

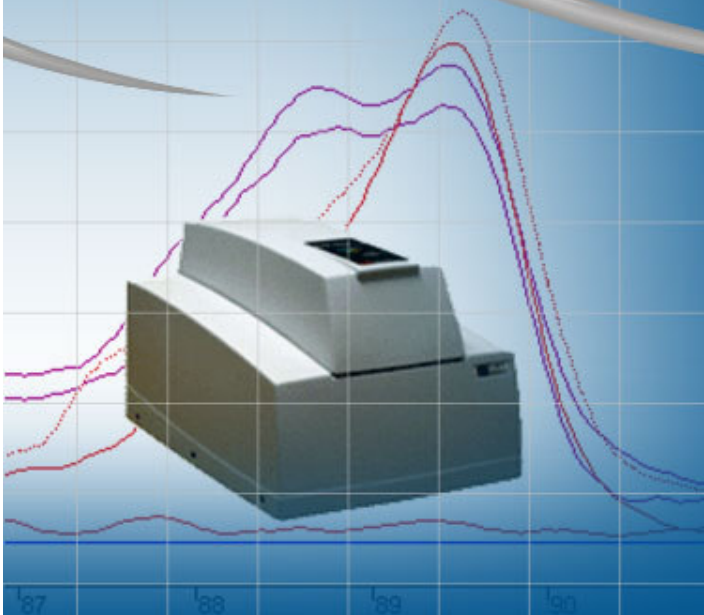


The Fitness of an Amateur Football Team: High Resolution Melt Genotyping

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Rugby

The demands of the game include:

- Acceleration
- Using upper body strength
- Changing direction
- Short sprints
- Fast striding
- Slow jogging
- Rapid recovery

And the ability to interchange between any of these activities on demand

As a result, all of the energy-producing systems within the body are placed under a certain degree of stress, and thus a rugby player needs to be an all-round athlete.



Short Term Energy

Strength and acceleration

- The phosphocreatine system provides most of the energy during maximum exercise for less than 20 seconds, and is central to the performance of the forwards during a whole rugby game (See Figure) and to the backs for short periods of the game (See Figure).
- The energy-supplying substrate, creatine phosphate, is found within the muscle fibres.
- Strength and acceleration are predominantly attributed to the fast twitch muscle fibres.



The primary role of the Forward is to accelerate at the opposing players and use their strength to break through the defensive line in order to gain distance toward the try line.



The role of the Back, primarily the winger, is to sprint past the opposition in a race toward the try line.

ACTN3 (R577X) Genotype – Strength and Acceleration

- Muscles are made up of two types, slow twitch and fast twitch.
- Slow twitch muscles contain more mitochondria and myoglobin which make them more efficient at using oxygen to generate energy without lactate build up, thereby, they can go for a long time before they fatigue, which is important for endurance sports.
- Both fibre types generally produce the same amount of force per contraction, but the fast twitch fibres produce that force more rapidly, making them an asset to power based players when there is a limited amount of time to generate maximal force.
- The distribution of fibre types will influence sports performance depending on whether the sport requires endurance or power.
- The ACTN3 gene produces a product called alpha-actinin-3, which is involved in muscle contraction and is predominantly found in fast twitch muscle fibres.
- A mutation in the ACTN3 gene prevents the production of alpha-actinin-3 in the fast twitch fibres. In elite sprint athletes the absence of this mutation gives them the advantage of producing more alpha-actinin-3 and thereby increase muscle power (Yang et al. 2003).

Intermediate Energy

Lactate levels

- The intermediate energy system which provides the majority of energy for a sustained performance lasting between 20 seconds and two minutes is known as anaerobic glycolysis. This is critical to a rugby player during the whole game (See Figure).
- Muscle glycogen is the initial substrate, lactate is the end product, and no oxygen is directly involved.
- While unable to produce as much energy per unit time as the phosphocreatine system (ie, unable to sustain maximum sprinting speed), it lasts considerably longer before intensity must be further reduced.
- However, build up of lactate creates fatigue and can hamper a players performance.



Much of the game consists of continuous wrestling of opposing players to the ground in order to halt their progress to the defending teams try line. This activity places great demand on anaerobic glycolysis.

