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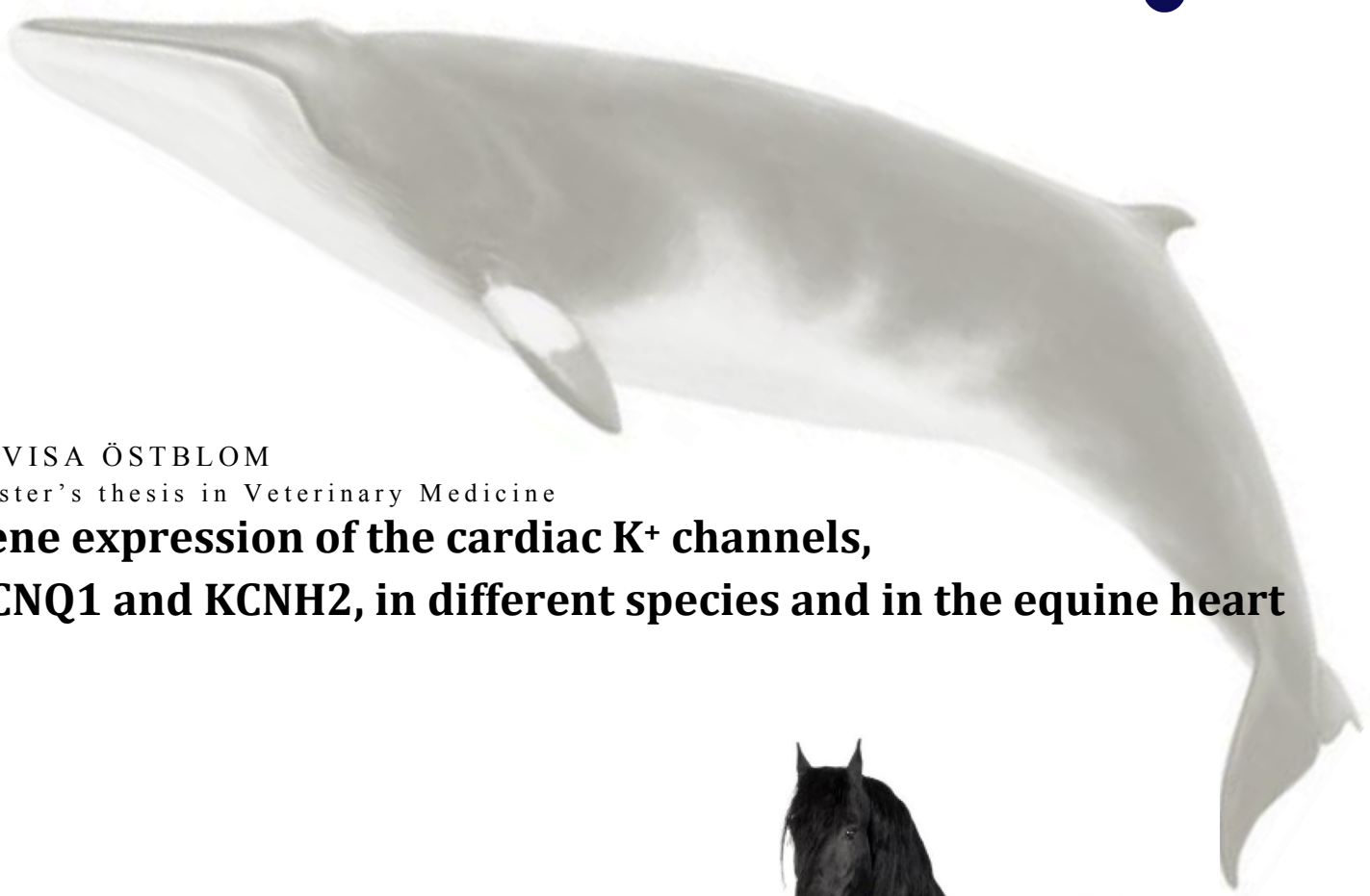
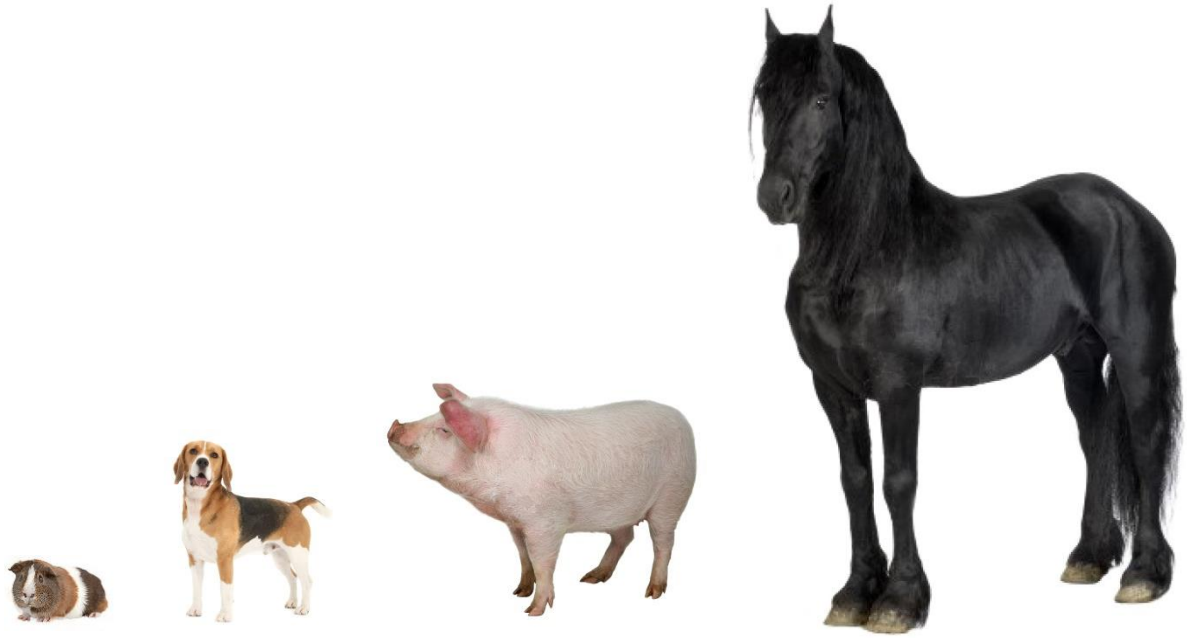


LOVISA ÖSTBLOM

Master's thesis in Veterinary Medicine

Gene expression of the cardiac K^+ channels, KCNQ1 and KCNH2, in different species and in the equine heart



Academic advisors: Dan A. Klærke and Philip J. Pedersen

Submitted: 28th of March 2014

Gene expression of the cardiac K^+ channels, KCNQ1 and KCNH2, in different species and in the equine heart

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Master's Thesis for the Degree in Veterinary Medicine

Faculty of Health and Medical Sciences, University of Copenhagen

Academic advisors: Professor Dan A. Klærke and Ph.D.-student Philip J. Pedersen

28th of March 2014

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Abstract

Even though mammals are a diverse order, they still share many physiological traits. Accompanying increasing body weight is a prolongation of the QT-interval. Prolonged repolarisation in humans is related to the long QT syndrome (LQTS) which is associated with sudden cardiac death. The two main causes for this condition are loss-of-function of the KCNQ1 and KCNH2 gene, which encode two repolarising K^+ channels. This study will by quantitative PCR investigate how KCNQ1 and KCNH2 are expressed in larger mammals, as it is hypothesised that the longer QT-interval in large species primarily is a regulatory adaption. The relative gene expression of KCNQ1 and KCNH2 in guinea pig, dog, pig, horse and minke whale in the left ventricle of the heart was studied with a novel method of normalising to genomic DNA.

It is also known that regional gene expression differences within the heart causes spatial voltage gradients which can facilitate initiation of arrhythmias. Therefore, the regional gene expression differences of KCNQ1 and KCNH2 within five areas of the equine heart was investigated. The Purkinje fibres of the heart were of special interest due to their unique ion channel expression. Laser Capture Microdissection was attempted for isolation of single Purkinje cells. False tendon Purkinje fibres were studied with histology.

It was concluded that the gene expression of KCNQ1 is high in guinea pig, significantly declining in dogs and significantly lower in pig, horse and minke whale. Gene expression of KCNH2 was highest in guinea pig, significantly lower in dogs and lowest in pig, horse and minke whale. The result indicates a regulatory mechanism of the KCNQ1 and KCNH2 between species. Within the equine heart, no significant differences in KCNQ1 gene expression were found between the tested regions. The regional distribution of KCNH2 expression within the equine heart showed that atriums and Purkinje fibres show a significantly lower gene expression than ventricles. The knowledge of regional electrophysiology within the heart will increase the possibility of cardiac disease treatment.

Key words: KCNQ1 – KCNH2 – action potential duration – gene expression

1 Abbreviations

AV =atrio-ventricular

bp = base pairs

bpm = beats per minute

Ca^{2+} = calcium ion

cDNA = complementary deoxyribonucleic acid

C_t-value = cycles to threshold-value

DNA = deoxyribonucleic acid

gDNA = genomic deoxyribonucleic acid

H_20 = water

H-E = Hematoxylin-Eosin

I_{Ks} = slowly activating delayed outward rectifying current

I_{Kr} = rapidly activating delayed outward rectifying current

I_{to} = transient voltage gated outward K^+ current

K^+ = potassium ion

LA = left atrium

LCM = Laser Capture Microdissection

LV = left ventricle

LQTS = long QT syndrome

Na^+ = sodium ion

PAS = Periodic acid-Schiff

PF = Purkinje fibre

qPCR = quantitative Polymerase Chain Reaction

RA = right atrium

RIN-value = RNA Integrity Number

RNA = ribonucleic acid

RNAse = ribonuclease

RPS18 = ribosomal protein S18

RV = right ventricle

SA =sino-atrial

2 Preface

This project is the thesis for my master's degree in veterinary medicine at the Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen. It has been carried out in collaboration with the biotech company Gubra and Ph.D.-student Philip J. Pedersen.

The project can contribute to a better understanding of the evolutionary adaptions to regulation in different species in heart electrophysiology. It may shed light on regulatory components of electrophysiology between species and within the equine heart.

I would like to thank everyone helping me with this project – for support and guidance, encouragement and assistance in study design, laboratory techniques, fault identification and sampling of tissues;

- My supervisor: professor M.D Dan A. Klærke, IKVH, University of Copenhagen
- My co-supervisor: Ph.D.-student, DVM Philip J. Pedersen
- Scientific advisor: Sarah Paulsen, Msc., Ph.D., research scientist.
- Support and reviewing: Emma Fredholm, DVM., Hanna Lähdekorpi, DVM.
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I hope that anyone interested in this subject find this thesis read-worthy.

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Lovisa Östblom

28th of March 2014 Frederiksberg, Denmark

3 Content

4 Introduction

Mammals represent an astonishingly diverse order which still share many physiological traits. The crucial pump function of the heart is responsible for distributing oxygenated blood throughout the body and thereby making life possible. The mammalian heart shares the basic anatomical appearance and function of pumping oxygenated blood out by the left ventricle, receiving blood from the body by the right atrium which is to be pumped into lungs by the right ventricle. The left atrium receives the oxygenated blood from the lungs and this is yet again pumped out by the left ventricle. The heart serves the same goal but cardiac function or performance must also correlate to the needs of the particular species. The different metabolic requirements are met by heart rate rather than a relative to body weight different heart size. Resting heart rate can in fact vary between 782 beats per minute (bpm) for a shrew (Spector 1956) to 12 bpm for a whale (Kanwisher & Senft, 1960).

The heart physiology is both very alike and very different between species. Equal enough for comparison between species, yet different enough to represent an individual adaption of a certain species. Large mammals has a longer contraction period of the heart than do smaller mammals (Rosati *et al*., 2008). This represents a slower repolarisation, a phenomenon which is associated to cardiac disease in human. A slow repolarisation enhances the risk of arrhythmias, a non-synchronic contraction of the heart, in humans. When the heart's pump function is impaired, it may result in sudden cardiac death.

Heart physiology does not only vary between different species, but also within the heart itself. The heart is an organ with many anatomical features. The sinoatrial (SA) node dictates the heart rhythm and distributes this signal to the atrium, which contract prior to ventricles. The AV node transmits the signal from the SA node to the ventricles with a slight delay, thereby allowing atriums to contract prior to ventricles. This will give a substantial contribution to filling of the ventricles and give an optimal hemodynamic efficiency. Purkinje fibres distribute the signal from the AV node in the ventricles, so ventricles contract synchronously.

The heart need thus to possess different physiological properties within itself. These differences create heterogeneities of different regions, which may play a role in arrhythmias. Cardiac rhythm disturbances are one of the most common causes of collapse in horses during exercise (Piercy *et al*., 1999) and studies of differences between species and regional differences within the heart will represent one piece in the puzzle towards a better understanding of arrhythmias and sudden cardiac death.

5 Background

5.1 Electrophysiology and the action potential interval

It is of greatest importance that the heart is synchronic in its contractions for a meaningful function. Contraction of the cardiac muscle cells is triggered by an electrical action potential. Cardiac cells are electrically linked to each other by gap junctions. Normal electrical activity in the heart will therefore give a coordinated propagation of excitatory stimuli in single cardiomyocytes from the sinoatrial (SA) node, via the atriums to the atrioventricular (AV) node and His' bundle to be distributed in ventricles through Purkinje fibres.

The membrane potential of a cardiac cell is determined by its relative permeability and gradient of the cell membrane to Na^+, Ca^{2+} and K^+. Action potentials arise as a result of the opening and closing (gating) of ion channels which permit ion fluxes across the cell membrane, creating the different phases of the action potential (Amin *et al*., 2010).

A single contraction and later relaxation consists of a series of events initiated by the pacemaker cell's spontaneous depolarisation, where after the signal is conducted from cell to cell throughout the heart. The action potential is determined by the sequential changes in the permeability of the plasma membrane for Na^+, Ca^{2+} and K^+. The depolarisation is due to opening of the Na^+ channels, causing Na^+ to enter the cell and thereby making the cell positively charged (phase 0). The Na^+ channels are quickly inactivated and partial repolarisation begins with opening of the transient outward K^+ channel (I_{to}) (phase 1). The Ca^{2+} influx is initiated together with a slower outward efflux of K^+ by slow delayed rectifier current I_{Ks} and I_{Kr} (phase 2). Ca^{2+} maintains the depolarisation during the plateau phase, which is intervened when the outwards potassium currents dominate to recreate the negative membrane potential (phase 3). The potential is restored to resting state, where the inward rectifying I_{K1} is dominating (phase 4) (Nerbonne & Kass, 2005).

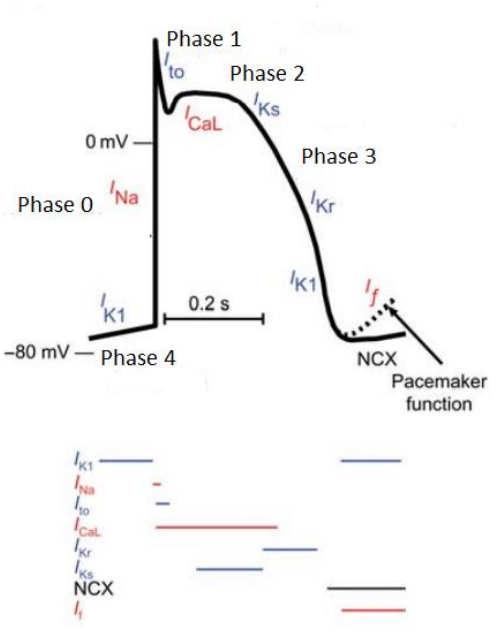

Figure 1. *The action potential and main cardiac ion currents.* The phase 0, 1, 2, 3 and 4 of the action potential and main cardiac ion currents of the action potential. Red, inward (depolarising) current; blue outward (repolarising) current. I_{K1}, inward-rectifier background K^+ current; I_{Na}, Na^+ current; I_{to}, transient outward K^+ current; I_{Ks}, slow delayed-recitifier K^+ current; I_{Kr}, rapid delayed rectifying K^+ current; NCX, sodium-calcium exchanger; I_f, 'funny' current. (modified after Michael *et al*., 2009).

5.2 Regional differences of cardiac action potentials

The action potential and transmission varies between anatomical regions to ensure a perfect timing from initiation of action potential in the pacemaker cells in the SA node to contraction of the atriums followed by contraction of the ventricles and an adequate resting period, allowing filling of the heart.

The action potential waveform differences ensure a unidirectional propagation of excitation. The SA node possesses unique abilities in order to control hearth rhythm. A funnily behaving Na^+/K^+ current, I_f, displays unique gating properties and enable a spontaneous initiation of cardiac electrical activity in pacemaker cells. The depolarisation is much slower in the pacemaker cells of the SA-node than seen in other cardiac regions (figure 2), likely caused by a not so prominent role of the Na^+-channel (Nerbonne & Kass, 2005). The slow depolarisation seen in the SA node will inactivate most Na^+ channels and decrease their availability for phase 0. The depolarised state of the action potential is instead mainly achieved by Ca^{2+} currents (Amin *et al.*, 2010).

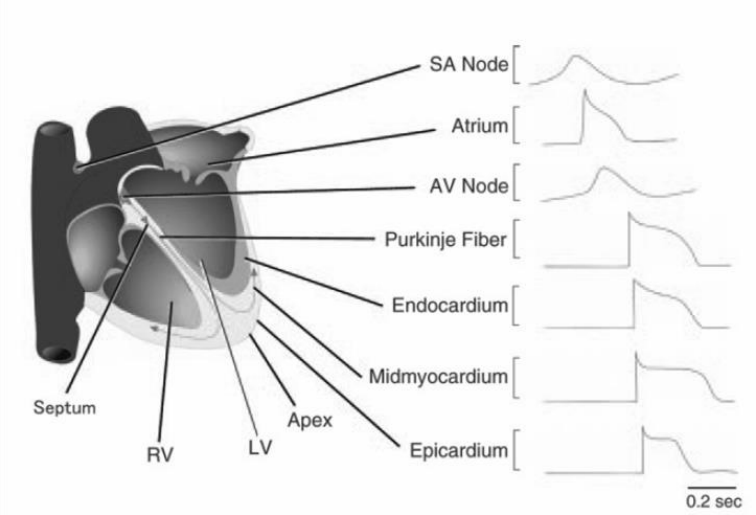

Figure 2. *Action potential waveforms in different cardiac regions of a human heart.* Figure shows the action potential waveform in sinoatrial (SA) node, atrium, atrioventricular (AV) node, Purkinje fibre, endocardium, midmyocardium and epicardium. Right ventricle (RV) and left ventricle (LV) are indicated (Nerbonne & Kass, 2005).

After initiation of the SA node a relatively slower conductance to the atrial-ventricular (AV) node allow atriums enough time to contract prior to ventricles. The atria displays a different action potential waveform than do the SA node with a fast phase 0, followed by transient repolarisation reflecting Na^+ channel inactivation and activation of fast transient voltage-gated outward K^+ current ($I_{to,f}$) (Nerbonne & Kass, 2005).

To ensure a fast conductance once the AV node initiate an action potential in the ventricles, distribution throughout the ventricle must be efficient over large areas. This fast and efficient conductance is due to Purkinje fibres (PF), which distribute the potential from the AV node. Even though PFs make a crucial part of heart function, they are not fully understood (figure 3) but are known to work differently than ventricular myocardium. The PFs have a prolonged action potential plateau and a known altered ion channel expression and faster conductivity (Qu *et al.*, 2007;

Sedmera & Gourdie, 2014; Vaidyanathan *et al*., 2013) with a constant refractory period independent of heartrate (Dale & Drury, 1932). Though different, they ought to resemble myocytes, as they are derived from these cells and differentiate foetally (Gourdie *et al*., 1995).

The I_{to} current changes in Purkinje cells compared to ventricles, because of regulatory changes of inactivation and recovery (Han *et al.*, 2000). The flow of sodium ions is responsible for the prolonged plateaus as well as the upstroke (Fearon & Gautier, 2007). Purkinje cells repolarise differently during phase 1 and the plateau through a limitied I_{Kr} current contribution (Dumaine & Cordeiro, 2007). Purkinje cells' local heterogeneity in conduction compared to myocytes is recognised as an important cause of cardiac arrhythmias (Han *et al*., 2000).

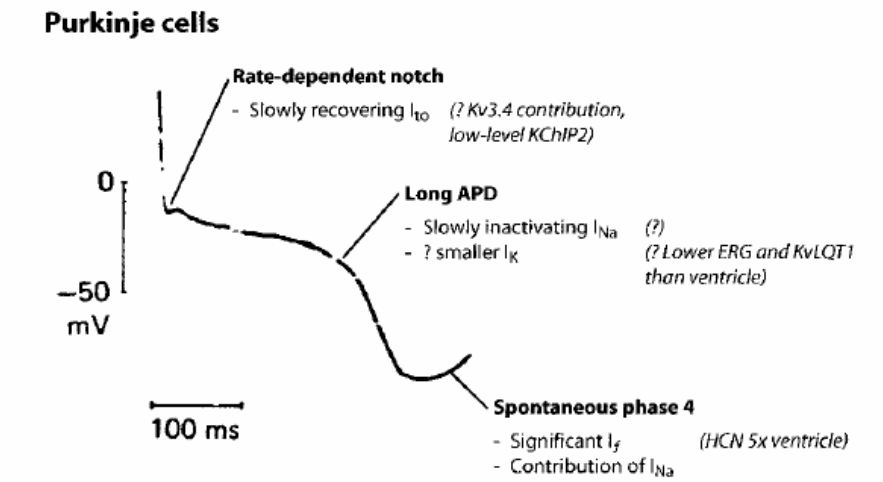

Figure 3. *Purkinje cell properties with possible regulations in the action potential currents in relation to ventricles in humans*. Special features of Purkinje fibres action potential are shown with speculations of the regulating cause. I_{Na}, Na^+ current; I_{to}, transient outward K^+ current; I_K, K^+ current; I_f, 'funny' current; ERG = KCNH2; K_vLQT1 = KCNQ1; K_v3.4, voltage-gated potassium channel protein 4; KChIP2, K_v channel-interacting protein 2; HCN, a group of genes encoding I_f. Specification of current in millivoltage (mV) and time in milliseconds (ms) (Schram, 2002).

The ventricular myocardium resemble the atrial action potential waveform, but have a significantly longer plateau (phase 2). Even within the ventricular myocytes, variations in action potential waveform are recognised (Nerbonne & Kass, 2005). The electrical activity changes dependent on location (epi-, endo-, midmyocardium). The heterogeneity in action potential waveforms in different cardiac regions reflects differences in ion channel expression levels (Nerbonne & Kass, 2005).

5.3 Cardiac ion channels and their genetic background

Ion channels are responsible for the previously described ion currents across the cell membrane. A wide range of different ion channels will shape the action potential. They are basically constituted of several proteins: α-subunits, β-subunits and/or accessory proteins, which operate together in

different conformations to regulate their permeability of ions (Roden *et al*., 2002). The assembly of these subunits regulate conformation changes between activated, inactivated and deactivated states.

The α-subunits create a selective ion pore which only transports specified ions through the channel. The principal subunits are linked to auxilary subunits that modify their expression, functional properties and subcellular localisation (Isom *et al.*, 1994). The functional role of the β-subunit is not fully understood, but they possess important regulatory functions. Deletion of β-subunits affects the channel's properties in numerous ways. Reported modifications include changes in channel inactivation as well as alternative interaction with extracellular matrix proteins, cell adhesion molecules and cytoskeletal linker proteins. They may also affect the channel with a direct physical action rather than a modulating function (Yu *et al*., 2005).

Na^+-, Ca^{2+}-, pacemaker-, transient outward- and delayed rectifier K^+-channels are voltage gated, meaning that they are regulated by the membrane potential. In order to sense voltage changes, region(s) sensitive to voltage register this and respond by changing conformation and thereby function of the ion channel complex (Roden *et al.*, 2002). The inactivated state is expressed by some voltage-gated ion channels like the Na+ channel active during phase 0 of the action potential. When inactivated, the channel does not conduct voltage.

Due to the voltage-dependence of the ion channels shaping the action potential, the inwards or outwards amplitude of ion currents are dependent of membrane potential and conductivity. Membrane potential changes is expected to result in a linearly response of the current amplitude. However, not all channels behave in this way – these channels alter conductivity in response to membrane potentials. Cardiac rectifying currents display this feature, possibly explaining why an outward current of K^+ ions is activated with a delay following initial depolarisation (Amin *et al.*, 2010). Due to their delay in activation they do not substantially contribute to the phase 0 or 1 of the action potential, but becomes important in later phases of the action potential (Roden *et al*., 2002).

The channels' functionality is encoded by multiple genes, as shown in table 1, and the actual current is dependent of the combined expressed pool of genes regulating the ions' permeability over cell membrane.

Current	α-Subunit	Gene	β-subunit(s)/accessory proteins	Gene
I_{Na}	$Na_v1.5$	*SCN5A*	β1	*SCN1B*
			β2	*SCN2B*
			β3	*SCN3B*
			β4	*SCN4B*
$I_{to,fast}$	$K_v4.3$	*KCND3*	MiRP1	*KCNE2*
			MiRP2	*KCNE3*
			KChIPs	Multiple genes
			DPP6	*DPP6*
$I_{to,slow}$	$K_v1.4$	*KCNA4*	$K_v\beta1$	*KCNB1*
			$K_v\beta2$	*KCNB2*
			$K_v\beta3$	*KCNB3*
			$K_v\beta4$	*KCNB4*
$I_{Ca,L}$	$Ca_v1.2$	*CACNA1C*	$Ca_v\beta2$	*CACNB2*
			$Ca_v\alpha2\delta1$	*CACNA2D1*
$I_{Ca,T}$	$Ca_v3.1$	*CACNA1G*		
	$Ca_v3.2$	*CACNA1H*		
I_{Kur}	$K_v1.5$	*KCNA5*	$K_v\beta1$	*KCNAB1*
			$K_v\beta2$	*KCNAB2*
I_{Kr}	$K_v11.1$	*KCNH2*	MiRP1	*KCNE2*
I_{Ks}	Kv7.1	*KCNQ1*	minK	*KCNE1*
I_{K1}	Kir2.1	*KCNJ2*		
I_f (pacemaker current)	HCN1-4	*HCN1-4*		

Table 1. *Genetic and molecular basis of cardiac ion currents* (modified after Amin *et al*. 2010).

Notably, β-subunits/accessory proteins do not exclusively correspond to distinct α-subunits but can actually link to diverse α-subunits. Therefore the true relationship between these components *in vivo* is debated.

5.4 Primary electrical heart disease and implication

The importance of ion channels is highlighted by the increased incidence of arrhythmias in either inherited or acquired heart diseases affecting the electrophysiological properties. Normal cardiac excitation and relaxation is dependent on the fine balance of ion currents passing through different membrane channels. Genetic defects, pharmaceuticals or structural abnormalities can alter the balance and cause severe arrhythmias leading to (sudden) cardiac death (Clancy & Kass, 2002). Genetic-caused arrhythmias are seen as a result of mutations in genes encoding ion channels, their accessory proteins, changes in expression levels or gating properties (Amin *et al*., 2010). This results in an altered electrophysiology which can be categorised into either a loss-of-function or a gain-of-function.

The consequence of electrophysiological imbalances is an abrupt change in heart rhythm which impairs pump function and deprives vital organs of oxygenated blood. A short state may result in syncope, whereas longer periods lead to death. Arrhythmias commonly associated with sudden cardiac death include ventricular tachycardia and ventricular fibrillation, both conditions where the heart fail to pump enough blood throughout the body's circulation system (George, 2013).

Pathologic electrophysiology of the heart can affect the initiation, conduction system, relative timing or contractile ability.

