Myoglobin expression in Notothenioid ancestor *Bovichtus variegatus* suggests loss is not cold adaptive

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Abstract

The icefish family *Channichthyidae* has been the focus of considerable research in myoglobin expression. It is known that none of the sixteen species studied produce myoglobin protein in their pectoral muscle, and six species do not produce myoglobin at all. This loss of expression has occurred by at least three separate events and two different mechanisms, and was thought be an adaptation to the cold waters of Antarctica. Four *Bovichtus variegatus* individuals were studied as the basal ancestor to the notothenioid suborder, and was compared to five notothenioids, a non-Antarctic notothenioid and an outgroup. Preliminary results suggest myoglobin expression is not cold adaptive as none of the species produced myoglobin protein in the pectoral adductor profundus muscle, including the outgroup, NZ Spotty (*Notolabrus celidotus)*. All species had myoglobin protein present in the heart tissue ranging in size from 14kDa to 16kDa, and could only be seen by comparing the red muscle and heart tissue of each species. The RNA was better quality than the gDNA but neither cDNA (RT product) or gDNA bound well to the primers, and only very faint bands could be seen on the gels. Further analysis should be undertaken to confirm the results of this study by purifying the samples to gain brighter bands on the gel and sequencing the product to check it is myoglobin.

Introduction

Notothenioid fish have many adaptations to survive in the stable cold oceans surrounding Antarctica with constant sea water temperatures of -1.86°C. These include increased fluid lipids, antifreeze glycoproteins, increased heart size and blood volume, and decreased blood viscosity, and these adaptations have been the focus of considerable research (Sidell et al, 1997). There has been a large amount of research done on the *Channichthyidae* family of icefishes with some species losing haemoglobin and myoglobin protein expression from their heart and oxidative skeletal tissues (Sidell et al, 1997). It was suggested that this loss was adaptive to the cold-water temperature with individuals having increased intracellular lipids to transport oxygen, and lower metabolic rates. They were expected to rely on the increased oxygen solubility of the water to compensate (Vayda et al, 1997). No notothenioids studied to date express myoglobin in their pectoral adductor profundus muscle (oxidative (red) skeletal muscle), and it is known that multiple independent mutation events throughout evolution of the notothenioid family have

produced a random pattern of expression of myoglobin expression (Vayda et al, 1997; Moylan and Sidell, 2000; Small et al, 2003).

Myoglobin (Mb) is an intracellular oxygen binding protein, that both stores and transports oxygen from capillaries to mitochondria in oxidative muscles, and gives red tissues their characteristic colour (Sidell, 1998; Moylan and Sidell, 2000; Vayda et al, 1997). Mb is a monomeric protein and therefore has a hyperbolic saturation curve, which would increase its affinity for oxygen at lower temperatures and release bound oxygen only at very low intracellular partial pressure (Sidell, 1998).

The Bovichtidae family is one of eight families in the Notothenioidei suborder within the Perciform order. They are the basal group consisting of the three genera *Cottoperca, Halaphritis* and *Bovichtus,* with high species diversity and dispersal (Mazzei, 2006). The generally accepted relationships between the Notothenioidei suborder are (Bovichtidae, (Nototheniidae, (Harpagiferidae, (Artedidraconidae, (Bathydraconidae, (Channichthyidae)))))) (Ritchie et al, 1997), with the Notothenioid suborder evolving in Antarctic waters around 25 million years (Sidell et al, 1997). *Bovichtus variegatus* (thornfish) are found between Wellington and the Sub-Antarctic Islands of New Zealand. The aim of this study is to determine whether this species has lost the expression of the myoglobin protein and mRNA with comparison to other Antarctic notothenioid species. The NZ Spotty *Notolabrus celidotus* within the *Labridae* family is used as an outgroup. The hypothesis for this study is that all species will have Mb protein and mRNA present in the heart tissue, and all (with the exception of NZ Spotty) will not express the Mb protein or mRNA in the pectoral muscle, but will retain the gene in the genomic DNA

Method

Species studied

Heart and pectoral adductor profundus muscle (oxidative (red) skeletal muscle) were analysed from four *Bovichtus variegatus* (BV) individuals (labeled 1 to 4), and five notothenioid species. These included *Chionodraco hamatus* (CH), *Pagothenia borchgrevinki* (PB), *Trematomus newnesi* (TN), *Trematomus eulepidotus* (TE) and *Trematomus bernacchii* (TB). The red pectoral muscle of a NZ black cod *Notothenia angustata* (NA) was also analysed as a non-Antarctic

notothenioid. Both heart and red muscle were analysed in the NZ Spotty *Notolabrus celidotus,* (NZ spot) as an outgroup. The Spotty belongs to a different family within the perciform order, and should be a suitable control for the study.

Tissue preparation

All tissues were kept at -80°C before use. Each sample was cut with a sterilized scalpel into two pieces, one for protein analysis and the other for RNA extraction. Each tissue was weighed to the nearest milligram, to be around 100 milligrams if there was sufficient tissue. The samples being used for protein analysis were transferred to an ice bucket to keep them cool, and the other samples were returned to the -80°C freezer to prevent degradation.

Part A: Myoglobin Protein Analysis

Protein Homogenization

The protein homogenization method was modified from Moylan and Sidell (2000). Between 10 milligrams and 140 milligrams of tissue was homogenized with nine times the volume of 20mM HEPES buffer (pH 7.8 at 4°C) to create a 10% (w/v) homogenate. Larger sized samples were placed into a glass flat bottom tube and ground twice for 30 seconds in the Tekmar Tissumizer with a rest period of 15 seconds on ice in between. Smaller samples were ground in a sterilized mortar and pestle (baked 4 hours at 140°C) with liquid nitrogen before the HEPES buffer was added. The homogenates were spun at 10,000 x g in the Eppendorf Centrifuge 5403 for ten minutes at 4°C. The supernatant was transferred to a new tube and both pellet and supernatant tubes were put into a refrigerator.

Bradford Assay

Protein concentrations were determined using the Bio-Rad Protein Assay method with the FLUOstar Optima and associated program (Software Version 2.00 R3) from BMG LabTech. All readings were done at 595nm and done in duplicate. A standard curve was created, using bovine serum albumin (BSA) as the standard, and water as a zero. Final concentrations of BSA were 2µg/ml to 20µg/ml, in increments of 2µg/ml, with a final volume of 200µl (160µl of standard and 40µl of Bradford reagent). 2µg/ml, 6µg/ml, 12µg/ml, 16µg/ml and 20µg/ml formed a linear line and were used as the standard curve points to determine protein concentration.

A dilution series was done using PB RM and TB H from 1:4 to 1:1000 with a final volume of 1000µl. 1:500 was seen the best dilution with absorbance readings around 0.7 at 595nm. Each sample was then diluted to 1:500 to optimize the absorbance readings to determine concentration.

The method for the Bradford assay follows the Microassay procedure for Microtiter plates in the Bio-Rad Protein Assay instructions. 40µl of dye reagent concentrate was added to each well, followed by 160µl of each diluted sample. Sarstedt (Aust) Microtest flat plates were used with BarSeal™ film covers. The plate was inverted once sealed to mix the reagents, before being incubated at room temperature for 5 minutes. The plate was run at the specified program including orbital mixing. The absorbencies were read and this method was repeated until the duplicates were within 0.05 to 0.1 of each other, and an average between the two was calculated.

Protein SDS PAGE (agarose gel electrophoresis)

A ratio of 6.5 protein sample to 3.5 SDS and BME buffer was created in a new tube to give 35µg of total protein in each well. The final volumes ranged form 10µl to 31µl. Three Invitrogen NuPAGE® 4-12% Bis-Tris 1.5 Gels were run. Each contained 5µl of a Fermentas PageRuler protein ladder as a marker, followed by Spot and CH tissues as controls. 1x 50mM MOPS SDS running buffer made of 50mM Tris Base, 0.1% SDS and 1mM EDTA was used. Each gel was run through Invitrogen 200M® dual power in Invitrogen SureLock™ Novex Mini-Cells for 70 minutes at 180 volts. The gels were then stained with Coomassie Blue Stain containing 0.1% Coomassie Brilliant Blue G for 30 minutes, and destained overnight in destain solution (10% acetic acid, 5% Methanol). Photographs were taken using Chemi Genius² Bio Imaging System and GeneSnap for analysis.

Part B: RNA and gDNA Analysis

RNA extraction

The method for RNA and gDNA extraction of heart and pectoral muscle was from the Trizol™ reagent instructions (Invitrogen life technologies). Invitrogen RNase Away (Molecular Bioproducts Inc, San Diego, CA) was used to create an RNase free workspace. The remaining tissue (up to 100 milligrams) of each sample was used and weighed to the nearest milligram.