MCT-1 (A1470T) polymorphism – Lactate levels and Fatigue

- Lactate is a product generated from the breakdown of glucose during the process of anaerobic metabolism. Lactate formed in the muscles during exercise is transported out to the liver through the blood stream where it is converted to a product called pyruvate in the presence of oxygen.
- During high levels of exercise, when oxygen levels are inadequate, lactate levels rise in the muscles and blood stream, reducing blood pH.
- Deficiencies in the transport of lactate in skeletal muscle can result in muscle cramping or fatigue (or both) upon exercise, compromising performance.
- Monocarboxylate transporter-1 (MCT-1) is involved in lactate transport and oxidative metabolism. Levels of MCT-1 increase with exercise in order to remove lactate from the muscles. A symptomatic deficiency in lactate transport can result in muscle injury during exercise and heat exposure.
- A mutation in the *mct-1* gene results in a reduced production of efficient MCT-1 enzyme, which observed in various cases results in a 40-50% rate of lactate transport compared with normal *mct-1* gene individuals (Merezhinskaya et al. 1999)

Long Term Energy

Recovery and endurance

- The aerobic system is not a major source of energy during most of the competitive phases of a rugby match.
- Nevertheless, a match is 80 minutes long, and thus nearly all the energy provided by the other two systems must be paid for aerobically before the match is over.
- Delivery of oxygen to the fatigued muscles replenishes stores of creatine phosphate and lowers levels of lactic acid.
- Aerobic fitness can be important to the backs who are required to cover greater distances during a game (See Figure).



Although the Backs are involved in power components of the game, half their time may be spent trailing their Forwards in anticipation of either attacking or defending. During this time the Backs will cover greater distances than the Forwards and therefore require a higher level of aerobic fitness.

HIF-1 (P582S) polymorphism – Recovery and Endurance

- A good supply of oxygenated blood to the muscles during exercise allows the muscles to expend more energy over a longer period of time, increasing performance.
- VO2 max is the maximum amount of oxygen you can use in one minute per kilogram of body weight. Fitter athletes have higher VO2 max values.
- VO2 max is affected by the ability of the cardiovascular system to transport oxygenated blood to muscle tissue.
- Angiogenesis is the process of growing new blood vessels, while erythropoiesis is the formation of new red blood cells. HIF-1 is a transcription factor that is regulated by hypoxia (low oxygen), initiating the processes of angiogenesis, erythropoiesis and metabolism.
- A variation in the *hif-1* gene causes an increase in gene expression associated with an increase in angiogenesis, erythropoiesis and metabolism. This can positively affect VO2 max before and after training improving performance (**Reference**).

ADRB-2 (Gln27Glu) polymorphism – Body Composition

- The human body is composed of a variety of different tissue types. The so-called 'lean' tissues, such as muscle, bone, and organs are metabolically active, while adipose, or fat tissue, is not.
- The average adult body fat is 15% - 18% for men and 22% - 25% for women.
- Catecholamines like adrenaline, binding to the beta-2 adrenergic receptor (ADRB2), activate lipolysis, the break down of fats, which is influenced by exercise and so regulates body weight.
- A variation in the *adrb-2* gene can result in reduced lipolysis and thereby increased body fat and body mass index (BMI) (20% of the population have the 27Glu variation).
- In previous studies, individuals with the mutation in both genes had 20 kg of excess body fat and 50% increase in fat cell size compared with individuals without the mutation or heterozygotes (Large et al. 1997).
- In individuals that exercised regularly, the variation had little influence on body fat composition.
- Aerobic exercise is a very effective way to decrease body fat, however, it must be combined with strength training to maintain or gain muscle mass.

Aims

- The aim of this study was to evaluate the fitness characteristics of several amateur rugby players and compare their physiology with their genetic profile of four sports associated genes.
 1. Power and Fatigue = ACTN3 (R577X)
 2. Lactate levels = MCT1 (A1470T)
 3. VO2max = HIF1 (P582S)
 4. Body Fat = ADRB2 (Gln27Glu)
- Evaluate the use of High Resolution Melt analysis to detect genotypes compared with FRET hybridization probes.

Methods

Methods

- A total of 12 amateur Rugby League players participated (6 Forwards and 6 Backs), ranging in age (20 – 42 years).

