearts respond similarly to verapamil, suggesting that these mutations are not involved in the generation of the cardiac myocyte action potential. Using RNAi constructs expressed through the GAL4- UAS system (Brand and Perrimon, 1993), we test the impact of RNAi knockdown of L-type and T-type voltage-gated Ca\textsuperscript{2+} channels in the heart. We compared flies bred at 25°C that express these constructs strongly with those bred at 18°C (only weakly expressing these constructs) and then subsequently switched to 25°C as adults. This should allow us to separate the effects of developmental knockdown with that of knockdown post-development. Unfortunately, this experiment showed no apparent differences in any of the RNAi lines despite previous reports of a strong effect of knockdown on the heart-beat’s frequency (Limpitikul et al., 2018). Finally, we show that forskolin, a known activator of adenyl cyclase (reviewed in Alasbahi and Melzig, 2012), causes a substantial upregulation of the heart-beat’s frequency that is consistent with protein kinase A-mediated phosphorylation of voltage-gated Ca\textsuperscript{2+} channels in cardiac myocytes.

In Chapter 4, we investigate the effect of carbenoxolone on the heart-beat of D. melanogaster and the physiology of individual cardiac myocytes. Carbenoxolone is a known blocker of connexin gap junctions (Rozental et al., 2001). Although it is used in numerous publications as a gap junction blocker of protostome invertebrates (e.g. Miriyala et al., 2018), such as nematodes and insects, we are not aware of an unequivocal demonstration that it is a blocker of pannexin gap junctions. We show that at low concentrations carbenoxolone reduced the heart-beat’s frequency of the adult D. melanogaster heart, and that at high concentrations it is capable of stopping
the heart-beat. At low concentrations, the effect of carbenoxolone on the variability of the heart-beat depend on the individual variability before application. The effects of carbenoxolone are reversible, at least at low concentrations. We show that two features of cardiac myocytes are consistent with strong gap junction inputs: synchronous action potentials in pairs of cardiac myocytes as revealed by paired electrophysiological recordings, and large depolarisations that trigger action potentials and that become subthreshold when the cardiac myocyte is hyperpolarised. Electrophysiology showed that carbenoxolone causes a rapid cessation of the cardiac myocyte action potential consistent with stopping the heart-beat. By adding verapamil, a known blocker of voltage-gated Ca\textsuperscript{2+} channels, we show that carbenoxolone causes cardiac myocytes to undergo a rapid increase in input resistance that cannot be explained through an ‘off target’ effect on voltage-gated Ca\textsuperscript{2+} channels.

5.2 The Importance of Interindividual Variability in the Adult *Drosophila melanogaster* Heart

Our findings from both Chapter 2 and Chapter 4 demonstrate that individuals differ in terms of their heart-beat’s frequency, and that individual cardiac myocytes differ in terms of the height and width of the action potential. Such individual differences have not been reported in previous studies of the adult *D. melanogaster* heart to our knowledge. The reason for this absence is likely due to three factors: (1) it has been assumed that all individuals, whether wild type or mutant, are similar and the differences in reported
measures of heart parameters (e.g. heart-beat’s frequency or contraction strength) are the result of random processes; (2) repeated measures of -eats have not been made either over the short or longer term; and (3) the statistical analysis performed in previous studies would be insufficient to detect individual differences. The combination of repeated measures coupled with an appropriate method of statistical analysis is crucial for the detection of individual differences in performance of an organ/tissue, physiological parameter, or behaviour. Statistical models must be able to incorporate ‘individual’ as a random factor, in addition to the typical fixed factors included in most statistical analysis. Such an approach requires generalised linear mixed models.

Generalised linear mixed models are widespread in studies of behaviour and ecology but are rare in physiology. Indeed, the concept of individual repeatability in behaviour is widespread within studies of behavioural ecology, though it is often termed ‘personality’ (e.g. Sih et al., 2015; Wolf and Weissing, 2012). Individual repeatability has previously been reported for adult D. melanogaster walking behaviour (Videlier et al., 2019). However, there appears to be limited repeatability in grooming behaviour in individual flies (Mueller et al., 2020). This suggests that the repeatability we observe in the adult heart may be part of a broader suit of behavioural and physiological traits in D. melanogaster that differ among individuals and are repeatable, but that not all behavioural or physiological traits that differ among individuals should be expected to be repeatable.

