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3D heart cell culture model from Zebrafish larvae for cardiac research

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Dissertation

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III) Abbreviation

ADP	adenosine diphosphate
AP	action potential
ATP	adenosine triphosphate
BPM	Beats per minute
BSA	Bovine Serum Albumin
BSU	Biological supply Unit
CCG	Clinical Commissioning Groups
CCM	cardiomyocyte clock mutant
CF	Contraction Frequency
CM	Compact myocardium
CuSO4	Copper Sulphate
CVD	Cardiovascualr disease
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate-Buffered Saline
E2 media	Embryo 2 media
ECG	Electrocardiogram
EDV	End dyastolic volume
ESV	End systolic volume
FAK	focal adhesion protein Kinase
FBS	Fetal bovine serum
GRK	G protein-coupled receptor kinase
HBSS	Hanks balanced salt solution
HDAC	histone deacetylace
hES-DC	human embryonic stem cell-derived cardiomyocytes
I2	Iodine
IHC	international conference of harmonisation
iPS-DC	induced pluripotent stem cell-derived cardiomyocytes
MAPK	Mitogen-activated protein kinase
MEF2	myocyte enhancer factor-2
MS222	98% Ethyl 3-aminobenzoate methanesulfonate
NaOCl	Sodium hypochlorite
NAS	new active substances
NHGRI	National Human Genome Research Institute
OCT	Optimal cutting temeprature
RT	Room temperature
ZF	Zebrafish
ZFHA	Zebrafish heart aggregate
β-AR	β-adrenergic receptor

IV) Abstract

Abstract

Introduction

Zebrafish (ZF) provide an excellent platform for modelling human cardiac arrhythmias since they have comparable action potential rhythms and have high sequence identity to humans for a number of ion channels. Previous studies showed that fish heart cells can be spontaneously propagated from embryonic heart progenitor cells to a mature 3D myocardium *in vitro*, termed Zebrafish heart aggregates (ZFHAs). Although generated from ZF larvae, ZFHAs show similar cellular architecture to adult zebrafish in terms of sarcomeric structures and cell-to-cell connections. Numerous studies have assessed the contribution of adrenergic tone in maintaining resting heart rate in adult and larval ZF although little is known about the electrophysiology of ZFHAs.

Method

To help further validate ZFHAs as a cardiac model we assessed the distinct differences in ECG pattern, contraction frequency and size under adrenergic stimulation with phenylephrine. These observations were compared to a control group of ZFHAs grown under normal culture conditions in high glucose DMEM. The development of ZFHAs involved the homogenisation of whole ZF larvae in accordance with schedule 1 methods stipulated by the home office. The first goal of this study was to distinguish the role of cardiac adrenergic receptor function in regulating contraction frequency (CF) and inotropic response in larval zebrafish compared to ZFHAs using the methods developed by Kopp et al., 2007. Cardiac function was observed using an inverted microscope and a high speed camera. Contraction frequency was measured by counting heart rate in a 20 second video and multiplied by three to get beats per minute (BPM). The size of aggregates was measured using imageJ calibrated against a 1mm graticule.

Results

ZFHAs in the control group showed gradual decreases in size and a gradual increase in CF between days 1 and 6 post homogenisation (d.p.h) Day1(Size=8191.9um^2 +- 1083.1, CF=45+-3 BPM) Day6 (Size7614.6um^2 +- 1428.3um^2 CF= 67+-12BMP) while the addition of phenylephrine caused an increase in size of ZFHAs and an increased trend in CF compared to control, Day1(Size=8191.9um^2 +- 1083.1, CF=52+-14 BPM) Day6 (Size7614.6um^2 +- 1428.3um^2 CF= 75+-22BMP). At 13 (d.p.h) a positive chronotropic change was observed in ZFHA in the presence of phenylephrine ($p=0.0026$, $n=12$) which aligned with a significant increase in size seen at 12 (d.p.h) ($p=0.0017$, $n=12$). Finally a positive correlation between size and contraction frequency ($r=0.2376$) in the presence of phenylephrine is seen, which was significant ($p=0.0415$, $n=74$) while control groups show no correlation between CF and size ($p=0.3333$, $n=253$). The chromogenic patterns of ZFHAs in response to phenylephrine showed positive inotropic responses as well as more defined ECG patterns.

Conclusion

These data suggest that ZFHA have similar developmental patterns of adrenergic receptors as larval ZF. It is also observed that adrenergic response to the agonist phenylephrine results in positive chrontropic and inotropic tone that maintains CF beyond 6 (d.p.h) and helps increase size. This response of ZFHAs to adrenergic stimulation could highlight important correlations between human and ZFHA response to chromptropic drugs developed for cardiac disease.

V) Declaration statement

Declaration Statement from Author

Portions of this work including and up to the entirety of this dissertation can be submitted in support of an application for another degree or qualification at the University of Manchester or any other university/institute of learning. The contents up to and including the entirety of this dissertation can also be published in any scientific journal. The conditions of submission of this dissertation have been agreed upon with the following supervisors:

- Dr Holly Shiels

Signature ..... Date 25/08/2016

- Dr Lisa Mohamet

Signature ..... Date 28/08/2016

I the author Jake Benjamin Ireland thus submit this dissertation under the above conditions stated in the deceleration statement

Signature  Date .....

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Manufacturing
Although the cell counting guides were not used in the end i would like to thank James Backhouse from the B15 Modelmaking Workshop at the university of manchester for taking the time to help design and produce some Jigs used for cell counting.

1 Introduction

1.1 Cardiac disease

Diseases affecting the heart and the circulatory system are known as cardiovascular disease (CVD). CVD was the second most common cause of death in the UK in 2014 and claimed the lives of 155,000 people (1 in 4 deaths) or 27% of all deaths in the UK. In addition to these mortalities 1.7 million episodes related to CVD and required hospital visits which places heavy burdens on the NHS. In the UK death before the age of 75 is considered a premature death and CVD caused 41,000 premature deaths in 2014. Since 1969 the CVD death rate has fallen by 74% across all age groups however, in recent years the number of hospital visits attributed to CVD has increased. When broken down into the types of cardiac disease its seen that since 2006 the highest increase in hospital visits of 30% is for diseases like arrhythmias, hypertension and other diseases related to ion channel disorders. Data collected from Clinical Commissioning Groups (CCGs) within the NHS in England has shown total cost of CVD to average £4.3 billion in 2014 with more than 370 million prescriptions being dispensed for CVD alone. Statistics from the BHF show this to be more than six times as many as issued in 1981. Since 1981 on average over 50% of all prescription are for drugs associated with ion channel manipulation such as β-blockers, positive inotropic drugs like Digoxin, anti-arrhythmic and anti-hypertensives. The increased prevalence of CVD in recent years correlates with the results of many lifestyle studies which indicate smoking, poor diets, lack of physical activity and increased alcohol consumption have contributing factors. These habits have proven consequences of obesity, high blood pressure, diabetes and higher than normal blood cholesterol (1). Education about the adversities of bad lifestyle habits has had a large role in the reduction of CVD since 1969, but the increased prescription numbers also indicate advances in understanding ion channel disorders and the development of pharmacology to delay or revers the effects of CVD has helped increase life expectancy also. The need for more advanced pharmacology means the need for novel drugs is an ever increasing demand. Although technology can improve computing power and analytical abilities pharmacology safety testing still requires the involvement of *in vivo* and *in vitro* models, and thats where Zebrafish come in.

1.2 Why zebrafish

Dr George Streisinger's gene linkage studies made the cover of the the 1981 issue of nature ("Cloned Zebrafish") which established a research community focusing on the developmental and genetic studies of the zebrafish (2). This community of researchers lead to the Systematic large-scale forward genetic screens for embryonic lethal mutations in Tübingen, Germany, and Boston, USA. This lead to the results of the 'Big Screen' being published in a single issue of Development, volume 123. There have been a large number of milestones since then including the establishment of the Trans-NIH Zebrafish Initiative which centralised a web-based database called ZFIN where all known mutations, and a whole genome sequencing project of zebrafish which began in 2000 (3). As the National Human Genome Research Institute (NHGRI) announced completion of the human genome project on April 14th 2003, models for human cardiac diseases quickly became desirable (4). Despite the pre-eminence of the rodent in modelling cardiac disease, several aspects of rodent electrophysiology limit its scope in modelling

normal ion channel activation and diseases affecting cardiac rhythm. Since 2003 There has been a large uptake in the genetics involvement of CVD epidemiology. An NCBI search of Genetics and CVD in 1998/99 revealed 7485 papers, a search as of today (August 2016) reveals 149515 papers with over 30,000 of those papers released in the last three years.

Zebrafish have primary been used to study organ development as gestational periods to a larvae are around 72 hours (5). There are a lot of features that make them a very desirable animal for studying heart disease. The embryos develop externally from there mother and because of there transparency you can visualise the heart directly. Genetic labelling and mutations can thus be observed directly which is also helped because ZFs small size means oxygen diffuses into their bodies in quantities sufficient to support early development (6). Compared to in vivo rodent models of cardiovascular development and disease, zebrafish are smaller and easier to house in large numbers, as well as having much larger numbers of offspring. Rodent models use in understanding ion channel function has aided in establishing electrophysiology in healthy and diseased states however when translating those findings to human biology there are limitations associated with rodent action potential (AP) patterns, cellular architecture and methods of observation. This stems form the high resting heart rate of rodent at around 500-700 beats per minute (bpm) to a human resting heart rate of between 60-70 bpm (7). ZF adults have a heart rate of 120-180 bpm which is more relatable to humans. There are also differences in rodents cellular architecture which have altered sarcomere structures and higher densities of ion channels (8). The action potential of a rodents humans and zebrafish (seen in figure 1.1) reveals maintained ventricular plateau phases in repolarization of the ventricle of in humans and zebrafish which is much shorter in rodents (9).

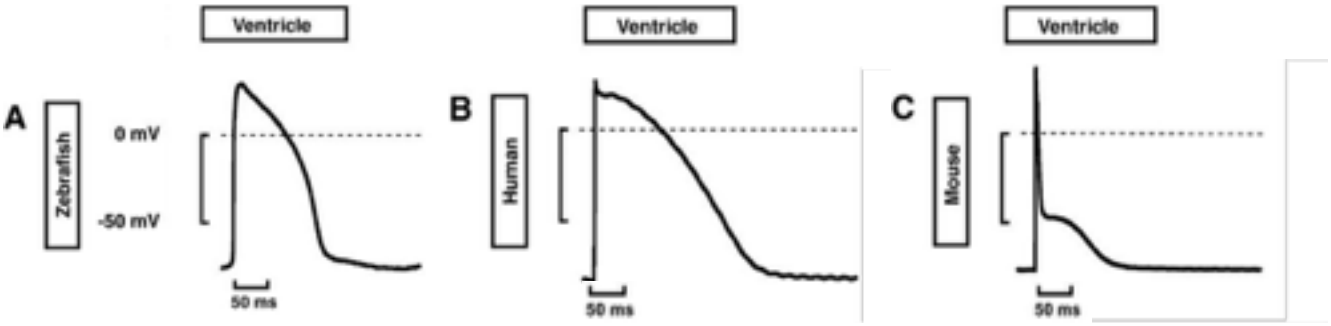


Figure 1.1: Ventricle Action Potential traces. A) Action potential of zebrafish ventricle. B) Action potential of human ventricle. C) Action potential of mouse ventricle. (adapted from MDE Research, 2014

1.3 Drug development

Drug development is an increasing field of research where the development of novel drugs need the reliable toxicology data to support them if they are to make it to market. European union directives like 86/609/EEC (10) and 2010/63/EU (11) focused on ensuring measures for the protection of animals used in science and research are established and that the difference between laws and regulations are not mistaken. The european agency for evaluation of medicinal products is an organisation dedicated to setting guidelines for the safe evaluation of new active substances (NAS) and indicates in certain guidelines the need for both in vivo and in vitro testing. A guideline published in 1997 by the commit for proprietary medicinal product (CPMP) (CPMP/986/96) indicates the potential QT as a secondary effect to some non-cardiovascular related drugs and shows that pre-clinical testing is a necessity for the development of drugs (12). Other guidelines like the 2005 guideline S7B by the international conference

of harmonisation (IHC) insists on the use of in vitro models (13). There are a number of cardiac models that are involved in safety pharmacology including human embryonic stem cell-derived cardiomyocytes (hES-DC) (14) and induced pluripotent stem cell-derived cardiomyocytes (iPS-DC) (15). These models are very expensive and currently a gap exists for a high throughput, cost and time effective cardiac models available for pre clinical testing. The indications by many papers is that once adequately validated, zebrafish have great potential for safety pharmacology testing as a way of identifying drugs that would have potential liabilities if selected for human trials (16).

1.4 Adrenergic receptor

β1-β2-adrenergic receptor (β-AR) agonist such as adrenaline or isoproterenol can have positive chronotropic and inotropic effects on the human heart. The mechanisms β-AR increase in contraction frequency involves the stimulation of linked G-protiens which increase adenylyl cyclase activity yielding higher levels of cAMP and thus increasing cardiac chronotropy and inotropy (17). Previous studies on adult ZF have shown there are abundant levels of β-AR and stimulation of these ARs has similar effects as in humans (18). Larval zebrafish begin to develop a chronotropic response to AR stimulation between 4 and 6 days post fertilisation (d.p.f) (19, 20) The roles of specific α and β-AR agonists and there effects in ZF beyond cardiac contraction frequency is poorly explored and less is known about larval ZF. Its seen that fish in general can have different affinities for AR receptors and a study by (21) showed that phenylephrine, a classic α-AR agonist, has similar binding abilities as noradrenaline dose for β-AR in catfish. Phenylephrine is often used in humans as a decongestant which causes local vasoconstriction (22). When administered through IV its effects involve increased blood pressure and reduced heart rate. Phenylephrine is said not to act directly on AR but is said to slow heart rate (bradycardia) through stimulation of vascular baroreceptors (23). There is contradiction to this form other studies by (24) which show phenylephrine increases heart rate due to the increases in blood pressure. A study by (25) shows that phenylephrine had a larger positive effect on increase contraction frequency in larval zebrafish than iosproteranol (a non-selective β adrenoreceptor agonist) (25). The results of this effect were discussed minimally in the paper but have indications that phenylephrine can have similar effect on cardiac tissue to β-AR.

1.5 Phenylephrine

Phenylephrine is a pharmaceutical that is an α1-adrenergic receptor agonist. Phenylephrine can be prescribed as a vasopressor to help increase the blood pressure of hypotensive patients by cause vasoconstriction through stimulation of α1-adrenergic receptors. Phenylephrine has be reported to have secondary effect of cardiac hypertrophy. Cardiac hypertrophy is normally considered an adaptive response to pressure or volume stresses in the heart which may be induced by the vasoconstrictive effects of phenylephrine. To understand how phenylephrine induces cardiac hypertrophy its important to identify the molecular events involved in the hypertrophic process. Phenylephrine's stimulation of the G proteins (Heptahelical receptor) has the initial effects of up regulating ATK through PI3K. ATK has the effect of silencing GSK-3 and promoting its translation to the cytoplasm. The use of phenylephrine can induce hypertrophy through the phosphorylation and silencing of GSK-3. Phosphorylation resistant mutants of GSK-3 become resistant to phenylephrine induced hypertrophy (26). Phenylephrine also acts to promote the activation of NF-AT transcription factors through the PLC intiatied release of calcium from the sarcoplasmic reticulum. Previous studies have shown that stimulation of the calciunurin/NF-AT

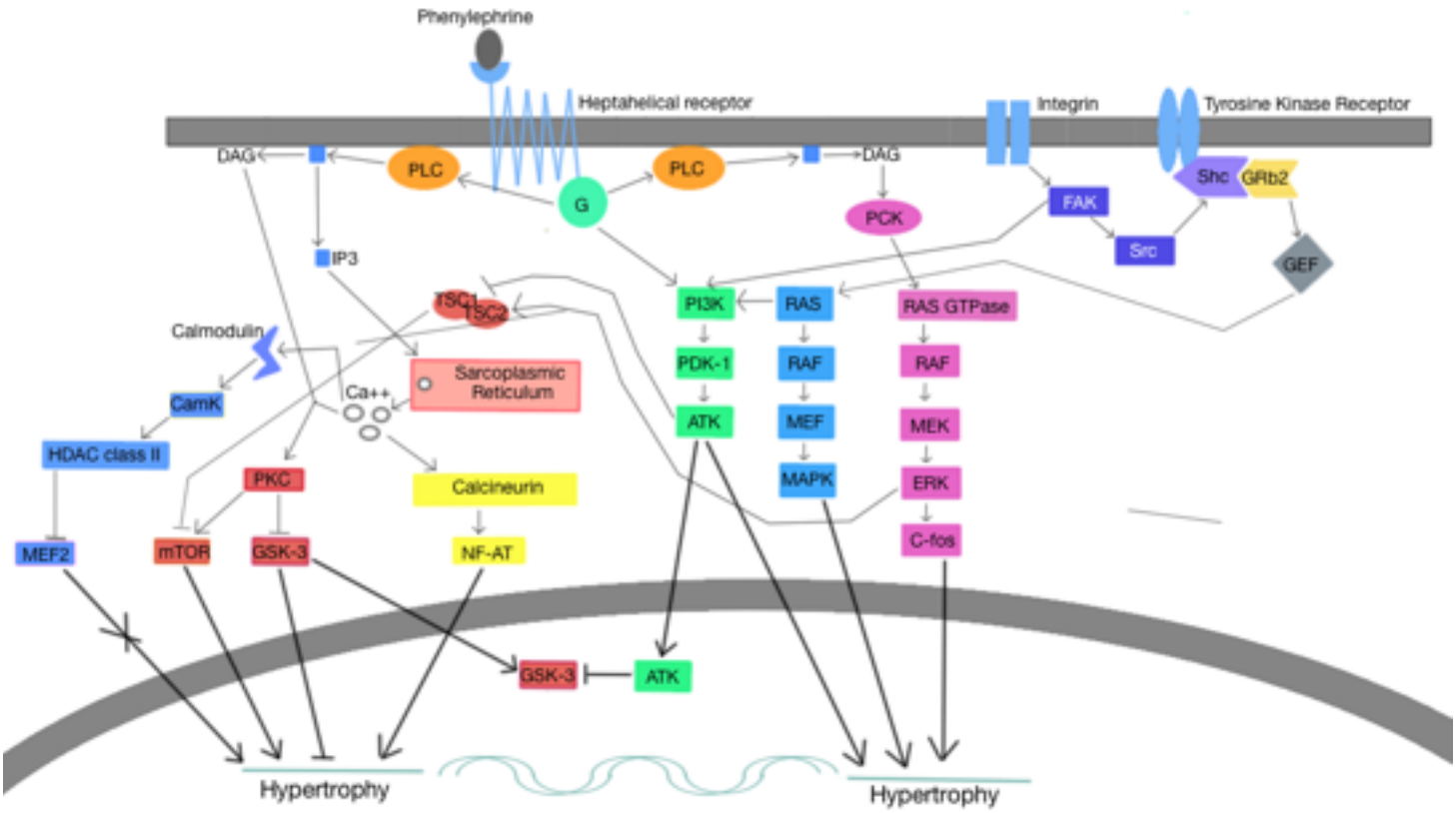


Figure 1.2: Illustration of the molecular pathways involved in phenylephrine induced hypertrophy. Stimulation of G-coupled proteins, integrin and tyrosine kinases can all lead to nuclear signalling associated with cardiac hypertrophy

pathway is sufficient to develop cardiac hypertrophy (27). Overstimulation with phenylephrine unregulated the release of calcium from the calcinerium and induces the activation of NF-AT. The release of calcium form the SR also has the stimulatory effect on calmodulin. The stimulation of calmodulin has the effect of phosphorylating the histone deacetylace enzyme (HDAC II) with CaMK. The phosphorylation of HDAC II reduces it binding affinity to myocyte enhancer factor-2 (MEF2) and results in reduced expression of HDAC genes. Reduction in HDAC transcription can increase cardiac hypertrophy as they serve a brake to myocardial growth signals (28). Mutant Ras genes have long been associated with increase proliferation in many types of cancer via Ras/Mitogen-activated protein kinase (MAPK) signalling (29). Phenylephrine can act to overstimulate the Ras/MAPK pathway and thus increasing the amount of available MAPK to translocating to the nucleus. MAPK is a kinase enzyme related to cellular functions including proliferation, mitosis and cell survival (30). The RAS pathway can be stimulated through many membrane receptors as shown by the stimulation of the tyrosine kinase receptor which activates the MAPK pathway through the stimulation of GEF. The Ras pathway can also be stimulated via phenylephrine binding to α1-adrenergic receptors. Previous studies have shown that phenylephrine binding has large up regulation of C-fos in the nucleus (31). Previous studies state C-fos proteins show a likely associated with cardiac hypertrophy through hormonal signalling to affect cardiac function (32). A previous study Phenylephrine can also stimulate cardiac hypertrophy through intern mediated signalling of focal adhesion protein kinase (FAK). These FAK proteins activate other secondary proteins including Src which can phosphorylate tyrosine kinase receptors which can have downstreamm signalling effects

on cell growth (33). Figure 1.2 illustrates that phenylephrine has the ability to induce cardiac hypertrophy through a number of signalling pathways. Phenylephrine signalling is shown to be possible in zebrafish from the previous study by (25). This study looks at cardiomyopathy in developing embryos meaning that the hypertrophic effect of phenylephrine on zebrafish larvae has yet to be explored.