Physiology Tests

Running-based Anaerobic Sprint Tests

- Participant completed six 35 m runs at maximum pace with a 10 sec turnaround.
- The time taken to complete each sprint was recorded along with weight.
- Power was calculated as: $\text{weight} \times \text{distance}^2 / \text{time}^3$
- Fatigue Index was calculated as : $(\text{maximum power} - \text{minimum power}) / \text{Total time for sprints}$
- Lactate levels were recorded before and after the test

VO2max (one mile jog)

- Participants jogged one mile at a steady pace.
- Time taken to complete the mile and heart rate were recorded at completion.
- VO2max was calculated as: $108.844 (\text{male}) - (0.1636 \times \text{weight}) - (1.438 \times \text{time}) - (0.1928 \times \text{heart rate})$

Percentage Body Fat (Yuhasz Test)

- Skin fold measurements were taken from the following sites:
 - abdomen, side chest, scapula, ileum, triceps, thigh

High Resolution Melts

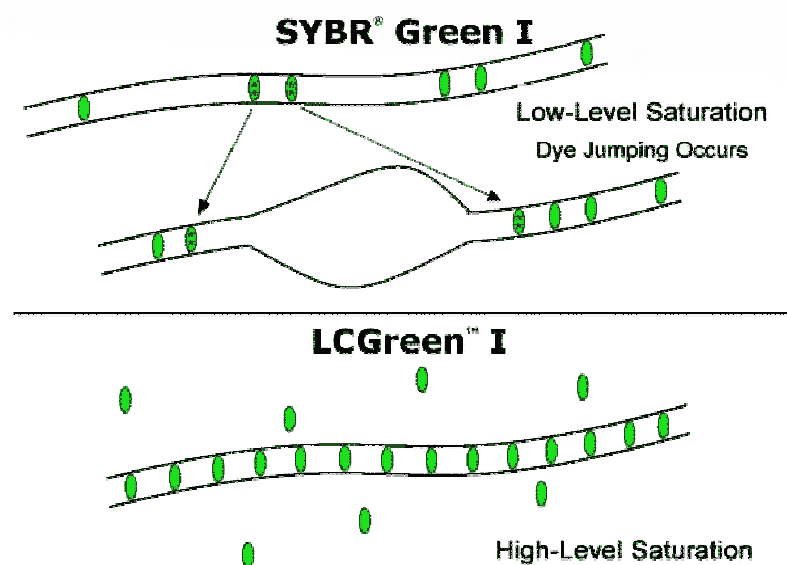


Figure from McKinney et al. 2004

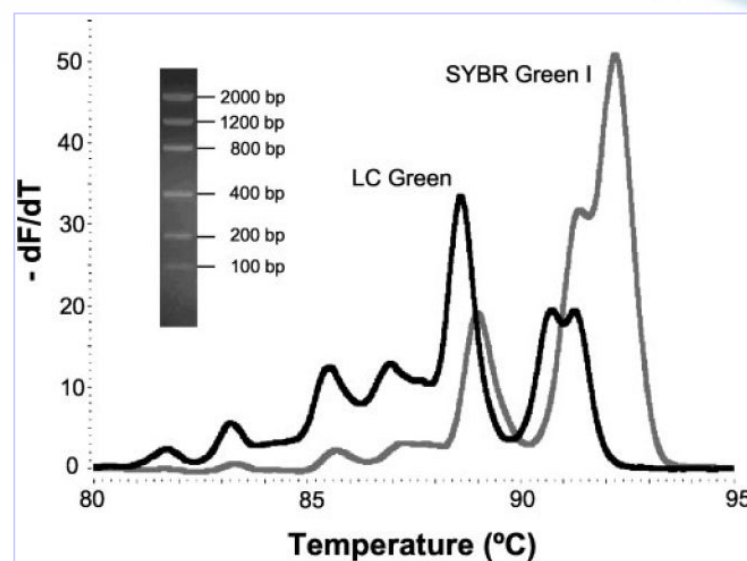


Figure from Wittwer et al. 2003

- LC Green is not toxic to amplification therefore it can be used at higher concentrations than SYBR Green (increased signal).
- LC Green saturates the DNA preventing dye redistribution during the melt, which is not the case for SYBR Green (increased resolution between genotypes).