Many studies of behaviour that report repeatable individual differences assume that such differences arise in natural populations either through differences
developmental environments or genetics. The differences that we observed among individual *D. melanogaster* hearts cannot be attributed to substantial differences in developmental environments because they lived in the same vials at the same temperature, with similar access to food, and with similar numbers of other individuals. However, developmental environments can have profound effects on behaviour, physiology and morphology even when differences superficially appear similar (West-Eberhard, 2003). Genetic differences may be present because mutations can accumulate in laboratory populations (Stanley and Kulathinal, 2016). Thus, it is possible that the differences we observe are the consequence of very small differences that arise during development or that they are due to genetic differences. Irrespective of its origin, it is important to recognise that we have only detected short term repeatability. Determining whether this repeatability extends to the longer term is important for understanding its implications for the *D. melanogaster* heart.

### 5.3 Towards the Development of a Preparation for Studying Electrical Events in the Adult *Drosophila melanogaster* Heart

A major aim of this thesis was to make a contribution to the development of electrophysiological techniques that can be used to study the electrical membrane properties of individual cardiac myocytes in the adult *D. melanogaster* heart. At the start of the thesis we envisaged that this would be through the development of an isolated cardiac myocyte preparation. Such a preparation would have the advantage that the
rhythmic activity of the cardiac myocytes would be prevented because this activity is due to the presence of central pattern generators located in the posterior region of the heart tube and the conical chamber that generate the anterograde and retrograde rhythms, respectively (Sláma, 2010). However, during the thesis a study was published that developed such a preparation and showed that it can be used to record the membrane properties of cardiac myocytes using whole cell patch clamp techniques (Limpitikul et al., 2018). In response to this publication, we altered our strategy to find a way to permit high quality sharp intracellular recordings of the electrical properties of the cardiac myocytes in the intact heart.

To this end, we explored the possibility of using a gap junction blocker to electrically isolate cardiac myocytes. Consequently, in Chapter 4 we tested whether carbenoxolone, a known blocker of connexin gap junctions in the deuterostome lineage (Rozental et al., 2001), is a suitable pharmacological agent to block gap junctions between cardiac myocytes in the *D. melanogaster* heart. We found that carbenoxolone has numerous features that are consistent with blocking the gap junctions in the *D. melanogaster* heart and is capable of stopping the heart-beat. Once applied, carbenoxolone permits the recording of cardiac myocyte responses to negative current pulses that allows the input resistance to be measured. In addition, the injection of positive current pulses can reveal the properties of voltage-gated currents in cardiac myocytes and can be sufficient to trigger action potentials without inputs from neighbouring cells through gap junctions (preliminary findings). This would allow complementary recordings of the electrical membrane properties of cardiac myocytes through both whole cell patch clamp in isolated cells and through sharp intracellular
electrodes in the intact heart. Moreover, both techniques allow the application of additional pharmacological agents that block specific channels, such as verapamil or nifedipine that block voltage-gated Ca\textsuperscript{2+} channels. The combination of both in vivo and in vitro techniques should allow for accurate measurements of currents and conductances and permit computational modelling of the individual properties of cardiac myocyte membranes. Such can approach has been particularly effective for studying the properties of photoreceptors in D. melanogaster, in which isolated photoreceptors can be whole cell patch clamped, intact photoreceptors recorded using sharp intracellular electrodes, and the results from both combined into computational models. We anticipate that the ease with which carbenoxolone can be applied to the semi-intact heart will make this a useful technique for future studies of the D. melanogaster heart.

5.4 OPEN QUESTIONS AND FUTURE WORK

The aim of this thesis was primarily to make contributions towards our understanding of the adult Drosophila melanogaster heart-beat that would further its use as a model system to understand the functioning of the cardiac systems, their physiology, and their molecular and genetic components. The development of the D. melanogaster heart as a model system has major implications for the reduction or replacement of preparations involving vertebrates. Although there are numerous questions raised by this thesis, many are highly relevant to this central aim and so we focus on those in the text below.
A primary question must be; are individual differences in heart-beat maintained over the long term? We observed individual differences among wild type flies in terms of the frequency of their heart-beat. These differences were repeatable indicating that they are maintained over the short term in the semi-intact preparation. These differences are important both for the response of the heart to environmental perturbations (e.g. temperature). Such individual differences have not previously been documented for the *D. melanogaster* heart. Indeed, studies of the heart have not considered individual repeatability in any context, to our knowledge. Instead, they have assumed that all flies within a population are identical, and that differences among individuals in the parameters of the heart-beat such as frequency or strength of contraction are the consequence of random variation. This is not the case for all studies of behaviour and physiology, however, and several insects including *D. melanogaster* are known to show repeatable behaviour and physiology (e.g. Videlier *et al.*, 2019). Individual differences are likely to have important implications for physiology, but may also allow the *D. melanogaster* heart to become a model system for the study individual differences, which is likely to be important for the study of individual differences in other physiological systems including that of mammalian hearts. Given this importance, it seems essential to determine whether these differences are maintained over the longer term remains unknown. One possibility is to image the adult heart through the cuticle (e.g. Andersen *et al.*, 2015), allowing multiple recordings spread over days or weeks in adult flies. Additionally, imaging the heart through cuticle would allow the heart-beat to be assessed before and after performing the dissection of the semi-intact preparation, revealing differences caused by the dissection. If individual differences are maintained
in the long term, then this must be considered as an important source of variation that should be considered in future studies.