Each sample was ground in a sterilized mortar and pestle with liquid nitrogen until a fine powder was formed. 1ml of Trizol™ reagent was added to each mortar and mixed with the ground tissue. The solution was transferred to an eppendorf tube and incubated at room temperature for 5 minutes to allow complete dissociation of nucleoproteins. 0.2ml Chloroform was added to each tube, before shaking in hand for 15 seconds. A further 3 minutes incubation at room temperature followed. The samples were then centrifuged in the Eppendorf Centrifuge 5810R at 11,500 x g for 15 minutes at 4°C to form three layers.

The upper aqueous phase was transferred into a new tube for RNA analysis, and the remaining organic phase was kept for gDNA analysis. 0.5 millilitres of isopropyl alcohol was added to the tube containing the RNA aqueous phase, and samples were incubated at room temperature for 10 minutes, before being spun at 11, 500 x g for 10 minutes at 4°C to precipitate the RNA. The supernatant was removed and the RNA pellet was washed once with 1ml of 75% ethanol, mixed by vortex for 5 seconds, and centrifuged at 7,000 x g for 5 minutes at 4°C. The RNA pellet was dried for 10 minutes in an RNase free cupboard before being redissolved in 100 µl RNase free water (DEPC-H_20) by passing the pellet through a pipette tip. The solution was then incubated at 60°C for 10 minutes before being transferred to the -80°C freezer.

DNA Extraction

The remaining organic phase from the RNA extraction was used to analyse the genomic DNA. 0.3 ml of 100% ethanol was added per 1 ml of Trizol™ reagent initially added. This was mixed by inversion and incubated at room temperature for 3 minutes. The samples were then centrifuged at 2000 x g for 5 minutes at 4°C. The supernatant was then removed and 1 ml of 0.1M sodium citrate was used to wash the pellet, and incubated at room temperature for 30 minutes with period mixing. A further 5 minutes of centrifugation at 2000 x g at 4°C followed. This wash was repeated a second time, and if there was a large amount of non-DNA material or a very large pellet, it was repeated a third time. 1.5ml of 75% ethanol was added to the pellet and incubated for 15 minutes at room temperature with periodic mixing, before being spun (2,000 x g, 5 minutes at 4°C). The supernatant was removed and the pellet air dried for 15 minutes. The pellet was dissolved in 8mM NaOH by pressing the pellet through the pipette tip, with 0.3ml NaOH for 50 mg of tissue, up to 1ml for 100mg tissue. 86 µl of 0.1M HEPES was then added per

1ml of NaOH, and 0.5M EDTA was added to make a concentration of 1mM. The resulting solution was centrifuged at 12,000 x g for 10 minutes to remove the insoluble material. The supernatant containing the gDNA was then transferred to a new tube, and this tube, plus the one containing the pellet of insoluble material were stored in the refrigerator.

RNA and gDNA concentration

BioLab NanoDrop® ND-1000 Spectrophotometer was used to measure the 260:280 ratio as a test of purity. The blank for RNA was DEPC- H_20. 2µl samples were placed on the tip, and read using the RNA-40 setting. A ratio of ~2 is 'pure' RNA. The blank for gDNA was 8mM NaOH as this is the solution the DNA was dissolved in. 2µl samples were placed on the tip and read using the DNA-50 setting. A ratio of ~1.8 is 'pure' DNA. The ratio of RNA is higher as there is a higher ratio of Uracil compared to Thymine. The value should be around this ratio, but not exact as the ratio varies with the composition of nucleotides. The NanoDrop also gave the concentration of the sample in ng / µl.

Total RNA and gDNA Yield

The final yield (µg/mg) was calculated by dividing the concentration from the NanoDrop reading by ten and by the milligrams of tissue initially used. The expected RNA yield is 1-1.5 µg /mg, and the expected gDNA yield is 2-3 µg /mg.

Total RNA Agarose Gel Electrophoresis to check integrity

The method was from Sambrook et al, 1989. An RNase free workspace was created and the tanks and combs were washed in 0.1M NaOH for 2 minutes and then rinsed with DEPC-H_20. 1% Agarose gels were made using 0.5% TBE buffer, with 1 gram of Agarose added to 100ml of 0.5x TBE Buffer. Each well contained 1-4µg of total RNA and 2µl of 6x RNA loading dye (30% glycerol, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF and 1ml DEPC- H_20), which were well mixed though a pipette tip before loading. 4µl of 0.24-0.95kb RNA ladder was used as a marker. The gels were run in 0.5x TBE Buffer at 120 volts for ~40 minutes. The gels were then stained in Ethidium Bromide (EtBr) for 30 minutes and washed in water before a photograph was taken under UV light. Two bands representing the ribosomal RNA, and background smearing representing the mRNA was expected in each lane if the RNA quality was high.

gDNA Agarose Gel Electrophoresis to check integrity

The method was from Sambrook et al, 1989. A 1% Agarose gel were made using 0.5% TBE buffer (1 gram of Agarose added to 100ml of 0.5x TBE Buffer). Each well contained 1-10 µg of gDNA and 2 µl of 6x DNA loading dye (0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF, 40% (w/v) sucrose and 1ml DEPC- H_20), which were well mixed though a pipette tip before loading. 10µl of high range ladder was used as a marker. The gel was run in 0.5x TBE Buffer at 150 volts for 40 minutes. The gel was then stained in Ethidium Bromide (EtBr) for 15 minutes and washed in water, before a photograph was taken under UV light. A single large band was expected in each lane if the quality of gDNA was good.

Reverse Transcription of RNA to cDNA

The method for reverse transcription was followed from the BioLine cDNA Synthesis Kit Instructions. A master mix was created on ice from 15µl Oligo (dT) 18 Primer Mix and 15µl 10mM dNTP Mix. 2µl of this master mix was added to new tubes. 1µg/µl RNA was added to each tube, and DEPC-H_20 was added to make the solution in each tube to 10µl. the samples were incubated at 65°C for 10 minutes and placed on ice for 2 minutes. A second master mix was created with 60µl of 5x BioScript Reaction Buffer, 15µl of 10u/µl RNase Inhibitor, 3.75µl of 200u/µl BioScript enzyme, and 71.25 µl of DEPC- H_20. After 2 minutes on ice, 10µl of this master mix was added to each tube and samples were incubated between 42°C and 50°C for 50 minutes, before the reaction was terminated by incubation at 70°C for 15 minutes before. The cDNA samples were then chilled on ice and placed in the refrigerator until the polymerase chain reaction (PCR) was started.

Polymerase Chain Reaction (PCR) on gDNA

Master mixes were made with the following ratios 2.5µl 10x buffer, 2.5µl 2mM dNTP, 5µl 1:50 Taq polymerase, 0.75µl 50mM $MgCl_2$, 1µl of 10µM forward (F) primer and 1µl of 10µM reverse (R) primer and 7.25µl DEPC - H_20. 20µl aliquots of this master mix were put into each tube, and 5µl of gDNA was added, resulting in a total volume of 25µl in each tube. The primer pairs used were Myo1 F and Myo3 R, and NMyo1F1 and NMyo3R1. The sequences are shown below in table 1. Myo1 and Myo3 are degenerate fish Mb primers that are known to work on

Notothenioids. NMyo primers are notothenioid specific and should work on the NZ Spotty as well, as the region is highly conserved.

Table 1: Primer Sequences and Location and Annealing Temperature

Primer Name	**Primer sequence**	**c. Primer Location**	**AT**
Myo1 F	GTN CTN AAR TGY TGG GG	+19 to +35	
Myo3 R	CCY GCY TTY TCN GYC ATN ACY TTA C	+323 to +347	
NMyo1F1	GTT TAT TCA CAG AGC ACC CAG AA	~140 nt in from mRNA beginning	56.1
NMyo3R1	GCC ATC ACG TTC CTC AGG GCA GTC	~ 100 nt from end of mRNA	64.7

Once the gDNA was added to the master mix aliquots, the tubes were placed in the Eppendorf Mastercycler ep gradient S with a temperature gradient from 50°C to 60°C, to determine the best annealing temperature. The PCR cycle began with a single cycle at 94 °C for 2 minutes, followed by 30 cycles of 30 seconds at 94°C, 20 seconds at 50-60°C gradient and 30 seconds at 72°C. Once 30 cycles were completed, the samples were kept at 72°C for 7 minutes followed by a hold at 4°C until the Agarose gel was ready to be run to prevent degradation. This was done using TN, CH and Spot heart tissue as a pilot to determine the best annealing temperature for further PCR's. The expected product size is ~451bp for Myo 1/3 primers and 431 for NMyo1F1/3R1 primers on gDNA (table 2).

Table 2: Products of PCR

PCR primer pair	**cDNA product size**	**gDNA product size**	**Tm**
Myo1/Myo3	329	~451	?
NMyo1F1/NMyo3R1	309	431	56.8

Agarose Gel Electrophoresis on PCR gDNA

The same method explained above for gDNA Agarose Gel Electrophoresis to check integrity (page 7) was used (Sambrook et al, 1989), with a 1% agarose gel. Each well had 2µl of 6x DNA loading dye and 5µl of PCR gDNA, which were well mixed though a pipette tip before loading. 3µl of Fermentas FastRuler™ DNA low range ladder was used as a marker. The samples were loaded in order of low annealing temperature to high annealing temperature to show which

temperature produced the brightest set of bands. The gel was run in 0.5x TBE Buffer at 180 volts for 30 minutes. The gel was then stained in Ethidium Bromide (EtBr) for 15 minutes and washed in water, before a photograph was taken under UV light.