High Resolution Melts - Strategy

In order to achieve HRM on a Real-Time platform you need:

- Temperature uniformity ($< 0.05^{\circ}\text{C}$)
- High resolution temperature control ($< 0.2^{\circ}\text{C}$ ramp)
- Sensitivity

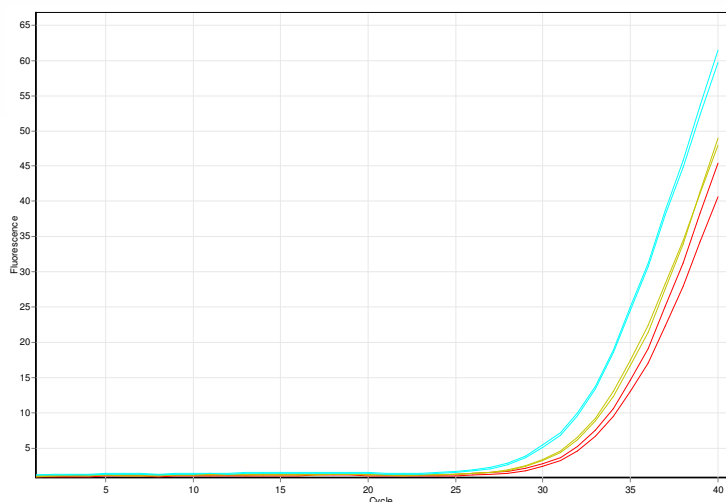
Considerations when running HRM:

- Product size (60 – 90 bp recommended)
- MgCl_2 conc. (1.5 – 3 mM recommended)
- Temperature dissociation difference between genotypes ($> 0.2^{\circ}\text{C}$ recommended)
- Melt domain (approx. 10°C recommended)
- Primer concentration (300 – 500 nM recommended)

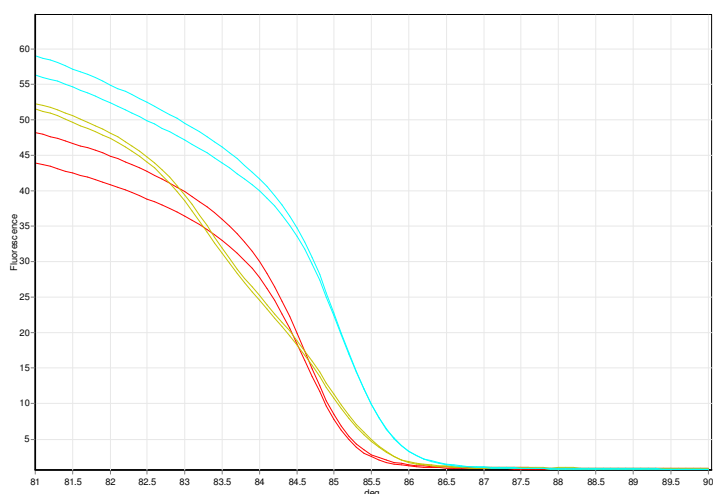
We ran a modified Rotor-Gene to achieve HRM.

Confirmed by running FRET hybridisation probe assays for each gene.