The symptom of arrhythmia has it's background in three electrophysiogical dysfunctions; abnormal automaticity, triggered activity and re-entrant excitation. Diseases causing arrhythmias in the structurally normal heart is commonly referred to as 'primary electrical disease'. The exact pathophysiology of triggering arrhytmias at a multicellular level is still unknown, but delayed repolarisation may increase the spatial voltage gradients sufficently enough to trigger repetitive excitation of ventricles (Wolf & Berul, 2008). Already mentioned, differences in the expression levels of ion channels underlie marked heterogeneity in action potential duration between different cardiac regions. The Purkinjefibers' difference in ion channel composition is thought to increase their potential of re-entry and thereby foci of arrhytmias (Boyden *et al.*, 2010). Changes in expression levels or gating properties of ion channels in pathologic conditions may exaggerate such regional heterogeneities and excitation waves may travel along a constant or different route to repeatedly excite cells (Nerbonne & Kass, 2005).

The action potentials of the heart can be measured by the electrocardiogram (ECG), which reflects the heart's electrical activity. The cardiac muscle cells works as dipoles which are created when ion currents flow across the cell membranes. This can be measured by an ECG, where the P wave represents the atrial depolarisation, the QRS complex the ventricular depolarisation and the T wave the ventricular repolarisation.

Focusing on genetic ion channels disorders, mutations affecting several different ion channels can lead to a similar phenotype of the action potential waveform and ECG. Such a phenotypically alike condition with various genetic backgrounds is long QT- syndrome (LQTS), a disorder first described in 1957 (Jervell & Lange-Nielsen, 1957) which affects 1 in every 2000-5000 human (Goldenberg & Moss, 2008; Saarel & Etheridge, 2014). It is characterised by a prolongation of the QT interval of the ECG caused by a longer plateau phase of the action potential (figure 4).

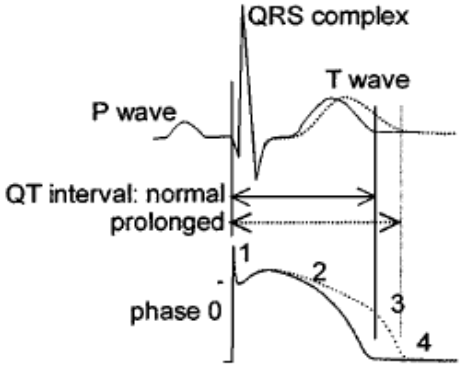

Figure 4. *Relation between electrocardiogram (top), QT-interval (middle) and action potential phases (bottom)* (Roden *et al*., 2002).

The action potential waveform changes due to Na^+, Ca^{2+}, K^+ current alternations or other unknown causes, which is caused by mutation in different genes in congenital LQTS (figure 5).

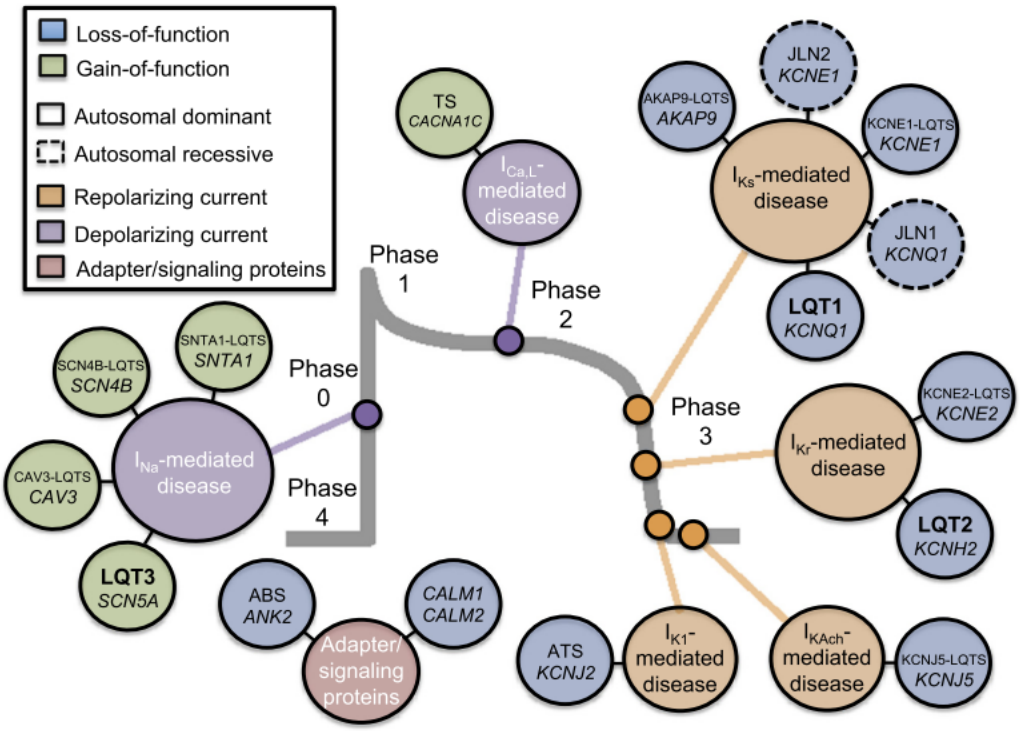

Figure 5. *Known congenital causes of LQTS affecting different phases of the action potential*. Mutations effect on ventricular cardiac action potential (purple), repolarisation (orange) or adaptor/signalling proteins (brown). Loss-of-function is indicated by blue and gain-of-function is indicated by green. Solid lines indicate a disorder that is inherited in an autosomal dominant way and dashed lines in an autosomal recessive way (Giudicessi & Ackerman, 2013).

The most prevalent dysfunctional current leading to LQTS is that of repolarising K^+ channels (table 2). Here, it is the α-subunits of the I_{Ks} (KCNQ1) and I_{Kr} (KCNH2) ion channel that are altered in most cases, rather than β-subunits and/or accessory proteins. This could be explained by gene length and relative risk of mutation; the KCNE1 gene is for example 1/5 the size of the KCNQ1 gene (Abbott *et al.*, 2007). Assuming a similar rate of mutation per base, this alone would make mutations less frequent in KCNE1. Both loss-of-function and gain-of-function mutations of KCNQ1 and KCNH2 are recognised, leading to long or short QT syndrome respectively– reflecting the I_{Ks}/I_{Kr} function of repolarisation.

Type	Occurrence (or % of genotyped)	Gene	Protein	Protein function	Affected current
Long QT Syndrome					
1	42%–54%	*KCNQ1*	$K_v7.1$	α-subunit I_{Ks} channel	I_{Ks} decrease
2	35%–45%	*KCNH2*	$K_v11.1$	α-subunit I_{Kr} channel	I_{Kr} decrease
3	1.7%–8%	*SCN5A*	$Na_v1.5$	α-subunit Na^+ channel	I_{NaL} increase
4	<1%	*ANK2*	Ankyrin-B	Adaptor protein	None
5	<1%	*KCNE1*	minK	β-subunit I_{Ks} channel	I_{Ks} decrease
6	<1%	*KCNE2*	MiRP1	β-subunit I_{Kr} channel	I_{Kr} decrease
7	Rare	*KCNJ2*	Kir2.1	α-subunit I_{K1} channel	I_{K1} decrease
8	Rare	*CACNA1C*	$Ca_v1.2$	α-subunit Ca^{2+} channel	$I_{Ca,L}$ increase
9	Rare (1.9% in one study)	*CAV3*	Caveolin-3	Component of caveolae (co-localizes with $Na_v1.5$ at sarcolemma)	I_{NaL} increase
10	<0.1%	*SCN4B*	$\beta4$	β-subunit Na^+ channel	I_{NaL} increase
11	Rare (2% in one study)	*AKAP9*	Yotiao	Mediates I_{Ks} channel phosphorylation	Inadequate I_{Ks} increase during β-adrenergic stimulation
12	Rare (2% in one study)	*SNTA1*	α1-syntrophin	Regulates Na^+ channel function	I_{NaL} increase
Short QT Syndrome					
1	Three families	*KCNH2*	$K_v11.1$	α-subunit I_{Kr} channel	I_{Kr} increase
2	Two case reports	*KCNQ1*	$K_v7.1$	α-subunit I_{Ks} channel	I_{Ks} increase
3	One family (two members)	*KCNJ2*	Kir2.1	α-subunit I_{K1} channel	I_{K1} increase
Familial Atrial Fibrillation					
—	One (small) family	*KCNE3*	MiRP2	β-subunit $I_{to,fast}$ channel	$I_{to,fast}$ increase
—	Three families	*KCNA5*	$K_v1.5$	α-subunit I_{Kur} channel	I_{Kur} increase
—	One family	*KCNH2*	$K_v11.1$	α-subunit I_{Kr} channel	I_{Kr} increase
—	Two families	*KCNE2*	MiRP1	β-subunit I_{Kr} channel (may modulate I_{Ks} channel)	I_{Ks} increase
—	One family	*KCNQ1*	$K_v7.1$	α-subunit I_{Ks} channel	I_{Ks} increase
—	One family	*KCNJ2*	Kir2.1	α-subunit I_{K1} channel	I_{K1} increase

Table 2. *Genetic basis of inherited cardiac diseases.* The KCNQ1 and KCNH2 gene is highlighted by a red box to point out the focus of this study (modified after Amin *et al*., 2010).

LQTS is seen despite no structural pathologies of the heart, and is usually recognised in non-elderly individuals. Its implication varies between asymptomatic conditions to sudden cardiac death, more recently also reported to occur in sudden infant death syndrome and intrauterine deaths (Crotti *et al*., 2014). However, the risk of sudden cardiac death is not equal for all individuals carrying the mutation, and different modifiers are recognised (Crotti *et al*., 2009). The biophysical function, type and location of the ion channel mutation may affect the risk of serious consequences. It should be noted, that the feature of a long QT interval does not only have genetic backgrounds, but also include acquired conditions such as myocardial ischemia, cardiomyopathies, hypokalemia, hypocalcemia, hypomagnesemia, autonomic influences, pharmaceuticals and hypothermia (Goldenberg & Moss, 2008).

5.5 KCNQ1 and slowly activating delayed outward rectifying current (I_{Ks})

The slowly activating delayed outward rectifying current slowly activates during the depolarising phase, shows no inactivation and deactivates slowly during repolarisation (Nerbonne & Kass, 2005).

In the ventricular I_{Ks} channel complex, it is generally accepted that the KCNQ1 (also called K_vLQT1) α-subunit $K_v7.1$ must assemble with minK (encoded by KCNE1) to produce the characteristic slowly activating I_{Ks} current. This has been experimentally proved in numerous mammals including guinea pig, horse and humans (Abbott *et al.*, 2007). When $K_v7.1$ assembles

with minK, activation is hereby decreased 5- to 10-fold together with regulatory changes leading to altered pharmacology of the complex. The possible regulatory role of other factors in relation to the KCNQ1 channel is less understood (Abbott *et al*., 2007) and no structural studies of this channel in complex with any other KCNE-family member is recognised (Van Horn *et al*., 2011). Apart from minK, is the I_{Ks} also dependent of yotiao, an anchoring protein important for spatial and temporal signalling (Li *et al*., 2012). It mediates interaction with cyclic adenosine monophosphatase dependent protein kinase A, and protein phosphatase 1, with regulates the I_{Ks} current in relation to β-adrenergic control (Marx *et al*., 2002). Mutations recognised in yotiao will also lead to prolonging of the action potential (Morita, 2013).

Nevertheless, gene mutations in the KCNQ1 gene itself can cause cardiac disorders which are recognised in humans. Loss-of-function of the KCNQ1 gene results in prolonged action potential duration and QT-interval (Amin *et al*., 2010), recognised as LQT1. KCNQ1 gain-of-function is linked to atrial fibrillation by a shortened action potential interval, which makes re-entry easier (Tsai *et al*., 2008).

5.6 KCNH2 and rapidly activating delayed outward rectifying current (I_{Kr})

The KCNH2 (ERG) gene encodes the α-subunit $K_v11.1$ of the channel responsible for I_{Kr}. I_{Kr} activation is slow during phase 0, but the $K_v11.1$ channel exhibit a fast, voltage-dependently inactivation. It results in a small outward K^+ current near the end of the action potential upstroke. As indicated by its full name, the channel is rapidly reactivated from the inactive state it reaches during depolarisation, and produces large I_{Kr} amplitudes during phase 2 and 3, making it responsible for repolarisation of most cardiac cells under normal circumstances. The channel is slowly deactivated independent of voltage prior to phase 4 (Amin *et al*., 2010).

MiRP1 (encoded by KCNE2) has been shown to interact with $K_v11.1$ channel function, altering its function and pharmacology. The *in vivo* role of MiRP1 is still under debate, in combination with the role of other MiRPs (2-4). It may be the case that most K_v channels display temporal heterogeneity of subunit composition, complicating both modelling of their functional impact on the ventricular action potential and design of current-targeted modulating drugs. The β-subunits may be the tail wagging the dog – possibly controlling various K^+ channels in a many ways (Abbott, 2012).

Independent of the possibly shifting composition of the channel responsible for I_{Kr}, mutations of KCNH2 gene have proven to seriously affect the current. The second most prevalent type of LQTS – LQT2 – is caused by a loss-of-function of KCNH2, causing action potential duration and QT-interval to increase. A gain-of-function mutation leads to the rare short QT-syndrome where repolarisation is shortened and re-entrant excitation waves more easily can induce atrial and/or ventricular arrhythmia (Amin *et al*., 2010).

5.7 Heart function in relation to species

The mammalian heart shares the basic anatomical features and serves the same, simple goal in all species by oxygenating the animals' tissues by acting as a pump.

Heart weight and body weight are nearly linearly related in mammals (Stahl, 1965) with a heart weight corresponding to 0.5-0.6% of body weight. The mammalian heart size is related to body mass in a relationship as following;

$y = 6.0x^{0.98}$, where y= heart weight (g) and x=body weight (kg). (Prothero, 1979)

As larger animals have a relative lower metabolic rate than do small mammals (White & Seymour, 2004), the efficiency of the pump must in some way be adjusted to correspond to need. Due to the constant relationship of heart size in relation to body weight, it is clear that larger mammals do not compensate their relative decreased need of oxygenation by relative reduced heart size. However, heart rate declines in relation to body weight from 782 bpm for a shrew, 280 bpm for a guinea pig (Spector 1956) to as low as 12 bpm for a whale (King *et al*., 1953).

The decreased metabolic need between species is thus addressed by pulse rather than relative heart size. There are other possible ways of adjustment, either directly related to the heart or indirectly. Decreased heart contraction efficiency would lower output, but cardiac myocytes is constructed identically without significantly differing in size (Loughrey *et al*., 2004) and a switch in contractile system is only recognised in animals with a resting heart rate above 300 bpm (Hamilton & Ianuzzo, 1991). Neither indirect causes, such as different blood volume (Altman & Dittmer, 1970) nor oxygen capacities of the blood seem altered. The haemoglobin concentration in the blood is quite independent of body size (Schmidth-Nielsen, 1984). In fact, many physiological properties of the heart are consistent, such as medium arterial pressure and minimal diastolic pressure (Elzinga & Westerhof, 1991; Holt *et al*., 1968).

Although differing in heart rate, the heart is only a reflection of metabolic rate; which is further empathised by the fact that the total number of heart beats/lifetime (Levine, 1997) and the total blood volume pumped/lifetime (Dobson, 2003) is constant between species. The preserved, basic anatomy and physiology of the heart and blood reflects the organ's necessary function to maintain a blood pressure capable of ensuring an adequate organ perfusion together with enough oxygen in relation to metabolism.

5.8 Structural and regulatory adaptions of the heart between species

The differences of heart function between species can be due either to the structural (anatomical) or regulatory (physiological) adaptions of the heart, or a combination of both (Rosati *et al*., 2008). Heart anatomy and physiology is in many aspects related to species' size. Like there are significant differences in heart rate between species, the QT-interval is also dependent of body size. The QT-interval is prolonged in larger mammals (Spector, 1956).

Another obvious difference is the absolute heart size difference between species. This lead to the question: – how shall a large whale with a distance of 50-80 cm from SA- to AV-node (James *et al*., 1995) be able to produce the astonishingly short AV transmission time (Meijler *et al*., 1992) seen in the enormous heart?

Structural differences between species' AV-node include blood supply changes, presence/absence of an *os cordis* and an increase with heart size, which however do not enlarge proportionally. The optimal PR interval, a measure for AV conduction time (Meijler, 1985), is associated with optimal ventricular hemodynamic efficiency. An excessively long PR interval will make the atria contract when AV-valves are closed, and thus not contribute to ventricular filling. If the PR interval is too short, atrial contraction would immediately be followed by ventricular systole, giving less time to ventricular filling. In both cases, stroke volume is compromised (Noujaim *et al.*, 2004). The PR interval increases from 40 msec in bat, 120 in dog to 350 msec in horses and elephants, and 450 msec in large whales. Comparing heart size to PR interval, they show stagnation in large animals (Meijler *et al.*, 1992). Even though the whale heart is >100.000 times larger than the bat, the PR interval is only increased 10 times (Meijler, 1985). The relation between size and function is still unexplained.

One of the solutions of transmitting signals lies in an effective conduction system throughout the working myocardium. The traits of the intra-atrial and inter-nodal conduction pathways is controversial, some claiming that Purkinje-like cells connect the SA- and AV-node, while other agree the atria is anisotropic (Sedmera & Gourdie, 2014). The relative need for Purkinje fibres may vary (Sedmera & Gourdie, 2014). In the ventricles of larger animals a pronounced network of PFs is seen (James *et al.*, 1995), thereby shortening the dissemination of the action potential over larger areas.

5.8.1 Electrophysiological differences between species

The electrophysiology of the heart differs – within the heart itself and between species, and in a way corresponding to physical changes.

Mammalian ventricular myocytes can display one of two action potential waveforms; either a spike and dome shape or a triangular shape. The first mentioned is seen in most mammals with the predomination of I_{Ks} and I_{Kr}. The triangular waveform is seen in small rodents, as an adaption to high heart rate with predominantly I_{to} and I_{Kur} being responsible for repolarisation in adults (Rosati *et al.*, 2008). Fetal mice show a current almost exclusively consisting of I_{Kr} but this is post-natally replaced by the I_{to} current (Wang & Duff, 1997; Wang *et al*., 1996).

The general inconsistency of the action potential's shape is due to different ion channels being reported in different species in combination with different expression levels of these channels (Nerbonne & Kass, 2005). It seems like a regulatory mechanism is the primarily factor for evolution between species, meaning that it is rather expression levels which change instead of function or composition of the ion channels (Rosati *et al.*, 2008). The KCNQ1 and KCNH2 are functionally

important genes only in larger mammals (Rosati *et al*., 2008) where I_K is composed of both I_{Ks} and I_{Kr} (Roden et al., 2002). The relative contribution of I_{Kr} and I_{Ks} in larger mammals also varies, for example has the dog a significantly greater contribution of I_{Kr} than rabbit, whilst I_{Ks} is similar. The rabbit is therefore more dependent on I_{Kr} (Dumaine & Cordeiro, 2007).

Conclusively, the repolarisation is different in different species. The regulation of the differences in repolarisation may be primarily achieved through different gene expressions between species. The repolarising K^+ currents encoded by KCNQ1 and KCNH2 might explain the difference of action potential duration seen between species in larger mammals. Species-differences of the gene expression have beforehand been recognised (figure 6), with guinea pigs showing a significantly higher expression of both KCNQ1 and KCNH2 than larger animals such as the dog (Rosati *et al*., 2008).

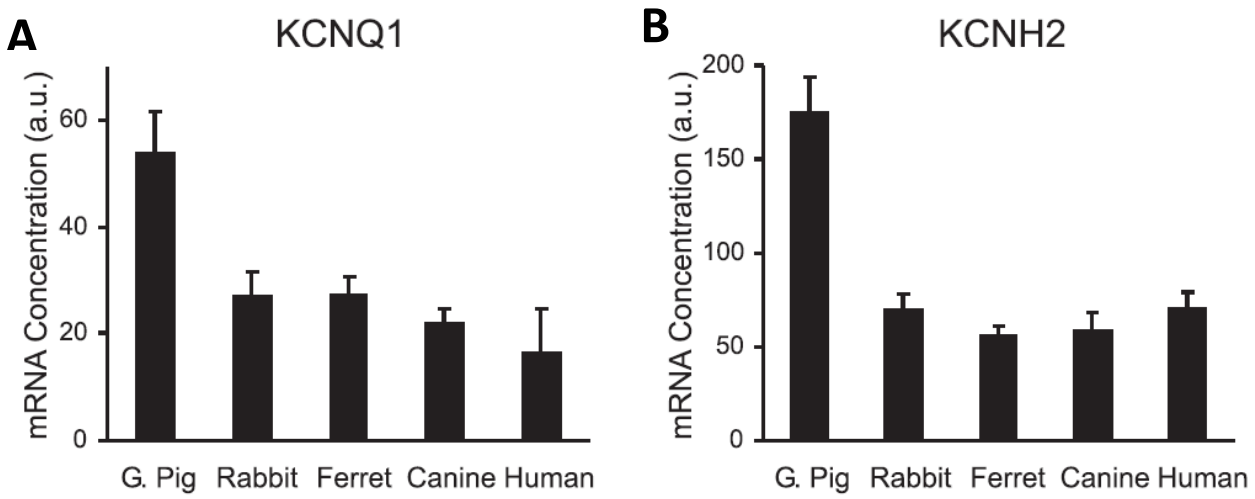

Figure 6. *Gene expression of KCNQ1 and KCNH2 between different species.* (A) KCNQ1 and (B) KCNH2 in left ventricular free wall in guinea pig (G. pig.), rabbit, ferret, dog and human. mRNA concentration is given in astronomical units (a.u.). Values are means ±SD, n = 3 (Rosati *et al*., 2008).

6 Aim

My aim with this study is to compare the cardiac KCNQ1 and KCNH2 gene expression level between guinea pig, dog, pig, horse and minke whale; i.e. compare mammals of very different sizes. The KCNQ1 and KCNH2 gene homology will be compared across species in order to design primers which can be used for all species in this study. The experiment will with quantitative PCR investigate the gene expression level with a novel method of normalising the relative gene expression to genomic DNA. This new method will be compared to normalising to the house-keeping gene ribosomal protein S18 (RPS18).

I further wish to compare the regional gene expression level of KCNQ1 and KCNH2 in the horse. Equine samples from the left and right atrium, left and right ventricle and Purkinje fibres will be compared. Purkinje cells will be isolated by Laser Capture Microdissection, which enable a precise sampling of single cells. The study will result in a relative gene expression comparison within the equine heart.

7 Method and materials

7.1 Tissue origin and handling

In order to investigate the repolarising potassium channels expression levels in species with increased body weight five different species where chosen: guinea pig, dog, pig, horse and minke whale. Heart tissue was derived from adult or nearly fully outgrown individuals. Guinea pigs were euthanized by cervical dislocation and hearts were immediately dissected out. Dogs were anesthetised with a tiletamin-zolazepam mix and thereafter euthanized by injection of pentobarbital. Pigs were made unconscious by CO_2 and de-blooded. One pig was however euthanized under anaesthesia by de-blooding the aorta. Horses were bolt-pistoled and de-blooded. Sampling varied between immediately sampling of heart tissue to a one hour delay after post mortem. The procedure carried out for the minke whale is not known. All tissues were collected with operators wearing gloves, using clean instruments treated with RNase Blaster Solution (Clontech). Samples from left atrium, left ventricle, right atrium and right ventricle were collected and immediately frozen on dry ice. False tendon Purkinje fibres running in left or right ventricle were also collected from the horse and frozen on dry ice. Some equine heart tissue samples were also immediately after euthanasia preserved in formalin.

Mice hearts were used in order to optimise an alternative preservation method for RNA intended for later use on Laser Capture Microdissection (LCM). Mice hearts were immediately after euthanasia by cervical dislocation preserved in a methacarn buffer consisting of 60% methanol, 30% chloroform and 10% glacial acetic acid.

7.2 Cryo-sectioning

Frozen tissue was mounted in Tissue-Tek® (Sakura), cut on freezing cryostat (Leica CM1850) with a temperature of -20°C and mounted on SuperFrost slides (Hounisen). Tissue-blocks were cut with varying orientation towards the knife since preservation of the endocardium on the slide proved difficult. It was easily detached from underlying muscle tissue depending on the cut angle of the knife. Blocks were cut with 0°, 90°, 135° and 180° orientation to the knife.

As it turned out that the Purkinje cells underlying the endocardium were not satisfactorily visible on slides from frozen sections, blocks were cut in several different thicknesses ranging from 12µm to 30µm in order to optimise the cutting procedure and preserve cells on slides.

7.3 Histological imaging and Laser Capture Microdissection

Sections intended for histological imaging of PFs on frozen equine heart tissue were mounted on SuperFrost slides, contra-stained and thereafter mounted with cover-glass by Pertex glue (Leica Biosystems). Sections intended for LCM were mounted on PEN Membrane Frame Slides (Arcturus) in the freezing cryostat chamber in a ribonuclease (RNA'se) free environment. They were stored in -20°C 70% ethanol and stained in room-temperatured cresyl violet for 5 minutes. After dehydration the slide was used for LCM.

Tissue intended for histological imaging was formalin-fixed and paraffin embedded. The fixation was performed as follows (table 3):

Solution	Concentration (%)	Time (hour:min)	Temperature (°C)
Formalin		0:05	40
Ethanol	70%	0:30	40
Ethanol	80%	0:30	40
Ethanol	96%	1:00	40
Ethanol	99.9%	5:30	40
Tissue-Clear® (Sakura)		5:00	40
Paraffin		6:00	60

Table 3. *Program for tissue-processing machine (Tissue-Tek® VIP™, Sakura) prior to embedding tissue in paraffin.*

Following formalin fixation, tissue was embedded in paraffin and sectioned with Microm HM340E (Thermo Scientific) with a thickness of 5 µm. Thereafter sections were mounted on SuperFrost slides, de-paraffined by 15 minutes in Xylene, dipped 2 x 3 min in 99% ethanol, 2 min in 96% ethanol, 2 min in 70% ethanol and rinsed in water for 5 min. Slides were hereafter stained with either Periodic acid-Shiff (PAS), cresyl violet or Hematoxylin-Eosin (H-E).

Periodic acid-Schiff stain was performed by oxidising slides in 0.5% periodic acid for 5 min, where after slides were rinsed in distilled water, stained in Schiff's reagent for 10 min, rinsed in water for 5 min, counter-stained in Hematoxylin for 30 seconds and rinsed in water. Cresyl violet stain was performed by staining slides in cresyl violet for 5 min and thereafter dipping in water 3 times. Hematoxylin-Eosin stain was performed by dipping slides in filtered Hematoxylin for 5 min, where after slides were rinsed in water for 5 min, counter-stained in 1% Eosin for 3 min and thereafter dipped 3 times in water. Hereafter the sections were dehydrated in 70%, 96%, 99% alcohol and incubated in Xylene, and finally mounted with cover-glass by Pertex glue.

Laser Capture Microdissection using a Veritas™ Microdissector was intended to be used for isolation of single cells (see section 8.1).

7.4 Gene comparison by the use of bioinformatics

The National Center for Biotechnology Information's (NCBI's) nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) was used for obtaining nucleotide sequences for the KCNQ1 and KCNH2 gene. KCNQ1 sequences were found for guinea pig, dog (predicted), pig (partial codons), horse (partial codons) and humane. Only the dog and human had known location on chromosome. KCNH2 sequences were found for guinea pig, dog, horse and human, with known chromosome location for dog, horse and human.

The sequences were aligned using NCBI's Basic Local Alignment Tool (BLAST) (http://blast.ncbi.nlm.nih.gov) for inter-species comparison (KCNQ1; appendix I, KCNH2; appendix II). To identify intron-exon boundaries these were identified using ClustalOmega from

The European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/clustalo) (KCNQ1; appendix III, KCNH2 appendix IV).

The sequences were thereafter mutually compared to each other by using the Base-by-Base software produced by Viral Bioinformatics Resource Center (http://athena.bioc.uvic.ca/virology-ca-tools/base-by-base).