1.6 Metabolism

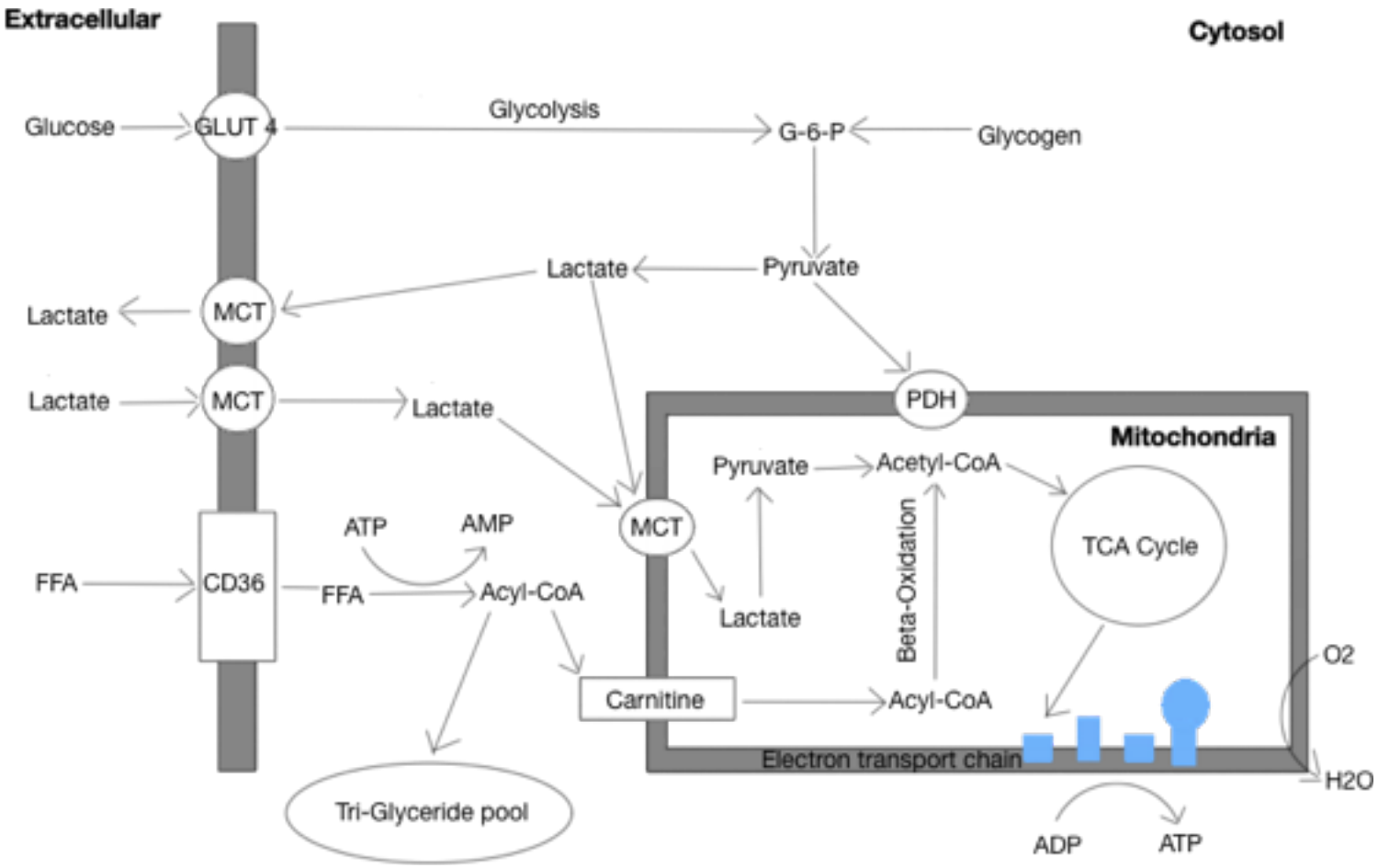


Figure 1.3: Cardiomyocyte metabolism. Diagrammatic representation of how a cardiomyocyte can utilise multiple substrates for metabolism.

Cardiac disease has a major association to metabolism and is often an overlooked factor in cardiac health. In recent years the gap between metabolism and cardiovascular health has begun to narrow with implementations of cardioprotective diets to reduce the onset of disease or to help minimise the effects of a cardiac event (34). Metabolism has been called the lost child of cardiology with a message that more needs to be known about the interplay between gene regulation and metabolism in the heart (35). Metabolism is the controlled regulation of energy release from fuels through mitochondrial oxidative phosphorylation where energy transfer produces a high energy phosphate bond between a phosphate ion and adenosine diphosphate (ADP). The heart is a continuously working organ meaning the production of adenosine triphosphate (ATP) must be continuously propagated. With limited ability to store energy in the form of triglycerides the heart must match the rates of ATP consumption with the rate of ATP production (35). The heart is a metabolic omnivore as it can use carbohydrates (glucose and

lactate), fatty acids, ketone bodies and even protein substrates as a fuel source (36) The metabolic flexibility of a cardiomyocyte is illustrated in figure 1.3. The heart can take these substrates, and in the presents of oxygen, can degrade them to produce ATP. The reasons why the heart has evolved to be a metabolic omnivore is inextricably linked to its continuos contraction and demand for energy. Cardiomyocytes also possess the ability to adapt to its substrate environment to select the most efficient substrate for ATP production (36). Cardiac metabolism can adapt based on factors like the circadian clock, the time of day including the light and dark cycles and the alternation between feeding and fasting periods (37, 38). The heart has the ability to possess "Metabolic memory" as shown in the diurnal fluctuations in metabolic flux seen in ex vivo rat hearts (36). Throughout the day the heart can anticipate, respond and even adapt to the metabolic availabilities which is lost in the hypertrophied heart (39). The adaption of metabolic flux in the heart can seen with alterations in cardiac rhythm based on the availability of fatty acids (40). A failing heart shows that the heart can have a preference for glycolysis as a metabolic source during heart failure (37) with further examples showing that metabolic flux can significantly reduce the utilisation and oxidation of fatty acids in cardiomyopathy (41). The ability of the heart to shift to carbohydrate metabolism is an important factor in cardiac hypertrophy. It is seen that the level of metabolic shift toward carbohydrate metabolism is actually proportional to the severity of hypertrophy while fatty acid oxidation has a negative correlation to the increase in ventricular wall thickening (42). The circadian clock has correlation to the O-GlcNAcylation of proteins which exhibit diurnal variations during periods of activity and rest. The removal of genes involved in circadian rhythms has the effect of variations in cardiac O-GlcNAc levels (43). Shift in light/dark cycles can have the effect of shifting the circadian rhythm of an organism which in turn has an effect on metabolic homoeostasis (44). Investigations using the cardiomyocyte clock mutant (CCM) mouse shows that the circadian rhythm can have effects on proteome expression and alterations in the vital metabolic pathways demonstrating that day and night cycles contribute to the metabolic abilities of the cardiovascular system (45). The hearts ability to shift its primary metabolic source is shown when the down-reuglation of fatty acid oxidative metabolism causes an up-regulation in glucose transporters and thus an ability to metabolise glucose (37). The metabolic flexibility of fed and fasting states has been reported (46, 37) showing the fasting state have 60-70% lipid oxidation and 15-20% carbohydrate metabolism. During fed periods this shifts and carbohydrates become the preferred source of energy with 50-75% carbohydrate metabolism and 20% due to lipid oxidative metabolism. The shifts in metabolic preference in fed and fasting states is insulin sensitive. Type 2 diabetes reduces a persons ability to be insulin sensitive and can alter cardiac metabolism as shown in previous studies (47). During gestation the embryonic heart has similar energy demands for contractility to aid in blood flow and oxygen diffusion. The heart is also developing with the proliferation of both muscle and non muscle tissues. Proliferating cells have a preference for glycolysis with lipid oxidation representing as little as 15% of metabolism. Glycolysis in proliferating cells could also be induced due to the hypoxic environment of the embryo (36). Evidence for the preference of glycolysis in hypoxic environments is supported by the work of (48) who shows the activity of the HIF-1a regulator is more abundant in hypoxic conditions. HIF-1a is a transcription factor which will bind to hypoxic response elements during hypoxia and up regulate the expression of glucose transporters (49)

When culturing cardiac tissue the common selection of metabolic substrate is glucose. The common use of high glucose medias means that cardiac tissue will have an abundance of glucose and has the ability to adapt to this environment in culture. Freeing cardiac tissue form an animal and studying ex vivo could cause cardiac damage in the same way cardiac failure dose. This may shift ex vivo tissue preferences to

carbohydrate metabolism. With a 3D cardiac aggregate of tissue perfusion of the inner tissue becomes a problem and hypoxia may induce the activation of HIF-1a and the up regulation of glucose transporters. These pathways may as well already be activated if the use of embryonic tissue is chosen. In short glucose is a good choice of metabolic substrate when culturing embryonic cardiac tissue however, glucose is not the only carbohydrate cardiac tissue can metabolise. Lactate has been labeled as the forgotten fuel in cardiology because of its low concentrations in the blood (1mM). Despite its low concentrations in resting states present in larger concentrations during active periods and in high stress situations like trauma or surgery (50). lactate is responsible for 30% of carbohydrate metabolism in the fed state and 10% in fasting states (36). Research into lactates abilities as a metabolic substrate in culture has focused on its abilities to purifying cardiomyocytes. This purification is achieved through starvation of other cell types that cannot utilise lactate. ZF have been shown to have similar lactate levels in the blood (51) indicating that lactate could be used as a good metabolic substrate for development of embryonic ZF cardiac tissue. Lactate may also be used as a potential purifying method of ZF cardiac tissue in culture.

1.7 ZFHA

ZF are a better cardiac model than rodents as they have similar contraction frequencies and action potential traces to humans. The preliminary work with ZF and rainbow trouts (*Oncorhynchus mykiss*) show that a 3D myocardium can spontaneously propagate from the separated cardiac progenitor cells of fish (52, 53). The demands for in vitro cardiac model in safety pharmacology show there is desire for a high throughput, cost and time effective cardiac model (54). Many drugs don't make it to market because they show undesirable effects during preclinical testing. Many current models are also ineffective or unreliable when translating the results to human biology. Some such models include human embryonic stem cell-derived cardiomyocytes (hES-DC) (14) and induced pluripotent stem cell-derived cardiomyocytes (iPS-DC) (55). These models are very expensive to make and don't represent adult cardiac tissue as the maturation of sarcomeres and other cellular architecture is limited (52). The development of a cardiac model called zebrafish heart aggregates (ZFHA) shows promising cardiac model representative of human cardiac tissue (54). ZFHAs have a unique ability to quickly mature sarcomere structures when freed form a ZF larvae which produces a phenotype more representative of adult ZF tissue than larval ZF tissue. The development of ZFHAs is built upon work form similar 3D cardiac models generated from salmonid larvae (rainbow trout) (54). The salmonid larvae aggregates mature into contractile syncytium that possess characteristic biomarkers, structural proteins and electrocardiogram data indicating they would make suitable in vitro assay systems (53, 54). Previous studies indicates that ZF may make a more reliable model of ion channel disorders in human (56). In addition to ZF being a more relevant model, there are a number of cardiac mutant lines (breakdance/ slomo) of ZF which could be used to make ZFHAs as well (57,58). The use of ZFHAs indicates they are a better cardiac model than current rodent models (54). They form a matured 3D spontaneously contracting syncytium which is more representative of cardiac tissue than current monolayer models (59) and finally they have similar cellular architecture and electrophysiology to human cardiomyocytes (54). ZFHAs show a future of inexpensive, easy to generate and high throughput cardiac models that could be adopted for cardiac safety pharmacology.

1.8 Study Aims and Objectives.

The overall aim of this study is to develop a better understanding of the characteristics of the relatively unexplored ZFHA. The development of a consistent protocol for the generation of ZFHAs should be established. The electrophysiology of ZFHA needs to be characterised to show that ZFHAs process the sustainability to be used as a in vitro assay for cardiac safety pharmacology.

This study is an experimental study with three experimental groups and one collective control group. The control group will use ZFHAs generated using a pestle & mortar with whole ZF Larvae suspended in trypsin/EDTA. ZFHAs generated in this way will be cultured in high glucose dulbecco's modified eagle medium (DMEM). The three experimental groups are based on the following questions:

1. Can the use of a classic alpha adrenergic receptor agonist “phenylephrine” be used to study the effect of alpha adrenergic receptor stimulation on ZF larvae and ZFHAs.
2. Phenylephrine causes cardiac hypertrophy in a number of organisms, can it also induce cardiac hypertrophy in ZF larvae and ZFHAs?
3. Can ZFHAs adapted the metabolic pathways to survive in glucose free DMEM supplemented lactate as an alternative metabolic food source.
4. Can a protocol be designed to generate ZFHA using a uniform technique that reduces the exposure of cardiac tissue to potentially damaging photolytic enzymes like Trypsin/EDTA.

The objective of the first question is to better understand the relationship cardiac tissue in ZF may have to alpha adrenergic stimulation. The association between beta adrenergic receptors and cardiac contraction frequency and force. Classic stimulation of these receptors in ZF with drugs like isoproterenol produce shortening of the ZF rhythm trace and increased inotropic forces. Previous studies in other fish species indicate that alpha adrenergic stimulation can have similar effects on chronotropy and inotropy.

The second objective will maintain the investigation of phenylephrine on cardiac tissue by looking at the hypertrophic effects it can have. Hypertrophy of cardiac tissue in humans is often associated with reduced cardiac function and often follows and ischemic event. In ZF embryos the growth of the heart is seen as proportional to the metabolic needs of the fish (60) however, the mechanisms of this growth are unknown. Cardiac growth in adult ZF is often a hypertrophic event induced by increased blood pressure and systolic volume. ZF embryo ventricles often start out as one cell layer thick and shortly after the chamber forms cells are proliferated for wall thickening. With previous studies showing increases in ZFHA size through the culture period it will interesting to better understand the hypertrophy effects phenylephrine may have on ZFHA growth. Induction of hypertrophy in ZFHAs is desirable because there microscopic sizes makes them difficult evaluate ECG data from.

ZF heart development focuses on the alterations of metabolic load to help increase stroke volume through bigger contractile forces rather than increases in contraction frequency. Lactate is commonly found in the blood and with cardiac tissue being a metabolic omnivore it should survive in culture on lactate alone. The third objective then is aimed at understanding how an alteration of metabolic food source may affect the growth and contractile abilities of ZFHA.

The current protocol involves homogenising whole ZF larvae into single or groups of cells with a micro tissue grinder. The purpose of this tissue grinder is to apply sheer forces via the fast movement of the solution the tissue is suspended in. In the current protocol this is trypsin/EDTA. The use of the tissue

grinder generates homogenates in a number of sizes and is inconsistent in the level of homogenisation the larvae are subject too. Development of a refined protocol is needed to help aid in the homogenisation of whole larvae to produce consistent sized homogenates without the loss or damage to cardiac tissue. The homogenisation of tissue using a collagenase enzyme instead of trypsin would help free the cardiac tissue form the ZF larvae by removal of the extracellular matrix without damaging the plasma membrane or connective membrane proteins between cardiomyocytes.

Study design protocols for each of the experimental groups can be found in Appendix I.

2 Materials & Methods

2.1 Ethical Statement

- The nature of the ethical review permissions.

The nature of the ethics involved in this study revolve around the correct housing and care of both adult and larval zebrafish, the methods of human killing and steps taken to reduce the stress of these animals during scientific procedures. All protocols using ZF throughout this study adhered to UK home office Animal (Scientific Procedures) Act 1986. All ZF larvae were collected and humanly killed between 1-5 d.p.f. Details of sterilisation and housing can be found throughout the material and methods section. Whenever ZF larvae were anaesthetised 98% Ethyl 3-aminobenzoate methanesulfonate (sigma) or commonly known as MS222 was used before homogenisation of the whole fish embryo was done using pestle&mortar or collagenase digestion. All adult ZF tissue was acquired from MS222 anaesthetised fish which had complete obliteration of the brain before dissection began.

Due to anaesthetisation and human killing of ZF before 5d.p.f no home office project or personal licence was needed or acquired. In the absences of home office project and personal licenses, Home office training for both the use of anaesthetic and the correct methods of humane killing was taught and reviewed every 12 weeks. All ZF eggs were laid and harvested and provided by the biological supply unit (BSU) breeding program at the University of Manchester. All adult ZF were anaesthetised and humanly killed by technical mangers with appropriate licensing and then delivered to us for dissection.

The housing and care of adult zebrafish at the university of manchester follows national guidelines set out in the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (61) and DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL (11). Institutional guidelines for care, housing and husbandry of zebrafish (*D. Rerio*) at UofM follows Reed&Jennings "Guidance on the housing and care of zebrafish *Danio rerio*" (62).

2.2 Study Bias

The topic of subject bias between groups was controlled by the random analysis of images and videos. Each image and video had a unique 8 digit alphanumerical ID which was separated from the group allocations during the analysis stages. Contractile frequency and size of aggregates was then measured without the unconscious assumption of how the aggregates would perform in each group.

2.3 Experimental Procedures

2.3.1 Zebrafish housing and husbandry

Adult zebrafish were obtained form the biological supply unit (BSU) (University of Manchester, UK) and are maintained in glass flow through tanks. The water entering these tanks has strict procedures for chlorine removal with carbon filters, Nitrate removal daily water dumps of 10%. In addition there are 2 different grades of filter for removing food waste and with a microbiological filter as well. Finally water is UV treated for killing bacteria. Snails are also used inside the tanks to reduce algae growth and aid with cleaning. These tanks are open air tanks containing 35 fish per tank with a male to female ratio of 3:4 respectively. Fish are fed Live artemia twice per day and ZM '300' powder once per day when used for

breeding. Marbles are used to encourage breeding as fish drop eggs over the marbles as it seems 'safe' to them, the marbles also protect the eggs from being eaten by the other fish. Eggs are collected 3-4 times per week with a separate tank each day with a max use of once per week. Besides the marbles while breeding the fish reside in a empty environment with no hides or covers.

2.3.2 ZFHA Generation

ZF larvae were collected 3-4 hours after fertilisation and sterilised before entering the lab. Larvae are sterilised twice at 6 and 24h.p.f with a buffered 100mg/ml sodium hypochlorite (BDH, UK) in DPBS x1 (w/o calcium or magnesium) (Sigma, UK). Larvae were then washed three times with DPBS after sterilisation. Larvae were hatched form there chorions 72h.p.f and split into groups of 15. Larvae where then aseptically homogenised through maceration of the whole embryo with a 0.2ml Wheaton™ Micro Tissue Grinders (Fisher Scientific, UK) submerged in 0.1% trypsin/EDTA at room temperature (RT). Before homogenisation takes place larvae are anaesthetised in 98% Ethyl 3-aminobenzoate methanesulfonate. The depth of anethstesia is assessed through the loss of voluntary moment and loss of reflex movement with the compressive rush of fluid from a pipette in the face and tail of the larvae. Cellular homogenates were then suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% heat inactivated-fetal bovine serum (FBS) (Gibco, UK). Antibiotics are supplemented to a 50ml aliquot of media to a final concentration of 200 units penicillin and 0.2mg streptomycin/mL (sigma, UK), 1mg/ml gentamicin, 0.005mg/ml Amphotericin B, 1mg/ml pipricillin and 0.01mg/ml Ciprofloxin. Homogenates from the 15 larvae were then centrifuged at 240rcf for 5mins. Cells were then resuspended in culture media and seeded into the well of a 24well plate with 1ml of media. Cells were then culture in 28°C at 2.5% CO_2 with a media exchange 24hours after seeding and every 48hours thereafter. Cell cultures were then observed for ZFHA every 24 hours.

2.3.3 Matrigel

Because of the ZFHAs having a 3D structure media exchanges often cause the aggregates to dissociate from the culture dish. To help ZFHA adhere, plate wells were coated with BD MatrigelTM Basement Membrane Matrix (BD Biosciences, UK) at concentration between 9-12mg/ml. Culture plates coated with matrigel were incubated at 28°C for 1hour to ensure an even coating. Plates would then be wrapped in Parafilm and placed in the fridge (4°C) for storage for up to 2 weeks.

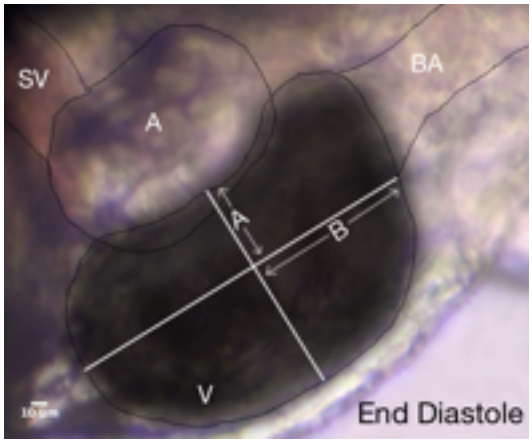
2.3.4 Stroke volume measurements

ZF larvae were collected 3-4 hours after fertilisation and sterilised (following the above protocol: ZFHA generation) before entering the lab. Larvae were then placed in embryo 2 (E2) media in 6 well plates (StarLab, UK). An experimental group was placed in E2 media supplemented with 0.1mM phenylephrine while the control group had larvae placed in E2 media only. Larvae were reared until they reach 120h.p.f (5days) at which point they are humanity killed with an overdose of MS222 at a concentration of 400ppm. Cardiac measurements were taken every 24 hours by horizontal visualisation of the ventricle when larvae are laid on there sides. Measurements of EDV and ESV of the ventricle were calculated from complete cardiac cycles of the ventricle captured in video using a sony high speed cam corder at 100 frames per second (FPS). The camcorder was attached to Nikon Ti-S inverted microscope. measurements of ESV, EDV and CF were taken every 24 hours.Determining ventricular perimeters and dimensions was accomplished using the fit to ellipse algorithm previously described in the literature (63, 64, 65, 66). This

algorithm identifies the centre of mass of the dawn perimeter and subsequently the best fitting ellipse. Major and minor axis were calculated as seen in figure 2.1. Values are collected and used in excel with he following equation for a prolate spheroid to determine ventricular volume (63, 65).

$$V = \frac{4}{3} \cdot \pi \cdot a \cdot b^2$$

Triplicates of EDV and ESV were taken every 20 seconds over a period of 1 minute to calculate mean stroke volume as the difference between ventricular EDV and ESV. CF as beats per minute (BPM) was measured and multiple with stroke volume to gather cardiac output. Larvae were kept in a fan assisted incubator set at 28°C with normal atmosphere and measurements were taken from larvae at an average RT of 23.49 ± 0.87°C.



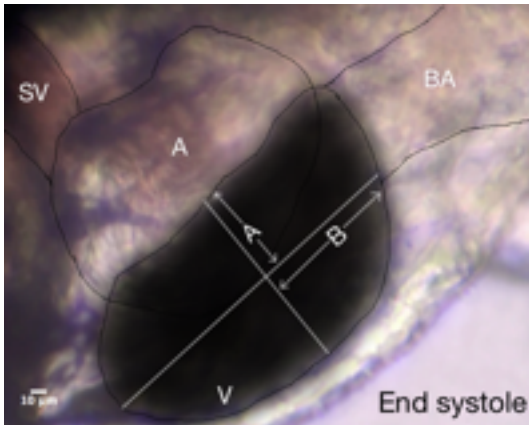


Figure 2.1: Example of Cardiac Ventricular volume measurements. A) Ventricular end diastolic volume measurement. B) Ventricular end systolic measurement.

2.3.5 Phenylephrine

All aggregates used for phenylephrine analysis were prepared as described above in ZFHA generation. Aggregates w placed in culture media supplemented with 1mM phenylephrine diluted (1:10 DMEM) to 0.1mM. This concentration was chosen based after a trials with other concentrations to determine a dose that induced a response on contraction frequency. This concentration is in keeping with other studies based on zebrafish larvae (67). Contraction frequency and size measurements were taken 24 hours after exposure to phenylephrine with daily media exchanges.