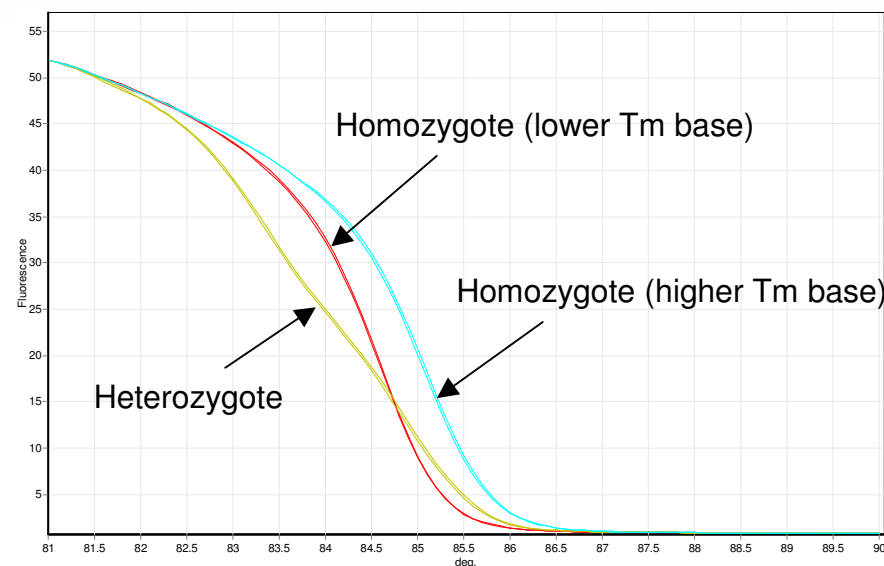
High Resolution Melts - Example



High Speed Run amplification for short products (<40 min)



Raw data for High Resolution Melts (80 – 90oC; < 15 min)

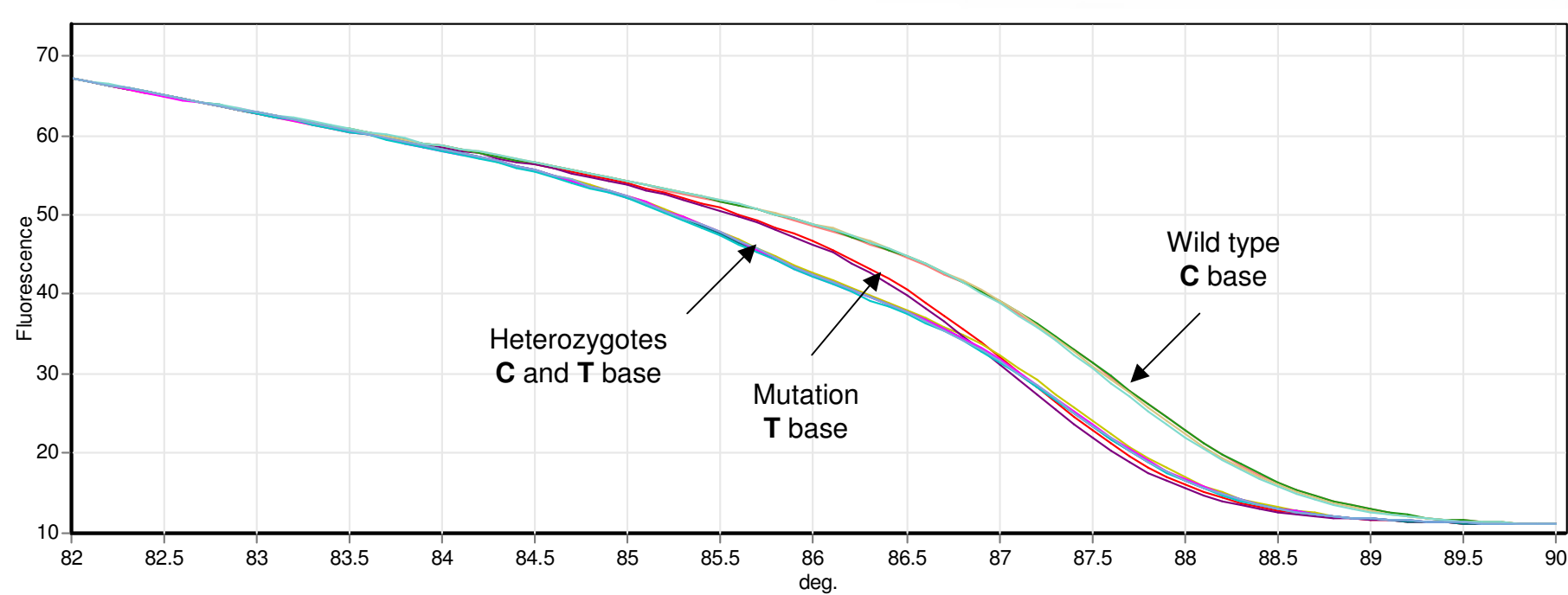


Normalised High Resolution Melt data

- Heterozygotes form heteroduplexes, which result in lower melt curves and two transition points.
- Homozygotes are differentiated by a change in melt curve represented by a base change: G > C > T > A.