7.5 Primer design and testing

Primer design was carried out with multiple criteria. The well preserved gene sequence made it possible to design primers which could be used on all species included in the study. Both KCNQ1 and KCNH2 show a high degree of homology independent of species, being between 80.72%-98.37% identical. Guinea pig, dog, horse and human sequences where used for alignment whereas dog, horse and human where used for BLAST against chromosome in seek for intron-exon borders to produce intron-spanning primers (this assures that the PCR is specific towards expressed genes and not genomic DNA). KCNQ1 were compared between dog and human splice-sites (appendix III), whereas KCNH2 where compared between dog, horse and human (appendix IV).

Universal primers were manually designed based on alignment result between species based on three criteria; highly conserved region between species, being intron-spanning and resulting in a 100-300 base pair (bp) long product. If a single nucleotide differed, the chosen one represented the most prevalent. These primers were thereafter analysed with OligoCalc (http://www.basic.northwestern.edu/biotools/OligoCalc) for hairpins, melting temperature and self-complementary. Equine primers were designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi).

In order to optimise the actual qPCR run, primers must work efficiently and should therefore preferably be designed with the following in mind;

- ✓ Primer-dimer formation

  Primers should not be complementary at their 3'-end, as this can cause primer-dimer artefacts which lower product yield. Primer-dimer formation can be adjusted for in the PCR run with annealing temperature changes.
- ✓ Self-complementarity

  Hair-pins near the 3'-end cause the primer to tie up, extend internally and thereby eliminating itself from the reaction.
- ✓ Stability of primers

  Melting temperature (T_m) is affected by the ratio of AT/GC and length. Primer should preferably be between 18-24 bp in length. Melting temperature ought to be 52-65°C and not deviate more than 5°C from each other. GC-content should be between 50-60%.
- ✓ Internal stability

  A high internal stability at the 5'-end and more unstable 3'-end effectively eliminates false priming.

- ✓ Unique primers

  The primers' sequence must not repeat in the template to avoid amplification of multiple, unspecific products.

  (Rychlik, 1995)

Primer	**Sequence 5'->3'**	**bp**	**T_m (°C)**	**GC-content**	**Concentration**	**Product lenght**
KCNQ1 universal forward	TGGACCTGGAAGGGGAGAC	19	61.0	63.2%	200 nM	199 bp
KCNQ1 universal reverse	CGCACCATGAGGTTGAGGT	19	58,8	57.9%	200 nM	
KCNH2 universal forward	CCCTCTACTTCACCTTCAGCAG	22	62.1	54.5%	200 nM	223 bp
KCNH2 universal reverse	TGGTGGAAGCGGATGAACT	19	56.7	52.6%	200 nM	
KCNQ1 equine forward	GGTGCTCATCACAGACATGC	20	59.4	55%	200 nM	177 bp
KCNQ1 equine reverse	ACGGTCAGCTGTTCGTAGGT	20	59.4	55%	200 nM	
KCNH2 equine forward	TCAGGTTTCCCAGTTCAT	20	59.4	55.0%	200 nM	230 bp
KCNH2 equine reverse	GAAAAGTCCTTGAGGTGCCTA	21	57.9	47.6%	200 nM	
RPS18 human forward	ATCACCATTATGCAGAATCCACG	23	53.5	43%	300 nM	168 bp
RPS18 human reverse	GACCTGGCTGTATTTTCCATCC	22	54.8	50%	300 nM	

Table 4. *Used primers with specification of sequence, length (basepair, bp), melting temperature(T_m), GC-content, used concentration in this study and expected product length.*

Primers were manufactured by Eurofins Mwg Operon (universal primers, KCNQ1 equine specific) or TAG Copenhagen (KCNH2 equine specific) and were delivered as dry substances.

The optimal primer-pair concentration was tested for each primer-pair. The primers were tested in concentrations of 100 nM, 200 nM, 250 nM and 300 nM. The choice of concentration level was decided upon Cycles to Threshold (Ct)-value (as low as possible), dissociation curve (only one

peak) and amplification plots (preferably reach a high level). The dissociation curves for the selected primers all showed a definite peak but unfortunately signs of primer-dimer formation in the negative control sample. The primer-dimer peak was however easily distinguishable from the targeted peak and was not observed in the presence of template.

7.6 RNA purification

RNA purification was done with precautions taken to RNA'ses. Operator was at all times wearing gloves and was working in a clean environment.

Tissue stored in -80°C was added to 1 ml trizol (5Prime) in homogenisation tubes (2 ml tubes containing tissuebeads). The FastPrep homogeniser (MP Biomedicals) was set to 6 m/s for 60 sec, and samples were homogenised by repeating this twice. The tubes were left in room temperature for 5 minutes and were thereafter centrifuged at 12.000 g at 4°C for 10 minutes. The red, soluble phase was transferred into a new tube and 100 µl 1-Bromo-3-Chloro-Propane (Sigma) was added and whirl mixed. After a centrifugation at 12.000 g at 4°C for 15 minutes a standard volume of 450 µl of the top, aqueous phase was transferred to a new tube and 500 µl isopropanol (Sigma) was added. The RNA was now precipitated at room temperature for 5 minutes, and was spun down with 12.000 g at 4°C for 8 minutes.

The RNA precipitate now formed a pellet and the supernatant was removed. RNA was washed by addition of 1 ml 75% ethanol and vortexed. After another centrifugation of 12.000 g at 4°C for 5 minutes the ethanol-wash was removed and the RNA-pellet briefly but thoroughly air-dried and re-suspended in 30 µl RNAse free H_20.

During initial optimisation of the RNA purification method, the recommended volume of tissue to trizol was used (100 mg tissue/1 ml trizol). However, despite perfect homogenisation this resulted in low RNA yields (5–200 ng/µl) and the volume of tissue was therefore increased. As an alternative to trizol purification, a kit "NucleoSpin® RNA" (Macherey-Nagel) was also tested according to manufacturers' instructions. Using this kit, RNA concentrations ranging between 10 200 ng/µl were obtained.

7.7 RNA quantification and quality control

Purified RNA was quantitatively measured using the NanoDrop 2000 (Thermo Scientific). Measured quantities were recorded and used for dilution calculations for BioAnalyser (Agilent Technologies) and cDNA synthesis.

The RNA integrity was assessed on a polyacrylamide-gel microelectrophoresis using a BioAnalyser (Agilent Technologies) and Agilent RNA 6000 Nano Kit with a quantitative range of 20–500 ng/µg. The procedure was performed according to manufacturers' instructions.

7.8 cDNA synthesis

Complementary-DNA (cDNA) synthesis was performed using the AffinityScript qPCR cDNA Synthesis Kit (Agilent Technologies) according to manufactures recommendations. In short, 10 µl first strand master mix, 1.5 µl oligo(dT), 1.5 µl random primers, 1 µl AffinityScript RT/TNase Block Enzyme mixture and 3 µg RNA were mixed and the volume where adjusted with H_20 to a total volume of 20 µl. The mix was spun down and run on a PCR machine (Stratagene™ Mx 3005P) as shown in table 5.

Time	Temperature	Reaction
5 min	25°C	Primer annealing
30 min	42°C	cDNA synthesis
5 min	95°C	Termination of reaction

Table 5. *cDNA synthesis reactions with settings (time and temperature).*

cDNA were stored at -20°C and diluted 30 times prior to qPCR.

7.9 Quantitative Polymerase Chain Reaction

Quantitative PCR were carried out with use of the Stratagene™ Mx 3005P and MxPro Software System (Agilent Technologies) and Brilliant II SYBR® Green qPCR Master Mix kit (Agilent Technologies). To one reaction was 12,5 µl MasterMix, 0.375 µl diluted ROX-reference dye (diluted 1000 times), 5 µl cDNA and primers in an optimised concentration (table 4) used. This mix was diluted with H_20 to achieve a volume of 20 µl/reaction. All reactions were run in triplicates. Settings for the actual qPCR-run are displayed below (figure 7). However, adjustments were done in the case of guinea pig KCNH2-run in order to minimise unspecific products seen during a run with 56°C annealing temperature. Guinea pig KCNH2 was therefore run with a 58°C annealing temperature and 20 seconds of elongation.

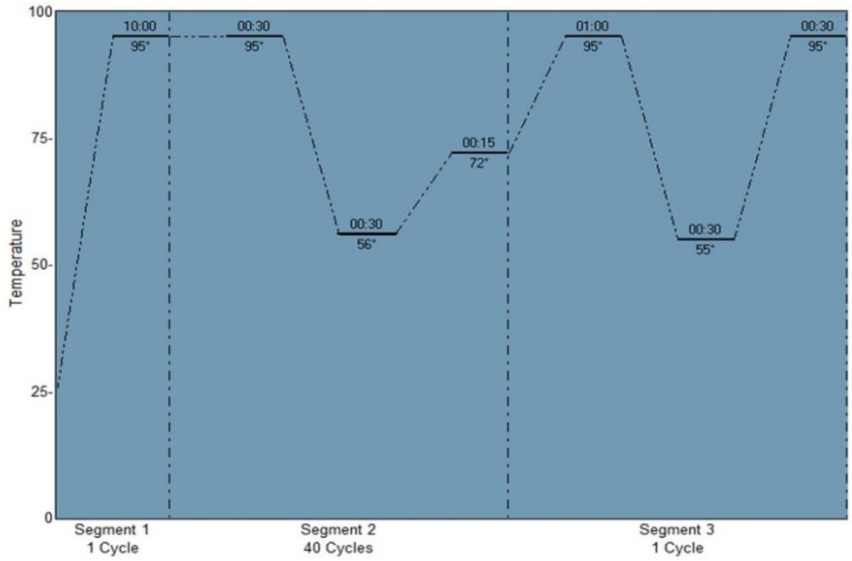

Figure 7. *Settings for all qPCR runs, except guinea pig KCNH2-run, showing temperature and time settings*. Time and temperature settings for segment 1 (initialisation step), segment 2 (denaturation, annealing and elongation) repeated for 40 cycles and segment 3 (T_m curve analysis), except from guinea pig KCNH2-run with 58°C annealing temperature and 20 seconds elongation temperature.

In order to adapt a precise approach for the analysis of quantitative data, a standard curve unique for each species and primer-pair was run with a serial dilution of template. cDNA was diluted 20 times and was thereafter subsequently diluted 2 times each step to a total of 7 aliquots. A no-template control was included. Each standard curve was run in triplicates.

It should be assured that the reaction performs at equal efficiency for high and low concentrations, and the subsequent comparison between species would be accurate. Automatic pipettes were used to minimise pipetting inaccuracies. If the aliquotting was accurate and efficiency does not change, the plot of generated C_t-value against the log of initial template will give a linear regression with a coefficient of -3.32. The amount of PCR product will double each cycle (in a 100% efficient reaction), giving a 10-fold increase of amplicon every 3.32 cycle during the exponential phase of amplification, thus the linear regression coefficient -3.32 ($\log_2$ 10 = 3.32). An efficiency level between 80-120% is generally acceptable (appendix VI). By comparing the C_t-values of unknown samples to the standard curve, a relative quantification of initial copy number can be done. This will enable a comparison between groups (species/heart sample), but not an absolute quantification. The relative quantification of initial copy number was compared to the efficiency by following equation;

$$y = \text{Efficiency coefficient} * \text{LOG}(x) + \text{constant}$$

where y = C_t-value, x = relative initial template concentration

The relative initial template concentrations were calculated by dividing the obtained C_t-value with the linear regression co-efficient and subtracting the deviate of the constant and the linear regression coefficient. The mean C_t-value of the samples' triplicates was used.

Gel electrophoresis was performed on samples to determine the size of amplified product, hereby confirming that the desired product was generated. The PCR products were separated on a 2% agarose (agarose powder, Gel Red (Biotum) and TBE buffer). Prior to loading, were samples mixed with DNA loading buffer (Sigma). Visualisations of bands were observed under ultraviolet light and were compared to GeneRuler^{TM} Low Range DNA ladder (Thermo Scientific) with bands ranging between 25 – 700 base pairs.

7.10 Normalisation of gene expression

The C_t-value of the experiment is a relative value which represents the cycle number at which the amount of amplified DNA reaches threshold level. The C_t-value was normalised by comparing the unknown gene to a reference gene in order to control the sample-to-sample variation that is not due to biological effect.

Genomic DNA is regarded contamination and not the desired product, but a small portion is difficult to avoid. If the contamination is assumed to be equal in all samples, this can be used for normalisation. ValidPrime^{TM} (TATAA Biocenter) universal assay for vertebrates is designed to

target one non-transcribed ultra-conserved region in the genome not present in pure cDNA preparations. These regions show much higher stability during evolution than other regions of gDNA, and are confirmed with a 100% homology for guinea pig, dog, pig and horse. The kit was used in accordance with manufacturer's recommendations.

Normalisation may also be achieved by comparison to the expression level of a house-keeping gene. Ribosomal protein 18 human primer sequences were used.

7.11 Statistical methods

The dataset were tested for outliers using the Grubbs' test. Significant outliers ($p > 0.05$) were excluded from the analysis. Data is presented in columns with error bars representing standard error of the mean. One-way ANOVA analysis with Bonferroni's Multiple Comparison post-testing was used for comparison of the different groups. Differences between groups were calculated with significance levels of 95%, 99% and 99.9%.

8 Results

8.1 Histological imaging and Laser Capture Microdissection

Myocardial samples from atriums and ventricles can easily be isolated by macroscopic sampling. In order to examine the gene expression of KCNQ1 and KCNH2 in pure populations of Purkinje cells, it was the idea to collect Purkinje cells from the equine heart using LCM. The experiments described in the following part were performed to 1) identify Purkinje cells 2) perform LCM on Purkinje cells.

The Purkinje cell identification was initially performed on frozen tissue to characterise the morphology and location of cells of interest, which were intended for later single cell isolation by LCM. Equine heart samples were stained in either cresyl violet, Hematoxylin-Eosin or Periodic Acid-Schiff to identify Purkinje cells between the endocardium and the myocardium. As cresyl violet is known to preserve RNA, this stain should be used for LCM. The H-E and PAS staining were used for morphological characterisation of the slide and provided a reference to the cresyl violet. As seen in figure 8, there could not be identified any intact cells beneath the endocardium in the frozen heart. Different approaches to cryo-sectioning and different stainings had no impact on the preservation of cells on the slide.

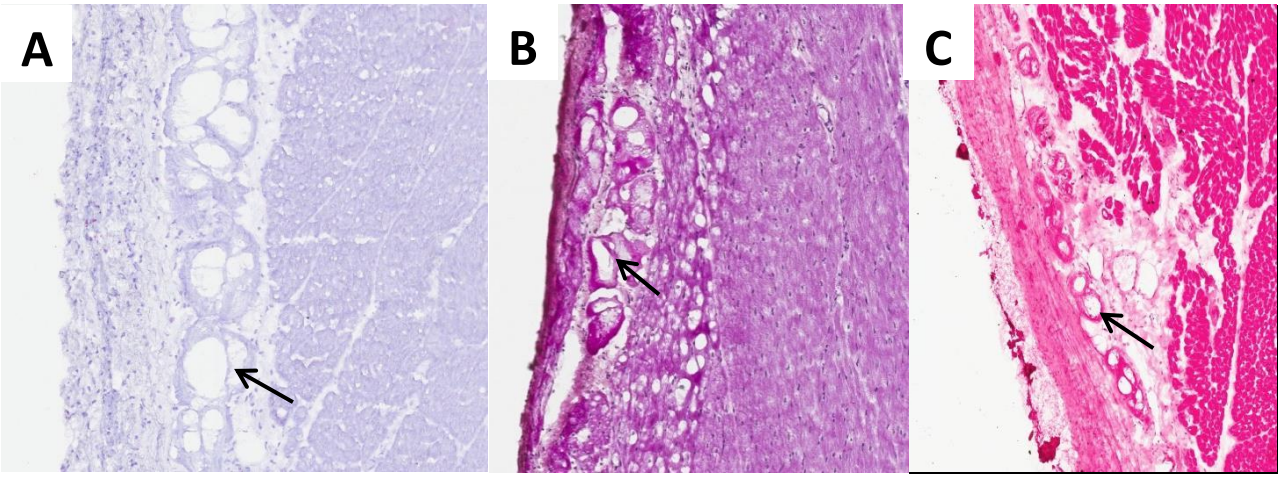

Figure 8. *Histological images of endocardium and underlying tissue from frozen equine heart in light microscope.* Endocardium (left in images), underlying remnants of Purkinje cells (arrows) and myocardium (right in images) are visualised. No Purkinje cell nuclei were observed. Sections are stained with (A) Cresyl Violet, (B) Periodic Acid-Shiff, (C) Hematoxylin-Eosin.

Next, the ability to collect cells using LCM equipment was tested. Single Purkinje cells from equines were to be cut out by LCM. Sections of frozen equine heart tissue stained in cresyl violet were used for this purpose. Simply looking at a glass slide without cover glass in the LCM microscope shows the endocardium followed by an area with disrupted tissue, followed by identifiable myocytes. Heart tissue mounted on MembraneSlides resulted in single cells being totally unidentifiable. This poor morphology was not improved neither by auto-focusing nor manually focusing on the slide (figure 9).

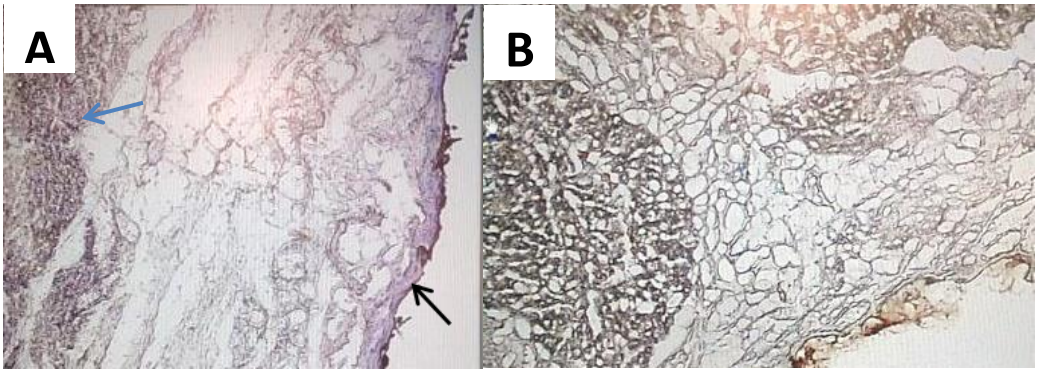

Figure 9. *Laser Capture Microdissection images of equine heart tissue stained in cresyl violet in light microscope.* (A) Visualisation of endocardium (black arrow) and myocardium (blue arrow) of a 12 µm thick section on a SuperFrost slide without cover-glass. Looking at slide in microscope (A) with cover glass, morphology was drastically improved. (B) Visualisation of heart section of a 12 µm thick section mounted on MembraneSlide.

Using the Veritas LCM equipment, tissue is cut out with a UV-laser and thereafter collected on a cap using an infra-red. The UV laser damages the tissue as it cuts through it, whereas the infra-red has no negative effect on tissue and hence RNA quality. The two lasers

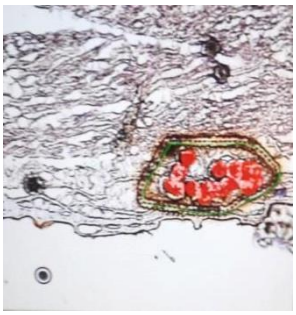

Figure 10. *Capture and cutting on LCM.* Usage of medium-power laser. Trial of laser-settings seen in the periphery as characteristic "donuts". Presence of burned areas along the cut-line is visualised.

were adjusted by regulating power and pulse intensity and time respectively. Cutting of the equine heart tissue was initially attempted with a low power UV laser. This did not successfully cut the area of interest, and more laser power had to be used. This burns the tissue intensively, but manages to loosen the area which could be attached to a cap. The burning of tissue decreased the precision of selecting the area of interest. Therefore, the cutting procedure would have been forced to be carried out with a larger margin to cells of interest – thereby risk the capturing of undesired cells.

As frozen tissue failed to point out the cells of interest, it was attempted to avoid the freezing process. It is known that formalin-fixation dramatically decreases RNA quality by cross-linking RNA and proteins and causing mutations. Therefore was an alternative preservation method for later use on LCM horse heart attempted on mice hearts. Two mice hearts was preserved in a methacarn buffer (figure 11) in an attempt to identify Purkinje cells lying beneath the endocardium, as they do in horses. The overall cell morphology of a heart preserved in methacarn buffer was good but no Purkinje cells were identified in the mouse. Purkine cells may not necessarily be present in mice hearts, but trials showed that the buffer did not preserve any RNA. The method was therefore discarded.

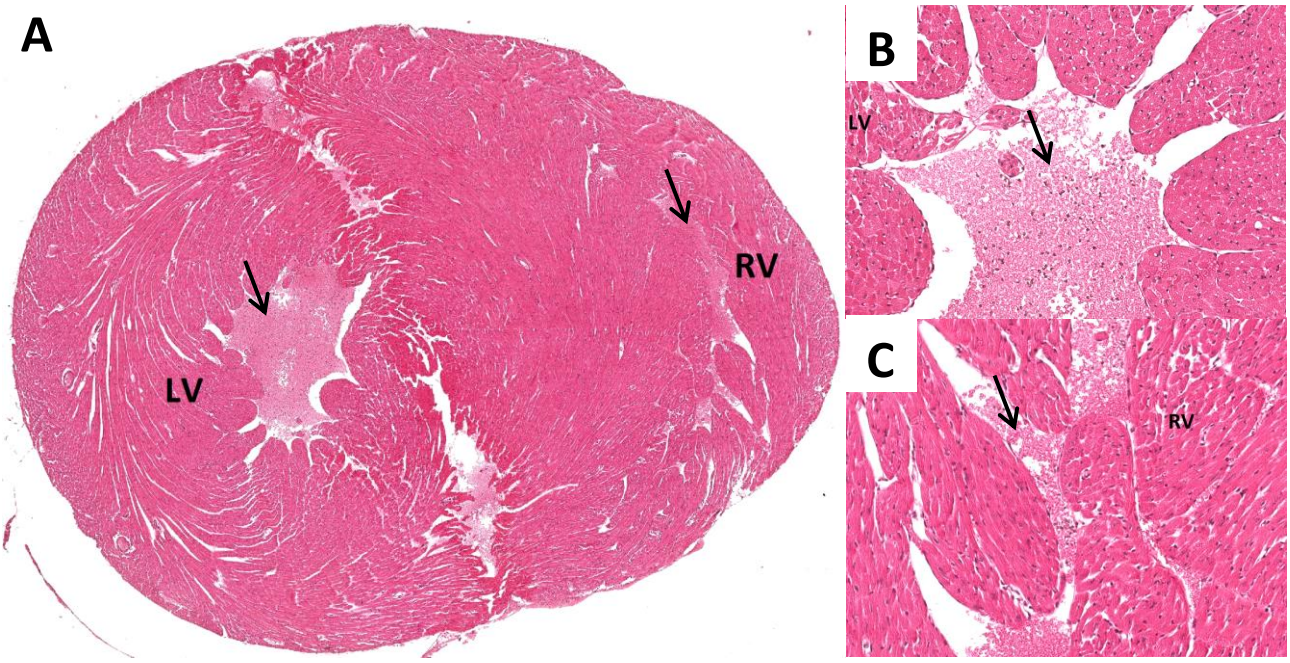

Figure 11. *Cross-section of mouse heart in light microscope fixated in methacarn buffer stained in Hematoxylin-Eosin.* (A) Cross-section of heart showing left ventricle (LV) and right ventricle (RV). The separation in between is artefact. Eryhrocytes in ventricles indicated by arrows. (B) Close up of endocardium of left ventricle (LV). (C) Close up of endocardium of right ventricle (RV).

As it was not possible to verify the existence of Purkinje cells on slides of frozen horse hearts, and an alternative method with hearts preserved in a methacarn buffer did not preserve RNA, LCM of single cells was abandoned.

An alternative to LCM for quantification of Purkinje cell gene expression is macro-dissection of false tendon Purkinje fibres (*trabeculae septum marginale*) found in the equine heart. These false tendons were histologically evaluated to determine their cell composition (figure 12, 13, 14). The formalin-fixated tissue shows an outstanding morphology with an exceptional preserving of Purkinje cells leaving no doubt in their placing, in contrary to frozen tissue (figure 12). The Purkinje cells are easily identifiable even in low magnification by their large size in comparison with surrounding tissue. The false tendon Purkinje fibre is consisting of the Purkinje cells in combination with connective tissue. There are no contractile myocytes found in the sections.

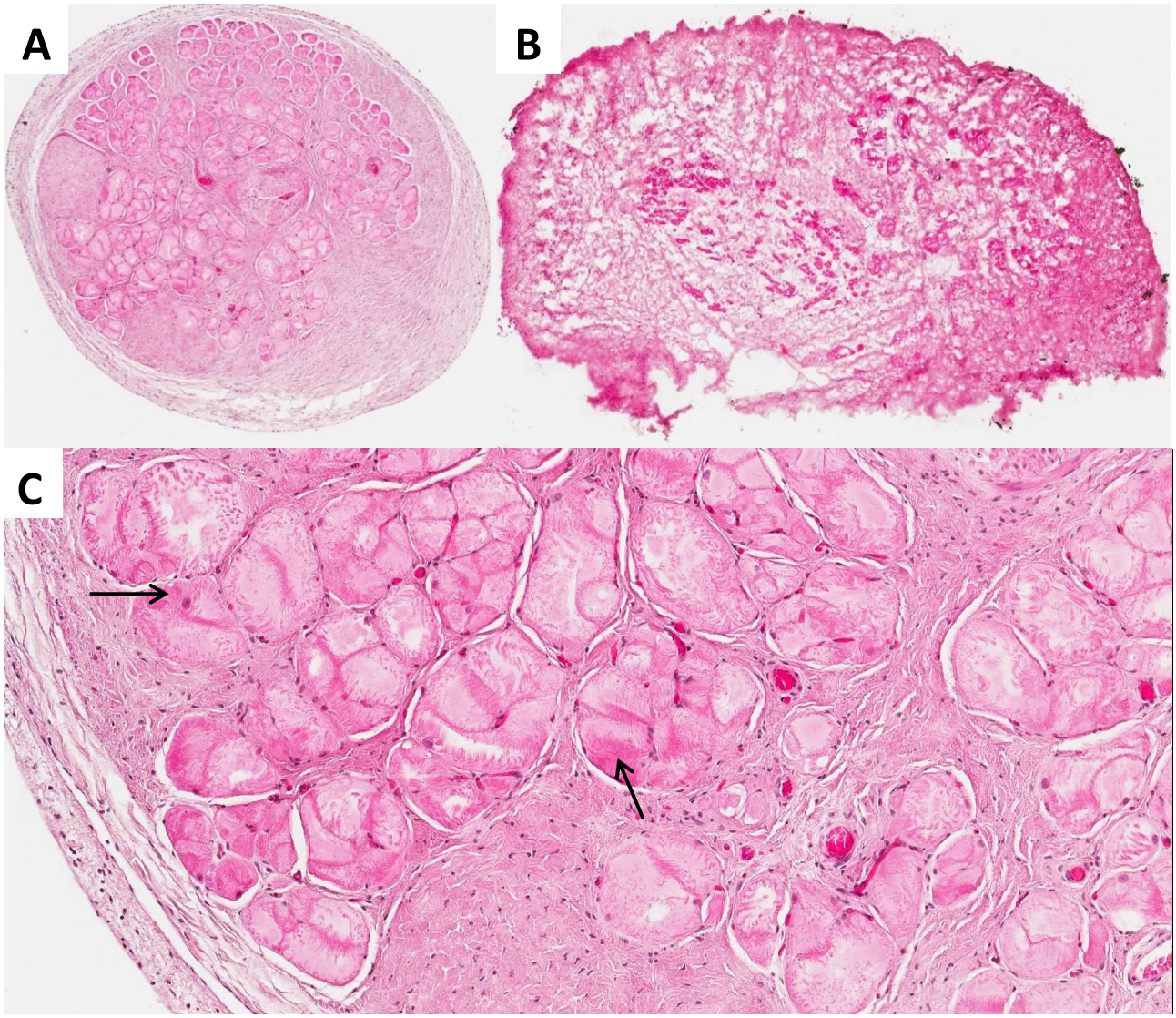

Figure 12. *Cross-sections of false tendon Purkinje fibre from horse in light microscope stained in Hematoxylin-Eoisin.* (A) Cross-section of false tendon Purkinje fibre fixated in formalin. (B) Cross-section of false tendon Purkinje fibre from frozen tissue. (C) Close-up of (A). Example of single Purkinje cells marked with arrow.