2.3.6 Lactate

ZFHAs used to measure lactate and metabolism were placed in DMEM culture media supplemented with 1% (v/v) of sodium-L-lactate 60% syrup (Sigma, UK). ZFHAs were cultured in lactate from homogenisation onwards. The concentration of lactate used was in keeping with other studies (68). Contraction frequency and size measurements were taken 24 hours after culturing in lactate with a media exchanges 24 hours after seeding and every 48 hours thereafter. Cell density was also measured after media exchange to asses the decrease in cell density with lactate as compared to control cultures.

2.3.7 Collagenase vs Trypsin/EDTA

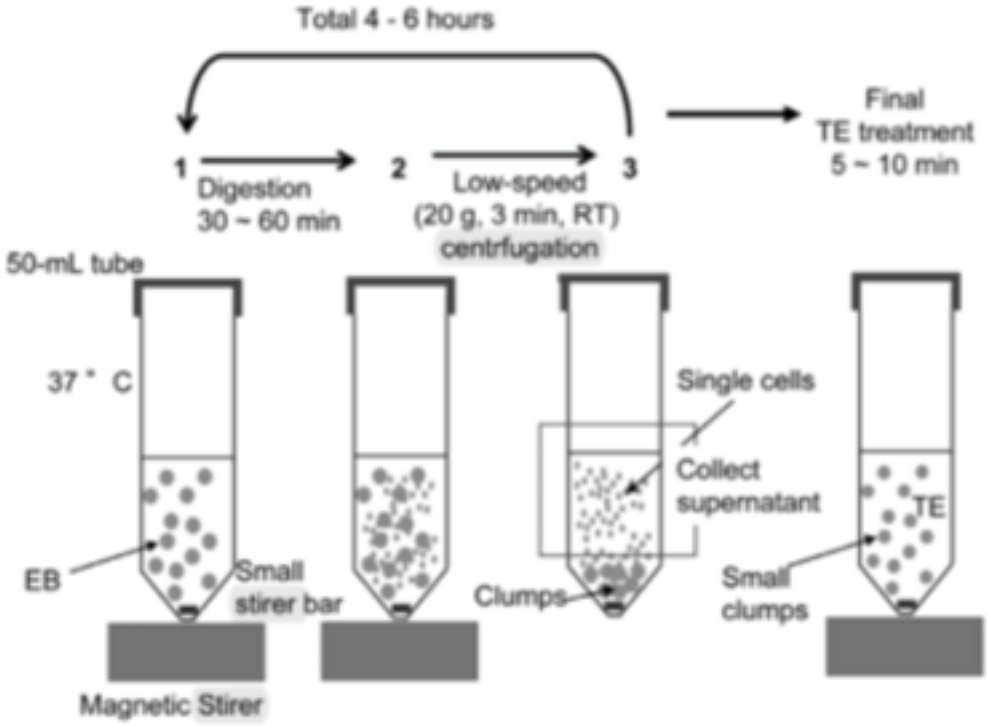


Figure 2.2: Image of collagenase method protocol: This diagram is borrowed from (Fukuda & Yuasa, 2013) and illustrates the protocol developed for collagenase digestion

Whole ZF larvae are first anaesthetised in MS222 until reflex movement is absent. Larvae are then quickly transferred to a 50ml fulcon tube containing 7ml of Hanks balanced salt solution (HBSS) with calcium and magnesium (Sigma,UK) supplemented with 7mg/ml Molar Collagenase type II (worthington, UK) and antibiotics at the following final concentrations 200 units penicillin and 0.2mg streptomycin/mL (sigma, UK), 1mg/ml gentamicin, 0.005mg/ml Amphotericin B, 1mg/ml pipricillin and 0.01mg/ml Ciprofloxin. Larvae are then vortexes with the aid of a magnetic stirring plate and a micro stirrer that fits in the bottom of the 50ml fulcon tube. Every 30mins the tube is centrifuged at 9.13rcf for 2mins to allow larger cell lysates to collect as a pellet while leaving smaller cells in the suspension. The supernatant is taken of and further centrifuged at 240rcf for 5mins. The collected cell pellet is then resuspended in 1ml of culture media and placed in an incubator with a gas permeable lid at 28°C and 2.5% CO2. The larger cell pellet in the 50ml fulcon tube is then resuspended in 7ml of collagenase supplemented HBSS to repeat the cycle again. This is repeated 6 times to ensure complete digestion and ultimate separation of the cellular homogenates into a consistent density. After six cycles if larger bodies of tissue still persist then the resuspension in collagenase is replaced with 0.5ml of trypsin/EDTA and allowed to vortex slowly for 1 minute. 3ml of media is then added to inactivate the trypsin and the cell pellet is collected and resuspended in culture media. The cell homogenates are then collected into one 15ml tube and centrifuged at 1000rpm for 5mins. The cell pellet is then resuspended in a volume culture media depending on the number of wells being seeded. The number of wells is based on the number of larvae used with 60 larvae being the maximum between four wells. 60 larvae is the set maximum based on the

need to have a collagenase solution between (0.1%-0.5% w/v) which will give between 50–200 U/mL of collagenase which will comfortable digest the weight equivalent of 60 larvae over 6 hours (Gibco, 2013).

2.3.8 Tissue Processing, Staining and Imaging

Tissue to be examined further for structural architecture includes Adult ZF whole hearts, skeletal muscle, whole larval ZF cardiac tissue and ZFHAs at days 4 and 6. Prior to embedding tissue is fixed for 5minutes in 10% Formalin (Sigma, UK) at RT and washed with DPBS. Tissue is then embedded in Optimal cutting temperature (OCT) compound (Tissue-Tek, NL). Tissue is then snap frozen in liquid nitrogen and stored in a -80°C freezer. Sectioning of the tissue is achieved using a cryostat (Cryotom Leica 350S) in serial 10µm sections set to 22°C and 26°C for chamber temperature and stage temperature respectively . Once adhered to adhesive microscope slides tissue is soaked in the following solutions with triple washes with DPBS after each solution, all steps are performed at room temperature (RT). Tissue is permeabilised using 0.1% Triton-X for 30minutes, blocking with 1% Bovine Serum Albumin (BSA) in DPBS for 1hour, staining with 0.1 uM rhodamine-phalloidin (TheromFisher Scientific, UK) Excitiation/Emission: 540/565 nm. Tissue on slides is then mounted using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, UK) Excitiation/Emission:360/460 nm. Slides are covered with rectangular cover slips, thickness 1.5 (ThermoFisher Scientific, UK) and sealed with generic nail polish. Images were collected on a Leica TCS SP5 AOBS upright confocal microscope using a 63x/0.90 HCX Apo L dipping lens objective with a range between 0 - 2.14x confocal zoom. The confocal settings were as follows, pinhole 1 airy unit, scan speed 1000Hz unidirectional, format 1024 x 1024. Images were collected using the following detection mirror settings; DAPI 410-479nm; Rodamine-Phallodin 551-667nm; Cy5 640-690nm using the 405nm (20%), 543nm (100%) and 633nm (100%) laser lines respectively. To eliminate cross-talk between channels, the images were collected sequentially. When acquiring Z-stacks the confocal software was used to determine the optimal number of Z sections with sections being 6um apart with 16% overlap between sections. Maximum intensity of images was attained by gain alterations to a point just below detection saturation.

2.3.9 Sterilisation

ZF eggs are disinfected when they enter the lab for two reasons, firstly is to eradicate protozoa that will have been transferred with the eggs as they are present in the system water. secondly is to reduce the overall abundance of bacteria and fungi that could potentially affect larval respiration and development (69, 70, 71, 72). The third and final reason is to eliminate pathogens and bacteria that may be present on the eggs that could eventually be transferred to the cell culture and potentially alter measurements of ZFHA. There is speculation about the over use of biocidal disinfectants and antibiotics regarding there potential to increased antibiotic resistance of bacteria (73). The common protocol for disinfecting zebrafish eggs involves washing eggs with DPBS 6h.p.f followed by a two cycle procedure of submerging for 5mins in bleach and then 5mins in PBS at 24h.p.f. The current protocol (74) uses a 306ppm Bleach dissolved in autoclaved dH2O. Once the eggs reach 24h.p.f the eggs are submerged in bleach for 5mins followed by a 5min wash in DPBS, this cycle is repeated twice.
There are are a number of bactrialcidal chemicals available that could be used for disinfection but it has become common place for sodium hypochlorite (bleach) to be used. Previous studies have indicated (75) the effectiveness of other compounds such as copper sulphate and iodine to be a better alternative for eradicating bacteria in the cell culture. Due to the persistence of a bacterial contamination in our primary

culture of ZFHA the disinfectant sterilisation steps of the current lab protocol were questioned and assessed. Two things the current protocol docent take into account is the pH of the bleaching solutions and the age of the zebrafish. Sodium hypochlorite often raises the pH of its solution and reduces the sterility efficiency of the bleach. Studies on other fish species show the resilience to disinfection is higher at early ages like 6h.p.f and often have less chance of developing deformations than if they were sterilised at an older age. As part of refining the protocol to find the most efficient disinfection all bleach is buffered in DPBS and eggs are disinfected at both 6 and 24h.p.f. The following protocols were assessed. The concentration of stock solutions were not assessed and all dilution calculations were based on label concentrations. Iodine (Sigma, UK), NaCl (RDH, UK) CuSO4 (RDH, UK)

At 6h.p.f eggs were submerged in the following:

1. 100ppm Iodine for 2.5mins - PBS for 5mins.
2. 100ppm Iodine for 5mins - PBS for 5mins.
3. 100ppm Iodine for 5mins - PBS 5mins - Iodine 5mins - PBS 5mins.
4. 100ppm NaOCl for 5mins - PBS 5mins.
5. 100ppm NaOCl for 10mins - PBS 10mins.
6. 100ppm NaOCl for 5mins - PBS 5mins - NaCl for 5mins - PBS 5mins.
7. 40mg/l CuSO4 for 5mins - PBS 5mins.
8. 60mg/L CuSO4 for 5ims - PBS 5mins.

At 24h.p.f eggs were submerged in the following:

1. 100ppm Iodine for 2.5mins - PBS for 5mins.
2. 100ppm Iodine for 5mins - PBS for 5mins.
3. 100ppm Iodine for 5mins - PBS 5mins - Iodine 5mins - PBS 5mins.
4. 100ppm NaOCl for 5mins - PBS 5mins.
5. 100ppm NaOCl for 10mins - PBS 10mins.
6. 100ppm NaOCl for 5mins - PBS 5mins - NaCl for 5mins - PBS 5mins.
7. 40mg/l CuSO4 for 5mins - PBS 5mins.
8. 60mg/L CuSO4 for 5ims - PBS 5mins.

2.4 Experimental outcomes and measurement techniques

The size and CF of ZFHAs were measure when viewed from above with on an nikon Ti-s inverted microscope attached to a Canon EOS 600d DSLR with a 3072x2304 pixel diameter of and a frame rate of 30 FPS.Contraction frequency of ZFHA was measured as the time interval necessary for 30 individual contractions to occur. This time interval divided by 60 and multiplied by the number of contraction (30) gave contraction frequency as BPM. ZFHA size was determined by hand drawing a line around the perimeter and measuring the enclosed area calibrated against a 1mm slide graticule. This was done using the software package ImageJ for MacOSx. To ensure consistency the pixel:micrometer ratio was established and kept consistent throughout all measurements.

2.5 Rational of anaesthetic used

Before culturing of the zebrafish larvae commences they are anaesthetised in a commonly used anestethic called 98% Ethyl 3-aminobenzoate methanesulfonate (sigma) (commonly known as MS222) (62). MS222 is made into an aqueous solution which can decrease the pH of the solution, therefore it is

buffered with sodium bicarbonate in a ratio of one part MS222 to two parts sodium bicarbonate (1:2) (74). Because of frequent use of MS222 a 500ml stock was made which was allocated into 15ml volumes and frozen at -20C. Exposure to light is minimised by wrapping tin foil around the allocates (74). It is recommended that an amount of 100-200 mg/L be used for anaesthetising zebrafish larvae (76). Two other literature sources recommend using 168mg/L of MS222 so this is the reason why we used this amount (74, 76). Because the zebrafish larvae used in this experiment are 3d.p.f there is no legal requirement to use an anaesthetic before culturing as they are not considered protected animals under the Animals (Scientific Procedures) Act 1986 until 5d.p.f or a time when they can participate in independent feeding (61). Because of the debate over the ability for zebrafish to experience pain and distress before 5d.p.f it was decided that an anaesthetic be used on the zebrafish larvae before culturing begins (77, 78, 79, 80, 81). In the process of deciding to use an anaesthetic it was agreed that our protocol should follow the criteria detailed in Animals (Scientific Procedures) Act 1986 as this is considered the correct guidance to follow when anaesthetising and humanity killing animals. We also adhere to annex IV of the "directive 2010/63/eu of the european parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes" which states if an animal is to be killed in a manner not described in annex IV then the animal must be unconscious and does not regain consciousness before death (11). The depth of anaesthesia is assessed as successful when voluntary and reflex movement of the larvae is non-existent and this is assessed before larvae are humanity killed (80, 82). Following schedule 1 of the Animals (Scientific Procedures) Act 1986 the larvae are humanity killed by way of complete maceration of the embryo (61).

2.6 Experimental Animals and group allocation

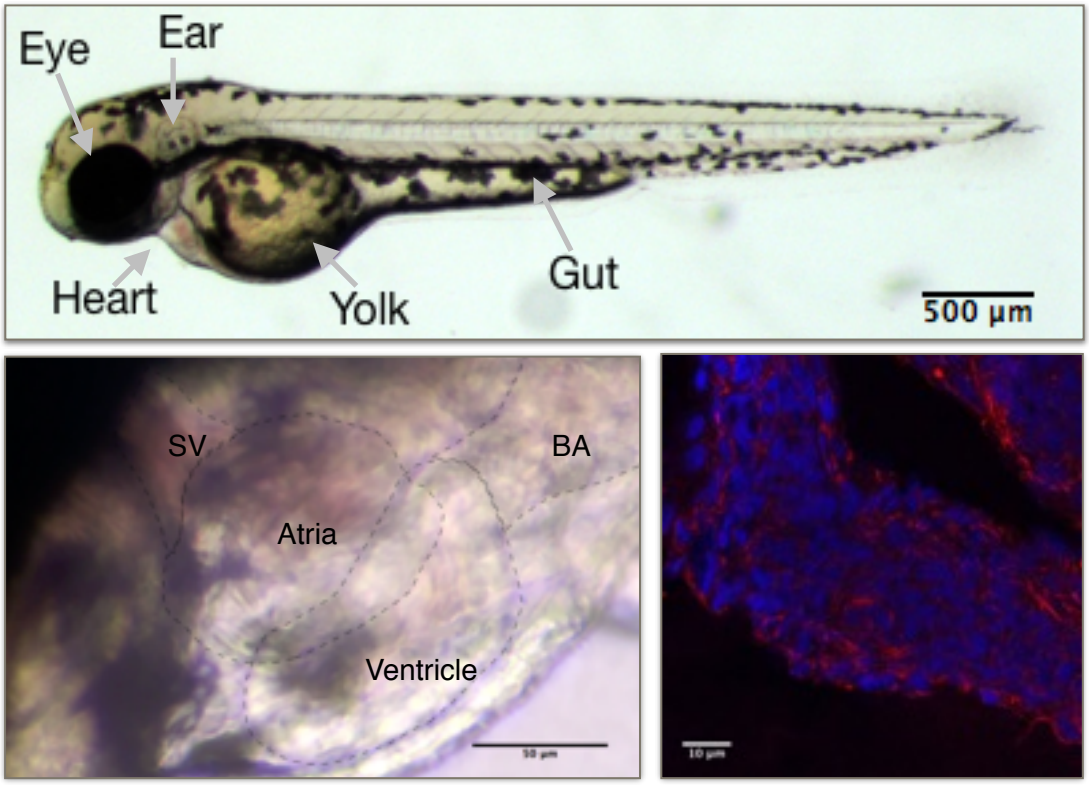


Figure 2.3: 72h.p.f wild type Zebrafish. A) Image of a 72h.p.f ZF with anatomical descriptions (Larvae image adapted form FishforPharma, 2016). B) Image of 72h.p.f ZF heart with anatomical guideline. C) Fluorescent confocal section of 72h.p.f ZF heart.

The experimental animals used are wild type ZF (Danio *rerio*) at 3d.p.f. These animals have an average length of 3.5mm when incubated at 28°C from 2h.p.f to 72h.p.f (this is assumed based on a 09:00 time of fertilisation through light activation). Using 15 ZF larvae at 72h.p.f per well of a 24 well plate generated an average of 3 ± 2 aggregates per well. All Zebrafish larvae went through the same sterilisation and washing procedures and were equally separated amongst the 6 wells in a 6 well plate during incubation periods (1-3d.p.f). Larvae with obvious malformations were excluded from testing but no other allocation bias was used when assigning larvae to groups.

2.7 Sample Size

As seen in the study design there are three experimental groups each of which measures the size and contraction frequency of ZFHAs. These measurements are taken every 24 hours and are taken over a period of 14 days. 14 days was chosen based on the average expected survival period of the aggregates due to either necrosis or loss of tissue with media exchange. ZFHAs have a lot of variance in size and contraction frequency and thus larger N numbers are required. Using the software G*Power for MacOSX it was calculated that the required N number for each day in each group would be 61. The acceptation of this however is the lactate group with measurements taken every 24hours for 7 days. this is due to ZFHAs reduced ability to survive in lactate.

2.8 Statistical Methods

The data collected includes the size and contraction frequency of the ZFHA from each group over a period of 14 days accept for the lactate group which had data collected over 7 days. Initially two questions below were asked when deciding what statistical tests to choose;

1. Are there any trends in size and contraction frequency from day to day?
2. Is there any difference in size and CF of ZFHAs between the treatment and control groups?

Before data is analysed its tested for normality with the D'Agostino & Pearson normality test, where the N numbers are too small for the D'Agostino & Pearson normality test the Shapiro-Wilk normality test is used instead using GraphPad Prism 7 for MacOSX. Normality testing and graphical representations of the data in histograms shows that the majority of data is normally distributed by rejecting the null hypothesis of the normality tests whereas some data dose not show normality by failing to reject the null hypothesis (normality test results and histograms can be seen in appendix II and III respectivly). Normality tests like the D'Agostino & Pearson and Shapiro-Wilk test are able to confirm if data is normally distributed but failure to confirm this dose not necessarily mean the deviation from the Gaussian ideal is severe enough to invalidate the statistical methods that are assumed by tests based on Gaussian distributions (such as t-tests and linear regression). In order to determine wether the data that fails to reject the null hypotheses is indeed non-normal data, it is tested using Minitab 17 for Windows. The collected data from each day in each group is subjected to an Individual Distribution Identification to find the distribution closest to the data sets.

We are interested to see if there is a difference in contraction frequency and size of ZFHAs in the different groups each day. Data that fails to reject the null hypothesis of the normality tests and shows affinity to another distribution is then analysed with the non-parametric mann whitney u test. Data that dose show normality is have comparisons between size and contraction frequency of the different groups analysed

using a parametric independent t-test. Trends in the data sets are then tested for trends using the Mann-Kendall trends test. This is test of data points over time to see if there is a significant monotonic relationship between the data points. Linear regression is done between the treatment group and the control to see if there is a significant difference between the slopes. A significant difference between slops would indicate a significant change between groups trends.

3 Results

3.1 Stroke volume calculations

Figure 3.1A shows that over five days phenylephrine has a positive result on increasing the ESV and EDV of ZF larvae. Phenylephrine also increases stroke volume as seen by the increased spacing between the EDV and ESV traces compared to the control group which has narrowing over five days of the gap between ESV and EDV. Average stroke volume per day are also shown in Figure 3.1B. Contraction frequency of ZF larvae in the phenylephrine group are consistently higher than the control group and appear to have more stabilised CF between days 3 and 5 whereas control groups decrease slightly between day 3 and 5. Cardiac output for phenylephrine groups are larger than control groups between days 3 and 5 which is consistent with higher contraction frequencies and larger stroke volumes.

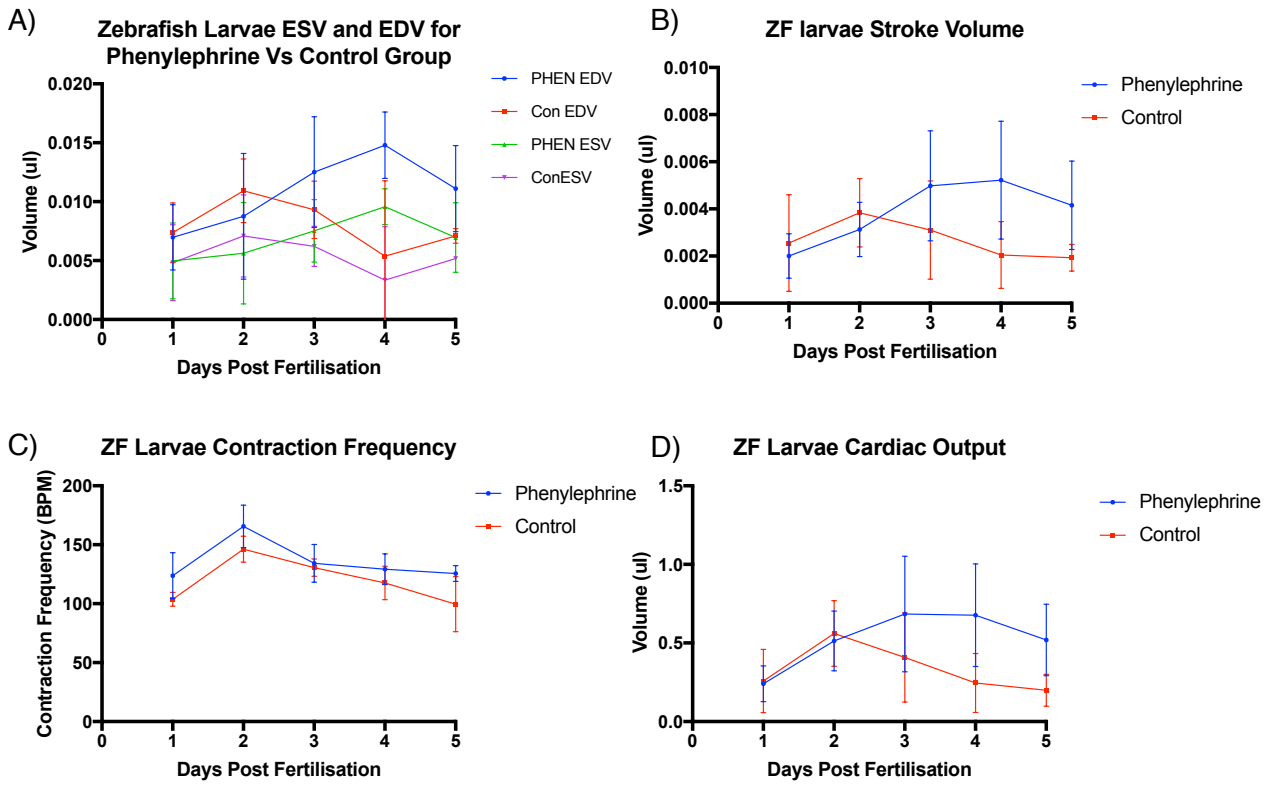


Figure 3.1. ZF Larvae cardiac function. A) end systolic volume (ESV) and end diastolic volume (EDV) time series. B) Stroke volume calculated as the difference between ESV and EDV. C) Contraction frequency of ZF larvae hearts. D) Cardiac output of ZF larvae calculated from the stroke volume multiplied by the contraction frequency. Both groups had a sample number of 5 over 5 days.