Results

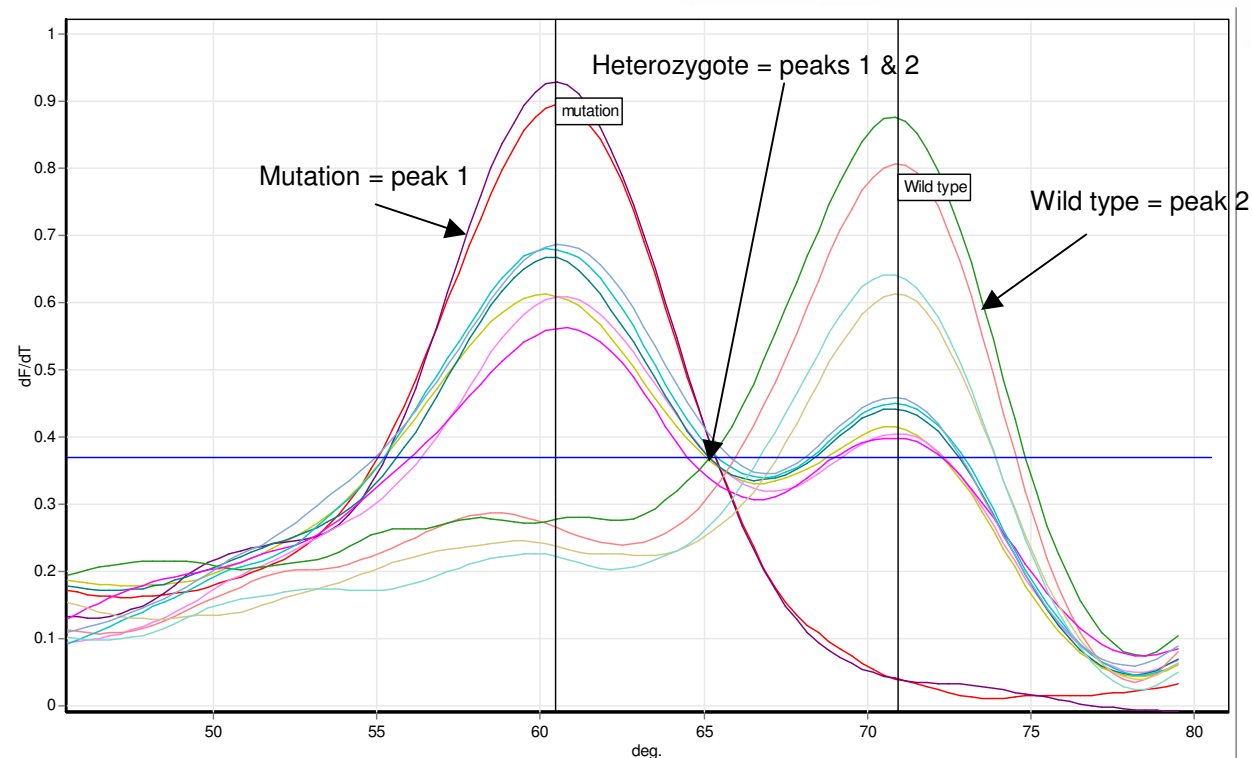
ACTN3 HRM (C → T)



C and T Tm = 87.33 & 85.72°C
C base Tm = 87.14°C
T base Tm = 87.63°C

FRET Result ACTN3 (C → T)

FRET results confirmed HRM results



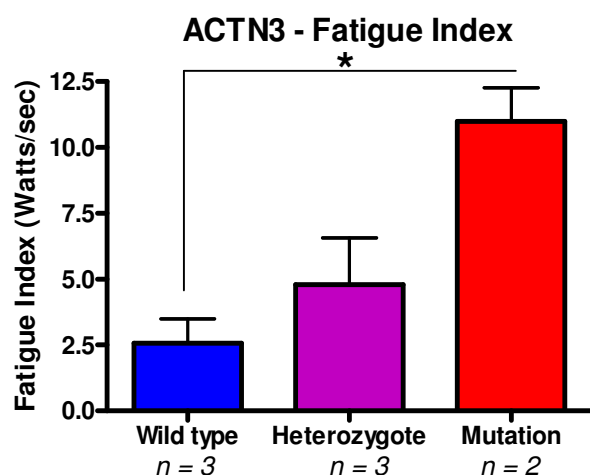
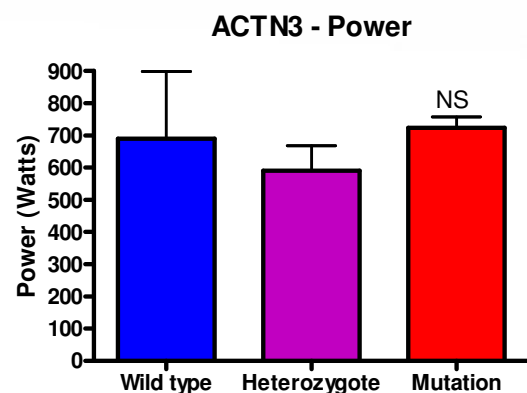
Probes were designed toward the wild type for ACTN3