The Purkinje cells run in bundles of the false tendon, where they are surrounded by connective tissue. This can be seen when false tendon Purkinje fibres were sectioned in a longitudinal manner (figure 13).

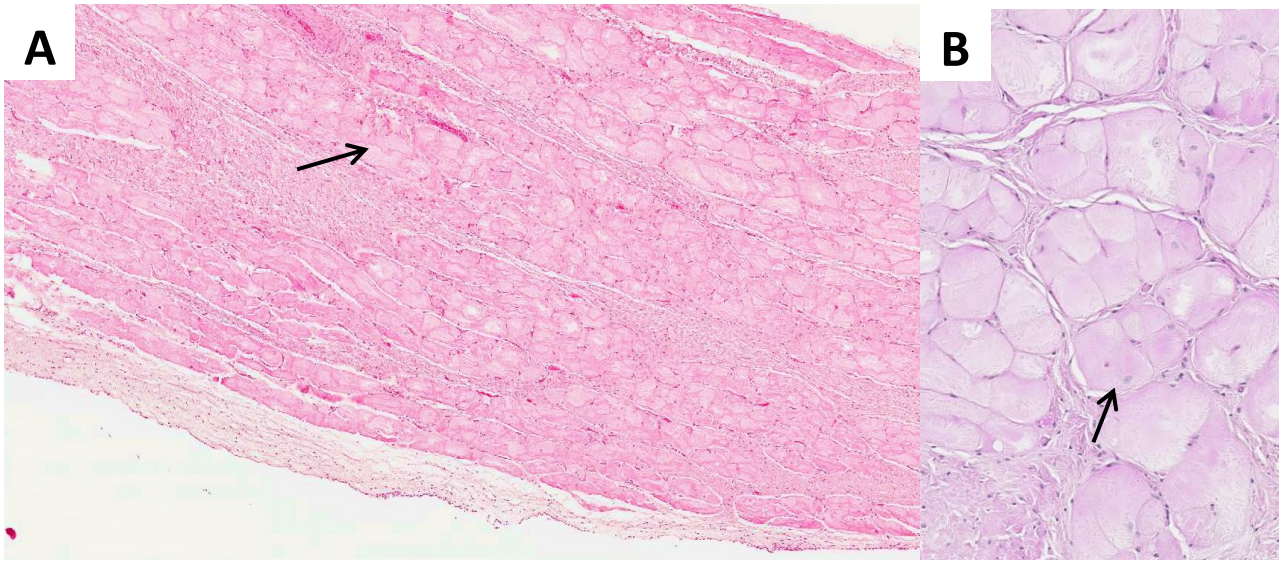

Figure 13. *False tendon Purkinje fibre from horse in light microscope fixated in formalin.* (A) False tendon Purkinje fibre from horse fixated in formalin. Purkinje cells are originated in bundles with inter-lying connective tissue (arrow). Stained with Hematoxylin-Eosin. (B) Purkinje cell (arrow) with a large, faint nuclei which can be compared to the connective tissues' relatively smaller nuclei. Stained in Periodic Acid – Shiff.

The presence of Purkinje cells beneath the endocardium and within the ventricular wall of formalin-fixated tissue was also investigated, as previously done in frozen sections. Sections spanning from endocardium to epicardium were examined in order try to visualise the sub-endocardial Purkinje cells (figure 14) and their integration into muscles (figure 15). It was possible to identify Purkinje cells beneath the endocardium as well as Purkinje fibres integrating the muscle of the left ventricle in a horse.

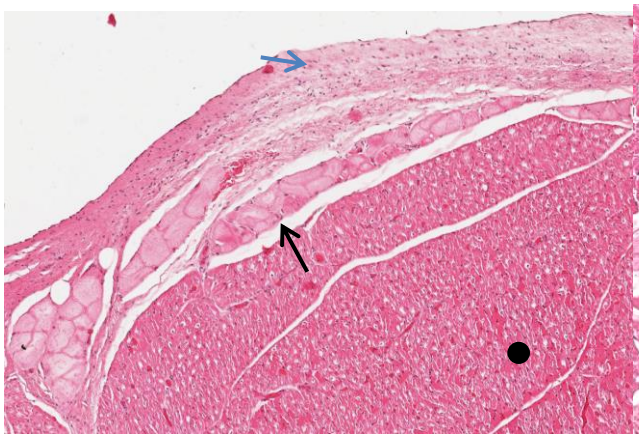

Figure 14. *Endocardium, Purkinje fibres and myocardium from horse in light microscope.* Endocardium (blue arrow) Purkinje fibres (black arrow) and myocardium (dot). Section stained with Hematoxylin-Eosin.

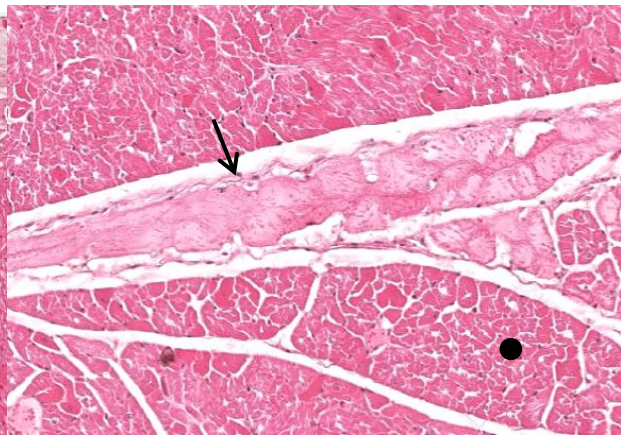

Figure 15. *Purkinje fibres in left ventricular wall from horse in light microscope.* Purkinje fibres (arrow) and myocardium (dot). Section stained with Hematoxylin-Eosin.

8.2 Gene homology, primer design and testing

The KCNQ1 and KCNH2 genes were compared across species in order to be able to design primers which could be used for all species in this study. The KCNQ1 and KCNH2 sequences are very well preserved between investigated species with a homology of 95% or higher for KCNQ1 (table 6) and 81%-98% for KCNH2 (table 7).

KCNQ1	**Guinea pig**	**Canine**	**Equine**	**Human**
Guinea pig	100%	94.92%	96.19%	95.93%
Canine	94.92%	100%	96.89%	96.72%
Equine	96.19%	96.89%	100%	98.19%
Human	95.93%	96.72%	98.19%	100%

Table 6. *%-degree of similarity of KCNQ1 gene between different species.*

KCNH2	**Guinea pig**	**Canine**	**Pig**	**Equine**	**Human**
Guinea pig	100%	81.17%	98.37%	91.34%	91.49%
Canine	81.17%	100%	97.56%	81.79%	80.72%
Pig	98.37%	97.56%	100%	98.37%	98.37%
Equine	91.34%	81.79%	98.37%	100%	91.69%
Human	91.49%	80.72%	98.37%	91.69%	100%

Table 7. *%-degree of similarity of KCNH2 gene between different species.*

Primers could be designed to only deviate a maximum of 1 nucleotide/investigated specie for KCNQ1 primers (appendix I) and with a 100% homology for KCNH2 primers (appendix II) for species which were investigated and used in this study (this will exclude human sequence which showed a single nucleotide deviation). The splice-sites were very well preserved between species and it was possible to obtain primers being intron-spanning (see section 7.5) in all tested species (appendix III and IV). The preservation seen across the investigated species makes it likely that it is also the case for the pig and minke whale. This was confirmed by existence of a definite, single peak in the qPCR dissociation curve along with a single band of expected length on gel electrophoresis.

Different primer concentrations were tested prior to gene expression studies to identify the optimal reaction setting (table 4). Gene of interest-primers were used in a 200 nM concentration. The primers showed a definite, single peak (figure 15), but also primer-dimer present in no template-control. The primer-dimer dissociation curve is easily distinguishable from target peak and was not present in template samples.

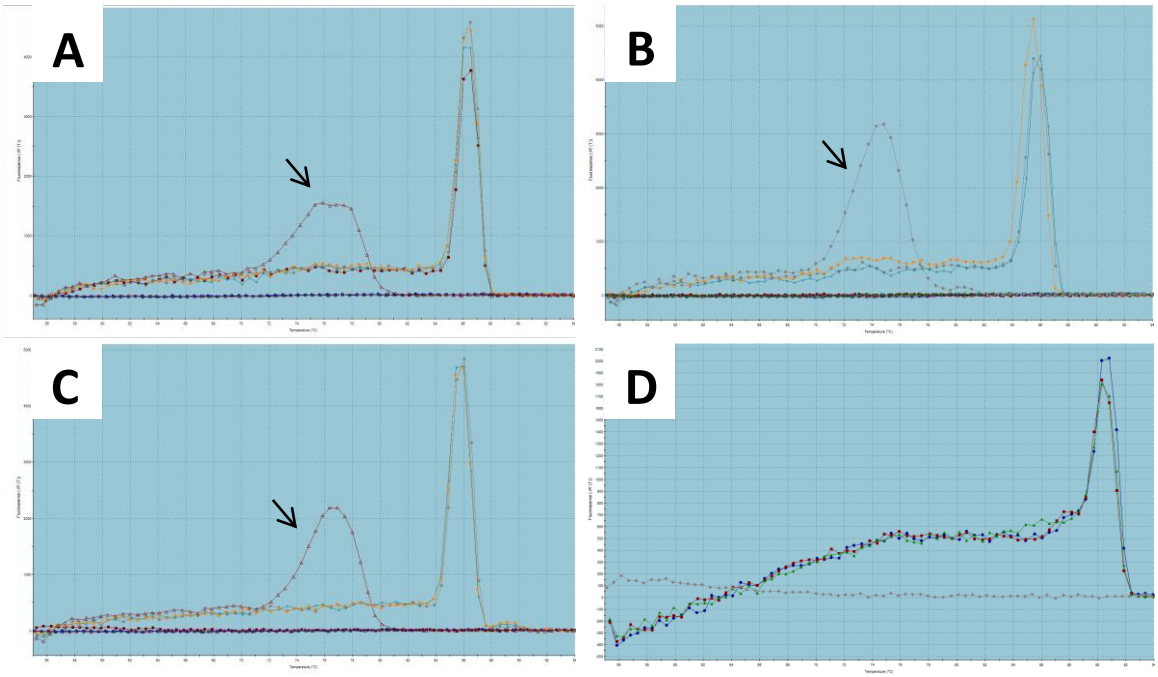

Figure 15. *Dissociation curves for used gene of interest-primers*. Dissociation curve for (A) KCNQ1-universal, (B) KCNH2-universal, (C) KCNQ1-equine and (D) KCNH2-equine. 3 primer-pairs show primer-dimer formation (arrows) in H_20 sample, which however is easily distinguishable from target peak.

Gel electrophoresis was performed on amplicons from the KCNQ1 and KCNH2 gene after qPCR run in order to determine the size of quantified product as a quality control for specificity of primers. Visualisations of bands were observed under ultraviolet light and were compared to a ladder with bands ranging between 25 – 700 bp. All qPCR-products showed a single band of expected length.

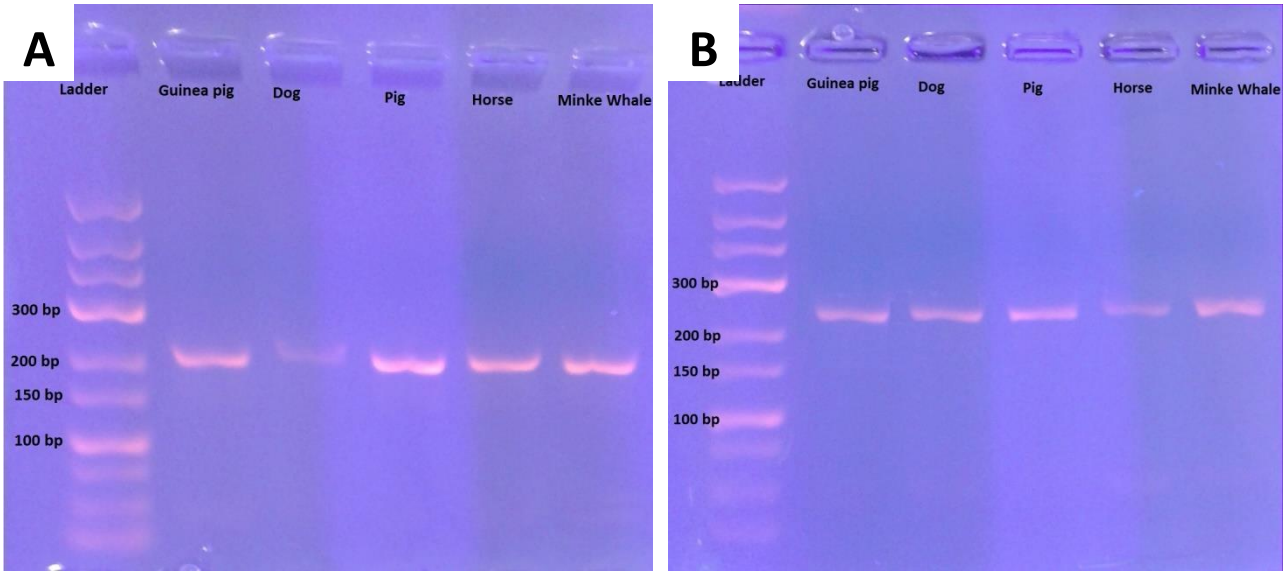

Figure 16. *Gel electrophoresis of qPCR products from guinea pig, dog, pig, horse and minke whale with universal KCNQ1 and KCNH2 primers.* Ladder spanning between 25-700 base pairs (bp), with the band corresponding to 100, 150, 200 and 300 bp specified in figure. (A) Visualisation of bands for universal KCNQ1-primer (expected length 199 bp). (B) Visualisation of bands for universal KCNH2-primer (expected length 223 bp).

8.3 RNA quantification and quality control

After extraction of RNA from heart tissue from guinea pig, dog, pig, horse and minke whale, the samples were analysed for quantity and RNA-integrity. The initial concentration ranged between 1300.8 ng/µl to 5733.8 ng/µl and the samples had RIN-values generally exceeding 7.5 (spanning from 5.9-8.8). However, this excluded 4 samples (guinea pig 2, 3, 4, 5) which expressed a high degree of degradation. They were repeatedly measured between RIN N/A to 2.5; although RNA concentration was high, and they were excluded due to their degradation. See appendix V for detailed data for all used samples.

8.4 Standard curve

Standard curves were used in order to relate the C_t-value to the actual efficiency of the setup. Unique standard curves were prepared for each primer and species (figure 17). The standard curves' efficiencies were within the accepted 80-120% (appendix VI) for all primer-species combination, except KCNH2-equine specific which had an efficiency of 131.2%. The R_{Square}-value should be as close to 1 as possible (appendix VI).

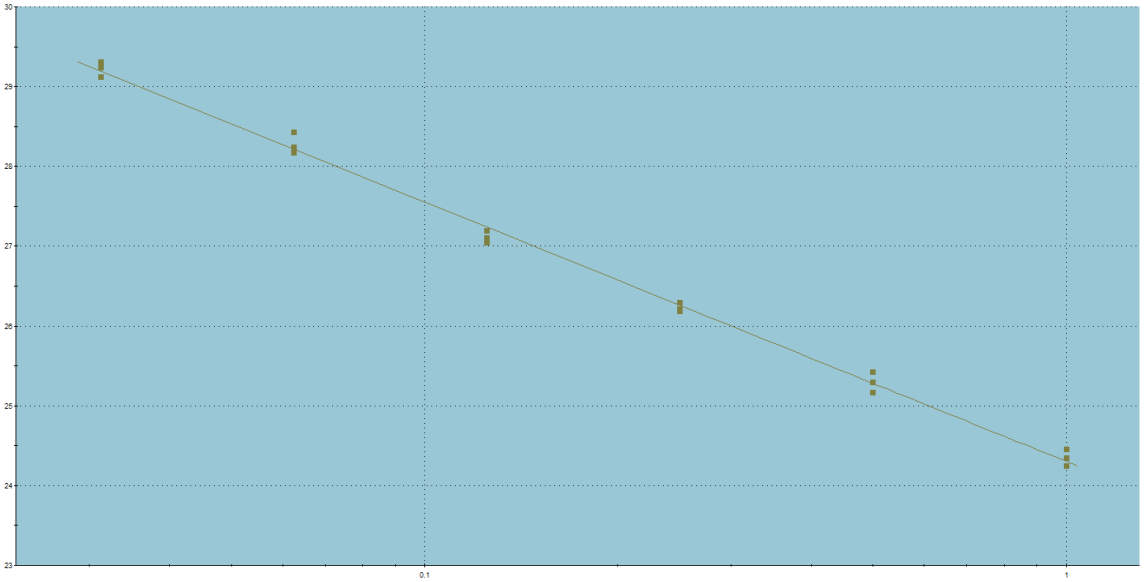

Figure 17. *Example of standard curve, showing standard curve for KCNQ1 minke whale.* y axis, C_t-value; x-axis, initial quantity (relative). The efficiency was calculated to 103.1% with a R_{Squre} value of 0.996.

8.5 Gene expression of cardiac KCNQ1

The relative gene expression of cardiac KCNQ1 was measured between guinea pig, dog, pig, horse and minke whale with qPCR. The KCNQ1 expression was normalised to gDNA (see section 7.10), which is believed to be equally present in all samples. The gene expression of cardiac KCNQ1 in left ventricle was concluded to be significantly higher in guinea pig than in dogs, pigs, horses and minke whale (figure 18). The dog in turn, is significantly different from the other species included in this study. The result of this study show a tendency towards that the larger the animal – the lower the KCNQ1 expression.

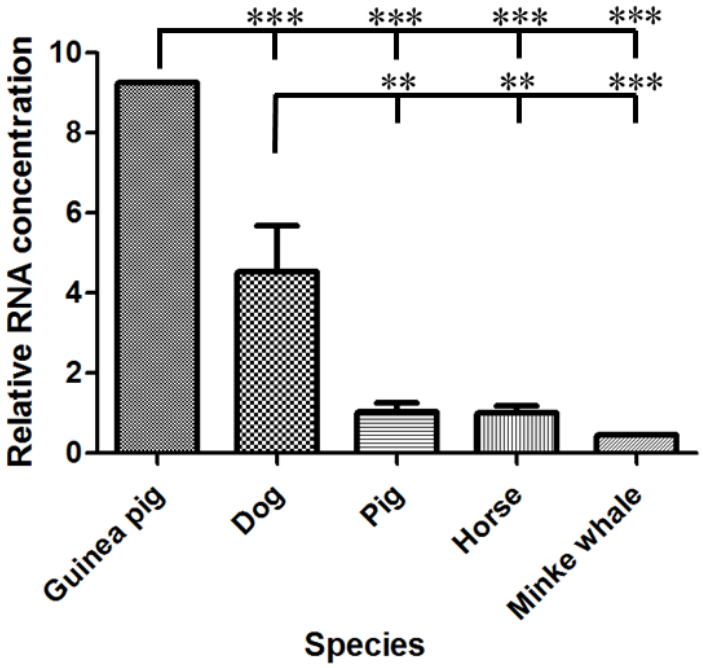

Figure 18. *The gene expression of KCNQ1 in left ventricle in relation to gDNA.* Guinea pig and minke whale: n=1; dog, pig and horse: n = 5. Error bars indicate standard error of the mean (SEM). Stars indicate significant gene expression differences between groups. * = P<0.05, ** = P<0.01, *** = P<0.001.

The KCNQ1 gene expression was further investigated in different areas in the equine heart (figure 19). Samples from left and right ventricle, left and right atrium and false tendon Purkinje fibres were compared. This was both normalised to gDNA and the house-keeping gene RPS18. There were not found any significant difference between different cardiac regions in the equine heart and no clear tendency of Purkinje fibres to display altered gene expression compared to the myocyte-samples. This was independent of method for normalisation.

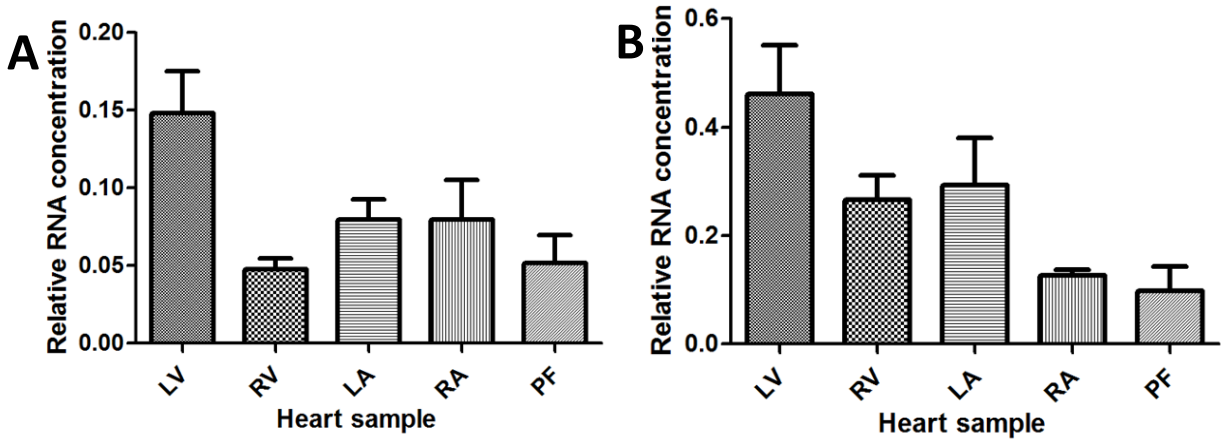

Figure 19. *Regional gene expression of KCNQ1 in equines.* (A) Regional gene expression of KCNQ1 in equines, normalised to genomic DNA. (B) Regional gene expression of KCNQ1 in equines, normalised to house-keeping gene RPS18. LV = left ventricle, RV = right ventricle, LA = left atrium, RA = right atrium, PF = Purkinje fibre. (A) RV, LA n = 4. LV, RA, PF n = 5. (B) RV, RA n = 4, LV, LV, PF n = 5. Error bars indicate standard error of the mean (SEM). There were no significant differences.

8.6 Gene expression of cardiac KCNH2

The relative gene expression of cardiac KCNH2 in left ventricle was measured between guinea pig, dog, pig, horse and minke whale by qPCR and normalised to gDNA. Guinea pig displays a significantly higher gene expression than the dog, pig, horse and minke whale (figure 20). The dog does furthermore express KCNH2 significantly higher than the pig and minke whale. A relatively high expression is seen in the horse, but this was however not statistically significant to dog, pig and minke whale.

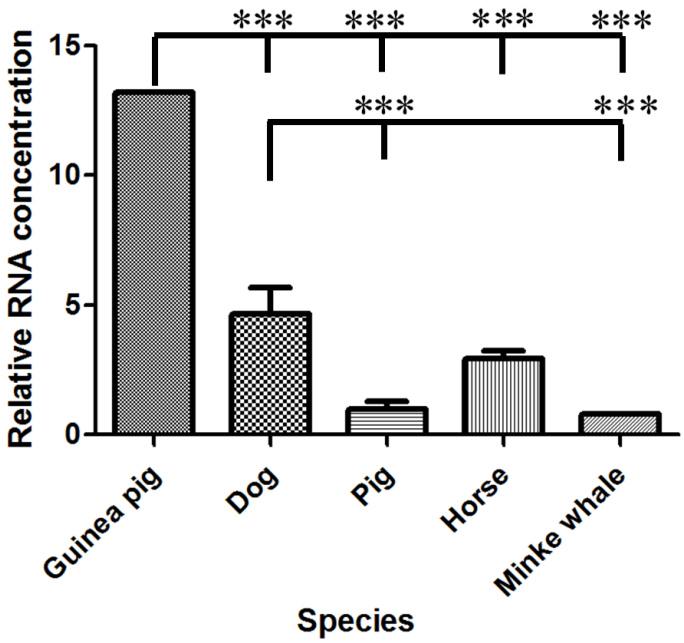

Figure 20. *Gene expression of KCNH2 in left ventricle in relation to gDNA.* Guinea pig and minke whale: n=1; dog, pig and horse: n = 5. Error bars indicate standard error of the mean (SEM). Stars indicate significant gene expression differences between groups. * = P<0.05, ** = P<0.01, *** = P<0.001.

The KCNH2 gene expression was also investigated within the equine heart (figure 21). It was here found that the atrium generally seems to express KCNH2 lower than the ventricles. The left ventricle expressed KCNH2 significantly higher in comparison with left and right atrium and Purkinje fibres. The right ventricle expressed KCNH2 significantly higher than right atrium and Purkinje fibres. The other dissimilarities were not statistically significant, but Purkinje fibres seemingly express a very low degree of KCNH2.

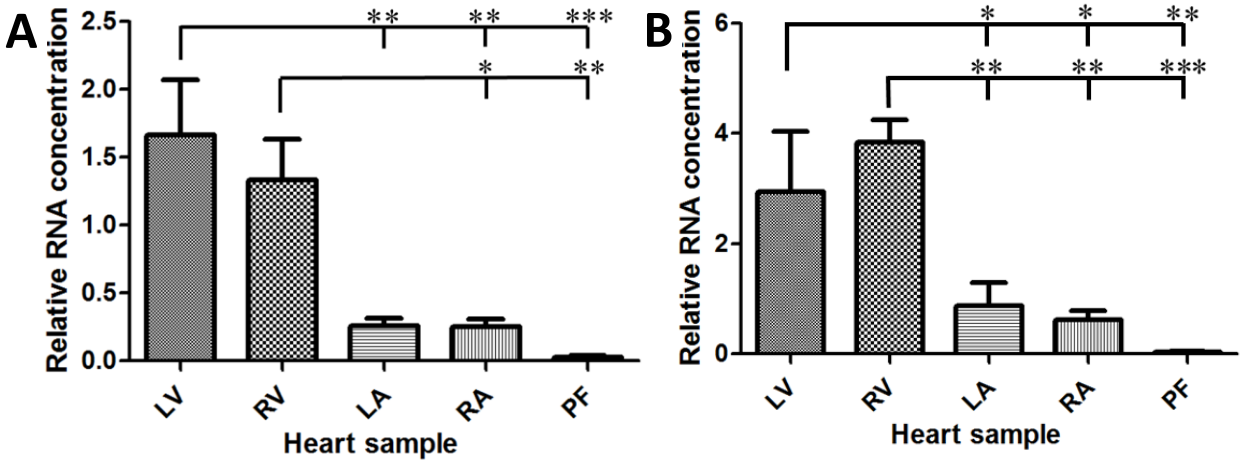

Figure 21. *Regional gene expression of KCNH2 in equines.* (A) Regional gene expression of KCNH2 in equines normalised to genomic DNA. (B) Regional gene expression of KCNH2 in equines normalised to house-keeping gene RPS18. Samples are obtained from left ventricle (LV), right ventricle (RV), left atrium (LA), right atrium (RA), Purkinje fibre (PF). (A) RV, LA n = 4. LV, RA, PF n = 5. (B) RV, LA, LV n =4. RA, PF n =5. Error bars indicate standard error of the mean (SEM). Stars indicate significant gene expression differences between groups. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

9 Discussion

The KCNQ1 and KCNH2 gene expression were studied in hearts from guinea pig, dog, pig, horse and minke whale. The action potential correlates linearly with body weight with a negative coefficient (Rosati et al., 2008). It is therefore expected that larger animals show a decreased gene expression of KCNQ1 and KCNH2 in conformance with a prolonged action potential interval. The cardiac gene expression of KCNQ1 and KCNH2 were investigated with quantitative PCR.