PCR and Agarose Gel Electrophoresis on PCR cDNA (RT Products)

The same method for PCR was followed as above (page 7) and gel electrophoresis on the products from the reverse transcription (RT) of cDNA was from Sambrook et al (1989). A similar master mix was used but with 2µl of cDNA with increased DEPC - H_20 to maintain the total volume at 25µl. 2µl of cDNA were added to 23 µl of master mix and the samples were placed into the Eppendorf Mastercycler ep gradient S and were run on the same program as the genomic DNA, also with a temperature gradient from 50°C to 60°C. This was done using TN, CH, Spot, BV 2 and TB heart tissue as a pilot run to determine the best annealing temperature for further samples. The gel was run in 0.5x TBE Buffer at 180 volts for 30 minutes. The gel was then stained in Ethidium Bromide (EtBr) for 15 minutes and washed in water, before a photograph was taken under UV light.

Control PCR and PAGE on Invitrogen Salmon Sperm DNA

A control PCR and agarose gel electrophoresis using commercial salmon sperm DNA were done by Jonci Wolff. This was compared to previous gels to compare DNA quality and the level of human error. The annealing temperature was also extended from 45°C to 60°C. The amount of dNTP's were reduced to 1µl of 2mM dNTP's and the magnesium concentration was increased to 1.5 µl of 50mM Mg, with 2 µl of 100ng/µl of Salmon sperm DNA in each tube. All other variables were the same as previous runs.

Results

For all gel figure headings, the symbols H represent heart (ventricular) tissue, and RM represent red muscle or pectoral adductor profundus muscle. The species names have also been shortened to initials. These are *Bovichtus variegatus* (BV1 to BV), *Chionodraco hamatus* (CH), *Pagothenia borchgrevinki* (PB), *Trematomus newnesi* (TN), *Trematomus eulepidotus* (TE) and *Trematomus bernacchii* (TB), NZ black cod *Notothenia angustata* (NA) and the NZ Spotty *Notolabrus celidotus* (Spot).

Part A: Myoglobin Protein

The absorbance readings from the Bradford Assay were averaged and the concentration was calculated using the formula from the standard curve. The absorbance readings at 595nm ranged from 0.411 for BV2 heart tissue to 0.934 for TN red muscle (table 3), with an average reading of 0.7214. The concentration formula was Concentration = (Absorbance-0.4255)/0.039. The two dilutions were then taken into account, so the value was multiplied by 500 for the 1:500 dilution of the sample, and by 1.25 for the dilution of the Bradford reagent. The final concentrations ranged from close to 0mg/ml for BV2 heart to 7.97mg/ml for TN red muscle. The amount to load was then calculated based on this concentration, and buffer was added to have a 6.5 sample: 3.5 buffer ratio with the remainder made up of DEPC-H_20. The final volume in each well ranged from 10µl to 31 µl (table 3).

Table 3: Calculations used in determining gel loading amounts

	SAMPLE	ABS	Conc from abs	[F] mg/ml	Amount for 35µg protein/ well (µl)	Water (µl)	Buffer (µl)	Final volume (µl)
RM	**PB**	0.724	7.48	4.68	7.5	2.3	5.25	15
	TE	0.621	4.90	3.06	11.4	1.6	7	20
	TN	0.934	12.74	7.97	4.4	2.1	3.5	10
	CH	0.733	7.71	4.82	7.3	2.5	5.25	15
	NA	0.7565	8.30	5.18	6.8	3.0	5.25	15
	TB	0.6775	6.32	3.95	8.9	0.9	5.25	15
	SPOT	0.6635	5.96	3.73	9.4	0.4	5.25	15
	BV1	0.5055	2.01	1.25	20.0	0.0	11	31
	BV2	0.5805	3.88	2.43	14.4	1.9	8.75	25
	BV3	0.7245	7.49	4.68	7.5	2.3	5.25	15
	BV4	0.7165	7.29	4.56	7.7	2.1	5.25	15
HEART	**TN**	0.891	11.67	7.29	4.8	1.7	3.5	10
	TE	0.84	10.39	6.49	5.4	1.1	3.5	10
	CH	0.7035	6.97	4.35	8.0	1.75	5.25	15
	TB	0.891	11.67	7.29	4.8	1.7	3.5	10
	PB	0.7665	8.55	5.34	6.6	3.2	5.25	15
	SPOT	0.864	10.99	6.87	5.1	1.4	3.5	10
	BV2	0.4115	-0.35	-0.22	20.0	0.0	11	31
	BV3	0.5085	2.08	1.30	20.0	0.0	11	31
	BV4	0.916	12.29	7.68	4.6	1.9	3.5	10

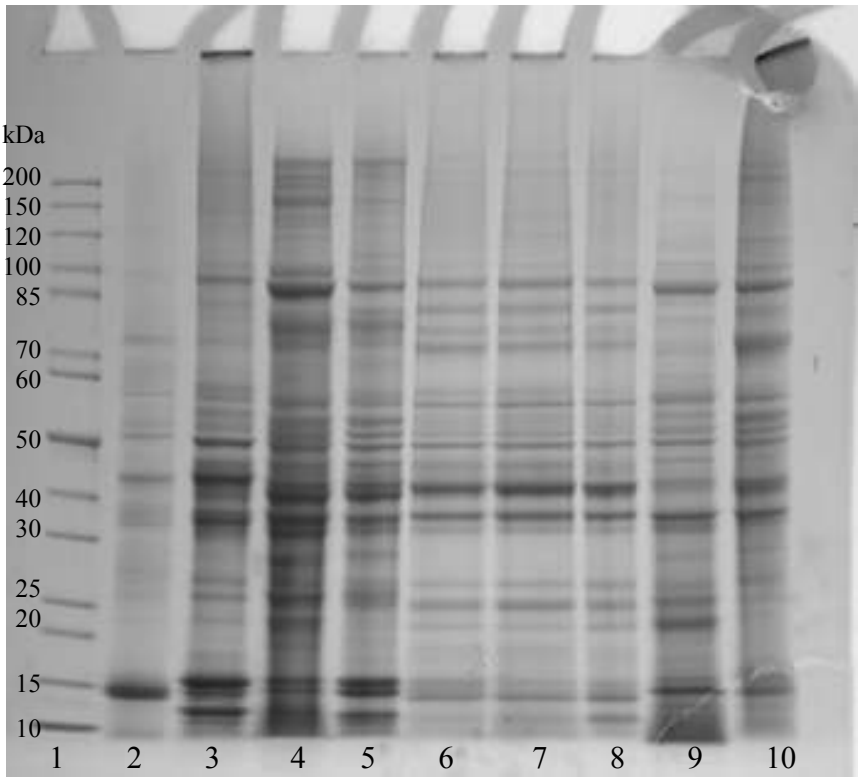

Figure 1: SDS PAGE gel Protein 1.

Lane 1, 5 µl PageRuler Protein ladder; lane 2, Spot H; lane 3, Spot RM; lane 4, CH H; lane 5, CH RM; lane 6, BV2 H; lane 7, BV3 H; lane 8, BV4 H; lane 9 PB H; lane 10 TB H

} Myoglobin ~16kDa

Each lane had 35µl of protein based on concentrations from the Bradford Assay but some lanes are paler than others. Each sample shows a range of protein bands from 200kD to around 10kD. Each sample generally has larger bands at 85kDa, 50kDa, 40kDa and 15kDa. The myoglobin protein band is located around 16kDa, but the actual size is different between species and ranges from around 14 to 16kDa. Spot H has a distinctive bright band around 14kDa and this bright band is used a marker for the myoglobin protein band. It appears that although there are many bands in this region, the band at 14kDa is absent from the Spot RM (lane 3). All three bovictid hearts (lanes 6, 7, 8) show a darker band at the bottom of group of bands around 14kDa, suggesting this species expresses myoglobin protein. Both PB and TB hearts also have a darker band which is likely to be myoglobin but is a bit higher than the bovictids, suggesting it is larger in size, around 15kDa. All the heart muscles on this gel show the myoglobin proteins. It is best to compare each species red and heart muscles to look for differences, rather than comparing species with each other.