A second question linked to the previous one is: how do the differences among individuals arise? The wild type flies that we studied in Chapter 2 were exposed to a similar developmental environment in terms of the vials in which they live, the amount of food they receive, temperature and the numbers of individuals with which they can interact. Although developmental environments can have profound effects on behaviour, physiology and morphology (West-Eberhard, 2003), the wild type flies we studied were exposed to similar environments throughout their lives. They are also likely to have similar genetic backgrounds. Given these similarities, the presence of individual differences may seem surprising. However, such individual differences are common within physiological systems. For example, differences among individuals are common even within single identified homologous neurons (e.g. Goldman et al., 2001). If such differences can arise in a heart tube with relatively few cardiac myocytes, it is likely that such differences also exist within mammalian hearts. Understanding how individual differences arise is likely to be more possible within D. melanogaster than in mammalian systems due to the genetic tractability and the ability to study heart development in the embryo, during larval growth and pupation, and in the adult. Non-invasive techniques may be particularly important in both detecting early differences in heart function and allowing them to be related to adult function.

A still unanswered question is: to what extent does the action potential differ among cardiac myocytes, and how does this influence heart function? Our
electrophysiological recordings of the cardiac myocyte action potential suggest that there is considerable variability among individuals and among cells within individuals. In part, this variability may be the product of shifts in the overall activity of the heart, which may be influenced by neuromodulators such as octopamine. Indeed, our own data using forskolin suggests that activation of the protein kinase A pathway is capable of considerably increasing the heart-beat’s frequency. Understanding the origins of individual variability in the cardiac myocyte action potential, and its interaction with broader mechanisms that affect the entire heart is likely to be important. Determining how the electrical properties of a single cardiac myocyte influences the overall heart-beat may be achievable using the adult *D. melanogaster* heart, where there are relatively few cardiac myocytes (in comparison with mammalian hearts) that can be recorded individually and in pairs. It will also be essential to understand how the action potential of single cardiac myocytes related to the simultaneous movement of the same region of the heart.

A further question is posed by the thesis: is carbenoxolone a general blocker of pannexin gap junctions? We have shown that carbenoxolone, a known blocker of connexin gap junctions, is capable of stopping the adult *D. melanogaster* heart. However, we have also emphasised the need for a clear and unequivocal proof that carbenoxolone is a blocker of pannexin gap junctions. Although numerous studies have used carbenoxolone as a gap junction blocker and some even use it as a confirmation that particular physiological properties can be attributed to gap junctions, to our knowledge proof is lacking. There is a clear need for using small molecular dyes that cross gap junctions, such as lucifer yellow, and paired electrophysiological recordings to
establish the presence of pannexin gap junctions. This would be most easily done within a lineage that expresses only pannexin gap junctions, a protostome lineage amenable to single cell electrophysiology such as an arthropod. The blockage of small molecule dye transfer and current pulses between cells by carbenoxolone would be sufficient proof, though off-target effects must always be considered when assessing the function of pharmacological agents. It may be possible to perform such experiments using the adult *D. melanogaster* heart because single cell electrophysiology is possible, and we have shown that paired recordings are feasible even from the beating heart. However, a potentially useful approach would be to use blebbistatin, a known myosin inhibitor (reviewed in Kovács *et al.*, 2004), that can be used to block the adult *D. melanogaster* heart-beat whilst leaving the cardiac myocyte action potential intact (Karen Ocorr *et al.*, 2017). This may facilitate paired recordings that allow for current and dye injection. The possibility of using carbenoxolone as an acute and reversible gap junction blocker would allow the adult *D. melanogaster* heart to be used for the study of gap junctions in cardiac systems, potentially allowing for the study of vertebrate diseases in which gap junctions are involved including atrial fibrillation. In turn, this may have implications for the reduction and replacement of vertebrates in the study of these diseases.


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