Validation of study design

To ensure a technical high reliability of the results, the quality and quantity of each RNA sample was assessed prior to cDNA synthesis and subsequent qPCR analysis. By quantifying the amount of RNA purified from each sample, the correct amount of RNA could be used for cDNA synthesis (which is highly sensitive to RNA overload), and furthermore, it was assured that all cDNA synthesis reactions contained the same RNA amount, thus providing a point of normalisation in addition to the subsequent qPCR normalisation. Noteworthy, the initial optimisation steps performed to purify RNA from heart tissue revealed that, despite thorough homogenisation it was difficult to extract RNA from this tissue indicating a general low RNA content in heart tissue.

The integrity of the RNA samples was also evaluated. The basis for this evaluation is that both quantification and qPCR analysis is very sensitive to RNA quality. Degraded RNA have a tendency to show false high concentration when measured on a spectrophotometer, and for most genes, the degree of degradation directly affects C_t-values (the higher the RNA quality the lower the C_t-value). Four samples (guinea pig 2, 3, 4, 5) were severely degraded. As degradation has severe effects on the quantitative expression analysis (Becker *et al*., 2010; Gingrich & Karlak, 2006), the degraded samples were excluded from the studies. It obviously limits the strength of the study that only one guinea pig and one minke whale was included, but due to time restraints and sampling difficulties this was what could be achieved. Importantly, an equal amount of RNA and only RNA with high quality has been used in this study, thus ensuring a high technical quality.

The actual study design can obviously impact the result. In order to be able to compare the gene expression levels between species, the method must be equally efficient independent of species. The approach of using non-species specific primers to compare between species is possible because KCNQ1 and KCNH2 are well preserved (have a high homology). Using species-unique primers would not increase the overall reliability of equal conditions, as different primers display different properties in relation to binding and efficiency (Rychlik, 1995). Species-unique primers would therefore not result in a 100% equal efficiency anyway, and would furthermore require sequencing for species with unknown gene sequences. Primer design is of utmost importance to ensure that the result can be compared in a justified way. The success of being able to design primers with sequences of only single nucleotide differences make the assumption of equally efficient primers justifiable. For the species included which did not have an annotated gene sequence an assumption of high homology was made. This assumption is based on the fact that the repolarising K^+ channels have shown to be highly conserved between species. The primer specificity to the gene in the pig

and minke whale was confirmed by a single, definitive peak in the dissociation curve with an alike T_m as well as a single band of expected length on agarose gel.

A standard curve determined the working range of primers and related the C_t-value to the efficiency. It is generally accepted that efficiency varies between 80-120%. As seen in appendix VI, the efficiency is similar between the species, although not identical. It would in practice be impossible to obtain an absolute equal standard curve. It is not only dependent on primer design but also pipetting accuracy (the ability to actually obtain a halving of template concentration each time). All standard curves used in the experiment lies within the generally accepted margin of variance, except for KCNH2-equine specific which showed an efficiency of 131.2%. It cannot be refused that this affects the result, but the data is only used for inter-comparison of KCNH2-equine specific and the efficiency conditions is equivalent between groups.

Normalisation of qPCR results is an important part of any qPCR analysis. It is generally recommended to normalise the expression level for the gene of interest to the expression of at least one normaliser. In the present study, two different normalisation strategies were tested and applied: normalisation to genomic DNA and to a house-keeping gene. Normalisation to gDNA was done using ValidPrime™ (TATAA Biocenter) universal assay for vertebrates. The assay is designed to target one non-transcribed ultra-conserved region in the genome not present in pure cDNA preparations. These regions show much higher stability during evolution than other regions of gDNA, and are confirmed with a 100% homology for guinea pig, dog, pig and horse. Normalisation to gDNA represents a novel method which can be used to compare gene expression between different species in a relative manner. The method relies on the fact that purified RNA samples should ideally be DNA free, but the co-purification of a small fraction is difficult to avoid. Assuming that the DNA content is equal in all RNA samples, the contaminating level of gDNA can be used for normalisation. To ensure a constant degree of DNA contamination in the present study, a standardised protocol (i.e. a fixed volume of the aqueous phase during trizol purification) was used.

Normalisation may also be done with a house-keeping gene which is constantly and highly expressed (Bilchick *et al*., 2006; Ou *et al*., 2010). A strategy of using primers designed for the human RPS18 protein for normalisation showed a similar result compared to the gDNA method, but only within species (the horse). Between species, this method of normalisation failed, likely because the sequences are too diverse for equal binding. Furthermore, it is not known if the different species express the house-keeping gene at the same level. That would make my results very much dependent on the reference instead of the actual gene of interest. Therefore, the results of this study suggest that the gDNA-method is more reliable in inter-species comparison.

From this study results' graphs of KCNQ1 and KCNH2 gene expression (section 8.5 and 8.6), it should be noted that there is a mismatch of the y-axis values between the horse left ventricle value inter-species comparison compared to equine-specific left ventricle and between normalisation to gDNA and RPS18. Though, relative values cannot be compared between different primers.

Cardiac gene expression of KCNQ1 and KCNH2 in different species

The result of this study supports the hypothesis that the prolonging of phase 3 of cardiac repolarisation between species of increasing body weight is achieved through a regulation in gene expression of KCNQ1 and KCNH2. This results in a longer contraction time (systole) of the ventricles which can be linked to the lower heart rate in large mammals.

The gene expression of KCNQ1 in the left ventricle is highest in guinea pigs and declining with increasing body weight with the lowest value observed in the minke whale. The KCNQ1 gene expression of left ventricle in the guinea pig is 2.2 times higher than in canines – comparable with previously reported 2.5 times increase compared to canine expression (Nerbonne & Kass, 2005). This is the first study to compare the gene expression in mammals larger than humans, and it can now be established that pigs, horses and minke whales has a lower gene expression of the KCNQ1 gene found in left ventricle of the heart compared to smaller animals like the guinea pig and dog.

The KCNH2 gene expression between species shows a similar pattern as KCNQ1, with a high gene expression in guinea pig. The gene expression is 2.8 times higher in the left ventricle of guinea pig than in canine, accentuated by the 3.2 times increase in guinea pigs in relation to dogs stated elsewhere (Nerbonne & Kass, 2005). The successive decrease in expression level in the horse and minke whale is not linearly with respect to body weight compared to the smaller species included in the study. The horse shows a relative high expression of KCNH2, but it is not significantly higher than the dog. It would be expected that the horse would have a lower expression than a pig. This may be attributed to the fact that primers and method may be more optimal in the horse, or that the true relationship of individual currents is more complex than previously expected. A different relative contribution of I_{Ks} and I_{Kr} is shown (Jost *et al.*, 2013; Lu *et al.*, 2001; Roepke & Abbott, 2006) and it is plausible that the horse might have a lower I_{Ks} to "compensate" for a higher I_{Kr} without actually altering the overall repolarising effect. The results can be seen is accordance with the fact that there is a limit for action potential prolongation (James *et al.*, 1995).

The next question is whether the I_{Kr} and I_{Ks} mainly are responsible for the repolarisation and action potential duration in all investigated species or whether other, possibly unidentified, currents have a more prominent role. There are known differences of currents between mammals, but the selection of species used for this comparative study of the repolarising K^+ channel α-subunits encoded by KCNQ1 and KCNH2 is based on evidence of their importance in different species. Guinea pig is the smallest known mammal where repolarisation is proven to be depending primarily on the I_{Kr} and I_{Ks} repolarising currents (Rosati *et al.*, 2008), which is in contrast to mice (Wang *et al.*, 1996). In larger mammals I_{Kr} and I_{Ks} have also been recognised as the main repolarisation currents in mammals larger than guinea pig (Rosati *et al.*, 2008). Therefore, different gene expression of KCNQ1 and KCNH2 between larger species does indicate a regulation of the repolarising currents not being needed in great numbers, which is in concordance with longer QT-interval. Alternatively, other (unknown) currents could be suggested as main repolarisers.

Gene expression of KCNQ1 and KCNH2 in the equine heart

There are clear differences in the shape of the action potentials in different cardiac regions (figure 2, Nerbonne & Kass, 2005), and the spatial voltage gradient within the heart may initiate arrhythmias. Cardiac rhythm disturbances are probably one of the most common causes of collapse in horses during exercise (Piercy *et al*., 1999). A possible contribution to this spatial gradient within the heart is repolarising K^+-channels. Samples from left and right ventricle, left and right atrium and Purkinje fibres were investigated in this study.

The study result show that gene expression varies between different cardiac regions. This was not statistically significant for KCNQ1, indicating it may be more equally distributed than other channels affecting the action potential. The right ventricle seemingly expressed a low level compared to left ventricle, which is in accordance to human heart where the right part of the heart is found to express KCNQ1 significantly lower (Luo *et al*., 2008). Purkinje fibres were hypothesised to express KCNQ1 to a low degree, as in the dog (Han *et al*., 2002) and rat (Ou *et al*., 2010), but it could not be statistically validated although this tendency was shown.

KCNH2 on the other hand shows significant gene expression differences in the horse. A lower gene expression is seen in atriums than in ventricles. This is in accordance with previous studies, showing a low percentage contribution of KCNH2 in human atriums (Bertaso *et al*., 2002) and lower expression of the left atrium in humans (Luo *et al*., 2008). Lu *et al*. (2008) did however find that expression were high in the right side of the heart, even in atria. On the other hand, extrapolating results between species has previously shown to be inaccurate. The low atrial expression is somewhat in contrast to the fast repolarisation phase in atria (Schram, 2002). This could indicate that the α-subunits of I_{Kr} and I_{Ks} are not up-regulated relative to ventricles, but that β-subunits may have an effect which increases the overall current and shortens the plateau phase of the action potential. In fact, it is shown that KCNE1 has a higher expression in atria (Zhang *et al*., 2012). Another possibility is that another current, like the I_{Kur} which is dominant in humans atria (Nerbonne & Kass, 2005), is the main repolarising current or that the plateau phase is not likewise maintained by I_{Ca}.

The Purkinje fibres of KCNH2 show a marked low expression level, which is in accordance with other studies emphasising their unique ion channel composition and relative lower expression of KCNH2 (Han *et al*., 2002). Han *et al*. (2002) also found the lower gene expression to correspond to a lower protein level of $K_v11.1$ in PFs relative to ventricle. The Purkinje fibres are hypothesised to show a longer action potential duration due to a limited I_K contribution (Schram, 2002), in accordance with this study.

Results and possible future studies in relation to study design

The overall results are in accordance with previously reported gene expression relationships. It should be noted that the present study only takes species and or area of the heart (atria, ventricle, PF) into consideration. Thus, other varying factors such as gender, breed, age or heart disease have

not been addressed but may be further investigated in the future. Differences in expression levels would be expected for different individuals, and it has for example been reported that there are significantly different mRNA levels of KCNQ1 and KCNH2 between males and females (Moric-Janiszewska *et al*., 2011). Age seemingly does also have an impact, with different mRNA levels of KCNQ1 and KCNH2 between not fully-grown individuals and adult individuals (Moric-Janiszewska *et al*., 2011).

The choice of investigating only the genes responsible for α-subunits of the ion channel may limit the evidence when trying to draw conclusions on the actual current differences between species/cardiac regions. But, although minK is confirmed linked to KCNQ1 α-subunit (Abbott *et al.*, 2007), it is not impossible that other β-subunits/accessory proteins can actually link to diverse α-subunits (Abbott *et al.*, 2007; Finley *et al*., 2002; Tinel *et al*., 2000). The true relationship between these components *in vivo* is debated, and it must also be expected that constant biological changes occur and initiates regulations.

It could furthermore be argued, that studying only the genetic expression may not truly reflect the absolute current. Will the gene expression level correspond to the actual level of proteins? The mRNA translation can potentially be regulated by factors not included in this study. The *in vitro* studies of ion channel composition is limiting because it isolates units out of their complex habitat. Tough, a correspondence between ion channel gene expression and the actual current amplitude has been shown for other cardiac currents (Rosati *et al.*, 2003) and the KCNQ1 and KCNH2 α-subunit expression have previously been shown to link to the protein expression (Han *et al*., 2002). Mutations in KCNQ1 alone is known to lead to decreased I_{Ks} current (Hoosien *et al.*, 2013), and presents an indication of direct gene-to-function effect between KCNQ1 and I_{Ks}.

Nevertheless, future studies on β-subunits and other modulating proteins ought to be investigated, as well as the effect of other ion channels on the action potential. The correlation between gene expression of KCNQ1 and KCNH2 and proteins should also be further studied. The gene expression can be regulated by post-transcriptional modifiers, RNA transport, translation or mRNA degradation rate, and direct measurements on protein levels of $K_v7.1$ and $K_v11.1$ would add further information.

Are KCNQ1 and KCNH2 important for arrhythmias in large animals?

The longer QT-interval in larger mammals involves a regulatory component of the main repolarising K^+ channels α-subunit $K_v7.1$ (KCNQ1) and $K_v11.1$ (KCNH2). This may not be the only cause of the longer QT-interval seen in large mammals. QT-prolongation stagnates in very large mammals such as whales (James *et al*., 1995), in accordance with the found expression levels of KCNQ1 and KCNH2 compared to body weight for the minke whale in this study.

An abnormal QT-interval, or ventricular repolarisation, causes differences in the refractory period of the myocytes and after-depolarisations can be propagated to neighbour cells. This phenomenon leads to ventricular arrhythmias.

The question whether larger species manages a long QT-interval without increased risk of arrhythmias is difficult to conclude. It is easier to produce atrial or ventricular fibrillation in a large heart compared to a small heart and therapeutic termination is more difficult in large hearts (James *et al.*, 1995). The stagnation in PR interval in whales requires a very rapid atrial depolarisation (James *et al.*, 1995). Maybe the AV node does not play an equally important role, leading to less protection against ventricular fibrillation in large mammals. Even though there are no reports of increased risk of arrhythmias in large animals – you only find what you're looking for. The adaption to a large heart involves, apart from an increased QT-interval, presumably many factors not known to date. The arrhythmia incidence in large animals has not been investigated and whether the main cause of death is cardiac failure and if cardiac failure is the most common, natural cause of premature death is not known.

Loss-of-function of the KCNQ1 and KCNH2 gene in humans is associated with LQTS, which greatly increases the risk of ventricular arrhythmias and sudden cardiac death. KCNQ1 and KCNH2 gene mutations is not as strongly associated with atrial arrhythmias, there are only sporadic reports of mutations of KCNQ1 and KCNH2 leading to atrial arrhythmias in humans (Amin *et al*., 2010). The investigated regional gene expression in the large, equine heart suggests that this could also be true for larger species. The fast atrial repolarisation will require large amplitudes of repolarising currents, whereas the somewhat slower ventricular repolarisation would require less. The gene expression of ventricles was high in this study, implicating that the I_{Ks} and especially I_{Kr} may be the main repolarisers of ventricles in equines. This would make the KCNQ1 and perhaps furthermost the KCNH2 gene important for ventricular arrhythmias in the large equine heart. A low atrial KCNH2 gene expression suggests that the I_{Kr} current not are the main repolariser in the equine atriums, and is less important for atrial arrhythmias. This is unless β-subunits or other modulating factors affect the I_{Kr} current to produce a faster repolarisation than seen in ventricles.

The Purkinje fibres show a very low gene expression of KCNQ1 and KCNH2, and they should therefore theoretically repolarise slowly associated with an increased risk of being foci of arrhythmias, which also has been suggested by Schram (2002). The local heterogeneity of KCNQ1 and KCNH2 gene expression compared to ventricles may exaggerate this risk. This is, if no other repolarising channels or modulators play an important role in PFs in larger species such as the horse.

Action potential prolonging drugs are efficient treating re-entrant cardiac arrhythmias, but can result is serious complications due to the QT-prolongation (Han *et al*., 2000). Blocking cardiac K^+ channels prolongs repolarisation and refractoriness, but excessive lengthening of repolarisation may induce life-threatening arrhythmias. The normal repolarisation is achieved by different potassium channels which create a strong safety reserve for repolarisation (Dumaine & Cordeiro, 2007). Thus,

inhibition of a single type of potassium channel does not necessarily lead to marked QT interval prolongation. However, in cardiac disease with one or more decreased effect of potassium channels, drugs targeting the other potassium channels may suddenly lead to unexpectedly long action potential prolongation and risk of arrhythmia (Veerman *et al.*, 2013). Humans have a smaller repolarisation reserve due to smaller I_{Ks} and I_{K1} expression, which shows when I_{Kr} is blocked and action potential prolonging is relatively longer than in dog models (Jost *et al.*, 2013). The relation between the capacities of the normal heart and the diseased heart empathises the need for much specified research in species-specific, gender-specific and disease-specific conditions. The numerous aspects involved complicate the approach of therapy guidelines in treatment of electrical heart disease.

LQTS is continuing to intrigue, with genotype-phenotype correlation show significant variability between individuals with identical mutations (Clancy & Kass, 2002). The clinical presentation is determined by complex interactions between causal genes, gene modifiers and environmental factors such as febrile state, adrenergic stimulation and signalling molecules. The biophysical function, type and location of the ion-channel mutation are currently emerging as important determinants of outcome in genotyped patients (Goldenberg & Moss, 2008). The solution towards the total understanding of arrhythmia incidence, QT-prolongation risk and drug intervention is still ahead of us.

The understanding of electrophysiological differences will give a better foundation on research on laboratory animals as a model for human cardiac disease. However, extrapolating these results even between similar sized animals is not reliable. Knowledge of ion channels and electrical heart function in our domestic species will make it possible to identify genetic electrical heart disease such as the congenital LQTS in veterinary patients. Sudden death in horses is not uncommon, usually without specific post-mortem findings (Lyle *et al*., 2010, 2011). The first equine patient recognised with LQTS is yet to come.

10 Conclusions

The gene KCNQ1 is very well preserved between species with a homology around 95% or higher between guinea pig, dog, human and horse. It is likely that it is equally homologous in the pig and minke whale, based on the fact that primers did indeed give rise to a PCR product with similar length and a specific dissociation curve. The intron-exon boundaries are seemingly also very well preserved between species, most so at the 3'-end.

Purkinje fibres are recognised in large species such as the horse, where they are consistently found beneath the endocardium, but could not be identified in the mice heart. The equine Purkinje fibres also spread out into ventricles. False tendon Purkinje fibres consist of Purkinje cells organised in bundles surrounded by connective tissue, blood vessels and endothelium. In the present study it became clear that freezing Purkinje cells will result in devastating changes, possibly by mismatching in freezing properties between endocardium, subendocardium and myocardium, where the Purkinje cells no longer will adhere to their surroundings. The fact that it was not possible to cryo-cut heart tissue and obtain an acceptable morphology for identification of Purkinje fibres ultimately hampered the idea to collect pure Purkinje cells by LCM, and this part of the project had to be discontinued.

It has been possible to determine the relative gene expression of KCNQ1 and KCNH2 in relation to gDNA with the use of non-species specific primers. The KCNQ1 gene expression is high in guinea pig, declining in the dog and low but mutually incomparable in the pig, horse and minke whale. In the equine heart, the KCNQ1 gene expression does not vary significantly. Purkinje fibres do however seemingly have low expressions in the horse.

KCNH2 sequences are accordingly to KCNQ1 very well preserved between the guinea pig, dog, pig, human and horse; being between 80%-98% identical. The intron-exon boundaries are very well preserved, especially at the 3'-end, in the dog, human and horse. The gene expression of KCNH2 is high in guinea pig, relatively lower in the dog and lowest in the pig, horse and minke whale. Within the heart, significant differences between ventricles, atriums and Purkinje fibres are seen. Left and right ventricle express a higher level of KCNH2 than left and right atrium (though not statistically significant for right ventricle vs. left atrium). The Purkinje fibre showed lower expression than ventricles and atrium, the latter however not significantly different.

The found results are in accordance with previously knowledge which empathize the declining of KCNQ1 and KCNH2 in larger mammals, as well as their lower expression in atria and Purkinje fibres compared to ventricles. The present results support the hypothesis that prolonging of the QT-interval in large species is due to a lower gene expression of KCNQ1 and KCNH2. The QT-prolongation is not linearly related to body weight from horse to whales (~500 kg:350 ms to ~30 000 kg:450 ms), but an upper restriction of QT-prolongation is seen. The physiological adaption to the whale's enormous heart involves other, unknown regulations.

11 Perspectives

Confirming that gene expression of KCNQ1 and KCNH2 differs between species and different cardiac regions highlight the importance of gene regulation in relation to cardiac adaption. The Purkinje fibres possess interesting features and their role in initiation of arrhythmias should be further investigated. Laser Capture Microdissection of these cells might be possible in future research projects if even more effort is spent on method optimisation.

Gene expression is a fine balance dependent on the physiological state, which varies between species, genders, age, individuals, disease state or other factors. Studies attributing these factors could be investigated in future studies of the gene expression of KCNQ1 and KCNH2.

In the future, other cardiac ion channels should be investigated in relation to their presence, gene expression and function in different species. Exploring the molecular mechanisms involved in mediating the many protein-protein interactions important in controlling the assembly, trafficking and functioning of the channel proteins complexes will provide new knowledge of the link between heart disease and electrophysiological abnormalities. A future study could focus on β-subunit expression. The actual protein level should also be attributed in addition to gene expression.

The regional differences in repolarising capacity are a known cause of arrhythmias, and these repolarising differences can be exaggerated in disease. Whether arrhythmias are relatively more common in species with longer QT-interval is not known. The electrophysiology of the horse includes a slower repolarisation than man and regional differences within the heart, which is why equines represent an interesting animal model for human arrhythmias. The research in electrophysiological differences will give a better foundation for research on laboratory animals as a model for human cardiac disease. However, extrapolating these results even between size similar animals is not reliable. Knowledge of ion channels and electrical heart function in our domestic species will make it possible to identify genetic electrical heart diseases such as the congenital LQTS in veterinary patients.

The full understanding of electrophysiology in the heart is still ahead of us. The study results in combination with present and future research of ion channels can in the future lead to a fulfilled knowledge of electrical differences within the heart and thus be used to target drugs with a high specificity. Ion channels are clinically distinguishable by different sensitivity to blocking drugs. It may in the future be possible to develop pharmaceuticals altering ion currents in the heart by target up- or down-regulating factors of gene expression or affect the ion channels complex by signalling molecules.

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13 Appendices

13.1 Appendix I

Comparisation of homology of the KCNQ1 gene for dog (*canis familiaris*; Acc no: XM_540790.2), man (*homo sapiens*; Acc no: AF482704.1), guinea pig (*cavia porcellus*; Acc no: EF204535.1) and horse (*equus caballus*; Acc no: NM_000218.2).

```
CLUSTAL O(1.2.0) multiple sequence alignment


canis      ATGGTCAACTCCGAGCGGAGGGACTCCCAGCCTTTGGCCTGGTATGCTGCCCAAACTGGG
homo       ------------------------------------------------------------
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------


canis      GATCTCCGTGTCCCCGCGTGGGAGGAATGCCGATCCCGGGCCCTGCCTAGCACCCCCGCC
homo       ------------------------------------------------------------
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------


canis      TGCGCGTCTGTGTCTGCTCTGGGAGCTCAGGGCAACTCTGCGTCCAGCCGCTGGAGGCCA
homo       ------------------------------------------------------------
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------


canis      CTGCTCGGGAGAGCACTATCCCTGCCTGAACAGCATGCCGGGCTGAACATGAACAGCATT
homo       ------------------------------------------------------------
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------


canis      CGGGGCATGCAGCCGGTGCTCAGCGAGGACGGGGGAGTGCCGCCTGCCATTGCCTATCTG
homo       ------------------------------------------------------------
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------


canis      ATTTTCAATGTCTTGAACAAGCCGGTGCTGTTAATCCTTTACCTGCCAGAGCTCCGGCCC
homo       ------------------------------------------------------------
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------


canis      CTGATGGCTCTGCTGCTCTTGGAGGTCCGATCAGCCGGGATGGGGACACTCACAAGAGGG
homo       ------------------------------------------------------------
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------


canis      TCACCTGGCATCCGGCCTCCGAAGCCCAGGCCCCCTGCGGCCTGTGCCGTGGCGGTCAGC
homo       -------------------------------------------GCGGCGGGGCTGGCAGC
cavia      ------------------------------------------------------------
```

```
equus      ------------------------------------------------------------

canis      CTGCTGCCTGGCAGCTCGGAGCCCACAGACAGGCCGGGCCTGATGATCTCCGTCTGTGGT
homo       AG--TGGCTGCCCGCACTGCGCCC-----------GGG-----CGCTCGCCTT-------
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      GAGAAAGACGGGGAAGATCCAGGTGCCCGCAGTGAGGACCGGCTGCA--TCTGT-CCACT
homo       --------CGCTGCAGCTCCCGGTGCC-GCCGCTCGGGCCGGCCCCCCGGCAGGCCCTCC
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      GTGCGCGGAGTCTTGAGCAGATGCACCTTCCCGGCTGCCAGCATCTGGACTGGAGAGAAG
homo       -------TCGTTATG-GCCGCGGCCTCCTCCCCG-------C-CCAGGGCCGAGAGGAAG
cavia      ------------ATG-GACGCGGCCTCGTCCCCG-------C-CCAGGACGGAGAGGAAG
equus      ------------ATG-GACGCGGCCTCCTCCCCG-------C-CCAGGGCCGAGAGGAAG
                        :** *..*. **. * **** *       *  *:**.* *....****

canis      CTTTTGGAGGGGGTCACTGTAGGGGCAGAGACCG----GTGTGCACCCTGATGCCACTGC
homo       CGCT-----------------GGGGTTGGGGCCGCCTGCCAGGCGCCCGGCGG-GGCAGC
cavia      CGCG-----------------GGGGCTGGGGCCGTCTGCTGGGCTCCCGGCGG-GGCAGC
equus      CGCT-----------------GGGGCTGGGGCCGCCTGCCGGGCGCCCGGCGG-GGCAGC
           *                   **** :*.*.***      . ** *** *. *  .*:**

canis      TACAACCACCTCCAAGCTTGGCCCCGCTGTCCCCTGGGAGGCCGAGTGTGGGGTCCAGAA
homo       G-----------CGGGCCTGGCCAAGAAGTGCCCCTTCTCGCT--------------GGA
cavia      G-----------CCAGCTTGGCCAAGAAGTGCCCCTTCTCGCT--------------GGA
equus      G-----------CGGGCCTGGCCAAGAAGTGCCCCTTCTCCCT--------------GGA
                       * .** *****..*.:** ***    :  *              *.*

canis      TCTGGACTTAAGGAGGGTCGAGGGGTTGGAAAGGTGCGAGCCCCCCAGCTCCCGCATGTC
homo       GCTGG-CGGAGGGCGGCCCG-GCGGGCGG-------CGCGCT-CTACGCGCCCATCGCGC
cavia      GCTGG-CTGAGGGTGGCCCA-GCGGGCGG-------CACACT-TTACGCGCCTGTCGCGC
equus      GCTGG-CTGAGGGCGGCCCG-GCGGGCGG-------CGCGCT-CTATGCGCCCATCGTGC
            **** *  *.** **  *. * **  **       *...*    . ** ** . .   *

canis      CTCCAAGCGCTCAGCTTGCTGGTTCTCCAGCCTGCTGGGTGT-G-CAGGTGCATACCCCC
homo       ---CCGGCGCCCCAGGTCCCGCGCCCCCTGCGTCCCCGGCCGCGCCCGCCG--CGCCCCC
cavia      CTCCAGGCGCCCTGAGTCCCGGGTCTCCCGCGCCCCCAGCGTCCCCCGCCG--CGCCCCC
equus      CTCCCGGCACCCAGGGGCCCGCGCTCCCCGCGTCTCCGGCCCCGCCGGCCG--CGCCCCC
              *..**.* * .    * *     ** **      .*      * *  *   .*****

canis      CAAT----GCGACCTCGGCACTGGGGGCCGGGATTCTGGAGCCTCTGATACTAATGAGCC
homo       AGTTGCCTCCGACCTTGGCCCG----------------------CGGCCGCCGGTGAGCC
cavia      TGCCGGGCTGGAGCTCGGCCCG----------------------CGGCCGCCGGTAAGCC
equus      CGCAGCCGCCGACCTTGGCCCG----------------------CGGCCGCCGGTGAGCC
            .        ** ** ***.*                       * *. .* ..*.****

canis      TTGTGTCCCACCCATCCAC---CTG----------------CTAGAG----------ACC
homo       TAGACCCGCGCGTCTCCATCTACAGCACGCGCCGCCCGGTGTTGGCGCGCACCCACGTCC
cavia      TTGACCCGCGCGTCTCCATCTACAGCGCGCGCCGGCCGCTGCTCGCGCGCACCCACATCC
equus      TCGACCCGCGCGTCTCCATCTACAGGACGCGCCGCCCGCTGCTCGCGCGCACCCACATCC
           * *:  * *.*  .****    *:*                * *.*         :**

canis      A-----------AAGGCGGCCTGG----CACCCCCTGCAGGC--GGCTTCGTGCAGCTGG
homo       AGGGCCGCGTCTACAACTTCCTCGAGCGTCCCACCGGCTGGAAATGCTTCGTTTACCAC-
```