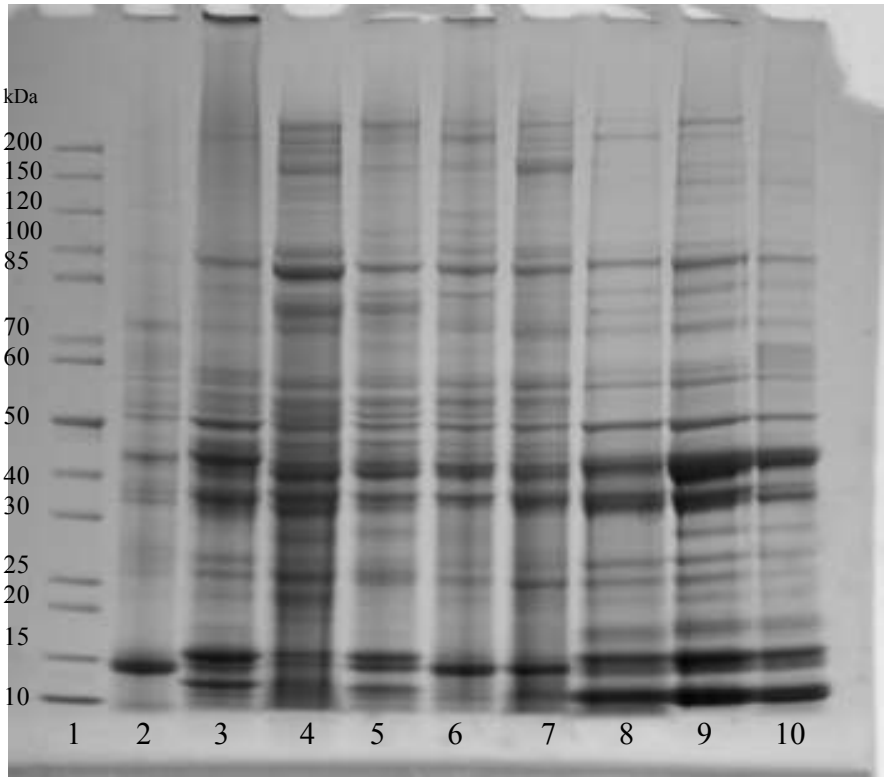

Figure 2: SDS PAGE gel Protein 2.

Lane 1, 5 µl PageRuler Protein ladder; lane 2, Spot H; lane 3, Spot RM; lane 4, CH H; lane 5, CH RM; lane 6, TN H; lane 7, TE H; lane 8, BV 1 RM; lane 9, BV 2 RM; lane 10, BV 3 RM

The first five lanes are the same as the previous gel and show the same pattern, with both Spot and CH hearts expressing the protein and both red muscles not. Both TN and TE hearts (lane 6 and 7) show a very distinctive bad around 15kDa showing myoglobin is also present in these hearts, while none of the bovichtid red muscles (lane 8, 9, 10) show a darker band at the bottom as seen on the previous gel (figure 1). This shows that the heart and pectoral muscle of the bovichtids is distinctly different, as expected.

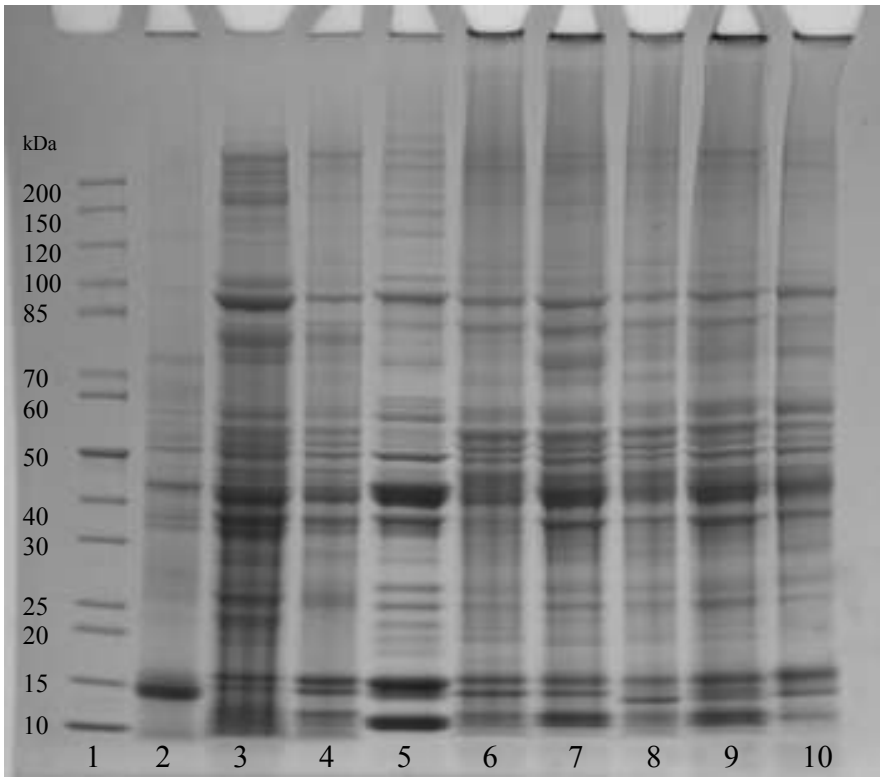

Figure 3: SDS PAGE gel Protein 3.

Lane 1, 5 µl PageRuler Protein ladder; lane 2, Spot H; lane 3, CH H; lane 4, CH RM; lane 5, BV 4 RM; lane 6, PB RM; lane 7, TB RM; lane 8, TN RM; lane 9, TE RM; lane 10, NA RM

Lane 4 is the BV4 RM (figure 3). When compared to lane 8 of figure 1, it appears the myoglobin band is not present, which is consistent with the three other bovichtid red muscle lanes in figure 2. PB and TB red muscle (lane 6 and 7) have a bright band at the same location at 15kDa, but when compared to heart muscle, it is a different location, suggesting myoglobin is not present. TN (lane 8) and NA (lane 10) have a brighter band around 14kDa, but is much paler than in the heart tissue and further analysis would be to be done to confirm if this band is present. Based on a comparison of the heart and red muscle, it appears the myoglobin is not present in the red muscle of either species. TE RM is known from previous studies to have no myoglobin present in the red muscle, but do have it in the heart tissue. This provides evidence that the bright band in the heart muscle (lane 7, figure 2) is the myoglobin protein, and should be found in between the two paler bands (lane 9 of figure 3) around 15kDa if it were present in the red muscle. Therefore, the heart tissue of all species analysed appear to express myoglobin, and all red muscle tissue shows an absence of myoglobin protein, including spot RM, which is surprising.

Part B: RNA and gDNA analysis

RNA SAMPLE		260:280	Conc ng/µl	Initial mg tissue	Yield µg/mg
BV 1	H	1.94	108.3	15	0.722
	RM	1.90	212.3	37	0.574
BV 2	H	2.01	160.7	19	0.846
	RM	2.02	126.2	52	0.243
BV 3	H	1.77	58	10	0.580
	RM	1.80	21	22	0.095
BV 4	H	1.98	262	49	0.535
	RM	1.88	161.6	89	0.182
Spot	H	1.82	257.5	20	1.288
	RM	1.97	212.5	36	0.590
PB	H	2.01	802.6	83	0.967
	RM	1.53	846.9	91	0.931
TB	H	2.06	1096.7	85	1.290
	RM	2.00	658	89	0.739
TE	H	2.06	1806.9	96	1.882
	RM	2.09	913.5	99	0.923
TN	H	2.06	471.9	60	0.787
	RM	2.08	1074.1	97	1.107
CH	H	2.03	672	89	0.755
	RM	1.72	55.7	91	0.061
NA	RM	1.95	318.6	96	0.332

Table 4: RNA Concentration and 260:280 ratios from NanoDrop

A ratio of around 2 is 'pure' for RNA. Most of the samples have ratios around 2 suggesting purity is high. However, PB RM has lower purity at 1.53, and BV3 H and CH RM are 1.73 and 1.72 respectively, which is also a bit low.

The expected RNA yield from the Trizol extraction is 1-1.5 µg per 1 milligram of tissue. Most of these values are less than this, around 0.7 to 0.9 µg/mg, with an average yield of 0.735 µg/mg.

Table 5: gDNA concentration and 260:280 ratios from NanoDrop

A ratio of around 1.8 is 'pure' for gDNA. Most of these values are between 1.6 and 2.0 suggesting purity is ok. BV3 H has a ratio of 8.25 so is not very pure at all, which may be due to the small initial sample size of 10 milligrams. TB RM has a low ratio of 1.0, which is unexpected due to the large tissue size of 89 mg.