3.3 ZFHA Generation

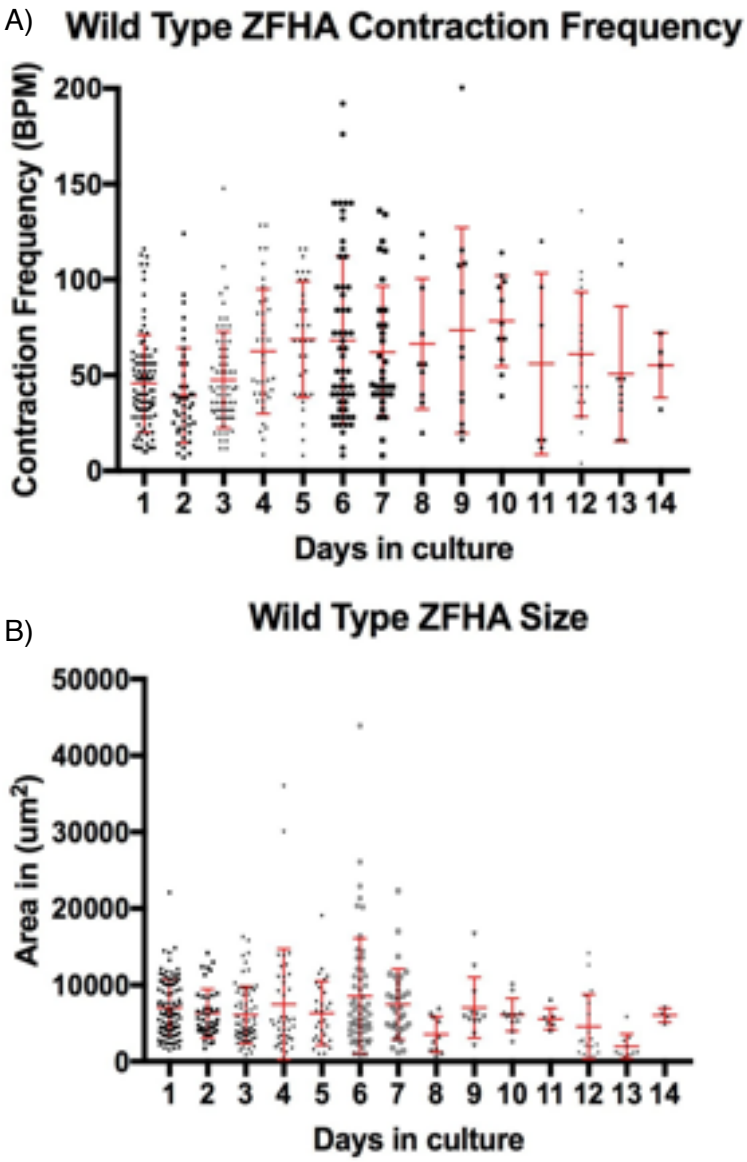


Figure 3.2. ZFHA contraction frequency and size data. A) Time series of contraction frequency for control ZFHA over 14 days. B) Time series of size for control ZFHA over 14 days.

Control ZFHA were grown for 14 days in high glucose DMEM. We analyse contraction frequency and measure the size of aggregates every 24hours. The means size of ZFHA on day 1 are (7007 ± 385.4, n=95) and reduced to (6043 ± 457.3, n=4) this is a 13.7% decrease but is not significant (p=>0.8972, Paired t-test) when comparing the two days. However, Trend analysis shows that there is a significant declining trend in the mean ZFHA size over 14 days (p=0.047, tau=0.209, Mann-Kendall trend test). The mean CF of ZFHA is (45.69 ± 2.57, n=97) on day 1 and increases over 14 days in culture (55.25 ± 8.50, n=4). Comparing the CF between day 1 and 14 is not significant (p=0.5395, Paired t-test) and shows no significant trend (p=0.331, tau=-0.407, Mann-Kendall trend test).

3.3 Phenylephrine

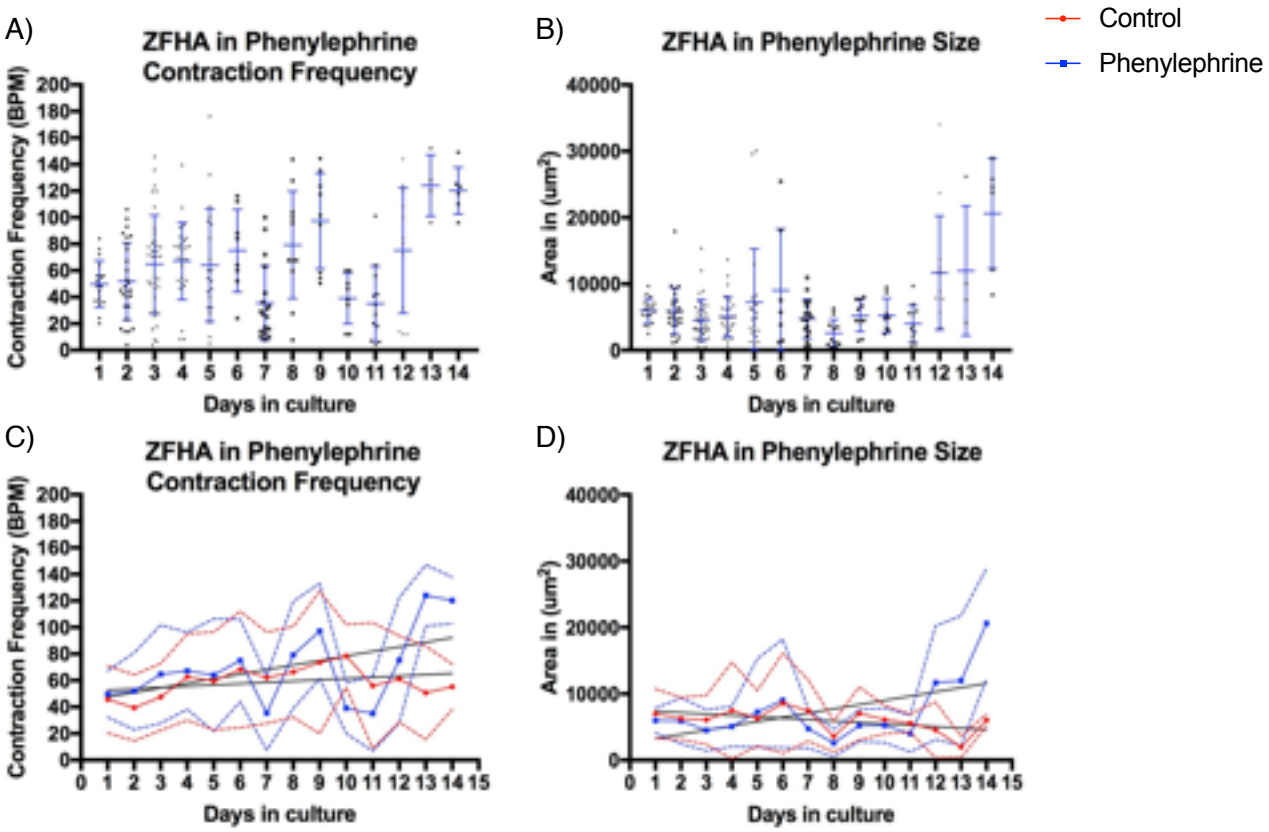


Figure 3.3. Contraction frequency and size data for ZFHA in phenylephrine. A) Time series of contraction frequency for control ZFHA over 14 days. B) Time series of size for control ZFHA over 14 days. C) Linear regression of ZFHA contraction frequency grown in culture media supplemented with phenylephrine compared to control. D) Linear regression of ZFHA size grown in culture media supplemented with phenylephrine compared to control.

ZFHA grown in phenylephrine have an increase in CF over 14 days in culture day 1(49.76 ± 4.263, n=17) day 14(120.2 ± 7.199, n=6). This is a significant increase in CF (p=0.0082, Paired t-test) however, there is no significant trend to the time series (p=0.079, tau=0.363, Mann-Kendall trend test). The size of the ZFHA also increases over the 14 days in culture, Day 1(5980 ± 452.7, n=17) Day 14(20607 ± 3391, n=6). This is also a significant increase (p=0.0170, paired t test) however, despite this increases in size there is also no significant trend in the time series (p=0.228, tau=0.253, Mann-Kendall trend test). In comparison to the control group it can be seen that both CF and size have a positive trendline whereas control data only has a positive trendline for CF and a negative trendline for size. Using Linear regression we can compare the slopes of the times series in the phenylephrine group compared tot he control group. A comparison of ZFHA CFs shows there is no significant difference between the incline of the slopes (P=0.1894) however, when comparing ZFHA size we see there is a significant difference (P=0.0063). Analysing the difference between ZFHA exposed to phenylephrine and the control group reveals that between day 13 -14 phenylephrine has a significantly higher contraction frequency than the control group. There is also a significant increase in average size of the ZFHAs when exposed to phenylephrine between days 12-14 (see appendix II: Normality tests)

3.4 Lactate

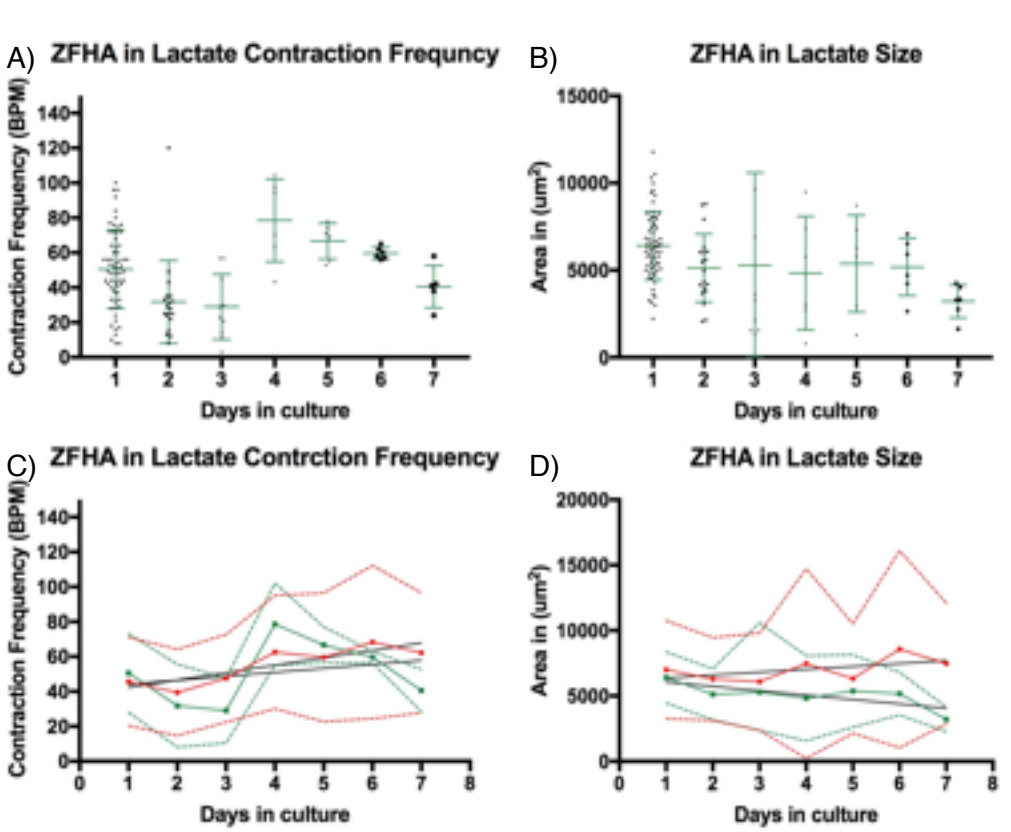


Figure 3.4. Contraction frequency and size data for ZFHA in Lactate. A) Time series of contraction frequency for control ZFHA over 14 days. B) Time series of size for control ZFHA over 14 days. C) Linear regression of ZFHA contraction frequency grown in culture media supplemented with lactate compared to control. D) Linear regression of ZFHA size grown in culture media supplemented with lactate compared to control.

ZFHA grown in lactate have an decrease in CF over 7 days in culture (Day 1: 50.56 ± 2.859, n=62, Day 7: 40.6 ± 5.418, n=5). This is not a significant decrease in CF (p=0.1580, Paired t-test) and there is no significant trend to the time series (p=1.000, tau=0.048 Mann-Kendall trend test). The size of the ZFHA also decreases over the 7 days in culture, (Day 1: 6408 ± 241.4, n=65, Day 7: 3213 ± 385.2, n=6). This is a significant decrease (p=0.0022 paired t test) However despite the significant decline in ZFHA size there is no significant declining trend in ZFHA size (p=0.239, tau=-0.429 Mann-Kendall trend test). When comparing to the control group to the lactate group over a 7 day culture it can be seen that both groups have inclining trends in CF but where the control group has an incline in size the lactate group has a decline size. Using Linear regression we can compare the slopes of the times series in the lactate group compared to the control group. A comparison of ZFHA CFs shows there is no significant difference between the incline of the slopes (P=0.6184) however, when comparing ZFHA size we see there is a significant difference (P=0.0199). Analysing the day to day difference between ZFHA cultured in lactate and the control group reveals no difference between the contraction frequencies of the two groups over 7 days in culture. There is a significant decrease in ZFHA size when cultured in lactate compared to control. (see appendix II: Normality tests).

3.5 Collagenase Digestion

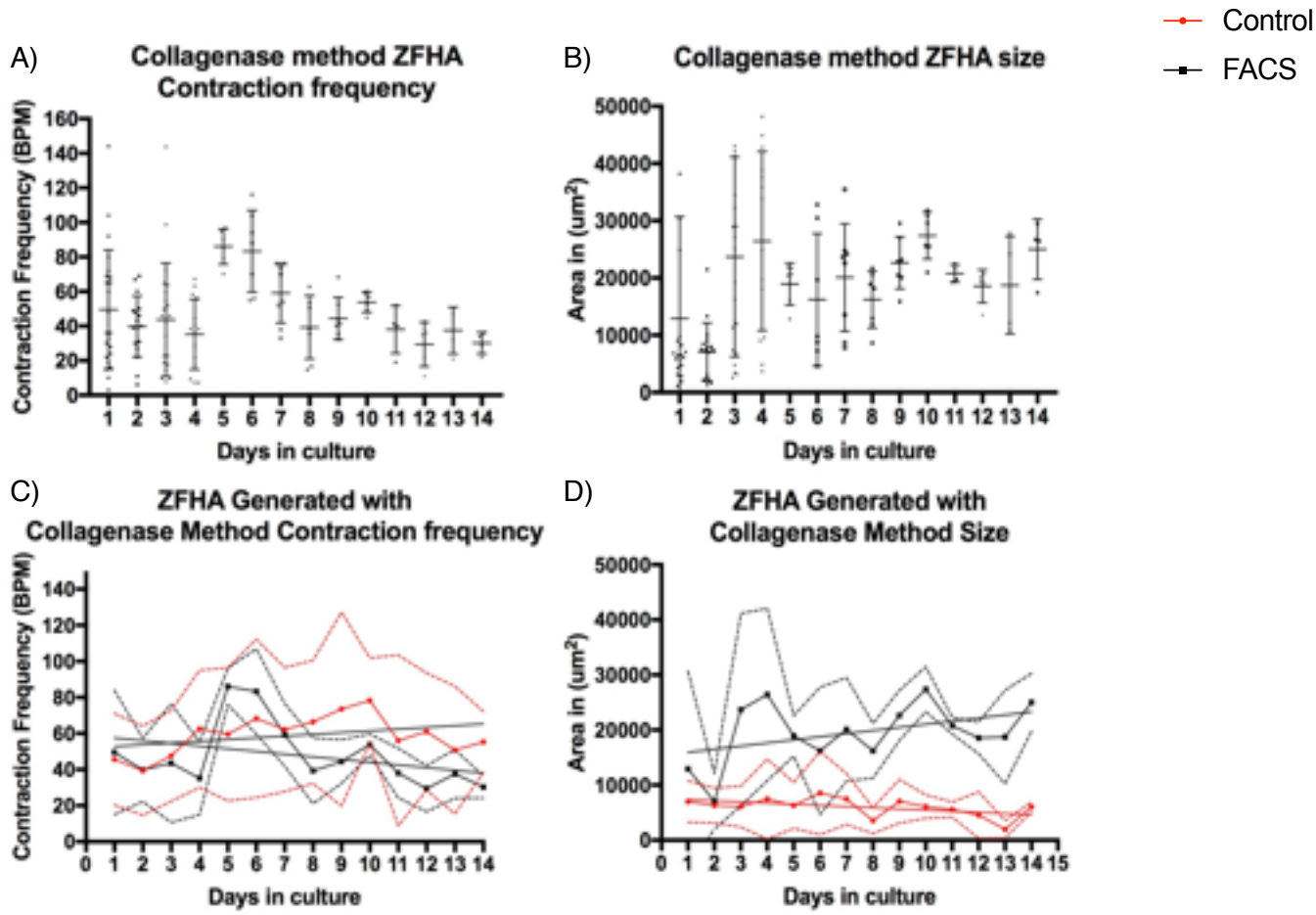


Figure 3.5. Contraction frequency and size data for ZFHA generated with the collagenase method. A) Time series of contraction frequency for control ZFHA over 14 days. B) Time series of size for control ZFHA over 14 days. C) Linear regression of ZFHA contraction frequency compared to control. D) Linear regression of ZFHA size compared to control.

ZFHA generate using the collagenase method have an decrease in CF over 14 days in culture (Day 1: 49.52 ± 7.211, n=23, Day 14: 30.25 ± 3.119, n=4). This is not a significant decrease in CF (p=0.1595, Paired t-test). There is also no significant trend to the decrease of ZFHA CF (p=0.062, tau=-0.385 Mann-Kendall trend test). The size of the ZFHA increases over the 14 days in culture, (Day 1: 12936 ± 3975, n=20, Day 14: 25027 ± 2620, n=4) however, this is not a significant increase (p=0.0864, paired t test). Despite this increases size being very large there is no significant positive trend in the time series (p=0.233, tau=0.253 Mann-Kendall trend test). When comparing the ZFHA generated with the collagenase method to the control method it can be seen that CF of the collagens group decrease where the CF of the control group increases. When comparing the sizes of ZFHA we can see that the control group decreases while the collagenase group increases. Using Linear regression we can compare the slopes of the times series for collagenase group compared to the control group. A comparison of ZFHA CFs shows there is no significant difference between the incline of the slopes (P=0.0778) however, when comparing ZFHA size we see there is a significant difference (P=0.0388). Analysing the day to day difference between ZFHA generated using the collagenase method compared to the control method reveals that the average means fluctuate enough that on days 4,5,10 and 12 the CF is significantly

different forms the control group. There is a significant decrease in ZFHA size between days 3-14 when generated using the collagenase method compared to control. The size difference is explained by the monolayer phenotype produced when ZFHA are generated using the collagenase method. (see appendix II: Normality tests).

3.6 Confocal microscopy

Stains used: Rhodamine-phalloidin, DAPI

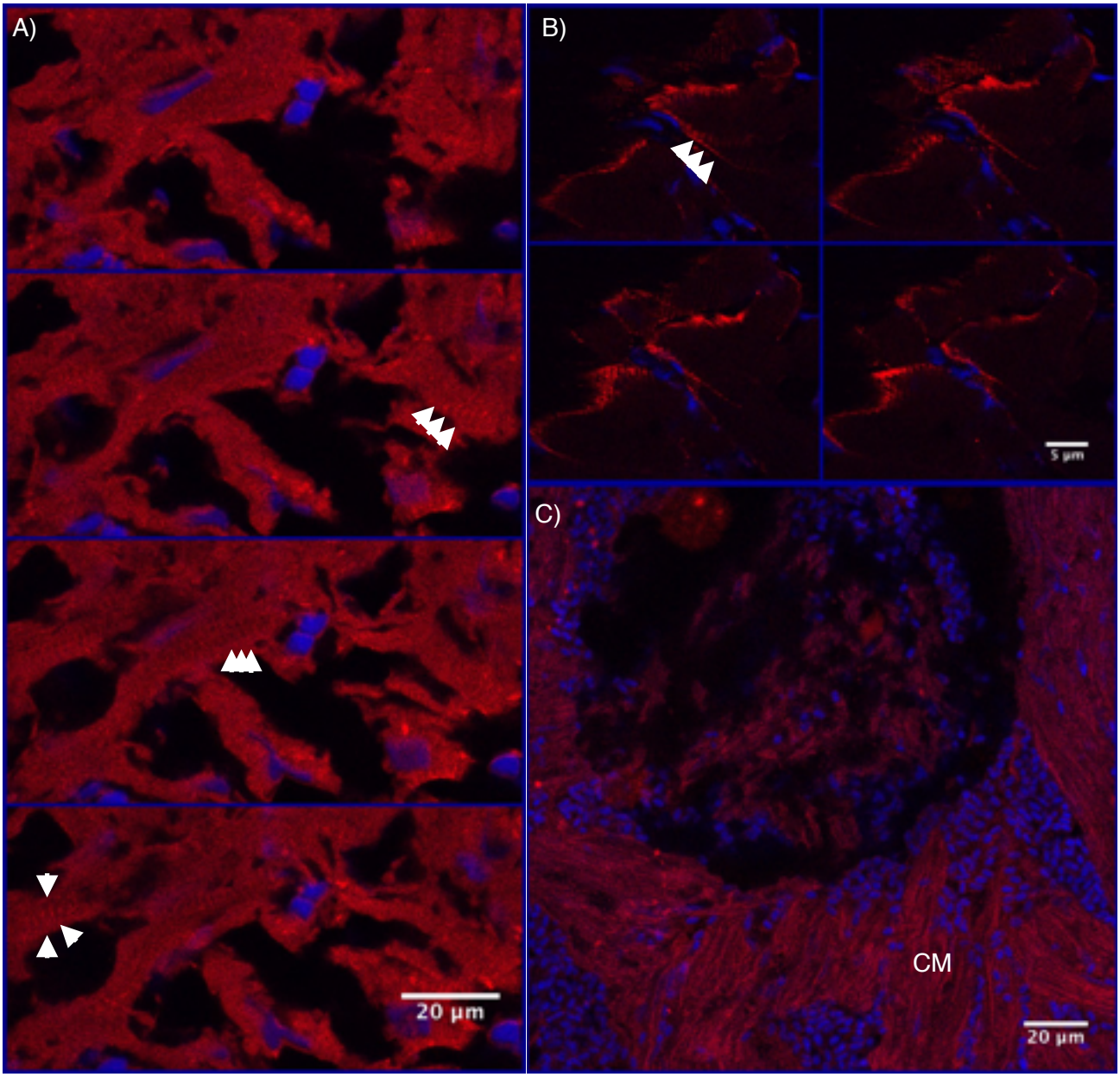


Figure 3.6. Confocal Images of adult zebrafish tissue. A) Adult ZF ventricular tissue showing the trabecular with visible striations (arrowheads), sections taken 6um apart. B) Adult ZF skeletal muscle tissue showing well ordered consistent striations (arrowheads), sections taken 12um apart. C) Adult ventricular tissue with compact myocardium (CM).