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cavia      AGGGCCGCGTCTACAACTTCCTCGAGCGCCCCACCGGCTGGAAGTGCTTCGTTTACCAC-
equus      AGGGCCGCGTCTACAACTTCCTCGAGCGCCCCACGGGCTGGAAGTGCTTCGTCTACCAC-
           *           *...*  *** *     .**.*  **:**.   *******  * *:

canis      CAGGCTTCAGCTTCCTCATCGTCCTGGTCTGCCTCATCTTCAGTGTCCTGTCCACCATCG
homo       --TTCGCCGTCTTCCTCATCGTCCTGGTCTGCCTCATCTTCAGCGTGCTGTCCACCATCG
cavia      --TTTGCCGTTTTCCTCATTGTCCTGGCCTGCCTCATCTTCAGCGTGCTGTCCACCATTG
equus      --TTCGCAGTCTTCCTCATCGTCCTGGCCTGCCTCATCTTCAGCGTGCTGTCTACCATCG
                  ..  ******** ******* *************** ** ***** ***** *

canis      AGCAGTATGTCGCCCTGGCCACAGGGACCCTCTTCTGGATGGAGATTGTCCTGGTGGTGT
homo       AGCAGTATGCCGCCCTGGCCACGGGGACTCTCTTCTGGATGGAGATCGTGCTGGTGGTGT
cavia      AGCAGTATGCCGCTCTGGCCACCGGGACCCTCTTCTGGATGGAGATTGTCCTGGTGGTGT
equus      AGCAGTATGTCACTCTGGCCACAGGGACCCTCTTCTGGATGGAGATCGTCCTGGTGGTGT
           ********* *.* ******** ***** ***************** ** **********

canis      TCTTTGGGACGGAGTATGTGGTCCGCCTCTGGTCAGCAGGCTGCCGCAGCAAGTATGTAG
homo       TCTTCGGGACGGAGTACGTGGTCCGCCTCTGGTCCGCCGGCTGCCGCAGCAAGTACGTGG
cavia      TCTTTGGGACAGAGTACGTGGTCCGCCTCTGGTCTGCAGGCTGCCGCAGCAAGTATGTGG
equus      TCTTTGGGACAGAGTACGCCGTCCGCCTCTGGTCAGCAGGCTGCCGCAGCAAGTACGTGG
           **** *****.***** *  ************** **.****************** **.*

canis      GCATCTGGGGGAGGCTGCGCTTTGCCCGGAAGCCCATCTCCATCATTGACCTCATCGTGG
homo       GCCTCTGGGGGCGGCTGCGCTTTGCCCGGAAGCCCATTTCCATCATCGACCTCATCGTGG
cavia      GCATCTGGGGCCGACTGCGTTTTGCCCGGAAGCCCATTTCCATCATCGACCTCATCGTGG
equus      GCATCTGGGGGCGGCTGCGCTTTGCCCGGAAGCCCATTTCCATCATTGACCTCATTGTGG
           **.******* .*.***** ***************** ******** ******** ****

canis      TCCTGGCCTCCATGGTTGTCCTCTGCGTGGGCTCCAAGGGGCAGGTGTTTGCCACCTCGG
homo       TCGTGGCCTCCATGGTGGTCCTCTGCGTGGGCTCCAAGGGGCAGGTGTTTGCCACGTCGG
cavia      TTGTGGCCTCCATGGTTGTCCTCTGTGTGGGCTCAAAGGGGCAAGTGTTTGCCACATCAG
equus      TTGTGGCCTCCATGGTTGTCCTCTGCGTGGGCTCCAAAGGGCAGGTGTTTGCCACCTCAG
           *  ************** ******** ********.**.*****.************ **.*

canis      CCATCAGGGGCATCCGCTTCCTCCAGATCCTGAGGATGCTGCATGTGGATCGCCAGGGAG
homo       CCATCAGGGGCATCCGCTTCCTGCAGATCCTGAGGATGCTACACGTCGACCGCCAGGGAG
cavia      CCATCAGGGGCATCCGCTTCCTCCAGATCTTGCGGATGCTACATGTGGACCGGCAGGGAG
equus      CCATCAGGGGCATCCGATTCCTTCAGATCCTGAGAATGCTGCATGTCGACCGCCAGGGAG
           ****************.***** ****** **.*.*****.** ** ** ** *******

canis      GTACCTGGAGGCTGCTTGGCTCTGTGGTCTTCATCCACCGCCAGGAGCTCATCACCACCT
homo       GCACCTGGAGGCTCCTGGGCTCCGTGGTCTTCATCCACCGCCAGGAGCTGATAACCACCC
cavia      GTACCTGGAGGCTTCTGGGCTCTGTGGTCTTCATCCATCGCCAGGAGCTGATCACCACTT
equus      GCACCTGGAGGCTGCTGGGCTCCGTGGTCTTCATCCACCGTCAGGAGCTGATAACCACCT
           * *********** ** ***** ************** ** ******** **.*****

canis      TGTACATCGGCTTCCTGGGCCTCATCTTCTCCTCGTACTTCGTGTACCTGGCCGAGAAGG
homo       TGTACATCGGCTTCCTGGGCCTCATCTTCTCCTCGTACTTTGTGTACCTGGCTGAGAAGG
cavia      TGTATATCGGCTTCCTGGGACTCATCTTCTCCTCCTACTTCGTGTACCTGGCTGAGAAGG
equus      TGTACATCGGCTTCCTGGGCCTCATCTTCTCCTCGTACTTCGTGTACCTGGCCGAGAAGG
           **** **************.************** ***** *********** *******

canis      ACGCCGTGAACGACTCGGGCCAAGTGGAGTTTGGCAGCTATGCAGATGCCCTGTGGTGGG
homo       ACGCGGTGAACGAGTCAGGCCGCGTGGAGTTCGGCAGCTACGCAGATGCGCTGTGGTGGG
cavia      ATGCTGTGAATGAATCAGGCCGTGTGGAGTTCGGAAGCTATGCAGATGCACTGTGGTGGG
equus      ACGCCGTGAACGAGTCGGGCCGTGTCGAGTTTGGCAGCTATGCAGATGCCCTTTGGTGGG
           * ** ***** ** **.****. ** ***** **.***** ******** ** *******

canis      GAGTGGTCACGGTCACCACCATCGGCTACGGGGACAAGGTGCCCCAGACGTGGGTTGGGA
```

```
homo        GGGTGGTCACAGTCACCACCATCGGCTATGGGGACAAGGTGCCCCAGACGTGGGTCGGGA
cavia       GGGTGGTCACAGTCACCACCATCGGTTACGGCGACAAGGTCCCCCAGACCTGGGTTGGGA
equus       GGGTGGTCACTGTCACCACCATTGGCTATGGAGACAAAGTGCCCCAGACGTGGGTCGGGA
            *.******** *********** ** ** ** *****.** ******** ***** ****

canis       AGACCATCGCCTCCTGCTTCTCTGTGTTTGCCATCTCCTTCTTTGCGCTCCCAGCGGGGA
homo        AGACCATCGCCTCCTGCTTCTCTGTCTTTGCCATCTCCTTCTTTGCGCTCCCAGCGGGGA
cavia       AGACGATTGCCTCCTGCTTCTCTGTCTTTGCCATCTCCTTCTTCGCGCTCCCAGCGGGGA
equus       AGACCATTGCCTCCTGCTTCTCCGTCTTCGCTATCTCCTTCTTCGCACTCCCGGCGGGGA
            **** ** ************** ** ** ** *********** **.*****.*******

canis       TTCTCGGCTCTGGCTTCGCCCTAAAGGTCCAGCAGAAGCAGAGACAGAAGCACTTCAATA
homo        TTCTTGGCTCGGGGTTTGCCCTGAAGGTGCAGCAGAAGCAGAGGCAGAAGCACTTCAACC
cavia       TCCTTGGCTCTGGGTTTGCCCTGAAAGTCCAGCAGAAGCAGAGGCAGAAGCACTTCAACC
equus       TCCTCGGCTCGGGCTTTGCCCTGAAGGTGCAGCAGAAACAGAGGCAGAAACACTTCAACC
            * ** ***** ** ** *****.**.** ********.*****.*****.******** .

canis       GGCAGATCCCGGCGGCAGCCTCGCTCATTCAGACGGCGTGGAGGTGCTACGCGGCCGAGA
homo        GGCAGATCCCGGCGGCAGCCTCACTCATTCAGACCGCATGGAGGTGCTATGCTGCCGAGA
cavia       GACAGATCCCAGCCGCTGCGTCACTCATCCAGACTGCATGGAGATGCTATGCTGCTGAGA
equus       GGCAGATTCCGGCGGCAGCCTCGCTCATTCAGACGGCGTGGAGGTGCTACGCAGCTGAGA
            *.***** **.** **:** **.***** ***** **.*****.***** ** ** ****

canis       ACCCCGACTCTTCCACCTGGAAGATCTACGTCCGGAAGCCCGCCCGGAGCCATGCCCTGC
homo        ACCCCGACTCCTCCACCTGGAAGATCTACATCCGGAAGGCCCCCCGGAGCCACACTCTGC
cavia       ACCCTGACTCTTCTACCTGGAAGATCTATGTGCGGAAGCCTGCCCGAAGCCACACGCTGC
equus       ATCCCGACTCCTCCACCTGGGAGATCTACGTGCGGAAGCCCTCCCGGAGCCACGCTCTGC
            * ** ***** ** ******.******* .* ****** *  ****.***** .* ****

canis       TCTCTCCCAGCCCCAAGCCCAAGAAGTCTGTCATGGTAAAGAGAAAGAAGTTCAAACAAG
homo        TGTCACCCAGCCCCAAACCCAAGAAGTCTGTGGTGGTAAAGAAAAAAAAGTTCAAGCTGG
cavia       TCTCTCCTAGCCCCAAACCAAAGAAGTCTGCTATGGTAAGGAAAAAGAAGTTCAAACCGG
equus       TCTCCCCCAGCCCCAAGCCCAAGAAGTCTGCCATGGTAAAGAAAAAAAAGTTCAAACTGG
            * ** ** ********.**.**********  .******.**.***.********.* .*

canis       ACAAGGACAACGGGGTGAGCCCCGGAGAGAAGATGCTCACGGTCCCCCACATCACGTGCG
homo        ACAAAGACAATGGGGTGACTCCTGGAGAGAAGATGCTCACAGTCCCCCATATCACGTGCG
cavia       ATAAGGATAATGGAGTGAGCCCTGGAGAGAAGATGCTCACAGTCCCCCACATCACATGTG
equus       ACAAGGACAATGGAGTGAGTCCCGGAGAGAAGACTCTCACGGTCCCTCACATCACGTGTG
            * **.** ** **.****  ** **********  *****.***** ** *****.** *

canis       AACCCGTCTCGGAGGAGCGGAGGCCAGACCACTTCTCCGTGGACAACTGTGACAGCTCTG
homo        ACCC---CCCAGAAGAGCGGCGGCTGGACCACTTCTCTGTCGACGGCTATGACAGTTCTG
cavia       ATCC---TCCAGAAGAGCGGAGGCCAGACCACTTCTCTGTTGATGGCTATGACAGCTCTG
equus       ACCTCGTCTCGGAGGAGCGGAGGCCAGACCATTTCTTGGTGGAGAGCTGTGACAATTCTG
            * *      *.**.******.*** .***** ****  ** ** ..**.*****. ****

canis       TGAAGAAGAGCCCCATGCTGCTGGAGGTGAGCACAGCCCACTTCATGAGAACCAACAGCT
homo        TAAGGAAGAGCCCAACACTGCTGGAAGTGAGCATGCCCCATTTCATGAGAACCAACAGCT
cavia       TGAGGAAGAGCCCCACACTGCTGGAAGTAAGCCCAACCCACTTCATGAGAACCAACAGCT
equus       TGAAGAAGAGCCCCACGCTGCTAGAAGTGAGCACGGCCCATTTCATGAGAACCAACAGCT
            *.*.*********.* .*****.**.**.***. . **** ******************

canis       TCGCCGAGGACCTGGACCTCGAGGGGGAGACGCTATTGGCGCCCATCACCCATGTGTCAC
homo        TCGCCGAGGACCTGGACCTGGAAGGGGAGACTCTGCTGACACCCATCACCCACATCTCAC
cavia       TTGCCGAGGATCTGGACTTGGAAGGGGAGACACTGTTGACCCCCATCACCCACGTGTCAC
equus       TTGCTGAGGACCTGGACCTGGAAGGGGAGACGCTGCTGGCTCCCATCACCCACGTGTCAC
            * ** ***** ****** * **.******** **. **.* ********** .* ****
```

```
canis       AGCTGCGGGAGCACCACCGGGCCACCATCAAGGTCATTCGGCGCATGCAGTACTTTGTAG
homo        AGCTGCGGGAACACCATCGGGCCACCATTAAGGTCATTCGACGCATGCAGTACTTTGTGG
cavia       AGCTGCGAGAGCACCACCGGGCCACCATCAAGGTCATCAGGCGCATGCAGTACTTTGTGG
equus       AGCTACGGGAGCACCATCGGGCCACCATCAAGGTCATTCGGCGCATGCAGTACTTTGTGG
            ****.**.**.***** ********** ******** .*.*****************.*

canis       CCAAGAAGAAATTCCAGCAAGCGCGGAAGCCCTACGATGTGCGGGACGTCATTGAGCAGT
homo        CCAAGAAGAAATTCCAGCAAGCGCGGAAGCCTTACGATGTGCGGGACGTCATTGAGCAGT
cavia       CCAAGAAGAAATTTCAGCAAGCGCGGAAGCCTTATGACGTGCGGGATGTCATCGAGCAGT
equus       CCAAGAAGAAATTCCAGCAAGCGCGGAAGCCCTATGATGTGCGGGACGTCATTGAGCAGT
            ************* ***************** ** ** ******** ***** *******

canis       ACTCCCAGGGCCACCTCAACCTCATGGTGCGCATCAAAGAGCTGCAGAGAAGGCTGGACC
homo        ACTCGCAGGGCCACCTCAACCTCATGGTGCGCATCAAGGAGCTGCAGAGGAGGCTGGACC
cavia       ACTCTCAGGGCCACCTCAACCTCATGGTGCGAATCAAAGAGCTACAGCGAAGGTTGGACC
equus       ACTCCCAGGGCCACCTCAACCTCATGGTGCGCATCAAAGAGCTGCAGAGAAGGCTGGACC
            **** ***************************.*****.*****.***.*.*** ******

canis       AGTCCATCGGGAAGCCCTCCCTCTTCATCTCCGTCTCAGAGAAGAGCAAGGACCGTGGGA
homo        AGTCCATTGGGAAGCCCTCACTGTTCATCTCCGTCTCAGAAAAGAGCAAGGATCGCGGCA
cavia       AGTCTATCGGGAAGCCCTCTCTATTCATCCCCATCTCAGAAAAGAGCAAGGACCGTGGCA
equus       AGTCCATCGGAAAGCCCTCCCTCTTCATCTCCGGCTCAGAAAAGAGCAAGGACCGCGGCA
            **** ** **.******** ** ****** **. ******.*********** ** ** *

canis       GCAACACGATTGGGGCACGCCTGAACCGAGTAGAAGACAAGGTGACACAGCTGGACCAGA
homo        GCAACACGATCGGCGCCCGCCTGAACCGAGTAGAAGACAAGGTGACGCAGCTGGACCAGA
cavia       GCAACACCATCGGTGCCCGCCTGAACCGGGTAGAAGACAAGGTGACACAACTGGACCAGA
equus       ATAACACCATCGGCGCCCGCCTGAACCGCGTGGAGGACATGGTGACGCAGCTGGACCAGA
            . ***** ** ** **.*********** **.**.****:******.**.**********

canis       GGCTGGTGCTCATCACGGACATGCTTCACCAGCTCCTCTCCTTGTACCATGGTGGCCCCC
homo        GGCTGGCACTCATCACCGACATGCTTCACCAGCTGCTCTCCTTGCACGGTGGCAGCACCC
cavia       GGTTGGTGGTCATCACAGACATGCTCCACCAGCTACTCTCCTTGCACCAAGGTGGACC--
equus       GGCTGGTGCTCATCACAGACATGCTGCACCAGCTGCTCTCCTTGCACCACGGCAGCCCCC
            ** *** . ******* ******** ******** ********* ** . ** .*..*

canis       CAGGT---GGCCGCCCCCCCAGCGGGAGCGGGGTCCAGGTGGTTCAGCCCTGCA------
homo        CCGGCAGCGGCGGCCCCCCCAGAGAGGGCGGGGCCCACATCACCCAGCCCTGCGGCAGTG
cavia       ----------------CCACAGTGGAGGTGGACCCCAAATGGTCCAGCCCTGTAGCGAAG
equus       CGGGC---GGCCGTCCCCCCAGCGGGGACGAGGCCCAAGTGGTCCAGCCCTGTGGT---G
                       **.*** *.... *..  *** .* .  ******** .

canis       ---GCTCCATCAACCCCGAGCTCTTCCTGCCCAGCAACACCCTGCCCACCTACGAACAGC
homo        GCGGCTCCGTCGACCCTGAGCTCTTCCTGCCCAGCAACACCCTGCCCACCTACGAGCAGC
cavia       ATGGCTCCATCCACCCTGAACTCTTTCTGCCCAGCAACTCCCTGCCCACCTACGAACAGC
equus       GCGGCTCCATCAACCCCGAGCTCTTCCTGCCCAGCAACGCCCTGCCCACCTACGAACAGC
               *****.** **** **.***** ************ ****************.****

canis       TAACTGTGCCCCGCAGGGGTCCCGAAGAGGGGTCCTGA----------------------
homo        TGACCGTGCCCAGGAGGGGCCCCGATGAGGGGTCCTGAGGAGGGGATGGGGCTGGGGGAT
cavia       TAACTGTGCCTCAAAGGGGCCCCGATGAGGCATCCTGA----------------------
equus       TGACCGTGCCCCACAGGGGCCCTGACGAGGGGTCCTGA----------------------
            *.** ***** .. ***** ** ** **** .******

canis       ------------------------------------------------------------
homo        GGGCCTGAGTGAGAGGGGAGGCCAAGAGTGGCCCCACCTGGCCCTCTCTGAAGGAGGCCA
cavia       ------------------------------------------------------------
equus       ------------------------------------------------------------
```

```
canis      ------------------------------------------------------------
homo       CCTCCTAAAAGGCCCAGAGAGAAGAGCCCCACTCTCAGAGGCCCCAATACCCCATGGACC
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       ATGCTGTCTGGCACAGCCTGCACTTGGGGGCTCAGCAAGGCCACCTCTTCCTGGCCGGTG
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       TGGGGGCCCCGTCTCAGGTCTGAGTTGTTACCCCAAGCGCCCTGGCCCCCACATGGTGAT
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       GTTGACATCACTGGCATGGTGGTTGGGACCCAGTGGCAGGGCACAGGGCCTGGCCCATGT
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       ATGGCCAGGAAGTAGCACAGGCTGAGTGCAGGCCCACCCTGCTTGGCCCAGGGGGCTTCC
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       TGAGGGGAGACAGAGCAACCCCTGGACCCCAGCCTCAAATCCAGGACCCTGCCAGGCACA
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       GGCAGGGCAGGACCAGCCCACGCTGACTACAGGGCCGCCGGCAATAAAAGCCCAGGAGCC
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       CATTTGGAGGGCCTGGGCCTGGCTCCCTCACTCTCAGGAAATGCTGACCCATGGGCAGGA
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       GACTGTGGAGACTGCTCCTGAGCCCCCAGCTTCCAGCAGGAGGGACAGTCTCACCATTTC
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       CCCAGGGCACGTGGTTGAGTGGGGGGAACGCCCACTTCCCTGGGTTAGACTGCCAGCTCT
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------
```

```
canis      ------------------------------------------------------------
homo       TCCTAGCTGGAGAGGAGCCCTGCCTCTCCGCCCCTGAGCCCACTGTGCGTGGGGCTCCCG
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       CCTCCAACCCCTCGCCCAGTCCCAGCAGCCAGCCAAACACACAGAAGGGGACTGCCACCT
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       CCCCTTGCCAGCTGCTGAGCCGCAGAGAAGTGACGGTTCCTACACAGGACAGGGGTTCCT
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       TCTGGGCATTACATCGCATAGAAATCAATAATTTGTGGTGATTTGGATCTGTGTTTTAAT
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       GAGTTTCACAGTGTGATTTTGATTATTAATTGTGCAAGCTTTTCCTAATAAACGTGGAGA
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       ATCACAGGCTGGGCTGGGCACTGCTCTCACCTTGGTTCCTGGGGCATCCATGGGGTCTCT
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      ------------------------------------------------------------
homo       CACAGACAGGACCCCTGCAGTTCCCCTGGAAGCAGTGCCCAGGTGGCTGTGGAATAGGAA
cavia      ------------------------------------------------------------
equus      ------------------------------------------------------------

canis      --------------------
homo       CGCTAAAAAAAAAAAAAAAA
cavia      --------------------
equus      --------------------
```

13.2 Appendix II

Comparisation of homology of the KCNH2 gene for dog (*canis familiaris*; Acc no: NM_001003145.1), horse (*equus caballus*; Acc no: NM_001193658.1, guinea pig (*cavia porcellus*; Acc no: Nm_001172973.1) and man (*homo sapiens*; Acc no: NM_000238.3).

```
CLUSTAL O(1.2.0) multiple sequence alignment
Canis       ------------------------------------------------------------
Equus       ------------------------------------------------------------
Cavia       ------------------------------------------------------------
Homo        CCGCGGCCGGGCCGAGCCGCAGGCGCTGCCCGAGCCGCGGGCGCTGGAGCGGCTGTCGGC


Canis       ----------------------------------------------------GCGGGGAC
Equus       ------------------------------------------------------------
Cavia       ------------------------------------------------------------
Homo        GCGGTGGCAGGCGGGAGCCAGGAGTCCGGGGCTCGGGCGGGCGGAGCGCAGCGCGGGGAC


Canis       GCGGCGGAGCAGGGCCCGTGTCTCGGCGGGGGCCGGCAGACAGGTGTGCGGGCGGCCGGG
Equus       ------------------------------------------------------------
Cavia       ------------------------------------------------------------
Homo        GCGGCGGAGCGGGCCCCGCGGCTCGGCGGGGGCCGGCAGACAGGTGTGCCGGCGGC-GGG


Canis       CGGCCCGAGCTCAGGCGGCTCCAGGCCCGGCCCGCGGTCAGGA-------GCCGGGAGCC
Equus       ------------------------------------------------------------
Cavia       ------------------------------------------------------------
Homo        CGGCTCGCGCTCAGGCGGCTCCAGGCCGGGCCCGGGGTCCGGAGCGGGGAGCCGGGAGCC


Canis       GAGCGGGGACCCCGCGCCCGCAACCTAGTCCCGGCCCCGCCCGTGCTCTGCTTGGCCGCG
Equus       ------------------------------------------------------------
Cavia       ------------------------------------------------------------
Homo        GAGCGAGGACCCGGCGCCCGCAGTCCAGTCTTGGCCGCGCCCGTGCTCGGCTTGGCCGCG


Canis       GGGTGCGGGGATCACGGCCGGGCCGGGCCACCCGAAGCCT-GTGTTGGGCCGGGCCGGGC
Equus       ------------------------------------------------------------
Cavia       ------------------------------------------------------------
Homo        GGGTGCGGGGACCACGGCCCGGCCGGGCCACCCGAAGCCTAGTGCTGGGCCGGGCCGGGC


Canis       CGGGGTGGGTGGGGGCCCGCCCGGCCCGCCCATGGGCTCAGGATGCCGGTGCGGAGGGGC
Equus       ----------------------------CCATGGGCTCAGGATGCCGGTGCGGAGGGGC
Cavia       ----------------------CCCGCCCATGGGCTCAGGATGCCAGTGCGGAGGGGC
Homo        CGGGGTGGGTGGGGGCCCGCCCGGCCCGCCCATGGGCTCAGGATGCCGGTGCGGAGGGGC
                                    ******************.************


Canis       CATGTCGCGCCGCAGAACACCTTCCTGGACACCATCATCCGCAAGTTTGAGGGCCAGAGC
Equus       CACGTCGCGCCGCAGAACACCTTCCTGGACACCATCATCCGCAAGTTTGAGGGCCAGAGC
Cavia       CACGTCGCGCCGCAGAACACCTTCCTGGACACCATCATCCGCAAGTTTGAGGGCCAGAGC
Homo        CACGTCGCGCCGCAGAACACCTTCCTGGACACCATCATCCGCAAGTTTGAGGGCCAGAGC
            ** ************************************************************


Canis       CGCAAGTTCATCATCGCCAACGCTCGGGTGGAGAACTGCGCCGTCATCTACTGCAACGAC
Equus       CGCAAGTTCATTATCGCCAACGCTCGGGTGGAGAACTGCGCCGTCATCTACTGCAACGAC
Cavia       CGCAAGTTCATCATCGCCAACGCCCGCGTGGAGAACTGCGCCGTCATCTACTGCAACGAC
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Homo        CGTAAGTTCATCATCGCCAACGCTCGGGTGGAGAACTGCGCCGTCATCTACTGCAACGAC
            ** ******** ********** ** ********************************

Canis       GGCTTCTGCGAGCTGTGCGGCTACTCGCGGGCCGAGGTGATGCAGCGGCCCTGCACCTGC
Equus       GGCTTCTGCGAGCTGTGCGGCTACTCGCGGGCCGAGGTGATGCAGCGGCCCTGCACCTGC
Cavia       GGCTTCTGCGAGCTGTGCGGCTACTCGCGGGCTGAGGTGATGCAGCGGCCCTGCACCTGC
Homo        GGCTTCTGCGAGCTGTGCGGCTACTCGCGGGCCGAGGTGATGCAGCGACCCTGCACCTGC
            ******************************** **************.************

Canis       GACTTCCTGCACGGGCCGCGCACGCAGCGCCGTGCGGCCGCGCAGATCGCGCAGGCCCTG
Equus       GACTTCCTGCACGGGCCGCGCACGCAGCGCCGCGCCGCCGCGCAGATCGCGCAGGCCTTG
Cavia       GACTTCCTGCACGGGCCGCGCACACAGCGCCGCGCCGCCGCGCAAATCGCGCAGGCCCTG
Homo        GACTTCCTGCACGGGCCGCGCACGCAGCGCCGCGCTGCCGCGCAGATCGCGCAGGCACTG
            ***********************.******** ** ********.***********. **

Canis       CTGGGCGCGGAGGAGCGCAAAGTGGAGATCGCCTTCTACCGGAAGGATGGGAGCTGCTTC
Equus       CTGGGCGCCGAGGAGCGCAAAGTGGAGATCTCCTTCTACCGGAAGGATGGGAGCTGCTTC
Cavia       CTAGGGGCTGAGGAACGCAAAGTGGAGATCGCATTCTACCGGAAAGATGGGAGCTGCTTC
Homo        CTGGGCGCCGAGGAGCGCAAAGTGGAAATCGCCTTCTACCGGAAAGATGGGAGCTGCTTC
            **.** ** *****.***********.*** *.***********.***************

Canis       CTGTGCCTGGTGGATGTGGTGCCCGTGAAGAACGAAGATGGAGCTGTCATCATGTTCATC
Equus       CTGTGCCTGGTGGATGTGGTGCCCGTGAAGAACGAGGATGGGGCTGTCATCATGTTCATC
Cavia       CTGTGTCTGGTGGACGTGGTACCTGTGAAGAACGAGGACGGGGCTGTGATCATGTTCATC
Homo        CTATGTCTGGTGGATGTGGTGCCCGTGAAGAACGAGGATGGGGCTGTCATCATGTTCATC
            **.** ******** *****.** ***********.** **.***** ************

Canis       CTCAACTTTGAGGTGGTGATGGAAAAGGACATGGTGGGGTCCCCGACCCATGACACCAAT
Equus       CTCAACTTCGAGGTGGTGATGGAGAAGGACATGGTGGGGTCCCCGGCCCGGGACACCAAT
Cavia       CTCAACTTTGAGGTGGTGATGGAGAAGGACATGGTGGGGTCCCCAGCTCGAGACACCAAC
Homo        CTCAATTTCGAGGTGGTGATGGAGAAGGACATGGTGGGGTCCCCGGCTCATGACACCAAC
            ***** ** **************.**********************..* *. ********

Canis       CACCGTGGTCCCCCCACCAGCTGGTTGGCCCCAGGCCGAGCCAAGACCTTCCGCCTGAAG
Equus       CACCGTGGCCCCCCCACTAGCTGGCTGGCCACAGGTCGGGCCAAGACCTTCCGCCTGAAG
Cavia       CACCGGGGGCCCCCCCACCAGCTGGCTGACACCTGGCCGTGCCAAGACCTTCCGGCTGAAG
Homo        CACCGGGGCCCCCCCCACCAGCTGGCTGGCCCCAGGCCGCGCCAAGACCTTCCGCCTGAAG
            ***** ** ******** ****** **.*..*:** ** ************** ******

Canis       CTGCCTGCGCTGCTGGCCTTGACCACCCGGGAGTCC---TCAGCGCGGCCAGGAGGTGTA
Equus       TTGCCTGCGCTGCTGGCCTTGACAGCGCGGGAGTCG---ACAGTGCGGCCAGGTGGCGCG
Cavia       CTTCCTGCGCTACTGGCCTTGACCACAAGGGAGTCGTCATCAGTGCGGCCAGGGGGCACA
Homo        CTGCCCGCGCTGCTGGCGCTGACGGCCCGGGAGTCG---TCGGTGCGGTCGGGCGGCGCG
             * ** *****.*****  **** .* .*******    :*.* **** *.** ** . .