The expected DNA yield from the Trizol extraction is 2-3 µg per 1 milligram of tissue. The average yield was less with 0.443 µg/mg.

gDNA SAMPLE		260:280	Conc. ng/µl	Initial mg tissue	Yield µg/mg
BV 1	H	1.64	99.9	15	0.666
	RM	1.61	46.8	37	0.126
BV 2	H	2.05	19.3	19	0.102
	RM	1.61	21.7	52	0.042
BV 3	H	8.25	2.6	10	0.026
	RM	2.00	416.1	22	1.891
BV 4	H	1.89	22.9	49	0.047
	RM	1.65	849.1	89	0.954
Spot	H	1.99	20.8	20	0.104
	RM	1.72	1230.6	36	3.418
PB	H	2.18	12.8	83	0.015
	RM	1.62	60.1	91	0.066
TB	H	1.73	149.3	85	0.176
	RM	1.00	0	89	0.000
TE	H	1.81	278.4	96	0.290
	RM	1.84	311.6	99	0.315
TN	H	1.83	32.6	60	0.054
	RM	1.84	290.6	97	0.300
CH	H	1.94	13.8	89	0.016
	RM	2.11	531.1	91	0.584
NA	RM	1.63	112.5	96	0.117

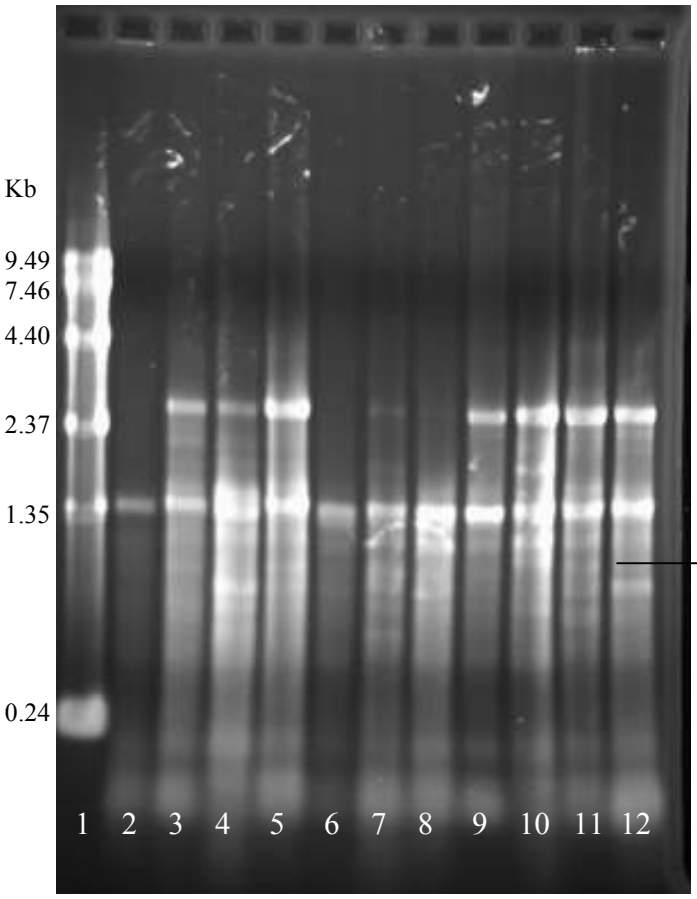

Figure 4: SDS PAGE total RNA 1

Lane 1, 5 µl PageRuler low range; lane 2, Spot H; lane 3, Spot RM; lane 4, TN H; lane 5, TN RM; lane 6, BV 1 H; lane 7, BV 1 RM; lane 8,BV 2 H; lane 9, BV 2 RM; lane 10, TE H; lane 11 TE RM; lane 12 NA RM.

Total RNA gels were run to show the integrity of the RNA. Good quality RNA should have two distinct bands at ~5kb (28S) and ~2kb (16S) which represent the two ribosomal RNA's (rRNA) and background smearing which represents the messenger RNA (mRNA). Spot H (lane 1), BV1 H (lane 6), and BV2 H (lane 8) all show some degradation with only one rRNA band seen (figure 4). The second band is very faint in the red muscle of BV1 (lane 7). All other bands show some degradation but generally the quality should be sufficient for analysis, as the two rRNA bands are distinctive with background smearing of smaller size. The two ribosomal RNA bands occur around 2.4kb and 1.35kb, which are smaller than expected. The mRNA size is around 1.5-3 Kb which is consistent with the band seen on the gel.

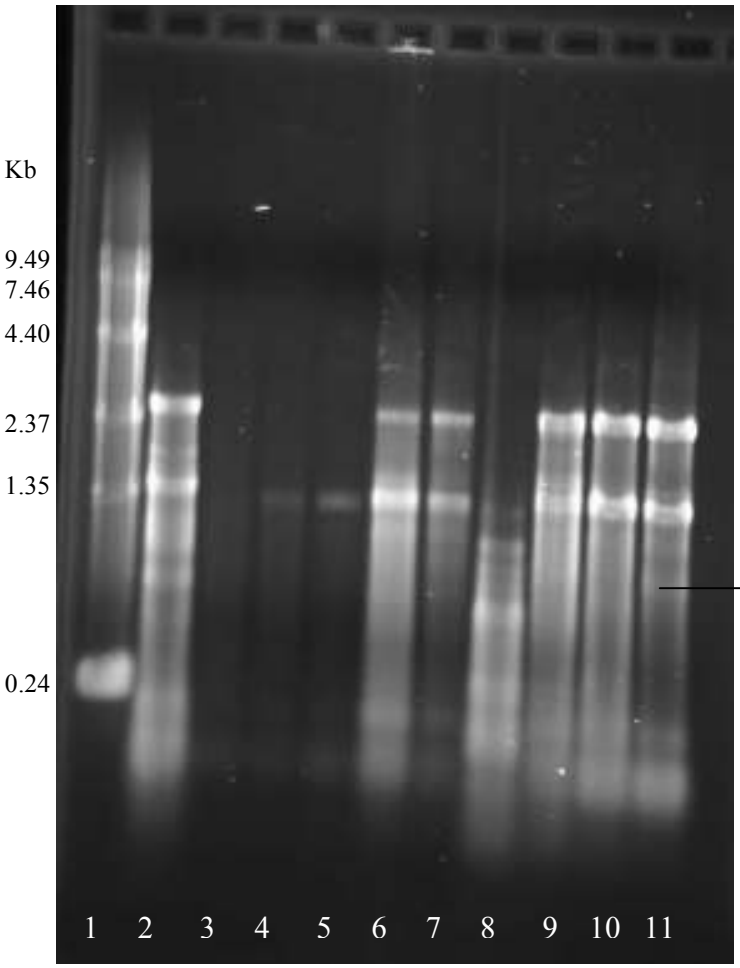

Figure 5: SDS PAGE total RNA 2

Lane 1, 5 µl PageRuler low range; lane 2, CH H; lane 3, CH RM; lane 4, BV 3 H; lane 5, BV 3 RM; lane 6, BV 4 H; lane 7, BV 4 RM; lane 8, PB H; lane 9, PB RM; lane 10, TB H; lane 11 TB RM

The two rRNA bands are at the same location as the previous gel around 2.4kb and 1.35kb (figure 5). CH H, BV4 H and RM, PB RM, and TB H and RM are all reasonably good quality with two rRNA bands and a background smear (figure 5). PB H (lane 8) has background smear of mRNA but no rRNA bands. The heart and red muscle of BV3 (lanes 4 and 5) are very degraded with a very pale band at 1.35 and little background smearing showing the quality is poor, and CH RM (lane 2) is heavily degraded and unlikely to be suitable for reverse transcription.

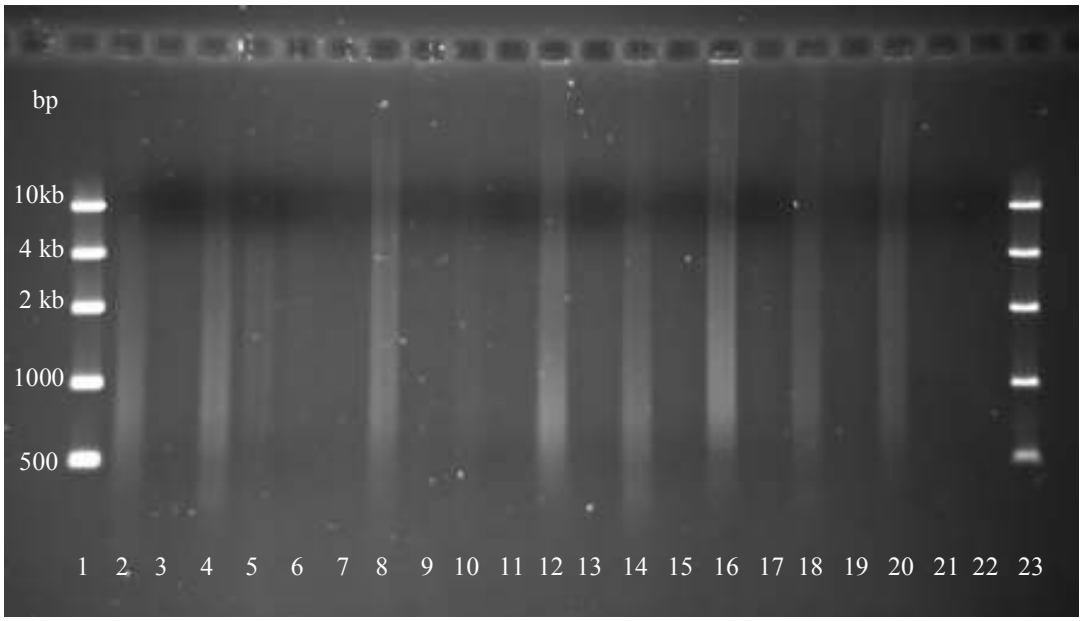

Figure 6: SDS PAGE of gDNA

Lane 1, 5 µl PageRuler low range; lane 2, Spot H; lane 3, Spot RM; lane 4, TN H; lane 5, TN RM; lane 6, BV1 H; lane 7, BV1 RM; lane 8, BV2 H; lane 9, BV2 RM; lane 10, BV3 H; lane 11, BV3 RM; lane 12, BV4 H; lane 13, BV4 RM; lane 14, CH H; lane 15, CH RM; lane 16, TE H; lane 17, TE RM; lane 18, PB H; lane 19, PB RM; lane 20, TB H; lane 21, TB RM; lane 22, NA RM; lane 23, 5 µl PageRuler low range