All adult tissue was dissected from 12 month old ZF. The Rhodamine-phalloidin staining for actin can be seen binding tightly to the actin filaments of the ventricular sarcomeres. This reveals striations in the tissue roughly 1.9-2.0um apart. The control tissue used in this study is adult ZF skeletal muscle which has abundant striations where Rhodamine-phalloidin has bound to the actin filaments in the tail muscles of the fish. Sections through the outer muscle of the ZF ventricle reveal compact regions of myocardium with frequent pours of blood vessels.

Stains used: Rhodamine-phalloidin, DAPI

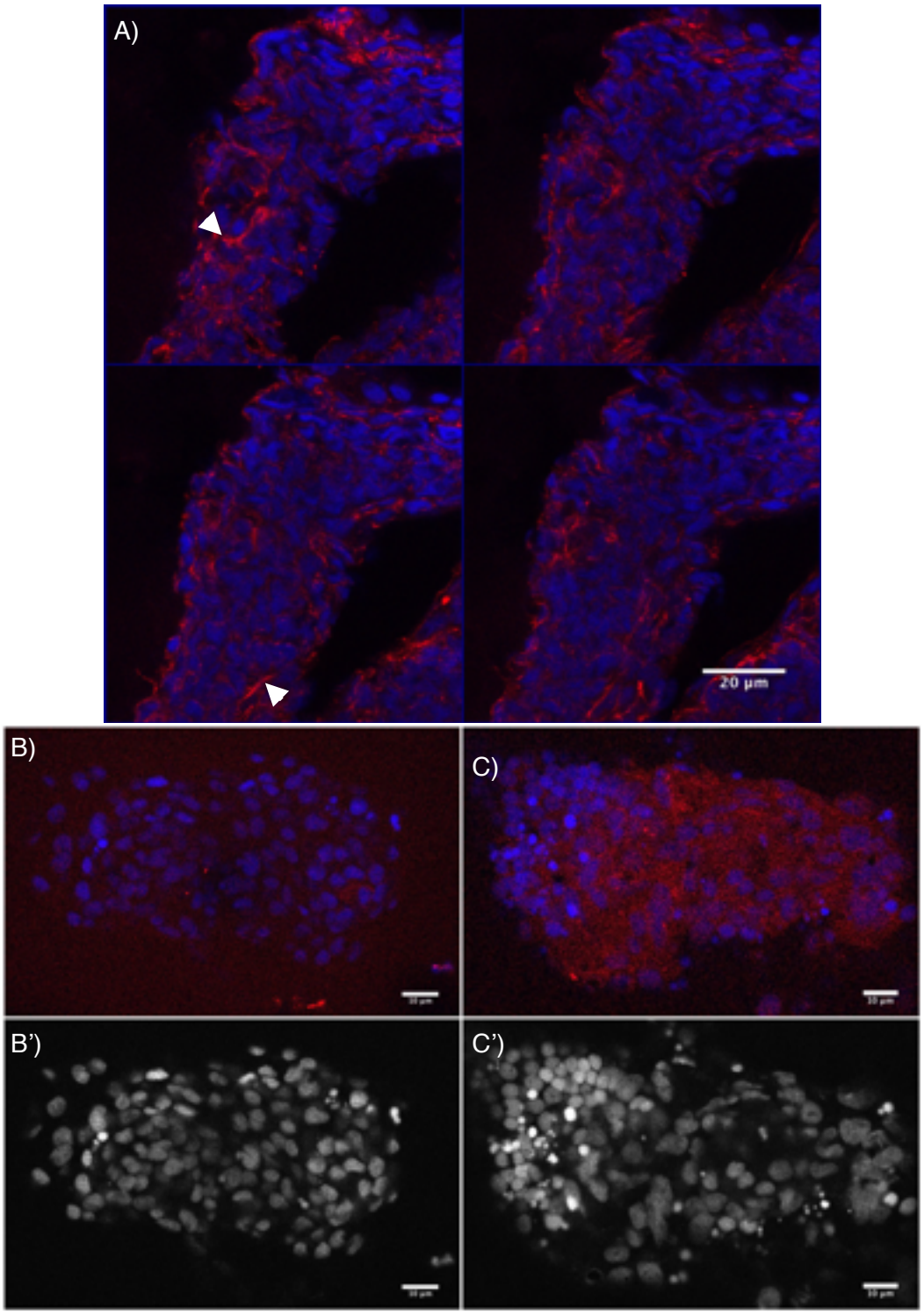


Figure 3.7. Confocal Images of larval zebrafish tissue. A) Larval cardiac tissue showing cardiac myofibrils (arrowheads) surrounding nuclei sections taken 18um apart. B) 4 day old aggregate with weak actin staining. C) 6 day old ZFHA with stronger actin staining. B') Nuclear staining of 4 day old ZFHA. C') nuclear staining of 6 day old ZFHA.

Larval tissue was collected from sections through whole 4 day old larvae. The Rhodamine-phalloidin staining for actin can be seen binding to regions of myofilaments that surround regions of densely compact nuclei. Staining of ZFHA has very week binding on day 4 aggregates. ZFHA that are 6days old and have been through the same staining technique have stronger binding but is still very week in

comparison to larval and adult ZF tissue. Using DAPI to stain nuclear material it can be seen from figures XXXX B′ and C′ that display compaction of cells between days 4 and 6 into a tighter aggregate of cells.

3.7 Sterilisation

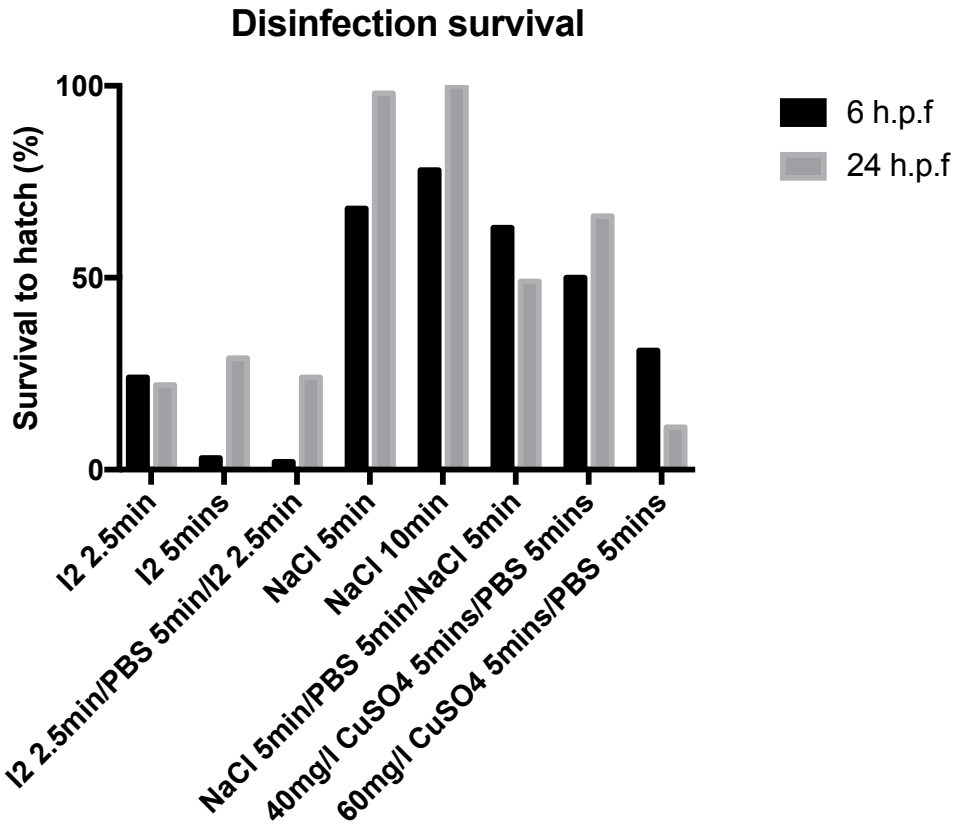


Figure 3.8. Comparison of survival to hatch (%) among wild type ZF in different biocidal solutions at 6 and 24 h.p.f.

Its found that iodine at concentrations needed to kill the bacteria is toxic to zebrafish eggs. Copper sulphate (CuSO4) was poor at removing bacteria and was would produce reduce hatch percentages to about 50% in 40mg/l and to about 11% in 60mg/l. It was seen that higher survival percentages were generally seen when eggs were disinfected at 24hours compared to 6hours. Buffered NaOCl had the best overall survival of ZF to reach hatching and was very good at removing visible signs of moving bacteria under a microscope. When ZF eggs are exposed to NaCl for 10 minutes in two 5minute periods separated by 5 minutes in PBS, we see are decrease in survival compared to the strait 10 minute exposure. It is thought that this difference is too do with agitation of the eggs and possible damage to the chorions during solution exchange.
It is worth noting that early in the study the use of autoclaved E2 media rather than the use of autoclaved synthetic salt water helped improve the survival of zebrafish eggs from roughly 20-30% survival to roughly 80-90% survival.

4 Discussion

4.1 The scientific implications form the results

ZFHA

Previous studies (Grunow) show that ZFHA had a decline in both size and CF over 7 days in culture with a significant decrease in size. This project has managed to increase the observational times of ZFHAs from 7 days to 14 days. In keeping with previous studies the size of my ZFHAs decreased but where CF of ZFHAs has previously been reported to decrease from 100 bpm to 67 ± 4.3 bpm, ours increased form an 45.69 ± 2.57 to 55.25 ± 8.49 bpm. Using the same time period as previous studies our data shows a contradiction to a reported decrease in ZFHA size over 7 as new data shows ZFHAs increase in size from 7007.2 ± 911.2 to 7469.6 ± 1121.9. The sample numbers of ZFHAs in this study have exceeded the sample numbers in previous studies however ZFHAs show large variance in both size and CF. It is seen that in this and previsouse studies there is a reduction of ZFHA size. An explanation for this could lie in the lack of perfusion to the inner cells of the 3D syncytium. Hypoxia has been shown to have a inducible effect on apoptosis in cardiomyocytes (83). The variance observed in contraction frequency is also relatable to the instability of heart rate in myocardial ischemia patients (84). Previous studies highlight the correlation between a reduced heart rate and reduced metabolism (85). This correlation dose not show casualty between a lowered metabolic level and a lower heart rate but the reduced fatty acid oxidative ability for a hypertrophic or damaged cardiac tissue means that limits on metabolism may lead to a reduction in contraction frequency over time.

Phenylephrine

The hypothesis of supplementing phenylephrine to the culture media is that ZFHAs will develop hypertrophy through stimulation of the α-AR, integrin mediated pathways and trypsin kinase receptors. The cardiac aggregate model that ZFHAs stemmed from used rainbow trout larvae which produced aggregates roughly three times the size of ZFHA. In the attempt to generate ZFHAs of similar size through phenylephrine induced hypertrophy an increase in size was seen after 12 days in culture. ZFHAs had similar aggregates size compared to control groups for the first 11 days in culture although there were more fluctuations since in the phenylephrine group. The fluctuations in size are seen between days 4 and 6 which corresponds with the time period needed for the development of AR in larval zebrafish. ZFHAs are generated when the larval ZF are 3d.p.f meaning the actual time we see fluctuations of size in response to phenylephrine occurs when cardiac tissue is reaching 7 days old. A possible explanation for the fluctuations in size observed after four days in culture may be due to the transdifferentiation of cardiac tissue when its freed from the larvae body. ZF possess the ability to regenerate cardiac tissue when its damaged which leads to an expression pattern of cardiogenisis genes similar to an embryonic larvae (86). The process of generating ZFHAs may induce a transdifferentiation of cardiac tissue which delays ZFHA response to phenylephrine until 4-6 days in culture. Hypertrophy of cardiac tissue after 12 days in culture is seen observed as increases in ZFHA cell size and cell numbers. Phenylephrine exposure also causes a significant increase in contraction frequency after 13 days in culture. Previous studies in humans and other organisms (XXXX) indicate that hypertrophy has an effect to increase heart rate variability and a reduction of frequency. Similar studies have also shown the possible hypos effects cause by hypertrophy can also result in lowered contraction frequency. A study by (Yin, Jin, He, & Yin, 2009) showed that phenylephrine can have a positive effect on contraction frequency as is seen here after 13 days in culture. Both CF and size of ZFHA when exposed to phenylephrine have trending increases between day 11 and 14. The correlation between increased size and increased contraction frequency is not supported by the literature. The progression of hypertrophy has been shown to have a proportional increase in cytosolic and membrane bound G protein-coupled receptor kinase (GRK) (87). A possible conclusion to the observed increase in contraction frequency in ZFHAs could be related to an increased ability of the ZFHA to respond to phenylephrine. Although phenylephrine is a classic α-AR it has been shown to have mild

stimulation of β-AR as well (88). β-AR are well known both chronotropic and inotropic response on cardiac tissue and the potential increase in GRK could correlate to an observable β-AR response seen in ZFHA.

Lactate

After heart failure the heart often adapts to a preferred carbohydrate metabolism (XXXX). When ZFHAs are freed form the body of ZF larvae they could indeed suffer cardiac damage and switch to a preferred carbohydrate metabolism as well. The shift to carbohydrate preference accounts for 50-75% of metabolic food source of which 30% is lactate. Culturing ZFHAs in a glucose free media supplemented with lactate means that ZFHAs will have only one metabolic substrate to choose. ZFHAs show a significant decrease in size ($p= 0.0022$, $N=69$, Paired t-test) over 7 days in culture. This reduction in size corresponds with the limited metabolic substrate availability and the apoptotic effects seen in starved cardiomyocytes (89). Contraction frequency of ZFHAs in lactate show a much narrower variance when compared to the control group. The fluctuations of CF in the lactate group is never significantly altered form the control group although the variability in CF indicates that lactate has an effect (36).

FACS

When comparing ZFHAs generate using the pestle & mortar method with trypsin/EDTA to the collagenase digestion method we see significant differences in ZFHA size. Trypsin is a common proteolytic used in cell culture to dissociate cells form an ECM or substratum through the digestion of cell surface proteins. The method of degrading cell surface protein can cause dysregulation to cell function and alterations to the expressed proteome (91). Most suppliers of trypsin such as sigma highlight the need to keep trypsin exposure times to a minimum as it can cause cellular damage (92). The use of trypsin in the generation of ZFHA dose not follow a specified period of exposure as the digestion time required for ZF larvae is largely discretional. This reveals a step in the protocol that has a variable period of which trypsin can cause cardiac damage. The development of the collagenase method was aimed at producing a consistent exposure time of cardiac tissue to a proteolytic enzyme. The collagenase methods also removes the potential damaging effect that trypsin can have on cardiac tissue. The larger sizes of ZFHA seen when generated using the collagenase method is due to monolayer phenotype. An initial hypothesis of why this monolayer phenotype might form is to do with the complete removal of ECM and other connective tissues between cells. This removal can essentially remove the natural ability of cells to actively maintain and reassemble its original tissue structure *in vitro* (93).

Confocal

Confocal imaging shows that in both adult cardiac and skeletal tissue of ZF there is organisation of actin filaments into the striations consistent with sarcomere structure. The binding of Rhodamine-phalloidin shows that sarcomere structures are present throughout the trabecular of the ZF ventricle. This binding shows that adult tissue ZF shows that it has a matured myocardium capable of consistent contractions. The matured myocardium is also seen with compact myocardium of the ventricle. When larval cardiac tissue is compared to the adult myocardium it can be seen that the binding of Rhodamine-phalloidin dose not reveal matured sarcomere structures nor dose it show compact myocardial tissue indicating muscle structure lacks organisation. Rhodamine-phalloidin binding of ZFHAs has a low signal on day 4 which is increased on day 6. The implications of this are actin filaments are disrupted during ZFHA generation but are quickly reassembled throughout the culture period. These findings are in keeping with previous work on ZFHA which shows maturation of sarcomere organisation of ZFHAs in culture (52).

Sterilisation

The results of the disinfection with iodine achieve the first goal by eliminating visible signs of moving bacteria under the microscope but are lethal to zebrafish eggs at the 100ppm concentration. Weaker concentrations of iodine were tested (data not shown) however all concentrations had the effect of death or visible malformations of ZF and had reduced ability to eliminate bacteria below 100ppm. Buffered NaOCl at a concentration of 100ppm is useful in eliminating the majority of the bacteria while maintaining the larval survival with few to no malformations. For visible signs of moving bacteria the best protocol

revealed to be disinfection for 10mins in NaOCl followed by 5mins PBS at both 6h.p.f and 24h.p.f. The results of this test also indicate that the current protocols of unbuffered 306ppm NaOCl at 24h.p.f may have a beneficial effect at removing protozoa and reducing bacteria and fungi but do not result in complete elimination of external bacteria. Copper sulphate test revealed a significant difference in the hatch rate between the two concentrations tested (40mg/l and 60mg/L). Copper sulphate had limited effects on its ability to remove visible signed of bacterial movement under the microscope at both 6 and 24h.p.f and had a larger mortality effect than buffered sodium hypochlorite did. The results of the copper sulphate test are similar to previous studies involving leather side chub (94). The implications of this test reveal that buffered sodium hypochlorite is the most effective biocidal solution and is most effective when zebrafish eggs are submerged in it for 10 minutes at 24h.p.f.

4.2 Study Limitations

This study was met with a few limitations including the lack of N numbers, bacterial contaminations and variability of data. The reasons for the lack of N numbers is based on the 25 week period set to complete the project in and also due to the over abitiose attempt to study multiple features of ZFHAs functionality. Using the available dat dose reveal some conclusions about ZFHA growth and contraction frequency over a period of 14 days but it should be known that in most instances the n numbers predicted by the software G*Power were not reached. The reasons N numbers were so hard to collect is based on a number of factors. The time period of the project was limited by 25 weeks and there was an ambitious number of areas that were researched. The lack of adhesion of ZFHa to the culture dish meant aggregate loss was a frequent occurrence which made it hard to maintain N numbers. Bacterial contamination was also assessed daily and at periods when bacterial was growth exponential the aggregates were retired through fear of collect bias or incorrect data.Despite the low N numbers on certain days the lack of N numbers docent nesserilly mean that the statistical test lacked power. The law of diminishing returns indicates that the statistical variance thats minimised through the use of larger n numbers is eventually negligible when reaching sample sizes of 30 (95). Its also for this reason why many tables used in statistics before the development of statistics software would often limit its tables to 30 degrees of freedom (96). Bacterial contaminations were persistent in the culture media as they often are in primary cultures (97). Process of elimination revealed the contaminations source was the aquarium water in the eggs are delivered in from the BSU. Analysis of the bacteria revealed the bacteria to be Achromobacter (98). The steps taken to prevent the growth of Achromobacter in the ZFHA culture included the supplementation of pipricillin and ciprofloxacin to the culture media as previously shown in a study by (Gray, Birmingham, & Fenton, 2010). The supplementation of these two new bacteria had positive effects at reducing bacteria growth and showed aggregation of the remaining bacteria instead of the biofilm phenotype seen without these antibiotics. The use of ciprofloxin required it to be diluted in HCL and caused a reduction in pH of the culture media if used in too larger quantities. This meant that ciprofloxin was limited to a concentration of 10ppm which reduced pH to an acceptable pH7.

4.3 The 3R's Implications

The focus of this study was to establish a better understanding of ZFHA in there development as a 3D cardiac model. The intended use of the model is for safety pharmacology testing to help establish early signs of a drug in R&D having adverse effects on cardiac rhythm or to minimise then required number of animals needed for pre-clinical testing of a proposed or current drug. The use of animals in research is a topic with ethical implications. The purpose of the 3R's is centred around more than ethics though and stretch there reach to involve the scientific question being asked and the economics of using animals in science. The development of an in vitro cardiac model form zebrafish could one day significantly reduce the needs of in vivo model in safety pharmacology which would have large economical benifits. The similarities seen between zebrafish and humans and the differences they have to mice also means that

ZFHAs might help in the better understanding of pharmacological effects on cardiac rhythm and might have more translational ability.

5 Conclusion

In conclusion this study investigated the electrophysiological, metabolic and hypertrophic characteristics of ZFHA and can shows that they are still a relevant cardiac model for safety pharmacology. ZFHAs show that they can stables there CF and size in culture over a period of 14 days and potentially longer. The use of phenylephrine as a hypertrophic agent on ZFHA produces increases in cell size and cell number causes increases in size that are similar to the salmonid aggregates of previous studies. Collagenase digestion reveals that there is no change in contraction frequency of ZFHA form the control group indicating they are still able to perform like ZFHA produce from the control method. The increased size in ZFHA due to a monolayer phenotype shows there are two ways to make ZFHA which may have beneficial applications depending of the type of investigation being performed. The development of the FACs method shows that ZFHA can be generated with a protocol that has a consistent exposure time to a digestive enzyme. ZFHA cultured in the presents of lactate confirms the findings of previous studies and shows that cardiomyocytes of ZF like other organisms can act as metabolic omnivores.

This project dose not signify and end point for this research and the study of ZFHA should continue. Much is left to be done before ZFHAs could be considered a stable in vitro cardiac model for safety pharmacology including investigations on how to improve the longevity of ZFHA in culture. Nearly all cardiac models used for safety pharmacology have data to support there response to drugs that may affect action potential traces as well as typical cardiac biomarkers indicating the use maturity and existence of cardiac tissue. Further investigation in to ZFHA should one day establish them as an appropriate model for in vitro testing and could help reduce the cost and use of animals in pharmacology testing.

6 Appendix

Appendix I: Study design protocols

Figure App 1.1. Phenylephrine (Larvae group)

This protocol has been designed using NC3R's Experimental Design Assistant. This protocol illustrates how zebrafish larvae are collected and allocated to either the control group or phenylephrine group. End diastolic volume (EDV) and end systolic volume (ESV) are measure to observe differences between the two groups. The difference between EDV and ESV determines the stroke volume of the ventricle. Together with contraction frequency, stoke volume can be used to measure cardiac output. Differences are then analysed using Prism GraphPad 7 to establish the statistical significance of the difference between the two groups.