Canis       GGCAGTGCGGGCGCCCCGGGGGCCGTGGTGGTGGATGTGGACCTGTCACCTGCGGTGCCC
Equus       GGCAGCACGGGGGCCCCCGGGGCTGTGGTGGTGGACGTGGACCTGACGCCTGCGGCGCCC
Cavia       GGTGGCTCCGGTGCCCCGGGGGCTGTGGTGGTGGATGTGGACCTGACGCCCGCGGCACCC
Homo        GGCGGCGCGGGCGCCCCGGGGGCCGTGGTGGTGGACGTGGACCTGACGCCCGCGGCACCC
            ** .*  * ** ***** ***** *********** *********:*.** **** .***

Canis       AGCCGCGAATCGCTGGCCCTGGATGAGGTGACGGCCATGGACAATCACGTGGCAGGGCTG
Equus       AGCAGCGAGTCGCTGGCCCTGGACGAGGTGACAGCCATGGACAACCACGTGGCGGGGCTT
Cavia       AGCAGTGAGTCACTTGCCCTGGACGAGGTGTCTGCCCTGGACAATCATGTGGCAGGA---
Homo        AGCAGCGAGTCGCTGGCCCTGGACGAAGTGACAGCCATGGACAACCACGTGGCAGGGCTC
            ***.* **.**.** ******** **.***:* ***.******* ** *****.**.

Canis       GGGCCGATGGAGGAGCAGCGTGCGCTGGTGGGCTCCAGCTCCCCACCTGCCGGTGCACCT
Equus       GGGCCGGCGGAAGAGCGCCGCGCGCTGGTGGGCCCCGGCTCGCCGCCCGCCTGTGCGCCC
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Cavia      ---CCGGCGGAGGAGCGGCGCGCCCTTGTGGCCCCGGGCTCACCGCCTAGCAGTGTGCCA
Homo       GGGCCCGCGGAGGAGCGGCGTGCGCTGGTGGGTCCCGGCTCTCCGCCCCGCAGCGCGCCC
              ** . ***.****. ** ** ** ****   * .**** **.**   * * * .**

Canis      GA---GCCACTCCCGTCACCCCGAGCACACAGCCTCAACCCTGATGCCTCAGGCTCCAGC
Equus      ATCCCG---CACCCGTCACCCCGGGCCCACAGCCTCAACCCCGACGCCTCGGGCTCCAGC
Cavia      GGCCCTCCACACACCTCACCCAGGGCCCACAGCCTCAACCCTGACGCCTCGGGCTCCAGC
Homo       GGCCAGC---TCCCATCGCCCCGGGCGCACAGCCTCAACCCCGACGCCTCGGGCTCCAGC
           .         :*.* **.***.*.** ************** ** *****.*********

Canis      TGCAGCCTGGCCCGGACGCGCTCCCGGGAGAGCTGCGCCAGCGTGCGCCGGGCCTCCTCA
Equus      TGCAGCCTGGCCCGGACACGCTCCCGGGAGAGCTGTGCCAGCGTGCGCCGCGCCTCATCA
Cavia      TGCAGCTTGGCTCGTACGCGCTCCCGGGAGAGCTGCGCCAGTGTCCGACGAGCCTCCTCG
Homo       TGCAGCCTGGCCCGGACGCGCTCCCGAGAAAGCTGCGCCAGCGTGCGCCGCGCCTCGTCG
           ****** **** ** **.********.**.***** ***** ** **.** ***** **.

Canis      GCTGATGACATCGAGGCCATGCGTGCAGGG---CTGCCCCCGCCACCTCGCCACGCCAGC
Equus      GCGGATGACATCGAGGCCATGCGCACCGGG---CTGCCCCCACCGCCACGCCATGCCAGC
Cavia      GCTGACGACATCGAGGCCATGCGTGCCGGAGCGCTGCCTCCACCACCGCGCCATGCCAGC
Homo       GCCGACGACATCGAGGCCATGCGCGCCGGGGGTGCTGCCCCCGCCACCGCGCCACGCCAGC
           ** ** ***************** .*.**.   ***** **.**.** ***** ******

Canis      ACCGGGGCCATGCACCCCCTGCGCGGCGGCCTGCTTAACTCCACGTCAGATTCCGACCTT
Equus      ACAGGGGCCATGCACCCCCTGCGCAGCGGCCTGCTTAACTCCACATCAGATTCGGACCTC
Cavia      ACGGGGGCCATGCACCCCCTGCGTAGTGGCCTGCTAAACTCCACCTCAGACTCGGACCTC
Homo       ACCGGGGCCATGCACCCACTGCGCAGCGGCTTGCTCAACTCCACCTCGGACTCCGACCTC
           ** **************.***** .* *** **** ******** **.** ** *****

Canis      GTGCGCTATCGTACCATTAGCAAGATTCCCCAAATCACCCTCAACTTTGTGGACCTCAAG
Equus      GTGCGCTACCGCACCATTAGCAAGATTCCCCAAATCACCCTCAACTTTGTGGACCTCAAG
Cavia      GTGCGCTACCGCACCATTAGCAAGATCCCCCAAATCACCCTCAACTTTGTGGACCTCAAG
Homo       GTGCGCTACCGCACCATTAGCAAGATTCCCCAAATCACCCTCAACTTTGTGGACCTCAAG
           ******** ** ************** **********************************

Canis      GGGGACCCTTTCCTGGCTTCACCCACCAGTGACCGGGAGATCATAGCACCCAAGATAAAG
Equus      GGCGACCCCTTCCTGGCTTCGCCCACCAGTGACCGGGAGATCATAGCACCCAAGATAAAG
Cavia      GGTGACCCTTTCTTGGCCTCGCCCACCAGTGACCGGGAGATCATAGCACCCAAAATAAAG
Homo       GGCGACCCCTTCTTGGCTTCGCCCACCAGTGACCGTGAGATCATAGCACCTAAGATAAAG
           ** ***** *** **** **.************** ************** **.******

Canis      GAGCGGACCCACAATGTCACTGAGAAGGTCACCCAGGTCCTGTCCCTGGGAGCTGATGTG
Equus      GAGCGGACCCACAATGTCACCGAGAAGGTCACCCAGGTCCTGTCTCTGGGTGCTGATGTG
Cavia      GAGCGAACCCACAATGTCACCGAAAAGGTCACCCAGGTCCTGTCCTTGGGCGCTGATGTG
Homo       GAGCGAACCCACAATGTCACTGAGAAGGTCACCCAGGTCCTGTCCCTGGGCGCCGACGTG
           *****.************** **.*********************  **** ** ** ***

Canis      CTGCCAGAGTATAAGCTGCAGGCACCACGCATCCACCGCTGGACCATCCTCCACTACAGC
Equus      CTGCCGGAGTACAAGCTGCAGGCGCCACGCATCCACCGCTGGACCATCCTGCACTACAGC
Cavia      CTCCCTGAGTACAAGCTGCAAGCGCCACGCATCCACCGCTGGACCATTCTGCACTACAGC
Homo       CTGCCTGAGTACAAGCTGCAGGCACCGCGCATCCACCGCTGGACCATCCTGCATTACAGC
           ** ** ***** ********.**.**.********************** ** ** ******

Canis      CCCTTCAAGGCAGTGTGGGACTGGCTCATCCTGCTGCTGGTCATCTACACAGCTGTCTTC
Equus      CCCTTCAAGGCCGTGTGGGACTGGCTCATCCTGCTGCTGGTCATCTACACGGCCGTCTTC
Cavia      CCCTTCAAGGCTGTGTGGGACTGGCTCATCCTACTGCTGGTCATCTACACGGCTGTCTTC
Homo       CCCTTCAAGGCCGTGTGGGACTGGCTCATCCTGCTGCTGGTCATCTACACGGCTGTCTTC
           *********** ********************.*****************.** ******

Canis      ACACCCTACTCGGCCGCCTTCCTGCTGAAGGAGACAGAGGAGGGCCCCCCGGCCCCCGAC
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Equus       ACGCCCTACTCGGCTGCCTTCCTGCTGAAGGAGACGGAAGAGGGCCCCCCGGCCACCGAC
Cavia       ACACCCTACTCGGCTGCCTTCCTGCTGAAGGAACCAGAAGAGGACGCCCAGACGGCTGAT
Homo        ACACCCTACTCGGCTGCCTTCCTGCTGAAGGAGACGGAAGAAGGCCCGCCTGCTACCGAG
            **.*********** *****************..*.**.**.*.* * *. .*  * **

Canis       TGTGGCTATGCCTGCCAGCCCCTGGCTGTGGTGGACTTCATCGTGGACATCATGTTCATT
Equus       TGTGGCTATGCCTGCCAGCCCCTGGCAGTGGTGGACCTCATCGTGGATATCATGTTCATC
Cavia       TGTGGCTATGCCTGCCAGCCACTGGCCGTGGTAGACCTCATCGTGGACATCATGTTCATC
Homo        TGTGGCTACGCCTGCCAGCCGCTGGCTGTGGTGGACCTCATCGTGGACATCATGTTCATT
            ******** *********** ***** *****.*** ********** ***********

Canis       GTGGACATCCTCATCAACTTCCGCACCACCTATGTCAATGCCAACGAGGAGGTGGTCAGC
Equus       GTGGACATCCTCATCAACTTCCGCACCACCTATGTCAATGCCAACGAGGAGGTGGTCAGC
Cavia       GTGGACATCCTAATCAACTTCCGCACCACCTACGTCAATGCCAACGAGGAAGTGGTCAGC
Homo        GTGGACATCCTCATCAACTTCCGCACCACCTACGTCAATGCCAACGAGGAGGTGGTCAGC
            ***********.******************* ****************.*********

Canis       CATCCTGGCCGCATTGCTGTCCACTACTTCAAGGGCTGGTTCCTCATTGATATGGTGGCT
Equus       CACCCTGGCCGCATCGCCGTCCACTACTTCAAGGGCTGGTTCCTCATCGACATGGTGGCT
Cavia       CACCCTGGCCGCATCGCCGTCCACTACTTCAAGGGCTGGTTTCTCATCGACATGGTGGCT
Homo        CACCCCGGCCGCATCGCCGTCCACTACTTCAAGGGCTGGTTCCTCATCGACATGGTGGCC
            ** ** ******** ** ********************** ***** ** ********

Canis       GCCATCCCCTTCGACTTGCTCATCTTTGGCTCTGGCTCTGAGGAGCTGATCGGGCTGCTG
Equus       GCCATCCCCTTTGACCTGCTCATCTTCGGTTCTGGCTCTGAGGAGCTGATCGGGCTCCTG
Cavia       GCCATCCCCTTTGACTTACTCATCTTTGGCTCTGGCTCTGAGGAGCTCATTGGGCTGCTG
Homo        GCCATCCCCTTCGACCTGCTCATCTTCGGCTCTGGCTCTGAGGAGCTGATCGGGCTGCTG
            *********** *** *.******** ** ***************** ** ***** ***

Canis       AAGACAGCGCGGCTGCTGCGGCTGGTACGCGTGGCAAGGAAGCTGGACCGCTACTCAGAG
Equus       AAGACGGCGCGGCTGCTGCGACTGGTGCGCGTCGCACGGAAGCTGGACCGCTACTCGGAG
Cavia       AAGACTGCTCGGCTGCTGCGGCTGGTGCGCGTGGCTCGGAAGCTGGACCGCTACTCGGAG
Homo        AAGACTGCGCGGCTGCTGCGGCTGGTGCGCGTGGCGCGGAAGCTGGATCGCTACTCAGAG
            ***** ** ***********.*****.***** ** .********** ********.***

Canis       TATGGGGCGGCTGTGCTCTTCCTGCTCATGTGCACCTTCGCGCTCATTGCACACTGGCTG
Equus       TACGGGGCAGCGGTGCTCTTCCTGCTCATGTGCACCTTTGCGCTCATCGCGCACTGGCTG
Cavia       TACGGGGCGGCGGTGCTCTTCCTGCTCATGTGCACCTTCGCGCTGATCGCCCACTGGCTG
Homo        TACGGCGCGGCCGTGCTGTTCTTGCTCATGTGCACCTTTGCGCTCATCGCGCACTGGCTA
            ** ** **.** ***** *** ***************** ***** ** ** ********.

Canis       GCCTGCATCTGGTATGCCATTGGCAACATGGAGCAGCCGCACATGGACTCCCGCATCGGT
Equus       GCTTGCATCTGGTACGCCATCGGCAACATGGAGCAGCCGCACATGGACTCCCGCATCGGC
Cavia       GCCTGCATCTGGTACGCCATCGGCAACATGGAGCAGCCGGACATGAACTCCCGCATCGGC
Homo        GCCTGCATCTGGTACGCCATCGGCAACATGGAGCAGCCACACATGGACTCACGCATCGGC
            ** *********** ***** *****************. *****.****.********

Canis       TGGCTGCACAACCTGGGGGATCAGATCGGCAAGCCCTACAACAGTAGTGGCCTGGGTGGC
Equus       TGGCTGCACAACCTGGGCGACCAGATCGGCAAGCCCTACAACAGCAGTGGCCTGGGTGGC
Cavia       TGGCTGCACAACCTGGGTGACCAGATTGGCAAGCCCTACAATAGCAGCGGCCTGGGTGGG
Homo        TGGCTGCACAACCTGGGCGACCAGATAGGCAAACCCTACAACAGCAGCGGCCTGGGCGGC
            ***************** ** ***** *****.******** ** ** ******** **

Canis       CCCTCCATCAAGGACAAGTATGTCACGGCCCTCTACTTCACCTTCAGCAGCCTCACCAGT
Equus       CCGTCCATCAAGGACAAGTATGTCACGGCCCTCTACTTCACCTTCAGCAGCCTCACTAGC
Cavia       CCGTCCATCAAGGACAAGTACGTCACGGCCCTCTACTTCACCTTCAGCAGCCTGACCAGC
Homo        CCCTCCATCAAGGACAAGTATGTGACGGCGCTCTACTTCACCTTCAGCAGCCTCACCAGT
            ** ***************** ** ***** ********************** ** **
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Canis       GTGGGCTTTGGCAATGTCTCCCCCAACACCAACTCGGAAAAGATCTTCTCCATCTGTGTC
Equus       GTGGGCTTCGGCAATGTCTCCCCCAACACCAACTCAGAGAAGATCTTCTCCATTTGTGTC
Cavia       GTGGGCTTTGGCAATGTCTCACCCAACACCAACTCAGAGAAGATCTTCTCCATCTGCGTC
Homo        GTGGGCTTCGGCAACGTCTCTCCCAACACCAACTCAGAGAAGATCTTCTCCATCTGCGTC
            ******** ***** ***** **************.**.************** ** ***

Canis       ATGCTCATCGGCTCCCTCATGTACGCCAGCATCTTCGGCAACGTGTCAGCCATCATCCAG
Equus       ATGCTCATTGGCTCCCTCATGTACGCCAGCATCTTTGGCAACGTGTCAGCCATCATCCAG
Cavia       ATGCTCATTGGCTCCCTCATGTACGCCAGCATCTTCGGCAACGTGTCGGCCATTATCCAG
Homo        ATGCTCATTGGCTCCCTCATGTATGCTAGCATCTTCGGCAACGTGTCGGCCATCATCCAG
            ******** ************** ** ******** ***********.***** ******

Canis       AGGCTGTACTCGGGCACTGCCCGCTACCACACGCAGATGCTTCGGGTGCGGGAGTTCATC
Equus       CGGCTATACTCGGGCACAGCCCGCTACCACACGCAAATGCTCCGGGTGCGGGAGTTCATC
Cavia       CGGCTGTACTCAGGCACAGCCCGCTACCACACACAGATGCTCCGCGTGCGTGAGTTCATC
Homo        CGGCTGTACTCGGGCACAGCCCGCTACCACACACAGATGCTGCGGGTGCGGGAGTTCATC
            .****.*****.*****:**************.**.***** ** ***** *********

Canis       CGCTTCCACCAAATCCCCAACCCCTTGCGCCAGCGCCTGGAGGAGTATTTCCAGCACGCC
Equus       CGCTTCCACCAGATCCCTAACCCGCTGCGCCAGCGCCTTGAGGAGTATTTCCAGCACGCC
Cavia       CGCTTCCACCAGATCCCCAACCCCCTGCGCCAGCGCCTGGAGGAGTACTTCCAGCATGCC
Homo        CGCTTCCACCAGATCCCCAATCCCCTGCGCCAGCGCCTCGAGGAGTACTTCCAGCACGCC
            ***********.***** ** **  ************* ******** ******** ***

Canis       TGGTCCTACACCAATGGCATCGATATGAACGCGGTGCTGAAAGGCTTTCCCGAGTGCCTA
Equus       TGGTCCTACACCAACGGCATCGACATGAACGCGGTGCTGAAGGGCTTCCCGGAGTGCCTG
Cavia       TGGTCCTACACCAATGGCATCGACATGAATGCGGTGCTGAAGGGCTTCCCTGAGTGCCTG
Homo        TGGTCCTACACCAACGGCATCGACATGAACGCGGTGCTGAAGGGCTTCCCTGAGTGCCTG
            ************** ******** ***** ***********.***** ** ********.

Canis       CAGGCGGACATCTGTCTGCACCTGAACCGCTCGCTGCTACAGCACTGCAAGCCTTTCCGA
Equus       CAGGCAGACATCTGCCTGCACCTGAACCGCTCGCTGCTGCAACATTGCAAGCCCTTCCGA
Cavia       CAGGCTGACATCTGCCTGCACCTGAACCGCTCACTGCTGCAGCACTGCAAGCCATTCCGA
Homo        CAGGCTGACATCTGCCTGCACCTGAACCGCTCACTGCTGCAGCACTGCAAACCCTTCCGA
            ***** ******** *****************.*****.**.** *****.** ******

Canis       GGGGCCACCAAGGGGTGCCTGAGGGCCCTGGCCATGAAGTTCAAGACAACACATGCACCG
Equus       GGGGCCACCAAAGGCTGCCTGCGGGCCCTGGCCATGAAGTTCAAGACGACACACGCACCG
Cavia       GGGGCCACCAAGGGCTGCCTTCGAGCCCTTGCCATGAAGTTTAAGACCACACACGCGCCA
Homo        GGGGCCACCAAGGGCTGCCTTCGGGCCCTGGCCATGAAGTTCAAGACCACACATGCACCG
            ***********.** ***** .*.***** *********** ***** ***** **.**.

Canis       CCAGGGGACACGCTGGTGCATGCTGGGGACCTGCTCACCGCCCTCTACTTCATCTCCCGG
Equus       CCAGGGGACACGCTGGTGCACGCCGGGGACCTGCTCACCGCCCTCTACTTCATCTCCCGG
Cavia       CCGGGGGACACGCTGGTGCACGCGGGGGACCTGCTTACTGCCCTGTACTTCATCTCCAGG
Homo        CCAGGGGACACACTGGTGCATGCTGGGGACCTGCTCACCGCCCTGTACTTCATCTCCCGG
            **.********.******** ** *********** ** ***** ************.**

Canis       GGCTCCATCGAGATCCTGCGGGGGGACGTCGTCGTGGCCATCCTGGGGAAGAATGACATC
Equus       GGCTCCATCGAGATCCTGCGGGGCGATGTCGTCGTGGCCATCCTGGGGAAGAATGACATC
Cavia       GGCTCCATCGAGATCCTGCGGGGTGATGTGGTTGTAGCCATCTTGGGGAAGAATGACATC
Homo        GGCTCCATCGAGATCCTGCGGGGCGACGTCGTCGTGGCCATCCTGGGGAAGAATGACATC
            ********************** ** ** ** **.****** ****************

Canis       TTTGGAGAGCCTTTGAACCTGTATGCTCGGCCTGGCAAGTCCAACGGGGACGTGCGGGCC
Equus       TTCGGAGAGCCTCTGAACCTGTATGCGCGGCCTGGCAAGTCCAATGGGGATGTGCGGGCC
Cavia       TTTGGAGAGCCTCTAAACCTGTATGCACGGCCTGGCAAGTCCAATGGGGATGTGCGAGCC
Homo        TTTGGGGAGCCTCTGAACCTGTATGCAAGGCCTGGCAAGTCGAACGGGGATGTGCGGGCC
            ** **.****** *.*********** .************* ** ***** *****.***
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Canis       CTCACCTACTGTGACCTACACAAGATCCACCGGGACGACCTGCTGGAGGTGCTGGACATG
Equus       CTCACCTACTGCGACCTGCACAAGATCCACCGGGACGACCTGCTGGAGGTGCTGGACATG
Cavia       CTCACCTACTGCGACCTGCACAAGATCCATCGGGATGACCTGCTGGAGGTGTTAGACATG
Homo        CTCACCTACTGTGACCTACACAAGATCC     TGCTGGACATG
            *********** *****.*********** ***** *************** *.******

Canis       TACCCTGAGTTCTCTGACCACTTCTGGTCCAGCCTGGAGATCACCTTCAACCTTCGGGAT
Equus       TACCCCGAGTTCTCCGACCACTTCTGGTCCAGCCTGGAGATCACCTTCAACCTTCGAGAC
Cavia       TACCCAGAGTTCTCAGACCACTTCTGGTCCAGCCTGGAGATCACCTTCAACCTTCGAGAT
Homo        TACCCTGAGTTCTCCGACCACTTCTGGTCCAGCCTGGAGATCACCTTCAACCTGCGAGAT
            ***** ******** ************************************* **.**

Canis       ACCAACATGATCCCTGGATCTCCCGGCAGCGCGGAGCTGGAGGGCGGCTTCAACAGACAG
Equus       ACCAACATGATCCCCGGCTCTCCCGGCAGCACAGAGCTGGAGGGCGGCTTCAACCGGCAA
Cavia       ACCAACATGATCCCGGGTTCTCCTGGCAGCACTGAGTTAGAGGGCGGCTTCAATCGGCAA
Homo        ACCAACATGATCCCGGGCTCCCCCGGCAGTACGGAGTTAGAGGGTGGCTTCAGTCGGCAA
            ************** ** ** ** ***** .* *** *.***** *******. .*.**.

Canis       CGCAAGCGCAAGCTGTCCTTCCGCAGGCGCACCGACAGGGACCCGGAACAGCCAGGGGAG
Equus       CGCAAGCGCAAGCTGTCCTTCCGCAGACGCACCGACAAGGACCCGGAACAGCCAGGGGAG
Cavia       CGCAGGCGCAAGCTGTCCTTTCGAAGGCGCACCGATAAGGACCCTGAACAGCCGGGGGAG
Homo        CGCAAGCGCAAGTTGTCCTTCCGCAGGCGCACGGACAAGGACACGGAGCAGCCAGGGGAG
            ****.******* ******* **.**.***** ** *.****.* **.*****.******

Canis       GTGTCGGCCTTGGGGCCGGGCCGGGCGGGGGCAGGGCCGAGTGGCCGAGGCCGGCCAGGG
Equus       GTGTCGGCCTTGGGGCCGGGCCGGGCGGGGGCAGGGCCCAGTAGCCGGGGCCGGCCAGGG
Cavia       GTGCCGGCCTTAGGGCCAGCCCGGGTGGGTGCAGGGCCCAGCAGCTGGGGCCGGCCGGGA
Homo        GTGTCGGCCTTGGGGCCGGGCCGGGCGGGGGCAGGGCCGAGTAGCCGGGGCCGGCCGGGG
            *** *******.*****.* ***** *** ******** ** .** *.********.**.