A single band of large size was expected in the genomic DNA gel. However, some lanes showed a background smear across the entire size range, while others showed nothing (figure 6). Spot H (lane 2), TN H (lane 4), BV2 H (lane 8), BV4 H (lane 12), CH H (lane 14), TE H (lane 18) and TB H (lane 20) all had the background smear (figure 6). This suggests that heart tissue was less susceptible to degradation and red muscle is more likely to become degraded over time. TN RM (lane 5) and BV3 H (lane 10) showed a very pale smear between 750 base pairs and 10kb.

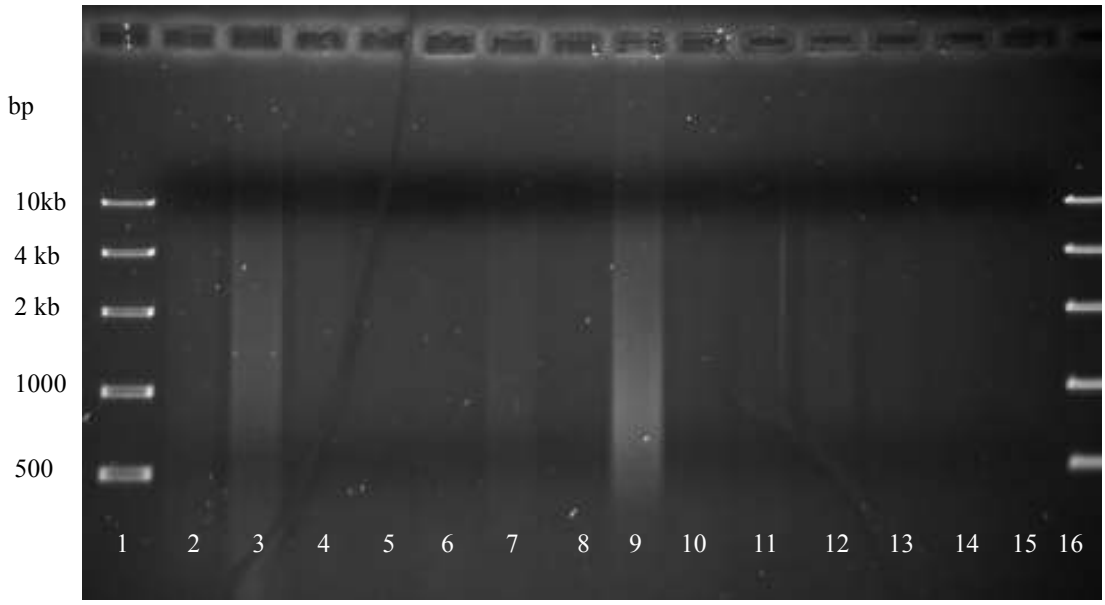

Figure 7: SDS PAGE gDNA

Lane 1, 4 µl PageRuler low range; lane 2, Spot RM; lane 3, TN RM; lane 4, BV1 H; lane 5, BV1 RM; lane 6, BV2 RM; lane 7, BV3 H; lane 8, BV3 RM; lane 9, BV4 H; lane 10, BV4 RM; lane 11, CH RM; lane 12, TE RM; lane 13, PB RM; lane 14, TB RM; lane 15, NA RM; lane 16, 4 µl PageRuler low range

The SDS PAGE for gDNA was re run for samples that were unsuccessful in the previous gel with 10µl of gDNA added to each well to make sure there was sufficient genetic material in the well. More bands were seen, but none of them were a single band of large size as expected (figure 7). Smears were not seen at all for BV1 RM (lane 5) or BV2 RM (lane 6), BV3 RM (lane8) or BV4 RM (lane 10) showing there was no genomic DNA in any of these lanes for red muscle in *Bovichtus variegatus* individuals. There was also no band seen for NA RM in lane 15.

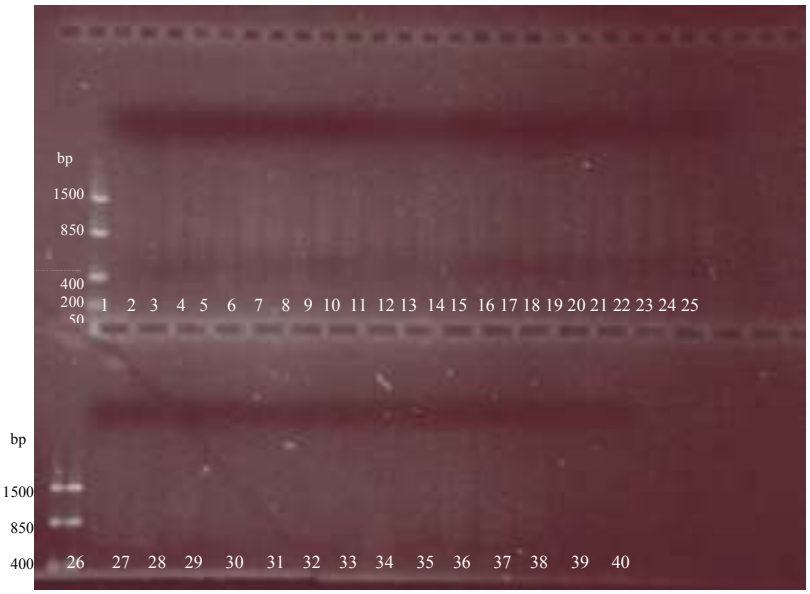

Figure 8: PCR on gDNA

Lane 1, 3µl PageRuler low range; lane 1, TN H (Myo); lane 2, Spot H (Myo); lane 3, CH H (Myo); lane 4, TN H (NMyo); lane 5, Spot H (NMyo); lane 6, CH H (NMyo); lanes 7-38 continued. Lanes 39 and 40 are negative controls.

This gel was run for the heart tissue of TN, CH and Spot using the two primer sets for a temperature range from 50°C to 60°C. A smear of DNA is seen across all lanes but no bands can be seen. They are either faded into the background smear and not bright, or the PCR did not work for reasons such as the gDNA is too degraded or a chemical was not at the correct soncentration. A primer dimer band can be seen around 50kb showing the primers did not anneal to the gDNA.