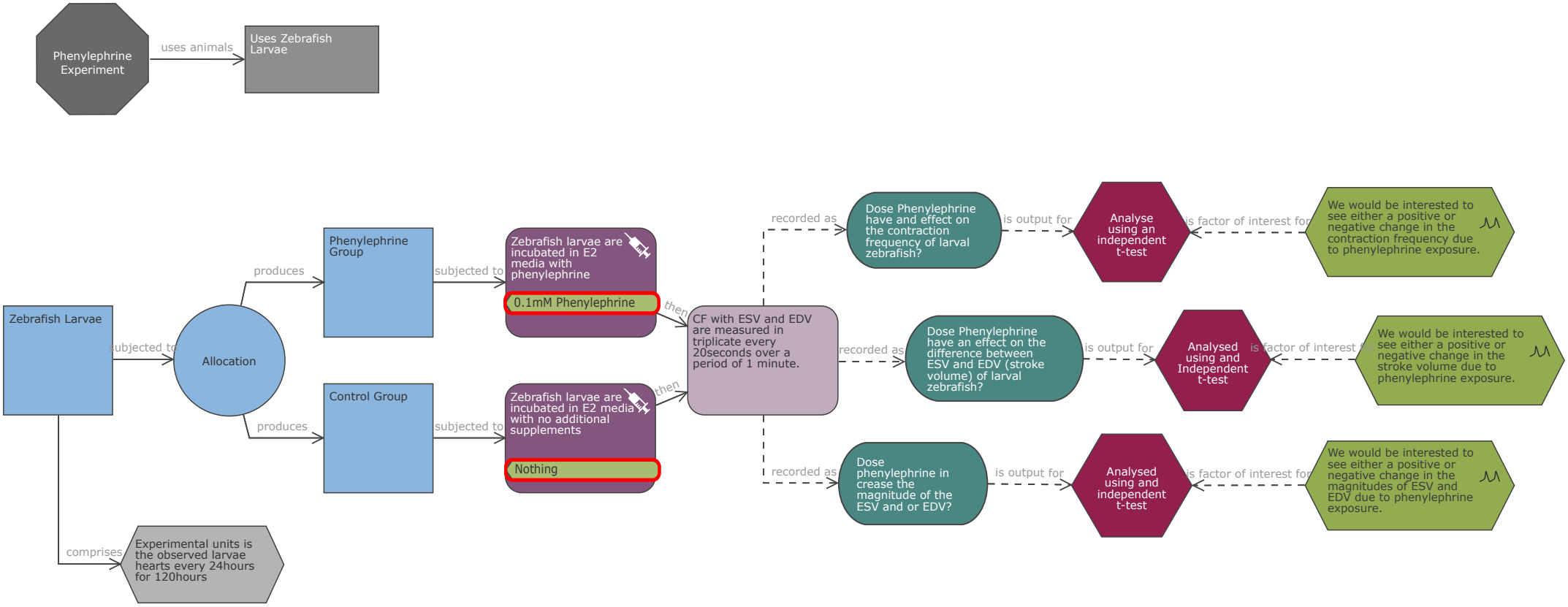


Figure App 1.2. Phenylephrine (ZFHA group)

This protocol has been designed using NC3R's Experimental Design Assistant. This protocol illustrates how zebrafish larvae are collected and allocated to either the control group or phenylephrine group. Contraction frequency and size of aggregates are measure to observe differences between the two groups. Differences are then analysed using Prism GraphPad 7 to establish the statistical significance of the differences observed.

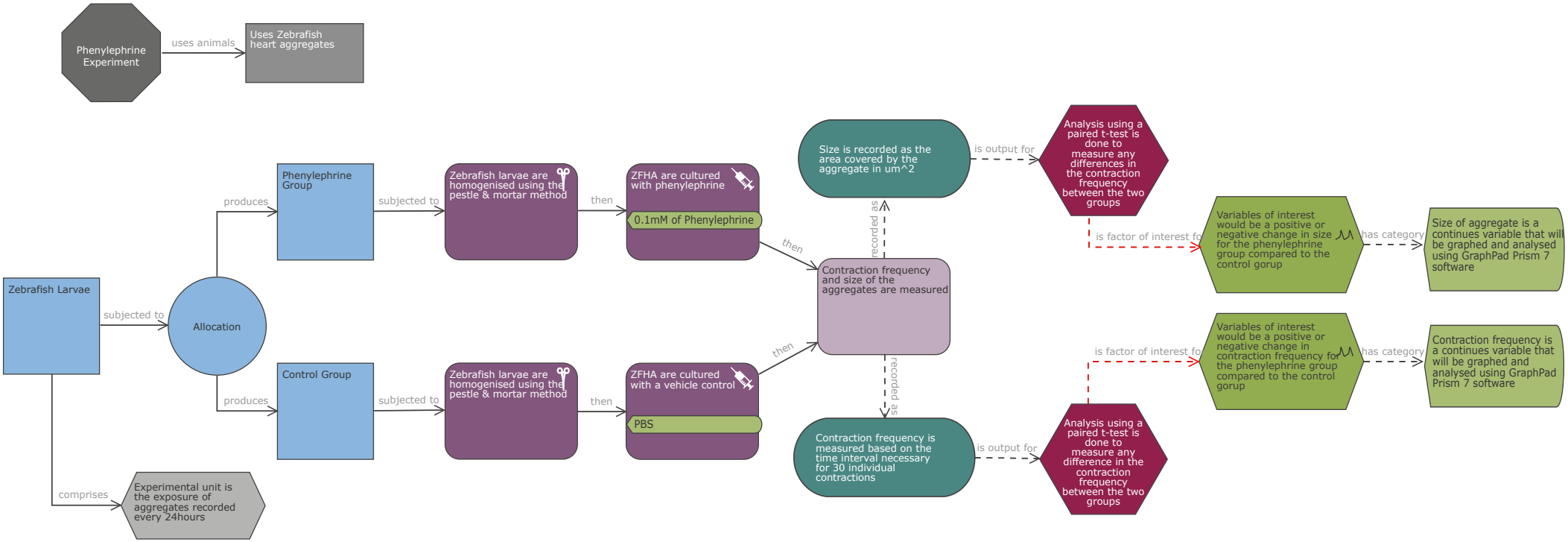


Figure App 1.3. Lactate (ZFHA Group)

This protocol has been designed using NC3R's Experimental Design Assistant. This protocol illustrates how zebrafish larvae are collected and allocated to either the control group or the lactate group. Contraction frequency and size of aggregates are measure to observe differences between the two groups. Differences are then analysed using Prism GraphPad 7 to establish the statistical significance of the differences observed.

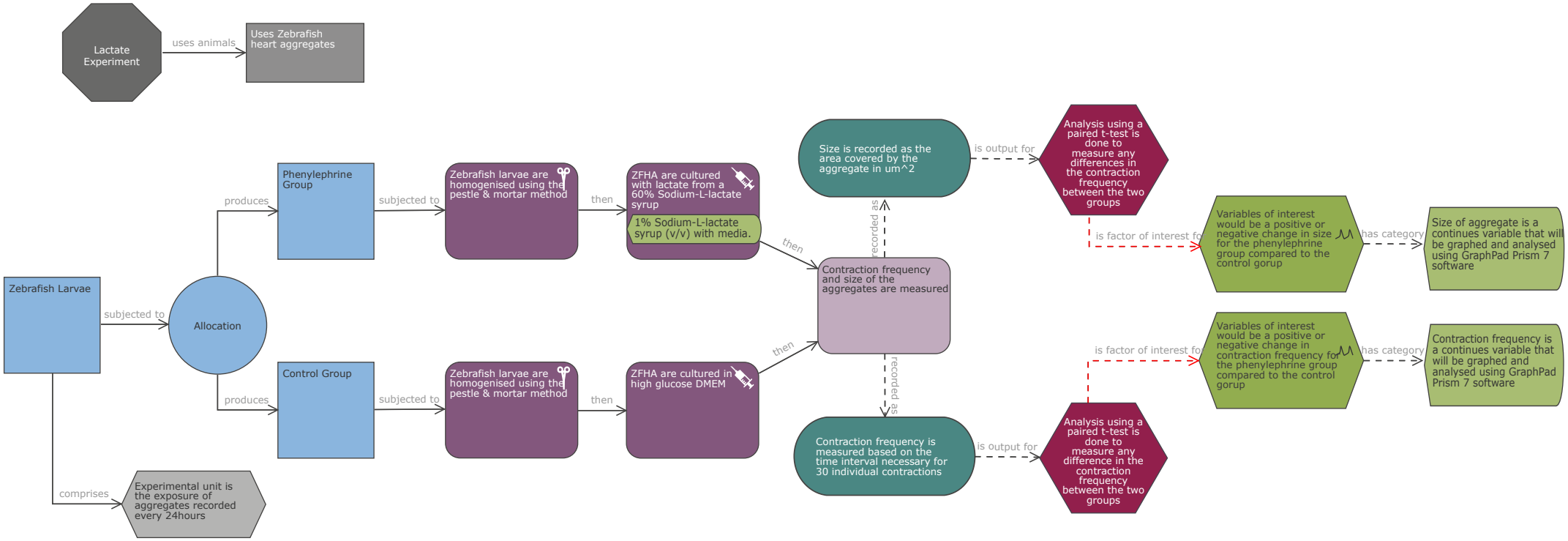
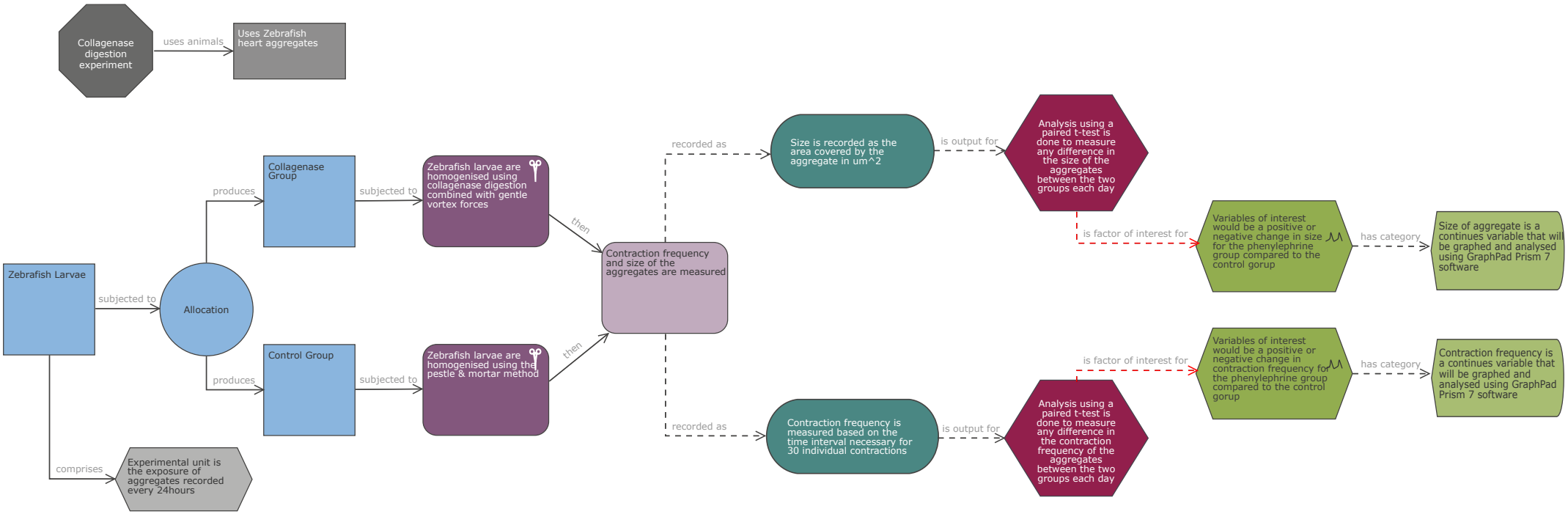


Figure App 1.4. Collagenase digestion method (ZFHA Group)

This protocol has been designed using NC3R's Experimental Design Assistant. This protocol illustrates how zebrafish larvae are collected and allocated to either the control digestion method or the collagenase digestion method. Contraction frequency and size of aggregates are measure to observe differences between the two groups. Differences are then analysed using Prism GraphPad 7 to establish the statistical significance of the differences observed.



Appendix II: Normality Test Tables

Group	Measurment	Day	D'Agostino-Pearson normality test		Shapiro-Wilk normality test		Distribution most favoured by minitab	P value of distribution	Resultant statistical test
			p Value	K2 Value	p Vaule	W Value			
Control	CF	1	0.0006	14.77	<0.0001	0.9221	Normal with johnson transformation	0.168	mann whitney u test
		2	0.0003	15.99	0.0008	0.8942	Normal with johnson transformation	0.919	mann whitney u test
		3	<0.0001	23.41	0.0001	0.9055	Normal with johnson transformation	0.67	mann whitney u test
		4	0.2819	2.532	0.1437	0.9554	Normal with Box-Cox Transformation	0.618	Independent t-test
		5	0.2833	2.522	0.3343	0.9632	Normal	0.461	Independent t-test
		6	0.023	7.548	0.0004	0.9071	Normal with johnson transformation	0.348	mann whitney u test
		7	0.1785	3.447	0.0165	0.9141	Normal with johnson transformation	0.296	mann whitney u test
		8	0.615	0.9722	0.5054	0.9356	Lognormal	0.822	Independent t-test
		9	0.0997	4.61	0.116	0.8895	Normal with Box-Cox Transformation	0.622	Independent t-test
		10	0.6871	0.7505	0.8609	0.9676	Normal	0.855	Independent t-test
		11		N too small	0.133	0.841	Smallest Extreme Value	0.191	Independent t-test
		12	0.583	1.079	0.8086	0.9726	Normal with Box-Cox Transformation	0.859	Independent t-test
		13	0.1166	4.298	0.0159	0.8033	Normal with johnson transformation	0.679	mann whitney u test
		14		N too small	0.6995	0.9473	Normal with Box-Cox Transformation	0.774	Independent t-test
	Size	1	0.0005	15.08	0.0001	0.9349	Normal with johnson transformation	0.15	mann whitney u test
		2	0.0427	6.306	0.002	0.9031	Normal with Box-Cox Transformation	0.617	mann whitney u test
		3	0.006	10.23	<0.0001	0.9037	Normal with johnson transformation	0.347	mann whitney u test
		4	0.0716	5.273	0.0025	0.8972	Normal with johnson transformation	0.571	mann whitney u test
		5	0.0219	7.638	0.0334	0.9216	Normal with Box-Cox Transformation	0.849	mann whitney u test
		6	0.0004	15.82	<0.0001	0.8717	Normal with johnson transformation	0.981	mann whitney u test
		7	0.0025	11.95	0.0171	0.9169	Normal with johnson transformation	0.942	mann whitney u test
		8	0.1688	3.558	0.1719	0.8905	Normal with Box-Cox Transformation	0.427	Independent t-test
		9	0.5979	1.029	0.4912	0.9395	Normal with Box-Cox Transformation	0.78	Independent t-test
		10	0.4363	1.659	0.178	0.8987	3-Parameter Weibull	0.487	Independent t-test
		11		N too small	0.4516	0.9123	Normal with Box-Cox Transformation	0.778	Independent t-test
		12	0.1412	3.916	0.0048	0.8089	Normal with johnson transformation	0.985	mann whitney u test
		13	0.0043	10.91	0.0091	0.7835	Normal with johnson transformation	0.869	mann whitney u test
		14		N too small	0.4024	0.8941	3-Parameter Weibull	0.405	Independent t-test

Table 1. Normality test table for control group. This table contains the results of the normality test performed on the collective data for each day the contraction frequency and size were measure for ZFHA in the control group. Data is tested for fit against 16 different distributions with he distribution of best fit being displayed here. Depending on the results of normality test and distribution fitting a statistical test is assigned for the analysis of this data set.

Group	Measurment	Day	D'Agostino-Pearson normality test		Shapiro-Wilk normality test		Distribution most favoured by minitab	P value of distribution	Resultant statistical test	Statistical test compared to control group
			p Value	K2 Value	p Vaule	W Value				
Phenylephrine	CF	1	0.8754	0.2661	0.9272	0.9772	Normal with Box-Cox Transformation	0.931	Independent t-test	0.25
		2	0.2902	2.474	0.2608	0.9535	3-Parameter Weibull	0.424	Independent t-test	0.0405
		3	0.6257	0.9378	0.3274	0.966	3-Parameter Weibull	0.415	Independent t-test	0.0193
		4	0.3944	1.861	0.1291	0.9393	Normal with johnson transformation	0.548	Independent t-test	0.5517
		5	0.0875	4.872	0.19	0.937	Normal with Box-Cox Transformation	0.976	Independent t-test	0.6559
		6	0.9421	0.1193	0.8153	0.9606	Normal	0.829	Independent t-test	0.438
		7	0.0561	5.761	0.0056	0.8382	Normal with johnson transformation	0.94	mann whitney u test	0.0016
		8	0.9636	0.07425	0.8667	0.9681	Normal with Box-Cox Transformation	0.729	Independent t-test	0.4391
		9	0.336	2.181	0.2791	0.9045	Normal	0.342	Independent t-test	0.2395
		10	0.6086	0.993	0.2286	0.8889	Normal	0.342	Independent t-test	0.0009
		11	0.108	4.451	0.2028	0.9083	Normal with Box-Cox Transformation	0.904	Independent t-test	0.3516
		12	0.3184	2.289	0.1962	0.9072	Smallest Extreme Value	>0.250	Independent t-test	0.3719
		13		N too small	0.962	0.9909	Normal	0.77	Independent t-test	0.009
		14		N too small	0.8552	0.9647	Normal	0.744	Independent t-test	0.0007
	Size	1	0.9906	0.01884	0.9963	0.9876	Normal	0.96	Independent t-test	0.4839
		2	<0.0001	18.67	0.0044	0.8703	Normal with johnson transformation	0.879	mann whitney u test	0.7329
		3	<0.0001	19.27	0.0011	0.8887	Normal with johnson transformation	0.927	mann whitney u test	0.0236
		4	0.0251	7.37	0.0679	0.9276	Normal with Box-Cox Transformation	0.905	mann whitney u test	0.3341
		5	<0.0001	22.54	<0.0001	0.6598	Normal with johnson transformation	0.656	Independent t-test	0.6825
		6		N too small	0.0942	0.8375	Normal with johnson transformation	0.91	Independent t-test	0.8169
		7	0.7377	0.6086	0.8254	0.9704	Normal	0.931	Independent t-test	0.0464
		8	0.4514	1.591	0.0995	0.8785	Exponential	0.604	Independent t-test	0.3257
		9	0.5863	1.068	0.1901	0.8944	Smallest Extreme Value	>0.250	Independent t-test	0.2005
		10	0.5205	1.306	0.1982	0.8823	Normal with Box-Cox Transformation	0.604	Independent t-test	0.4535
		11	0.6013	1.017	0.4784	0.9385	Normal with Box-Cox Transformation	0.81	Independent t-test	0.128
		12	0.0029	11.72	0.0087	0.8087	Normal with johnson transformation	0.741	mann whitney u test	0.002
		13		N too small	0.2117	0.8454	Normal with Box-Cox Transformation	0.794	Independent t-test	0.004
		14		N too small	0.1662	0.8529	Normal	0.244	Independent t-test	0.0074

Table 2. Normality test table for Phenylephrine group. This table contains the results of the normality test performed on the collective data for each day the contraction frequency and size were measure for ZFHA in the phenylephrine group. Data is tested for fit against 16 different distributions with the distribution of best fit being displayed here. Depending on the results of normality test and distribution fitting a statistical test is assigned for the analysis of this data set.