Canis       GGGCCGTGGGGGGAGAGCCCATCCAGTGGCCCCTCCAGCCCGGAGAGCAGTGAGGATGAG
Equus       GGCCCGTGGGGGGAAAGCCCGTCCAGTGGCCCCTCCAGCCCTGAGAGCAGTGAGGATGAG
Cavia       ACGCCATGGGGGGACAGCCCATCCAGTGGCCCCTCCAGCCCCGAAAGCAGTGAAGATGAG
Homo        GGGCCGTGGGGGGAGAGCCCGTCCAGTGGCCCCTCCAGCCCTGAGAGCAGTGAGGATGAG
            .  **.******** *****.******************** **.********.******

Canis       GGCCCAGGCCGCAGCTCCAGCCCCCTCCGCCTGGTGCCCTTCTCCAGCCCCAGGCCCCCC
Equus       GGCCCAGGCCGCAGCTCCAGCCCCCTCCGCCTGGTGCCCTTCTCCAGCCCCAGGCCCCCC
Cavia       GGTCAGGGCCGCAGCTCCAGCCCACTGCGCCTGGTGCCCTTCTCCAGCCCAAGGCCCCCT
Homo        GGCCCAGGCCGCAGCTCCAGCCCCCTCCGCCTGGTGCCCTTCTCCAGCCCCAGGCCCCCC
            ** *..*****************.** ***********************.********

Canis       GGAGAGCCACCGGGTGGGGAGCCCCTGACGGAGGATGGTGAGAAGAGCAGCGACACCTGT
Equus       GGAGAGCCGCCGGGTGGGGAGCCCCTGATTGAGGACTGCGAGAAGAGCAGTGACACATGT
Cavia       GGGGAGCCGCGGGGCGCAGAGACCCTGACCGAGGATGGTGACAAG---AGCGACACATGC
Homo        GGAGAGCCGCCGGGTGGGGAGCCCCTGATGGAGGACTGCGAGAAGAGCAGCGACACTTGC
            **.*****.* *** * .***.******  *****  * ** ***   ** ***** **

Canis       AACCCGCTGTCAGGCGCCTTCTCTGGAGTGTCCAACATCTTCAGCTTCTGGGGGGACAGT
Equus       AACCCGCTGTCAGGCGCCTTCTCGGGAGTGTCCAACATCTTCAGCTTCTGGGGGGATAGT
Cavia       AACCCACTGTCTGGCGCCTTTTCTGGGGTGTCCAACATTTTCAGCTTTTGGGGGGACAGT
Homo        AACCCCCTGTCAGGCGCCTTCTCAGGAGTGTCCAACATTTTCAGCTTCTGGGGGGACAGT
            ***** *****:******** ** **.*********** ******** ******** ***

Canis       CGGGGCCACCAGTACCAGGAGCTGCCTCGCTGCCCTGCCCCCACCCCTAGCCTCCTGAAC
Equus       CGGGGCCGCCAGTACCAGGAGCTGCCTCGCTGCCCCGCCCCCGCCCCCAGCCTCCTCAAC
Cavia       CGGGGCCGCCAGTATCAGGAGCTGCCACGATGTCCCGCCCCTGCCCCCAGCCTCCTCAAC
Homo        CGGGGCCGCCAGTACCAGGAGCTCCCTCGATGCCCCGCCCCCACCCCCAGCCTCCTCAAC
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          *******.****** ******** **:**.** ** ***** .**** ******** ***

Canis     ATCCCCCTCTCCAGCCCATGCCGGCGGCCCCGGGGCGACGTGGAGGGCAGGCTGGATGCT
Equus     ATCCCTCTTTCCAGCCCTGGCCGGCGGCCCCGGGGCGATGTGGAGAGCAGGCTGGACGCC
Cavia     ATCCCTCTGTCCAGCCCTGGCCGGCGGCCCCGGGGCGATGTGGAGAGTAGGCTGGATGCC
Homo      ATCCCCCTCTCCAGCCCGGGTCGGCGGCCCCGGGGCGACGTGGAGAGCAGGCTGGATGCC
          ***** ** ********  * ***************** ******.* ******** **

Canis     CTCCAGAGGCAACTCAACAGGCTGGAGACCCGGCTGAGTGCAGACATGGCCACTGTCCTG
Equus     CTTCAGAGGCAGCTTAACAGGCTGGAGACGCGGCTGAGTGCAGACATGGCCACCGTCCTG
Cavia     CTGCAGCGCCAGCTCAACAGGCTGGAGACCCGGCTGAGTGCGGACATGGCGACTGTCCTG
Homo      CTCCAGCGCCAGCTCAACAGGCTGGAGACCCGGCTGAGTGCAGACATGGCCACTGTCCTG
          ** ***.* **.** ************** ***********.******** ** ******

Canis     CAGCTGCTGCAGAGGCAGATGACGCTGATCCCACCTGCCTACAGTGCTGTGACCACCCCG
Equus     CAGCTACTGCAGAGACAGATGACACTGGTCCCTCCAGCCTACAGTGCTGTGACCACCCCG
Cavia     CAGCTGCTACAGAGACAGATGACACTGGTCCCACCTGCCTACAGTGCTGTGACCACTCCA
Homo      CAGCTGCTACAGAGGCAGATGACGCTGGTCCCGCCCGCCTACAGTGCTGTGACCACCCCG
          *****.**.*****.********.***.**** ** ******************** **.

Canis     GGGCCTGGGCCCACCTCCACCTCCTCTCTGCTGCCTGTCAGCCCCATCCCCACCCTCACC
Equus     GGGCCCGGCCCCACCTCCACCTCCCCTCTCCTGCCTGTCAGCCCCATCCCCACTCTCACC
Cavia     GGGCCCGGCCCCGCTTCCACCTCTCCCTTGCTGCCCATCAGCCCCATCCCCACCCTCACC
Homo      GGGCCTGGCCCCACTTCCACATCCCCGCTGTTGCCCGTCAGCCCCCTCCCCACCCTCACC
          ***** ** ***.* *****.**  *  *  **** .********.******* ******

Canis     CTGGACTCGCTTTCTCAGGTTTCCCAGTTCATGGCGTTCGAGGAGCTCCCCCCGGGGGCC
Equus     CTGGATTCGCTTTCTCAGGTTTCCCAGTTCATGGCGTGCGAGGAGCTCCCTCCGGGGGCC
Cavia     CTGGACTCGCTTTCTCAGGTTTCCCAGTTCGTGGCGTTCGAGGAGCTTCCGCCCGGAGCT
Homo      TTGGACTCGCTTTCTCAGGTTTCCCAGTTCATGGCGTGTGAGGAGCTGCCCCCGGGGGCC
           **** ************************.******  ******** ** ** **.**

Canis     CCAGAGCTCCCCCAAGACGGCCCCCCTCGACGCCTCTCCCTACCGGGCCAGCTGGGGGCC
Equus     CCAGAGCTTCCCCAAGACGGCCCCACTCGACGCCTCTCCCTGCCGGGCCAGCTGGGGGCC
Cavia     CCAGAGCTCCCCCAAGATGGCCCCACTCGACGCCTCTCCCTGCCCGGCCAGCTGGGGGCC
Homo      CCAGAGCTTCCCCAAGAAGGCCCCACACGACGCCTCTCCCTACCGGGCCAGCTGGGGGCC
          ******** ******** ******.*:**************.** ***************

Canis     CTCACCTCCCAGCCTCTGCACAGACATGGCTCAGACCCAGGCAGTTAGTGGGGCTGCCTG
Equus     CTCACCTCCCAGCCCCTGCACAGACACGGCTCAGACCCGGGCAGTTAGTGGAGCTGCCCA
Cavia     CTTACCTCCCAGCCCCTGCACAGACACGGCTCAGACCCGGGGAGTTAGTGAGGCTGTCTG
Homo      CTCACCTCCCAGCCCCTGCACAGACACGGCTCGGACCCGGGCAGTTAGTGGGGCTGCCCA
          ** *********** *********** *****.*****.** ********..**** * .

Canis     GTGTGGACATGTGGCTCACCCAGGGTTCAGCGCACTGCCTGGGCCCCTCCCCTCAGAGGC
Equus     CTGTGGACACGTGGCTCAC-----------------------------------------
Cavia     GTGTGAACACGTGGCTCACCAGGGT-TCCTGCACTGCC-TGGGCCCCTCCCCTCAGAGGC
Homo      GTGTGGACACGTGGCTCACCCAGGGATCAAGGCGCTGC-TGGGCCGCTCCCCTTGGAGGC
          ****.*** *********

Canis     CCTGCCCGGGAGGCCCTGGCCGAGACAGGGGAGAGGACCAAGGACCGTGAAGGCATAGCC
Equus     ------------------------------------------------------------
Cavia     CCTG--------------------------------------------------------
Homo      CCTGCTCAGGAGGCCCTGACCGTGGAAGGGGAGAGGAACTC-------GAAAGCACAGCT


Canis     CCTCCCC-----------------------------------------------------
Equus     ------------------------------------------------------------
Cavia     ------------------------------------------------------------
```

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Homo       CCTCCCCCAGCCCTTGGGACCATCTTCTCCTGCAGTCCCCTGGGCCCCAGTGAGAGGGGC

Canis      ------------------------------------------------------------
Equus      ------------------------------------------------------------
Cavia      ------------------------------------------------------------
Homo       AGGGGCAGGGCCGGCAGTAGGTGGGGCCTGTGGTCCCCCCACTGCCCTGAGGGCATTAGC

Canis      ------------------------------------------------------------
Equus      ------------CCGGAGGCACCCGGCCCTGGGCCTTAGGCACCTCAAGGACTTTTCTGC
Cavia      ------------------------------------------------------------
Homo       TGGTCTAACTGCCCGGAGGCACCCGGCCCTGGGCCTTAGGCACCTCAAGGACTTTTCTGC

Canis      ------------------------------------------------------------
Equus      TATTTACTGCTCTTATTGTTAAGGATAATAATTAAGGATCATATGAATAATTAATGAAGA
Cavia      ------------------------------------------------------------
Homo       TATTTACTGCTCTTATTGTTAAGGATAATAATTAAGGATCATATGAATAATTAATGAAGA

Canis      ------------------------------------------------------------
Equus      TGCTGATGACTATGAATAATAAATAATTATCCTGAGTAAAAAAAAAAAAAAA--------
Cavia      ------------------------------------------------------------
Homo       TGCTGATGACTATGAATAATAAATAATTATCCTGAGGAGACTCCAAAAAAAAAAAAAAAA

Canis      --
Equus      --
Cavia      --
Homo       AA
```

13.3 Appendix III

Primers are indicated by bolded, underlined and enlarged text.

Splicesites dog KCNQ1 (Acc no: XM_540790.2) against chromosome 18 (Acc no: NC_006600.3): 25 ranges

ATGGTCAACTCCGAGCGGAGGGACTCCCAGCCTTTGGCCTGGTATGCTGCCCAAACTGGGGATCTCCGTGTCCCCGCGTGGGAGGAATGCCGATCCCGGG
CCCTGCCTAGCACCCCCGCCTGCGCGTCTGTGTCTGCTCTGGGAGCTCAGGGCAACTCTGCGTCCAGCCGCTGGAGGCCACTGCTCG**GG**AGAGCACTATC
CCTGCCTGAACAGCATGCCGGGCTGAACATGAACAGCATTCGGGGCATGCAGCCGGTGCTCAGCGAGGACGGGGGAGTGCCGCCTGCCATTGCCTATCTG
ATTTTCAATGTCTTGAACAAGCCGGTGCTGTTAATCCTTTACCTGC**CAG**AGCTCCGGCCCCTGATGGCTCTGCTGCTCTTGGAGGTCCGATCAGCCGGGA
TGGGGACACTCACAAGAGGGTCACCTGGCATCCGGCCTCCGAAGCCCAGGCCCCCTGCGGCCTGTGCCGTGGCGGTCAGCCTGCTGCCTGGCAGCTCGGA
GCCCACAGACAGGCCGGGCCTGATGA**TCTCCGTCTGTGC**TGAGAAAGACGGGGAAGATCCAGGTGCCCGCAGTGAGGACCGGCTGCATCTGTCCACTGTG
CGCGGAGTCTTGAGCAGAT**G**CACCTTCCCGGCTGCCAGCATCTGGACTGGAGAGAAGCTTTTGGAGGGGGTCACTGTAGGGGCAGAGACCGGTGTGCACC
CTGATGCCACTGCTACAACCACCTCCAAGCTTGGCCCCGCTGTCCCCTGGGAGGCCGAGTGTGGGGTCCAGAATCTGGACTTAAGGAGGGTCGAGGGGTT
GGAA**AGGTG**CGAGCCCCCCAGCTCCCGCATGTCCTCCAAGCGCTCAGCTTGCTGGTTCTCCAGCCTGCTGGGTGTGCAGGTGCATACCCCCCAATGCGAC
CTCGGCACTGGGGGCCGGGATTCTGGAGCCTCTGATACTAATGAGCCTTGTGTCCCACCCATCCACCTGCTAGAGACCAAAGGCG**G**CCTGGCACCCCCTG
CAGGCGGCTTCGTGCAGCTGGCAGGCTT**CAG**

CTTCCTCATCGTCCTGGTCTGCCTCATCTTCAGTGTCCTGTCCACCATCGAGCAGTATGTCGCCCTGGCCACAGGGACCCTCTTCTGGATGGAGATTGTC
CTGGTGGTGTTCTTTGGGACGGAGTATGTGGTCCGCCTCTGGTCAGCAGGCTGCCGCAGCAAGTATGTAGGCATCTGGGGGAGGCTGCGCTTTGCCCGGA
AGCCCATCTCCATCATTGACCTCATCGTGGTCCTGGCCTCCATGGTTGTCCTCTGCGTGGGCTCCAAGGGGCAGGTGTTTGCCACCTCGGCCATCAGCGG
CATCCGCTTCCTCCAGATCCTGAGGATGCTGCATGTGGATCGCCAGGGAGGTACCTGGAGGCTGCTTGGCTCTGTGGTCTTCATCCACCGCCAGGAGCTC
ATCACCACCTTGTACATCGGCTTCCTGGGCCTCATCTTCTCCTCGTACTTCGTGTACCTGGCCGAGAAGGACGCCGTGAACGACTCGGGCCAAGTGGAGT
TTGGCAGCTATGCAGATGCCCTGTGGTGGGGAGTGGTCACGGTCACCACCATCGGCTACGGGGACAAGGTGCCCCAGACGTGGGTTGGGAAGACCATCGC
CTCCTGCTTCTCTGTGTTTGCCATCTCCTTCTTTGCGCTCCCAGCGGGGATTCTCGGCTCTGGCTTCGCCCTAAAGGTCCAGCAGAAGCAGAGACAGAAG
CACTTCAATAGGCAGATCCCGGCGGCAGCCTCGCTCATTCAGACGGCGTGGAGGTGCTACGCGGCCGAGAACCCCGACTCTTCCACCTGGAAGATCTACG
TCCGGAAGCCCGCCCGGAGCCATGCCCTGCTCTCTCCCAGCCCCAAGCCCAAGAAGTCTGTCATGGTAAAGAGAAAGAAGTTCAAACAAGACAAGGACAA
CGGGGTGAGCCCCGGAGAGAAGATGCTCACGGTCCCCCACATCACGTGCGAACCCGTCTCGGAGGAGCGGAGGCCAGACCACTTCTCCGTGGACAACTGT
GACAGCTCTGTGAAGAAGAGCCCCATGCTGCTGGAGGTGAGCACAGCCCACTTCATGAGAACCAACAGCTTCGCCGAGGACC***TGGACCTCGAGGGG***
GAGACGCTATTGGCGCCCATCACCCATGTGTCACAGCTGCGGGAGCACCACCGGGCCACCATCAAGGTCATTCGGCGCATGCAGTACTTTGTAGCCAAG
AAGAAATTCCAGCAAGCGCGGAAGCCCTACGATGTGCGGGACGTCATTGAGCAGTACTCCCAGGGCC***ACCTCAACCTCATGGTGCG***CATCAAAGA
GCTGCAGAGAAGGCTGGACCAGTCCATCGGGAAGCCCTCCCTCTTCATCTCCGTCTCAGAGAAGAGCAAGGACCGTGGGAGCAACACGATTGGGGCACGC
CTGAACCGAGTAGAAGACAAGGTGACACAGCTGGACCAGAGGCTGGTGCTCATCACGGACATGCTTCACCAGCTCCTCTCCTTGTACCATGGTGGCCCCC
CAGGTGGCCGCCCCCCCAGCGGGAGCGGGGTCCAGGTGGTTCAGCCCTGCAGCTCCATCAACCCCGAGCTCTTCCTGCCCAGCAACACCCTGCCCACCTA
CGAACAGCTAACTGTGCCCCGCAGGGGTCCCGAAGAGGGGTCCTGA

Splicesites human KCNQ1 (Acc no: NM_000218.2) against chromosome 11 (Acc no: NC_006600.3): 16 ranges

ATGGCCGCGGCCTCCTCCCCGCCCAGGGCCGAGAGGAAGCGCTGGGGTTGGGGCCGCCTGCCAGGCGCCCGGCGGGGCAGCGCGGGCCTGGCCAAGAAGT
GCCCCTTCTCGCTGGAGCTGGCGGAGGGCGGCCCGGCGGGCGGCGCGCTCTACGCGCCCATCGCGCCCGGCGCCCAGGTCCCGCGCCCCCTGCGTCCCC
GGCCGCGCCCGCCGCGCCCCCAGTTGCCTCCGACCTTGGCCCGCGGCCGCCGGTGAGCCTAGACCCGCGCGTCTCCATCTACAGCACGCCGCCCGGTG
TTGGCGCGCACCCACGTCCAGGGCCGCGTCTACAACTTCCTCGAGCGTCCCACCGGCTGGAAATGCTTCGTTTACCACTTCGCCGT

CTTCCTCATCGTCCTGGTCTGCCTCATCTTCAGCGTGCTGTCCACCATCGAGCAGTATGCCGCCCTGGCCACGGGGACTCTCTTCTGGATGGAGATCGTG
CTGGTGGTGTTCTTCGGGACGGAGTACGTGGTCCGCCTCTGGTCCGCCGGCTGCCGCAGCAAGTACGTGGGCCTCTGGGGGCGGCTGCGCTTTGCCCGGA
AGCCCATTTCCATCATCGACCTCATCGTGGTCGTGGCCTCCATGGTGGTCCTCTGCGTGGGCTCCAAGGGGCAGGTGTTTGCCACGTCGGCCATCAGCGG
CATCCGCTTCCTGCAGATCCTGAGGATGCTACACGTCGACCGCCAGGGAGGCACCTGGAGGCTCCTGGGCTCCGTGGTCTTCATCCACCGCCAGGAGCTG
ATAACCACCCTGTACATCGGCTTCCTGGGCCTCATCTTCTCCTCGTACTTTGTGTACCTGGCTGAGAAGGACGCGGTGAACGAGTCAGGCCGCGTGGAGT
TCGGCAGCTACGCAGATGCGCTGTGGTGGGGGGTGGTCACAGTCACCACCATCGGCTATGGGGACAAGGTGCCCCAGACGTGGGTCGGGAAGACCATCGC
CTCCTGCTTCTCTGTCTTTGCCATCTCCTTCTTTGCGCTCCCAGCGGGGATTCTTGGCTCGGGGTTTGCCCTGAAGGTGCAGCAGAAGCAGAGGCAGAAG
CACTTCAACCGGCAGATCCCGGCGGCAGCCTCACTCATTCAGACCGCATGGAGGTGCTATGCTGCCGAGAACCCCGACTCCTCCACCTGGAAGATCTACA
TCCGGAAGGCCCCCCGGAGCCACACTCTGCTGTCACCCAGCCCCAAACCCAAGAAGTCTGTGGTGGTAAAGAAAAAAAAGTTCAAGCTGGACAAAGACAA
TGGGGTGACTCCTGGAGAGAAGATGCTCACAGTCCCCCATATCACGTGCGACCCCCCAGAAGAGCGGCGGCTGGACCACTTCTCTGTCGACGGCTATGAC
AGTTCTGTAAGGAAGAGCCCAACACTGCTGGAAGTGAGCATGCCCCATTTCATGAGAACCAACAGCTTCGCCGAGGACC***TGGACCTGGAAGGGGAG***
ACTCTGCTGACACCCATCACCCACATCTCACAGCTGCGGGAACACCATCGGGCCACCATTAAGGTCATTCGACGCATGCAGTACTTTGTGGCCAAGAAG
AAATTCCAGCAAGCGCGGAAGCCTTACGATGTGCGGGACGTCATTGAGCAGTACTCGCAGGGCC***ACCTCAACCTCATGGTGCG***CATCAAGGAGCT
GCAGAGGAGGCTGGACCAGTCCATTGGGAAGCCCTCACTGTTCATCTCCGTCTCAGAAAAGAGCAAGGATCGCGGCAGCAACACGATCGGCGCCCGCCTG
AACCGAGTAGAAGACAAGGTGACGCAGCTGGACCAGAGGCTGGCACTCATCACCGACATGCTTCACCAGCTGCTCTCCTTGCACGGTGGCAGCACCCCCG
GCAGCGGCGGCCCCCCCCAGAGAGGGCGGGGCCCACATCACCCAGCCCTGCGGCAGTGGCGGCTCCGTCGACCCTGAGCTCTTCCTGCCCAGCAACACCCT
GCCCACCTACGAGCAGCTGACCGTGCCCAGGAGGGGCCCCGATGAGGGGTCCTGA

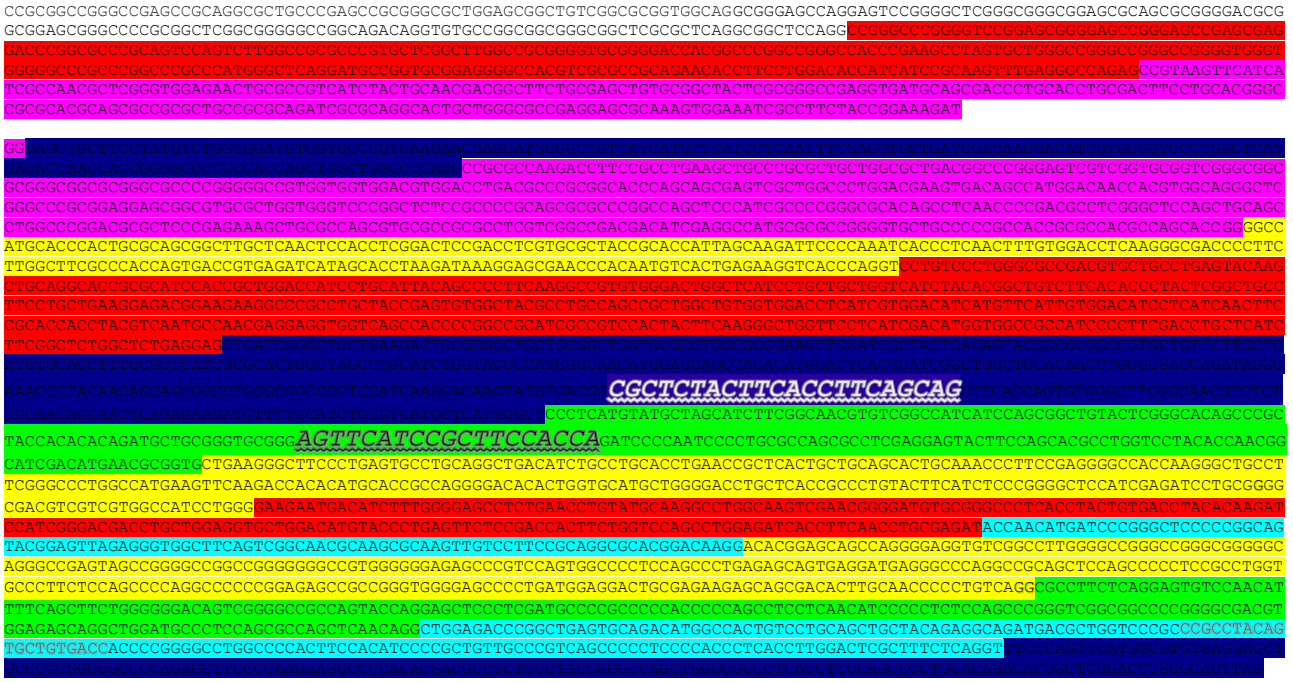
13.4 Appendix IV

Primers are indicated by bolded, underlined and enlarged text.

Splicesites dog KCNH2 (Acc no: NM_001003145.1) against chromosome 16 (Acc no: NW_003726086.1): 15 ranges

Splicesites horse KCNH2 (Acc no: NM_001193658.1) against chromosome 4 (Acc no: NW_001867145.1): 15 ranges

Splicesites human KCNH2 (Acc no: NM_000238.3) against chromosome 7 (Acc no: NC_018918.2): 15 ranges



13.5 Appendix V

Samples with specification of species, animal number, sample type, RNA concentration and RIN-value.

Species	Animal no.	Sample type	Concentration (ng/µl)	RIN-value
Guinea pig	1	LV	4691.7	8.4
Guinea pig	2	LV	3296.7	2.3
Guinea pig	3	LV	5733.8	2.5
Guinea pig	4	LV	4768.7	2.4
Guinea pig	5	LV	4143	2.3
Dog	1	LV	1925.1	7.9
Dog	2	LV	1386.1	8.5
Dog	3	LV	784.4	8.3
Dog	4	LV	1300.8	7.9
Dog	5	LV	2723.5	8.3
Pig	1	LV	4054.4	8.1
Pig	2	LV	3911.5	7.2
Pig	3	LV	4491.6	8.1
Pig	4	LV	3455.4	7.3
Pig	5	LV	3162.7	6.1
Horse	1	LV	3232.3	8.7
Horse	2	LV	2693.6	8.5
Horse	3	LV	2010.2	8.3
Horse	5	LV	1997.3	8
Horse	6	LV	2592.1	8.5
Horse	2	RV	1045.5	7.8
Horse	3	RV	1385.4	8.2
Horse	4	RV	799.9	7.9
Horse	5	RV	1974.2	6.8
Horse	6	RV	99.1	7.9
Horse	2	LA	2271.4	8.5
Horse	3	LA	2595.2	7
Horse	4	LA	2218.8	7
Horse	5	LA	2414.2	6.6
Horse	6	LA	1862.8	7.6
Horse	2	RA	2900.3	8.8
Horse	3	RA	2051.6	8.3
Horse	4	RA	3324.9	5.9
Horse	5	RA	2408.3	7
Horse	6	RA	2863.6	7.1
Horse	2	PF	2784.4	7.5
Horse	3	PF	1583.3	6.7
Horse	5	PF	1072.3	6.4
Horse	6	PF	1694.1	7.5
Horse	7	PF	935.9	7.1
Minke whale	1	LV	2846.6	7.2

13.6 Appendix VI

Standard curve equations, $R_{squares}$ and efficiencies.

St curve	Equation	R_{square}	Efficiency
Guinea pig, KCNQ1	Y = -3.249*LOG(X) + 27.55	0.973	Eff = 103.1%
Dog, KCNQ1	Y = -3.656*LOG(X) + 29.87	0.975	Eff = 87.7%
Pig, KCNQ1	Y = -3.800*LOG(X) + 24.99	0.972	Eff = 83.3%
Horse, KCNQ1-universal	Y = -3.208*LOG(X) + 25.92	0.992	Eff = 105.0%
Minke whale, KCNQ1	Y = -3.250*LOG(X) + 24.30	0.996	Eff = 103.1%
Guinea pig, KCNH2	Y = -3.834*LOG(X) + 29.18	0.948	Eff = 82.3%
Dog, KCNH2	Y = -3.326*LOG(X) + 24.89	0.975	Eff = 99.8%
Pig, KCNH2	Y = -3.074*LOG(X) + 25.60	0.990	Eff = 111.5%
Horse, KCNH2-universal	Y = -3.197*LOG(X) + 25.06	0.983	Eff = 105.5%
Minke whale, KCNH2	Y = -3.197*LOG(X) + 23.49	0.971	Eff = 105.5%
Guinea pig, gDNA	Y = -3.116*LOG(X) + 34.06	0.896	Eff = 109.4%
Dog, gDNA	Y = -3.262*LOG(X) + 30.39	0.609	Eff = 102.6%
Pig, gDNA	Y = -3.131*LOG(X) + 33.42	0.784	Eff = 108.6%
Horse, gDNA	Y = -3.210*LOG(X) + 31.55	0.852	Eff = 104.9%
Minke whale, gDNA	Y = -3.723*LOG(X) + 29.76	0.928	Eff = 85.6%
Guinea pig, RPS18	Y = -3.404*LOG(X) + 33.08	0.916	Eff = 96.7%
Dog, RPS18	Y = -3.875*LOG(X) + 18.96	0.961	Eff = 81.2%
Pig, RPS18	Y = -3.412*LOG(X) + 20.48	0.954	Eff = 96.4%
Horse, RPS18	Y = -3.801*LOG(X) + 19.16	0.978	Eff = 83.3%
Minke whale, RPS18	Y = -3.385*LOG(X) + 17.45	0.996	Eff = 97.4%
Horse, KCNQ1-equine	Y = -3.722*LOG(X) + 29.61	0.929	Eff = 85.6%
Horse, KCNH2-equine	Y = -2.748*LOG(X) + 31.12	0.977	Eff = 131.2%