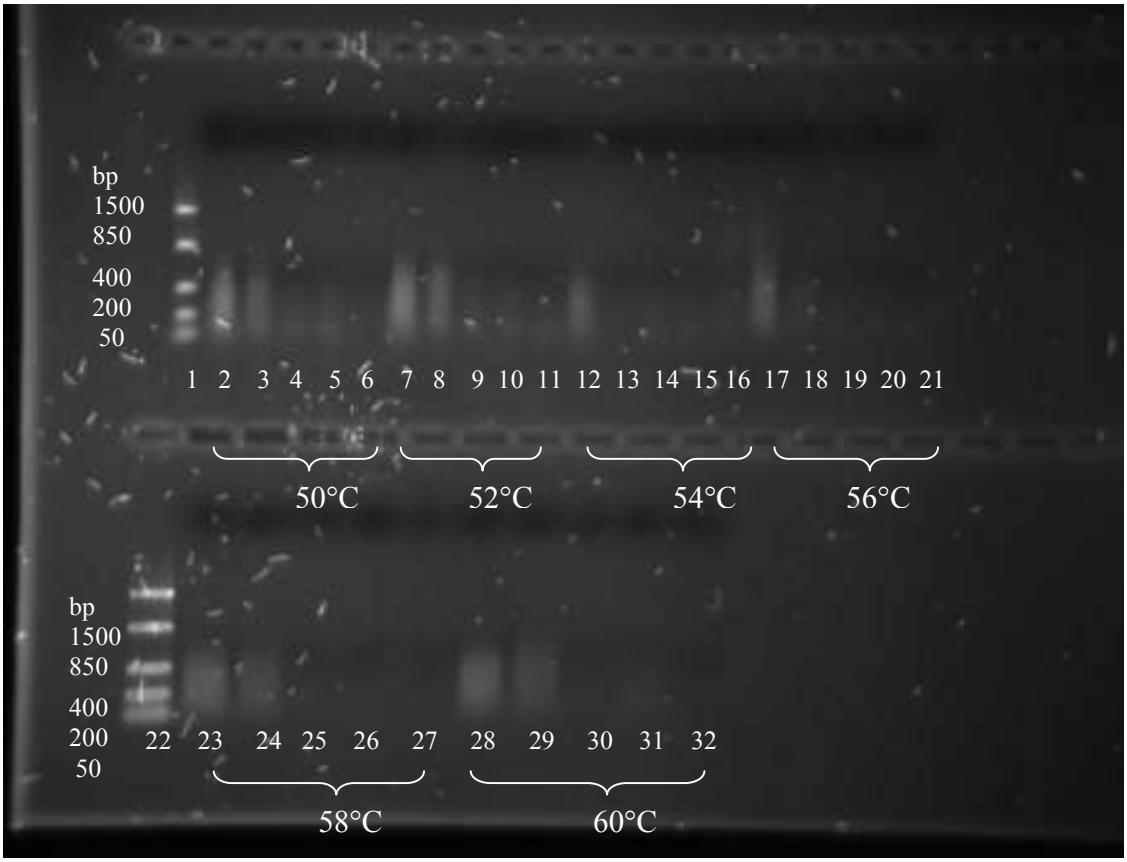

Figure 9: SDS PAGE cDNA (RT product)

Lane 1, 3 µl Fermentas FastRuler DNA ladder low range; lane 2, TN H; lane 3, CH H; lane 4, Spot H; lane 5, BV 1 H,; lane 6, TB H; lane 7, TN H; lane 8, CH H; lane 9, Spot H; lane 10, BV 1 H; lane 11, TB H; lane 12, TN H; lane 13, CH H; lane 14, Spot H; lane 15, BV 1 H; lane 16, TB H; lane 17, TN H; lane 18, CH H; lane 19, Spot H; lane 20, BV 1 H; lane 21, TB H; lane 22, 3 µl Fermentas FastRuler DNA ladder low range, lane 23, TN H; lane 24, CH H; lane 25, Spot H; lane 26, BV 1 H; lane 27, TB H; lane 28, TN H; lane 29, CH H; lane 30, Spot H; lane 31, BV 1 H; lane 32, TB H.

This gel was run over a temperature gradient from 50°C to 60°C for five different species to work out the best annealing temperature for the Myo 1/ 3 primers. TN and CH heart muscle tissue produced a dominant smear over the gel at most temperatures, but the bands within this smear of Mb cDNA were faded into the smear or not present. No bands were seen for Spot H, BV 1 H or TB H over the temperature range. A primer dimer band can be seen around 50bp showing the primers did not bind successfully to the cDNA. It is also possible that the reverse transcription process did not work as it was unable to be tested.

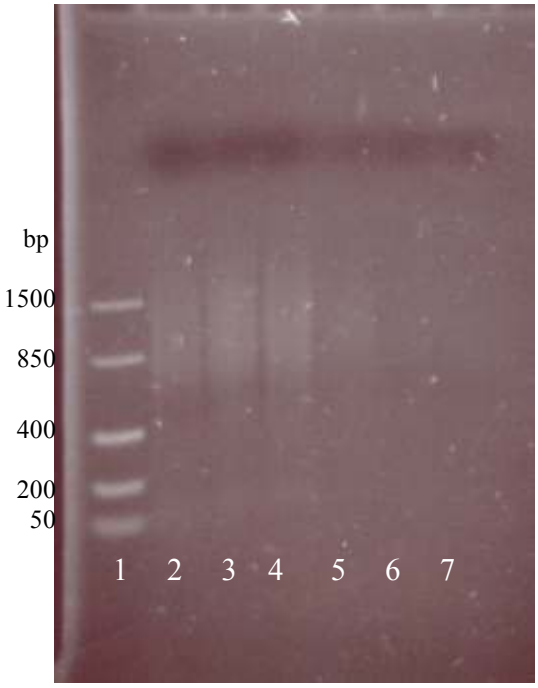

Figure 10: Control PCR with salmon DNA

Lane 1, 3 µl Fermentas FastRuler DNA ladder low range; lane 2, 45°C; lane 3, 48°C; lane 4, 51°C; lane 5, 55°C; lane 6, 58°C; lane 7, 60°C;

This control gel was run using 100ng/µl of Invitrogen Salmon Sperm DNA and Myo 1/3 primers. It should have shown clear bands. The temperature gradient should have shown which annealing temperature was best for this primer set. This gel was testing the primer quality and master mix, and also seeing if DNA and RNA quality were too poor for previous analysis. Again, the bands were not seen clearly but may be present within the smear. A faint primer dimer band can be seen around 50kb showing PCR did not work on the salmon sperm DNA.

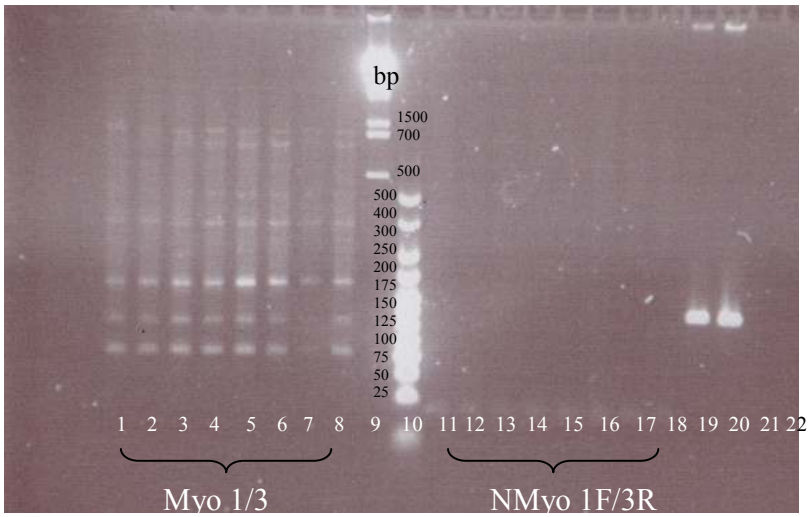

Figure 11:Salmon DNA control 2.

Lanes 1 to 8 contain salmon sperm DNA and Myo 1/3 primers with a temperature gradient from 45°C to 60°C; lane 9 λ Eco RI ladder; lane 10 Bioline Hyperladder V, lanes 12 to 18 contain salmon sperm DNA and NMyo 1F/3 R primers with a temp gradient from 45°C to 60°C; lanes 19 and 20 are a positive control, and lanes 21 and 22 are a negative control.

This gel was run by Jonci Wolff as a control. The Myo 1/3 primers to the left of the ruler worked well but were not very specific as they are degenerate primers and a short sequence, producing many bands from 1500bp to 100bp. The Mb gDNA product size using Myo1/3 primers is ~451 and there are faint bands in this area suggesting these primers did work. The NMyo primers did not work however, as no bands were seen except the primer dimer band at 50bp in every lane. The expected size for a band of Mb here was 431bp. Both positive controls worked showing PCR was successful, and both negatives were clear showing there was no contamination.

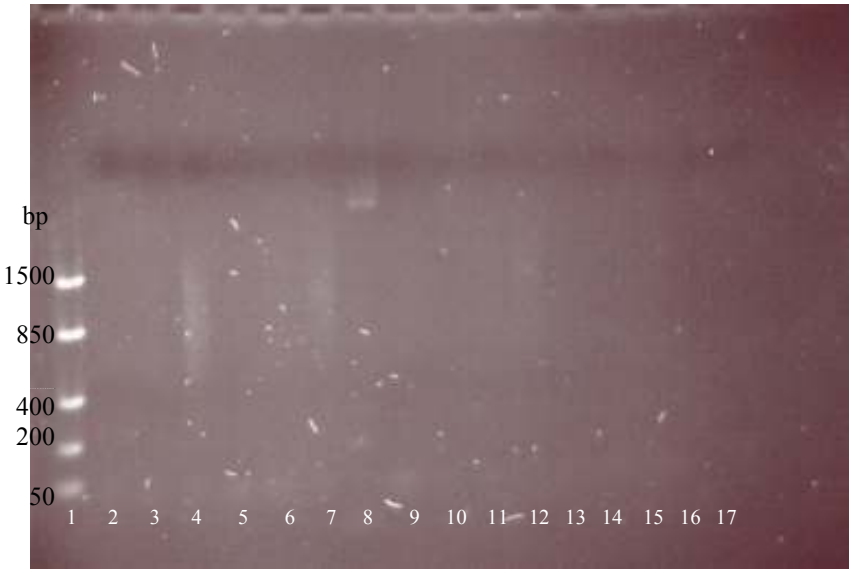

Figure 12: Repeat control expt incl salmon. Lane 1, 3 µl Fermentas FastRuler DNA ladder low range; lane 2, BV 1 H (Myo); lane 3, Spot H (Myo); lane 4, Salmon (myo); lane 5, BV 1 H (NMyo); lane 6, Spot H (NMyo); lane 7, Salmon (Nmyo); lane 8, positive control; lane 9, negative control. Lanes 2 to 9 using Jonci's ingredients for master mix. Lane 10, BV 1 H (Myo); lane 11, Spot H (Myo); lane 12, Salmon (myo); lane 13, BV 1 H (NMyo); lane 14 Spot H (NMyo); lane 15, Salmon (Nmyo); lane 16, positive control; lane 17, negative control. Lanes 10 to 17 using Victoria's ingredients for master mix.