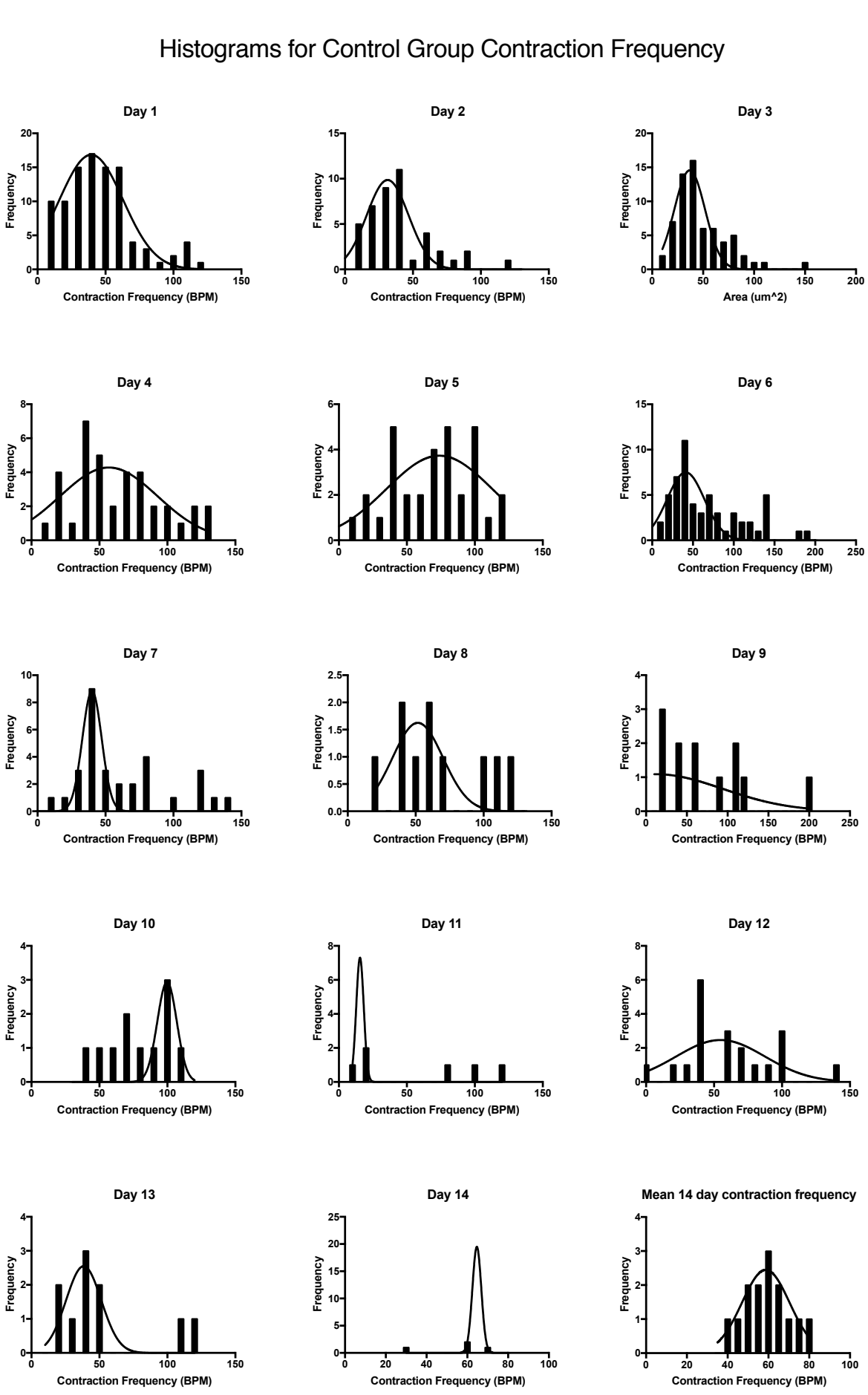
Group	Measurment	Day	D'Agostino-Pearson normality test		Shapiro-Wilk normality test		Distribution most favoured by minitab	P value of distribution	Resultant statistical test	Statistical test compared to control group
			p Value	K2 Value	p Vaule	W Value				
Lactate	CF	1	0.8112	0.41837	0.5132	0.982352	Normal	0.762	Independent t-test	0.0759
		2	<0.0001	34.5308	<0.0001	0.663733	Normal with johnson transformation	0.796	Independent t-test	0.1363
		3	0.6441	0.87993	0.4408	0.929272	Normal with Box-Cox Transformation	0.858	Independent t-test	0.0227
		4		N too small	0.5263	0.922877	Normal with Box-Cox Transformation	0.492	Independent t-test	0.1842
		5		N too small	0.2697	0.880131	Normal with Box-Cox Transformation	0.437	Independent t-test	0.7555
		6		N too small	0.7124	0.946567	Normal with Box-Cox Transformation	0.893	Independent t-test	0.8116
		7		N too small	0.7053	0.945543	Normal	0.429	Independent t-test	0.1572
	Size	1	0.3544	2.075	0.616	0.9849	Normal with Box-Cox Transformation	0.862	Independent t-test	0.7658
		2	0.6955	0.7261	0.3851	0.9493	Normal with Box-Cox Transformation	0.677	Independent t-test	0.255
		3	0.0133	8.647	0.0101	0.7734	Normal with johnson transformation	0.943	mann whitney u test	0.1675
		4		N too small	0.8162	0.9595	Normal with Box-Cox Transformation	0.807	Independent t-test	0.5033
		5		N too small	0.9338	0.9767	Normal	0.851	Independent t-test	0.749
		6		N too small	0.9751	0.9854	Normal with Box-Cox Transformation	0.943	Independent t-test	0.3642
		7		N too small	0.6095	0.9338	Normal	0.671	Independent t-test	0.0053

Table 3. Normality test table for lactate group. This table contains the results of the normality test performed on the collective data for each day the contraction frequency and size were measure for ZFHA in the lactate group. Data is tested for fit against 16 different distributions with he distribution of best fit being displayed here. Depending on the results of normality test and distribution fitting a statistical test is assigned for the analysis of this data set.

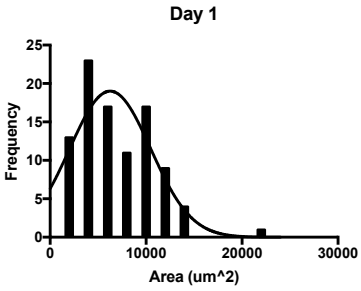
Group	Measurement	Day	D'Agostino-Pearson normality test		Shapiro-Wilk normality test		Distribution most favoured by minitab	P value of distribution	Resultant statistical test	Statistical test compared to control group
			p Value	K2 Value	p Vaule	W Value				
Collagenase Method	CF	1	0.0736	5.218	0.084	0.9247	Normal with Box-Cox Transformation	0.776	Independent t-test	0.8385
		2	0.9194	0.1681	0.8714	0.9732	Normal	0.877	Independent t-test	0.4321
		3	0.0036	11.25	0.0221	0.8971	Normal with johnson transformation	0.532	mann whitney u test	0.5618
		4	0.4276	1.699	0.3427	0.9367	Normal	0.517	Independent t-test	0.0007
		5		N too small	0.7275	0.9484	Normal with Box-Cox Transformation	0.886	Independent t-test	0.0184
		6		N too small	0.5689	0.9321	Normal	0.64	Independent t-test	0.1498
		7	0.3998	1.834	0.1374	0.8659	Normal	0.177	Independent t-test	0.8577
		8		N too small	0.5384	0.9285	Normal	0.56	Independent t-test	0.0526
		9		N too small	0.2336	0.8816	Normal with Box-Cox Transformation	0.91	Independent t-test	0.0964
		10		N too small	0.5587	0.9272	Normal with Box-Cox Transformation	0.678	Independent t-test	0.0069
		11		N too small	0.5131	0.9157	Normal with Box-Cox Transformation	0.49	Independent t-test	0.4136
		12		N too small	0.5966	0.93	3-Parameter Weibull	>0.500	Independent t-test	0.0032
		13		N too small	0.515	0.9161	Normal	0.455	Independent t-test	0.8681
		14		N too small	0.6298	0.936	Normal with Box-Cox Transformation	0.693	Independent t-test	0.0539
	Size	1	<0.0001	29.55	<0.0001	0.6099	Normal with johnson transformation	0.524	Independent t-test	0.7445
		2	0.0069	9.956	0.0105	0.8495	Normal with johnson transformation	0.139	mann whitney u test	0.7351
		3	0.4152	1.758	0.121	0.9297	Normal with Box-Cox Transformation	0.497	Independent t-test	0.0001
		4	0.1369	3.976	0.1917	0.9159	Smallest Extreme Value	>0.250	Independent t-test	<0.0001
		5		N too small	0.0867	0.8191	Normal with johnson transformation	0.892	Independent t-test	<0.0001
		6		N too small	0.1284	0.8521	Normal with Box-Cox Transformation	0.487	Independent t-test	0.1347
		7	0.9645	0.07231	0.3668	0.9118	Normal	0.261	Independent t-test	0.0002
		8		N too small	0.459	0.9187	Normal	0.432	Independent t-test	0.0002
		9		N too small	0.9318	0.975	Normal with Box-Cox Transformation	0.855	Independent t-test	<0.0001
		10		N too small	0.52	0.922	Normal with Box-Cox Transformation	0.527	Independent t-test	<0.0001
		11		N too small	0.577	0.927	Normal with Box-Cox Transformation	0.507	Independent t-test	<0.0001
		12		N too small	0.0331	0.755	Normal with johnson transformation	0.759	mann whitney u test	0.0001
		13		N too small	0.2421	0.8548	2-Parameter Exponential	>0.250	Independent t-test	0.002
		14		N too small	0.1932	0.8392	Normal		Independent t-test	0.0046

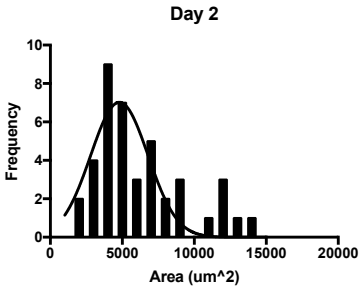
Table 4. Normality test table for the collagenase digestion group. This table contains the results of the normality test performed on the collective data for each day the contraction frequency and size were measure for ZFHA in the collagenase digestion group. Data is tested for fit against 16 different distributions with he distribution of best fit being displayed here. Depending on the results of normality test and distribution fitting a statistical test is assigned for the analysis of this data set.

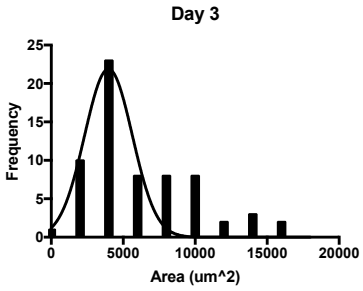
Appendix III: Histograms

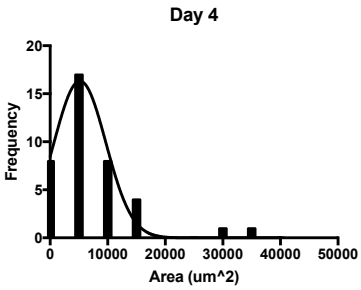


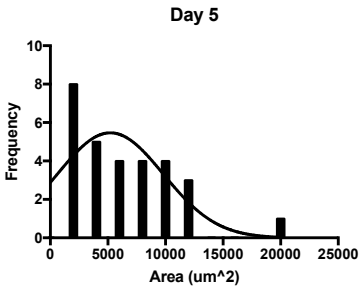
Histograms for Control Group Sizes

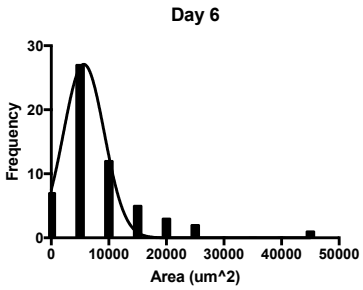


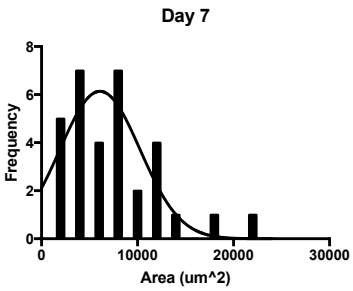


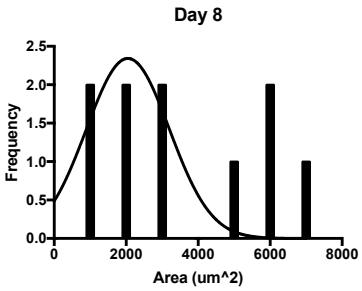


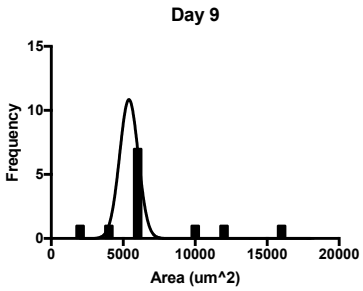


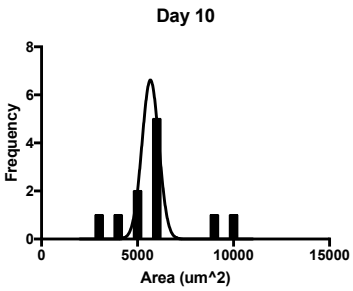


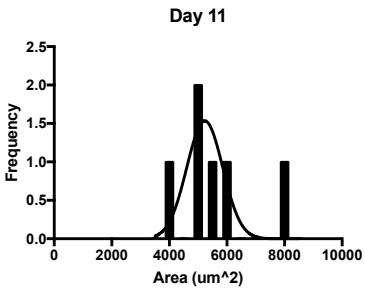


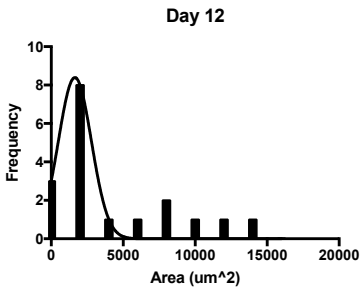


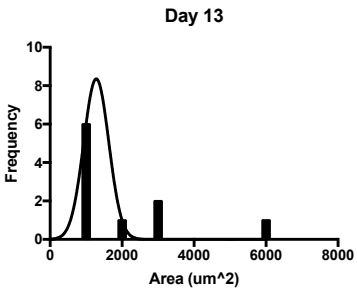


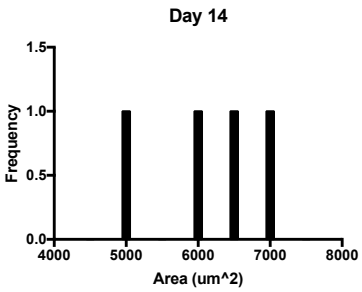


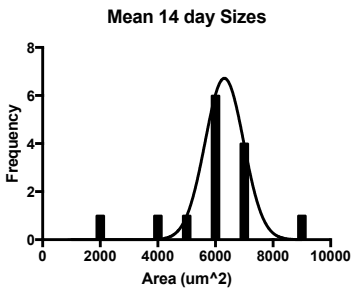




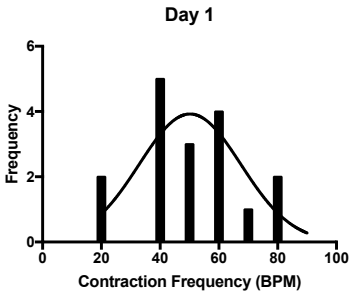


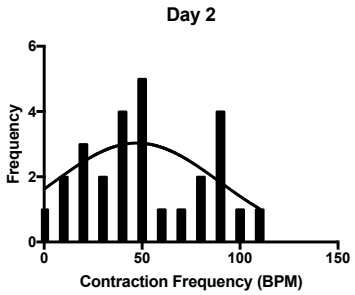


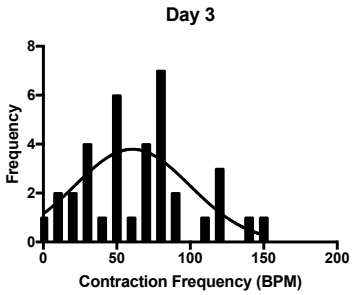


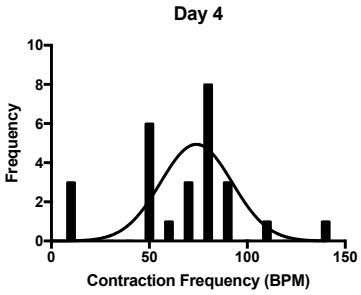


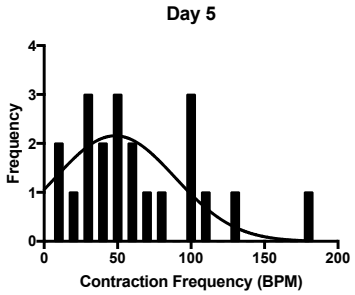
Histograms for Phenylephrine Group Contraction Frequency

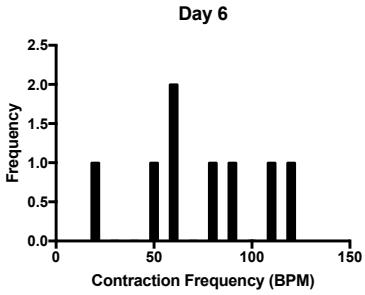


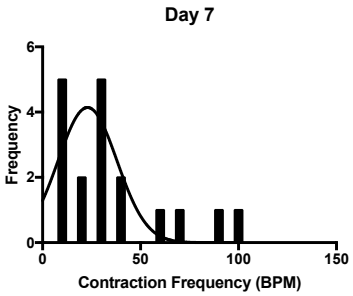


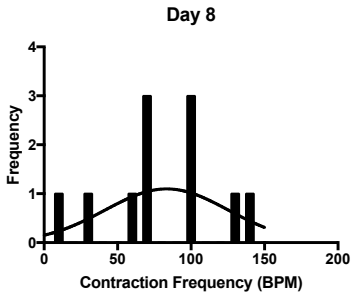


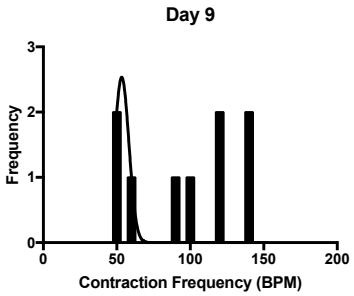


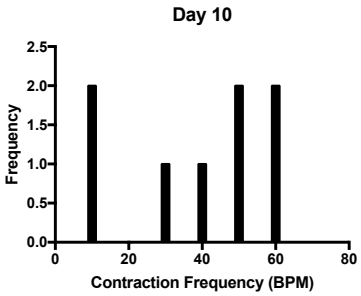


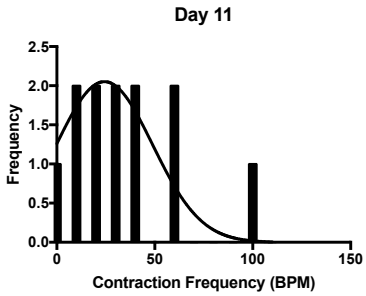


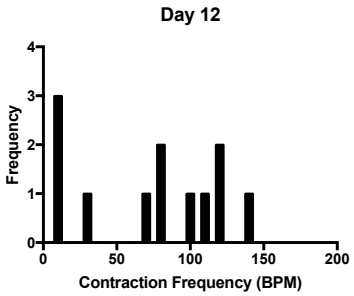


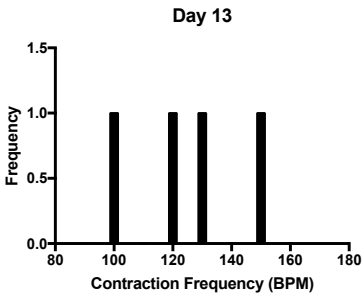


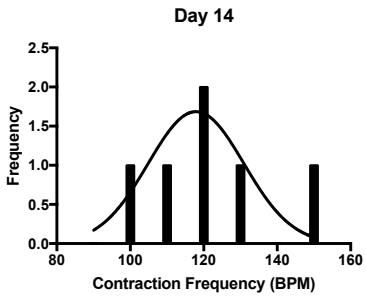


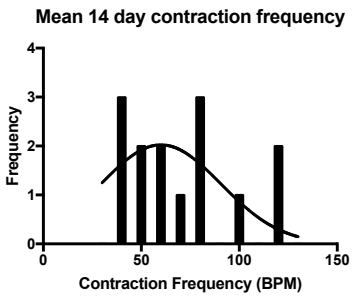




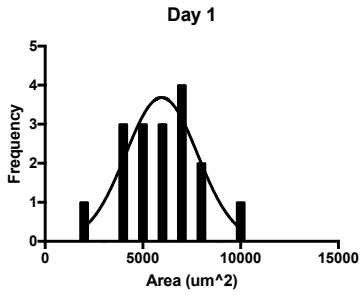


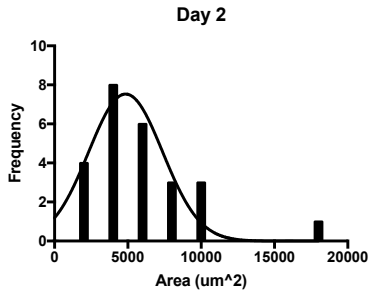


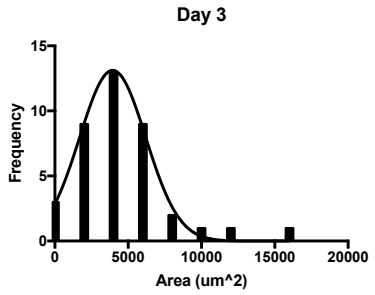


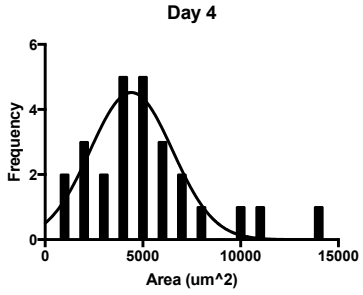


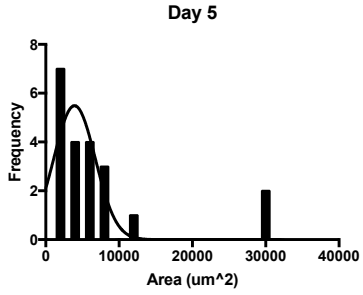
Histograms for Phenylephrine Group Sizes