This gel was expected to show similar bands to the gel above (figure 11), particularly in lanes 7 and 15, however the bands were extremely faint. Because the bands are present but faint, it appears the PCR has worked, just not very well. Faint bands can be seen in lane 3, lane 7 and possibly lane 6 around 1500bp, and in lane 11 around 2000bp. A primer dimer band can also be seen in most lanes at 50bp. The positive control in lane 8 did work and shows bands at 3 kb, 200bp and 50bp, and as there was nothing in the negative control lanes, there was no contamination. Lanes 10 to 17 are much fainter than lanes 2 to 8 suggesting one of the reagents may be a different concentration or not added correctly to the master mix, as this used separate master mix ingredients.

Discussion

Protein analysis

Myoglobin protein was expressed in the heart tissue of all species studied, and was not expressed in the pectoral (red) muscle of any species, including the NZ Spotty outgroup, *Notolabrus celidotus*. This was unexpected as the Spotty is not in the notothenioid suborder. It is therefore suggested that myoglobin expression in the pectoral muscle was lost before the radiation of the Notothenioids. The average absorbance readings from the Bradford Assay was 0.7214, with final concentrations ranging from close to 0mg/ml for BV2 heart tissue to 7.97mg/ml for TN red muscle. The BV2 heart tissue was only 15 milligrams and BV3 H was 12 milligrams in size and it is likely these extremely small sample sizes resulted in the protein concentration being very small. On average, heart tissue has a higher concentration of protein (5.16mg/ml) than the red muscle (4.61). The SDS PAGE for proteins was run using a crude protein mix. The gel percentage was low resulting in the smaller sized bands being very close to each other, making analysis difficult. It would be beneficial to run the gel again using a higher percentage to try to separate the bands out for easier comparison. Alternatively, it may be beneficial to cut the bands out of the gel and purify that section and re run the gels to separate the bands further. The bands also differ in size based on the composition so it is difficult to compare species with each other and know which band represents the myoglobin protein. Therefore, it would be beneficial to sequence the band to check that it is correct to have conclusive results.

RNA analysis

RNA extraction using the Trizol method was successful for most samples. The NanoDrop ratios should be around 2 for 'pure' RNA. The average reading was slightly lower than this at 1.937, which is still close suggesting the purity is generally good. However, PB red muscle has lower purity at 1.53, and BV3 heart and CH red muscle are 1.73 and 1.72 respectively, which is also a bit low. CH and PB red muscles had a large initial sample size so it was expected that the purity would be relatively good, but may have been contaminated with the phenol phase or the RNA pellet was not completely dissolved. All other samples look relatively pure with ratios very close to 2. The expected RNA yield from the Trizol extraction is 1-1.5 µg per 1 milligram of tissue. The average yield was about half of this at 0.735 µg/mg. A low RNA yield from the Trizol extraction can be due to incomplete homogenization of samples or the pellet not being

redissolved completely. RNA gels were run to show the integrity of the RNA. Good quality RNA should have two distinct bands at ~5kb (28S) and ~2kb (16S) which represent the two ribosomal RNA's (rRNA) and background smearing which represents the messenger RNA (mRNA). Spot, BV1 and BV2 heart tissue all show some degradation with only one rRNA band seen. All three of these samples had initial tissue samples of less than 20 milligrams, which is likely to be an error source. PB heart had an mRNA background smear but no rRNA bands. The heart and red muscle of BV3 were degraded with a very pale band at 1.35kb and less background smearing showing the quality is poor, and CH red muscle was heavily degraded and unlikely to be suitable for reverse transcription. All other bands showed some degradation but both rRNA bands were clear with background smearing of smaller size. The two ribosomal RNA bands occur around 2.4kb and 1.35kb, which were also smaller than expected. The mRNA was around 1.5 - 2kb, which is the average size for mRNA suggesting the quality is good. RNA degradation from the Trizol extraction method can be caused by RNase's, but as some RNA was seen in all lanes of the gel, this is unlikely. It is more likely that the samples were not kept at a low enough temperature, as they were kept on ice for considerable periods of time while analysis was being performed, rather than in a freezer.

Several polymerase chain reactions (PCR) and agarose gels were run on a smaller sample of species using the reverse transcription products (cDNA), and were generally unsuccessful. The primers did not bind to the mRNA at any of the annealing temperatures within the gradient from 50°C to 60°C. TN and CH heart muscle tissue produced a dominant smear over the gel at most temperatures, but the bands within this smear of Mb cDNA were faded or not present. No bands were seen for Spot H, BV 1 H or TB H over the temperature range. A primer dimer band can be seen around 50bp showing the primers did not bind successfully to the cDNA. It is also possible that the reverse transcription process did not work and the smear is the mRNA, as the products of reverse transcription are unable to be tested. The test was successful when run on commercial salmon sperm DNA, suggesting the RNA may have been too degraded for PCR even though the quality is high, based on NanoDrop 260:280 ratio and concentrations. When the gel was run using positive and negative controls, the positive showed clear bands while only a primer dimer band was seen in the negative, suggesting the PCR worked and that there was no contamination in the samples. Some very pale bands were seen on a couple of the samples, particularly the

salmon sperm DNA suggesting that the PCR is working, but not completely. It would be beneficial to re run the samples using different master mixes and possibly different primer sets to try to gain brighter bands with less background smearing. Although the temperature gradient was included in the program, it may also be helpful to try different program lengths at each temperature to allow more time for the primer and dNTP's to bind to the mRNA.

Genomic DNA analysis

All species that have been studied previously are known to retain the gene in the genomic DNA even when the protein is not expressed, which is what was expected of the species in this study. The NanoDrop ratios show that the samples are not close to pure DNA with an average ratio of 2.092, when pure DNA has a ratio around 1.8. When the samples were run on a gel, a background smear was seen, rather than a distinctive band. This suggests the gDNA has been degraded, and could be caused by old samples, and freeze and thaw cycles. Samples were snap frozen in liquid nitrogen after the initial sample was divided in two to prevent this and then kept in a -80°C freezer, so this was unlikely. Another source of degradation could be that once the supernatant containing RNA was removed, the remaining solution was put into the refrigerator and not a freezer. The yield was also much lower than expected with only is 0.443 µg/mg when 2-3 µg per 1 milligram of tissue was expected. A low yield can be caused by incomplete homogenization of the tissue or the final DNA pellet not completely redissolved in NaOH. There was a large amount of non DNA material in many of the samples and a third wash with sodium citrate was done, but may not have been sufficient. It is likely that the bands were very pale due to a combination of insufficient washing and the DNA not being completely redissolved. As the gDNA was degraded, the polymerase chain reaction with Myo 1/3 and NMyo 1F/3R was also unsuccessful, with no bands seen. Further analysis was not done on the gDNA, and cDNA was used instead.

Recommendations

Further analysis should be done on the mRNA and cDNA to confirm the results from the protein analysis. It would be useful to purify both protein and cDNA samples before PCR is run again. More samples should be used as there was a large amount of variation between the four bovichtids and it is likely this is true for all species, although this is difficult as most fish are from

Antarctic waters. It would also be good to sequence both protein using the Maldi-TOF analysis method to get the amino acid sequence, and also the cDNA to check the primers bound to the correct location. As the protein bands are similar in size but not exact, sequencing the band would provide absolute evidence whether myoglobin is present in the heart and pectoral muscle of each species. This could then be used infer evolutionary relationships between species. Species that do not have myoglobin should have compensatory mechanisms to transport oxygen and it is hypothesized that some species have increased numbers of mitochondria with fluid membranes, and this could be a useful direction for the work to progress.

Conclusion

Preliminary results based on the protein gels suggest that the loss of myoglobin expression is not cold adaptive as it is not present in the pectoral adductor profundus (red) muscle of any of the nothenioid species sampled including the basal ancestor *Bovichtus variegatus* and the NZ Spotty outgroup, *Notolabrus celidotus.* This suggests it is not specific to the notothenioid suborder, and was lost earlier within the Perciform order before the radiation of the Notothenioids. The expression of myoglobin is selectively neutral in the pectoral muscle, with selective advantages in the heart muscle of these species.

Acknowledgements

I would like to thank my supervisor Dr Victoria Metcalf for her assistance with this project in designing the method, providing tissue samples and equipment, and also for her time. I would also like to thank Jonci Wolff for his help with the PCR method and analysis of the gels.

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