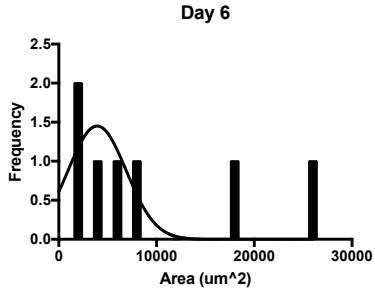


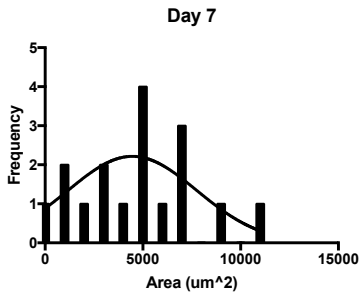


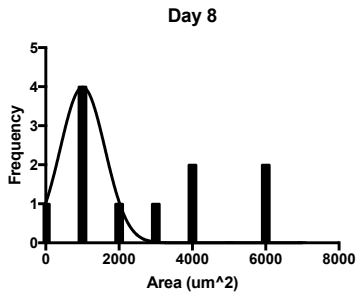


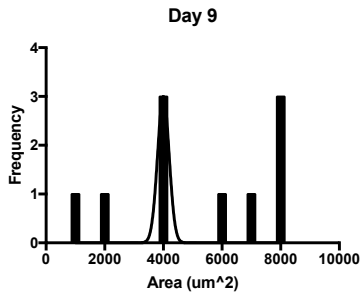


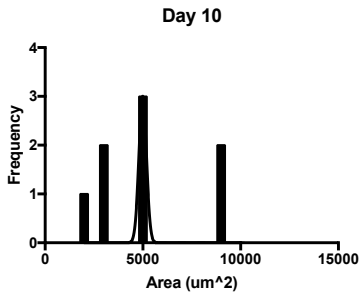


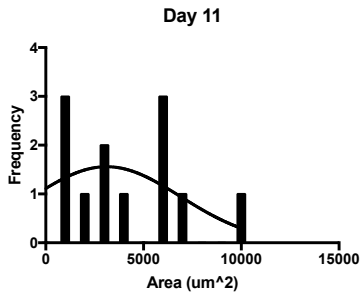


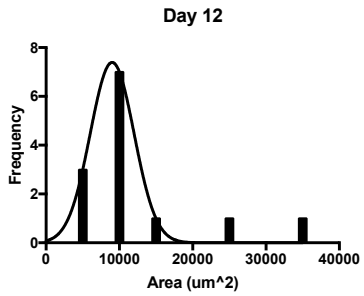


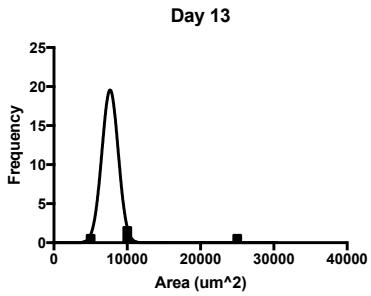


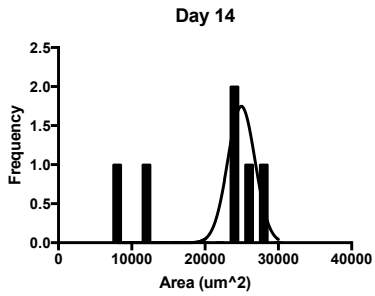


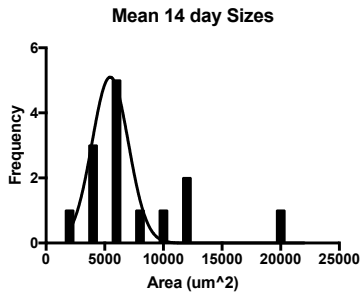




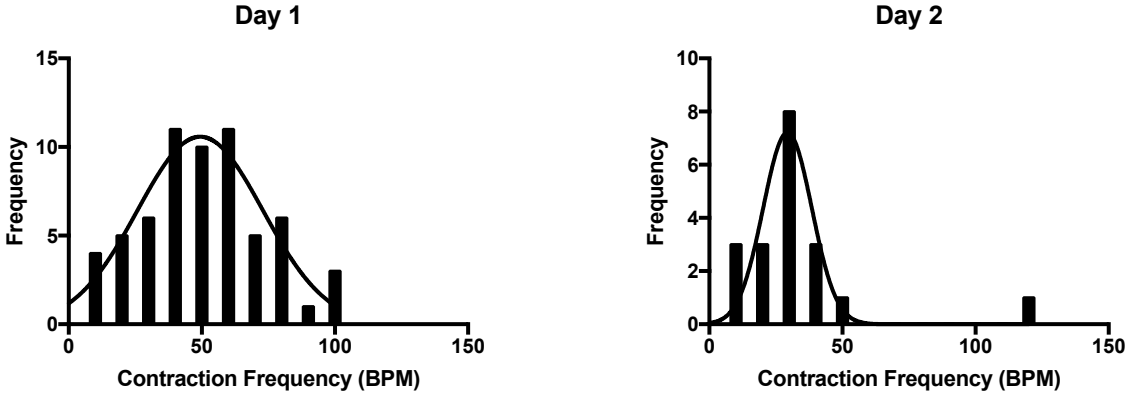


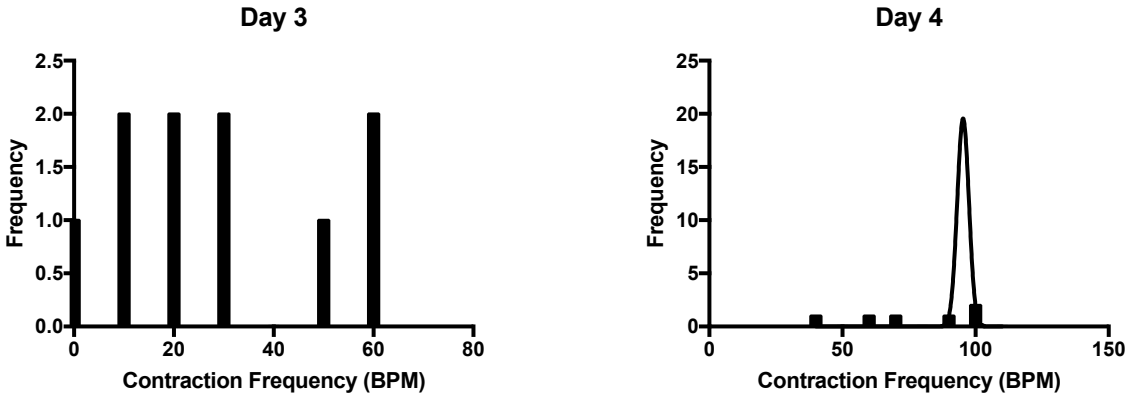


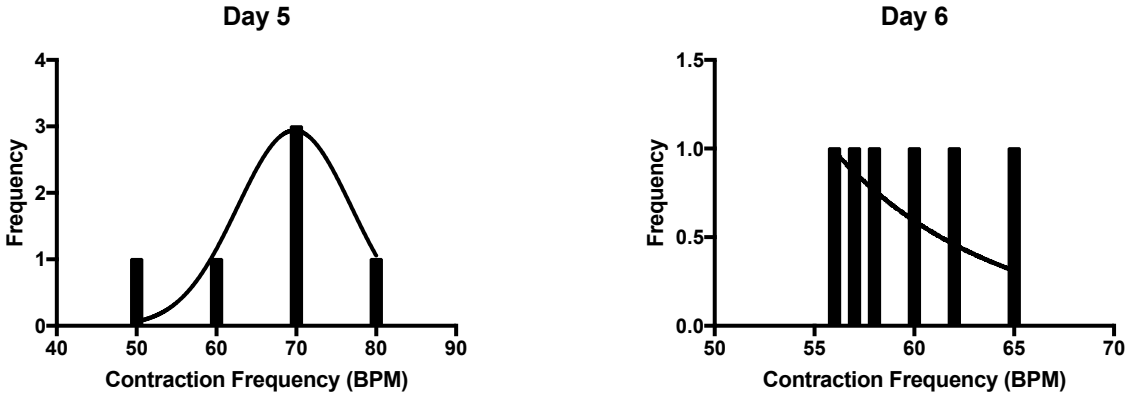


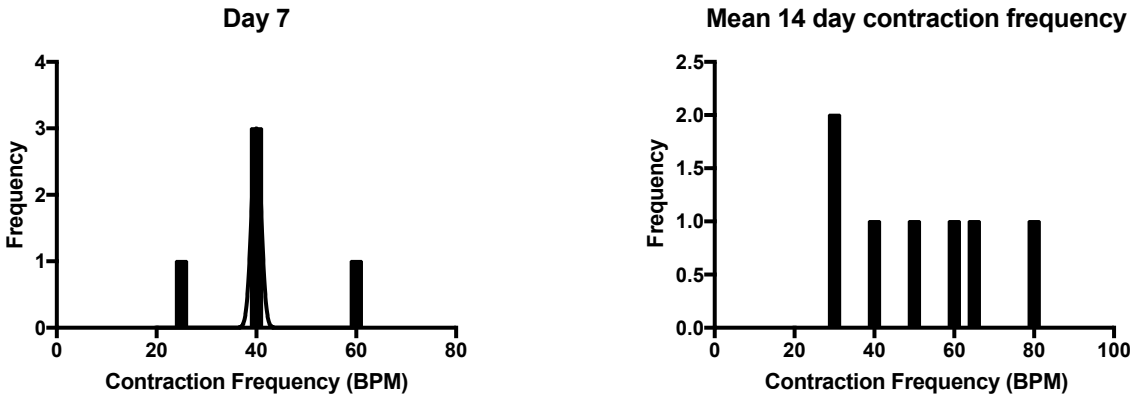


Histograms for Lactate group Contraction Frequency

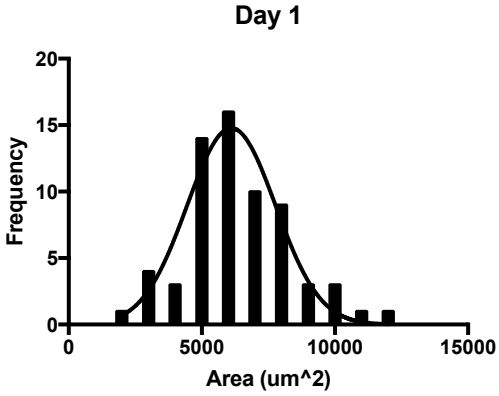


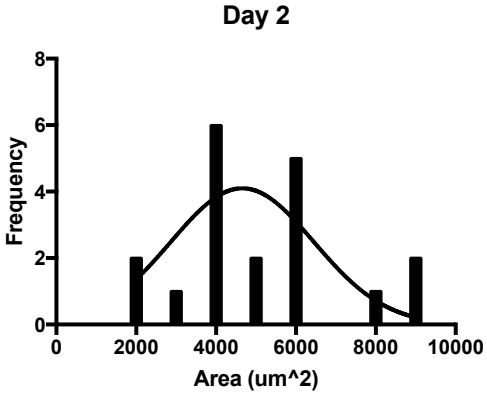


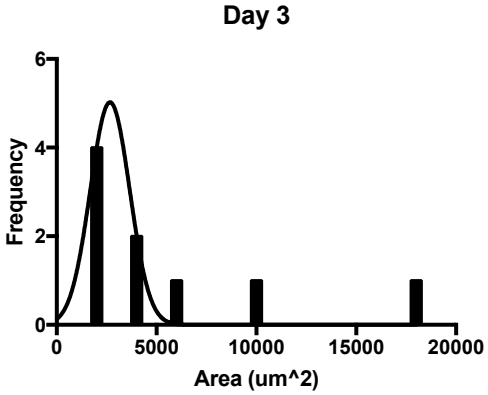


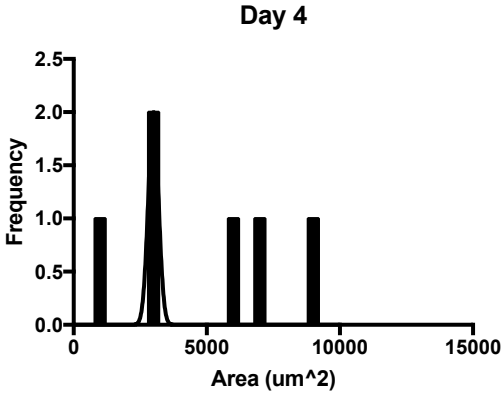


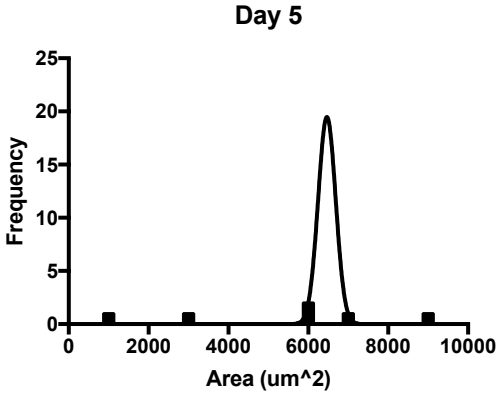
Histograms for Lactate Group Sizes

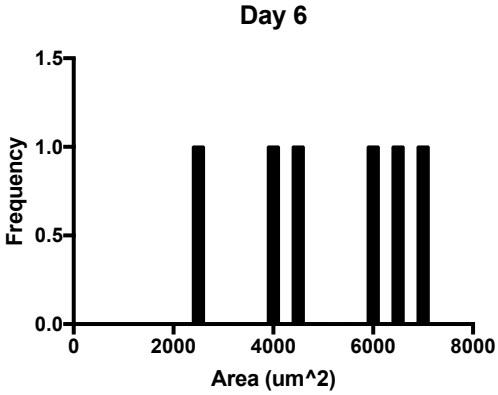


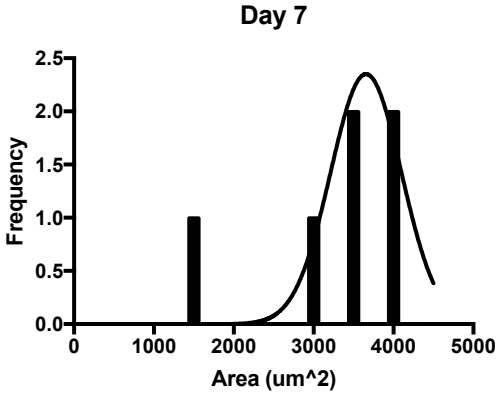


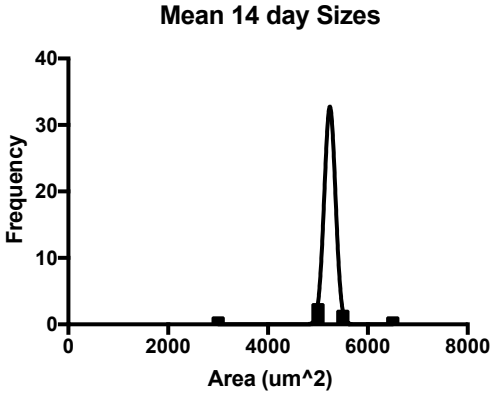




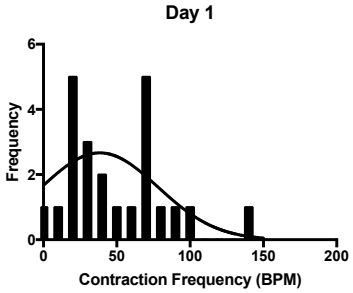


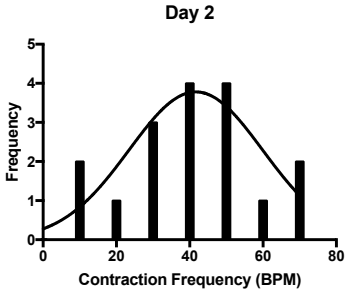


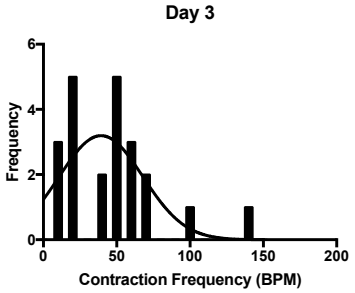


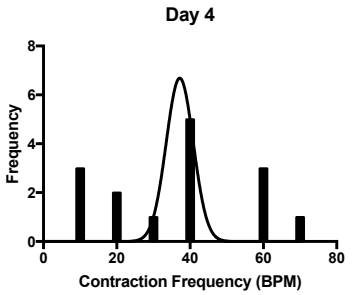


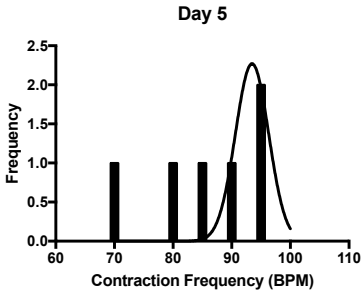
Histograms for Collagenase Method Group Contraction Frequency

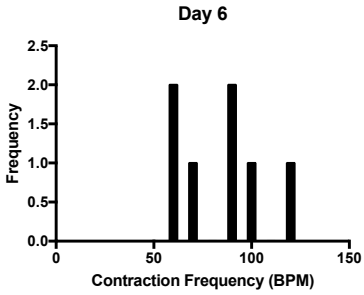


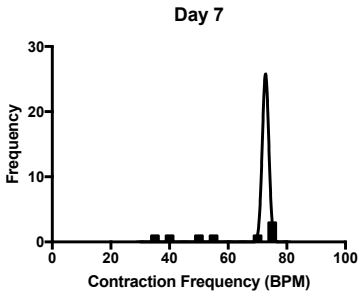


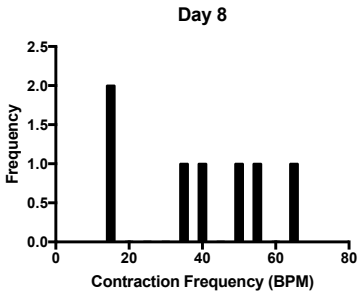


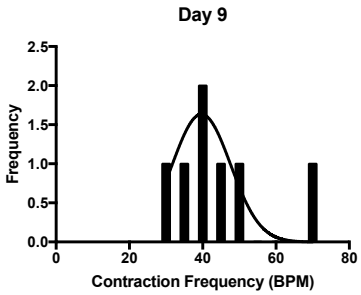


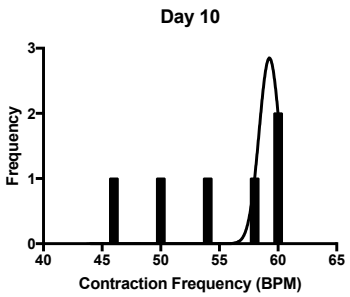


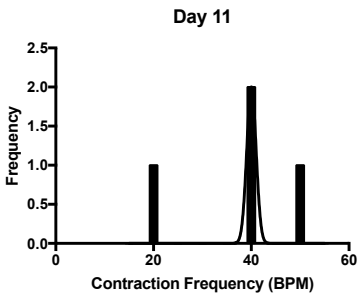


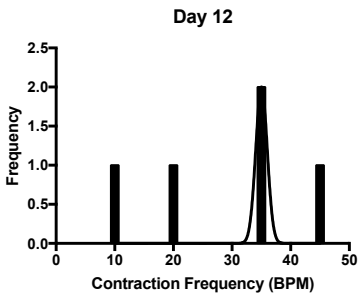


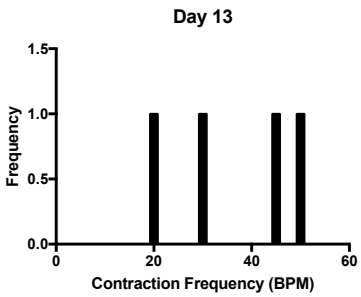


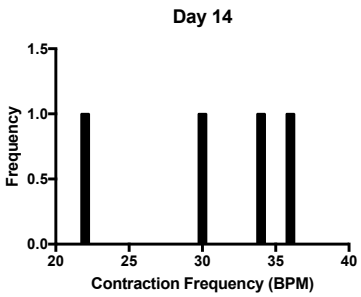


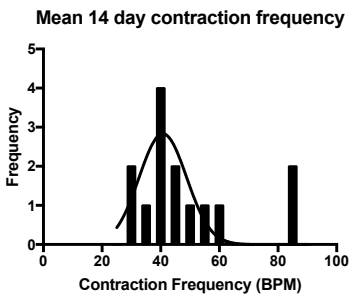




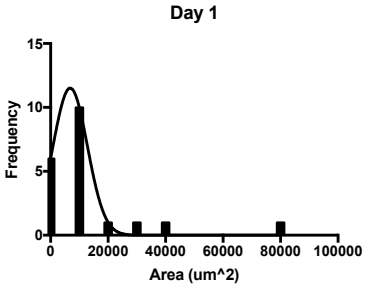


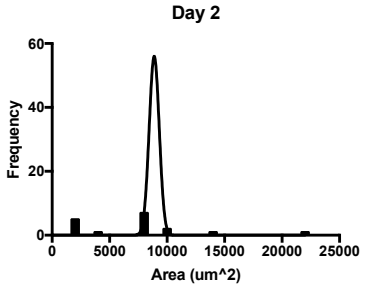


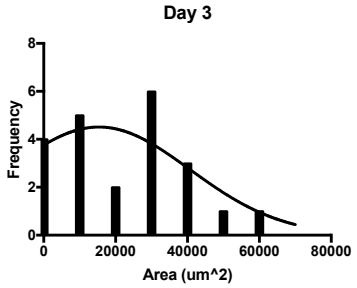


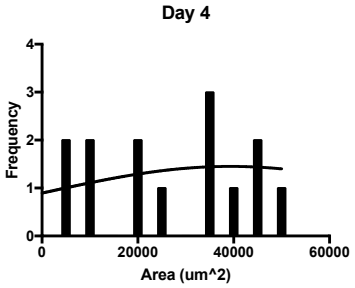


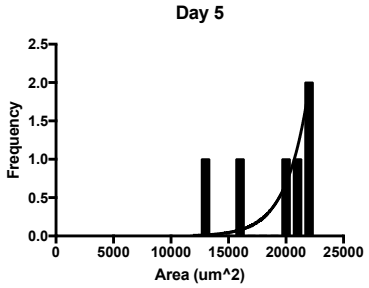
Histograms for Collagenase Method Group Sizes

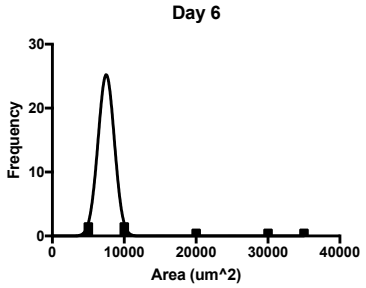


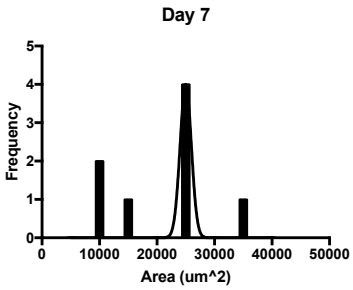


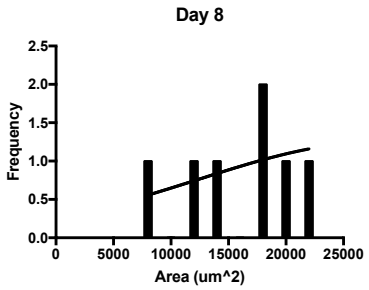


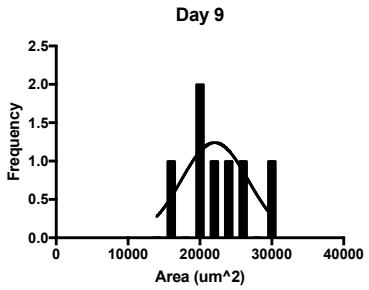


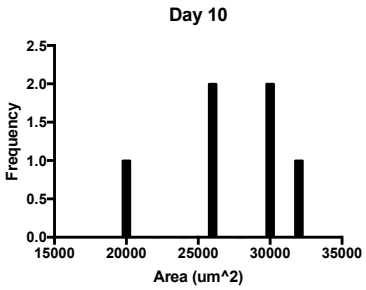


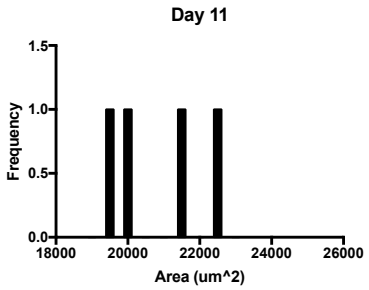


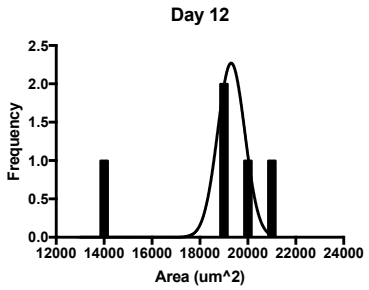


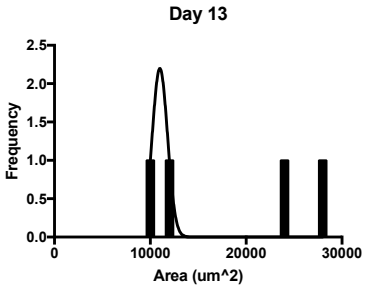


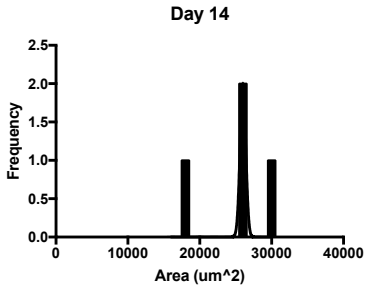


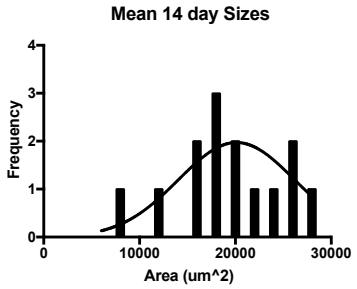












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