Wild type = 71°C

Mutation = 60°C

Power Performance

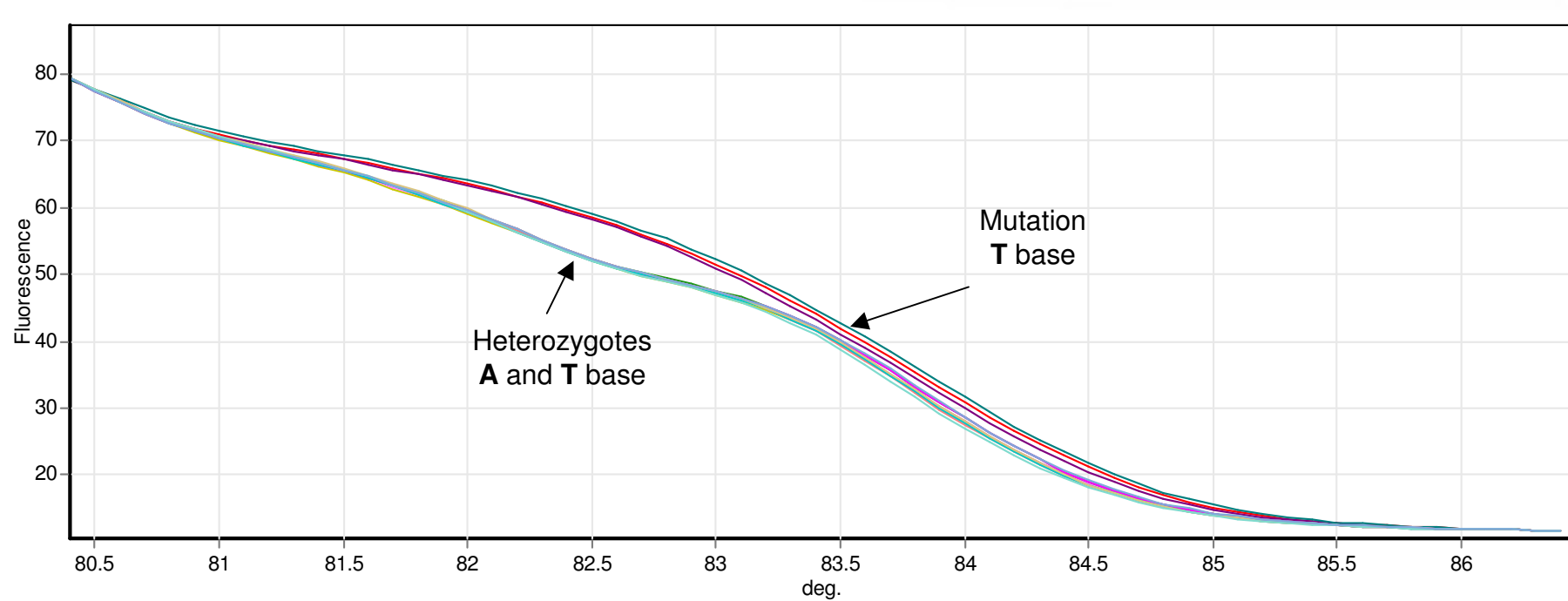
ACTN3 (R577X) polymorphism



* $P < 0.05$, NS = not significant

- There was no relationship between Power and ACTN3 genotype.
- There was a significant difference in Fatigue index between genotypes.
- 577X mutants fatigued more than 577R wild types.
- Although power did not relate to genotype the extent to which power lasted did.

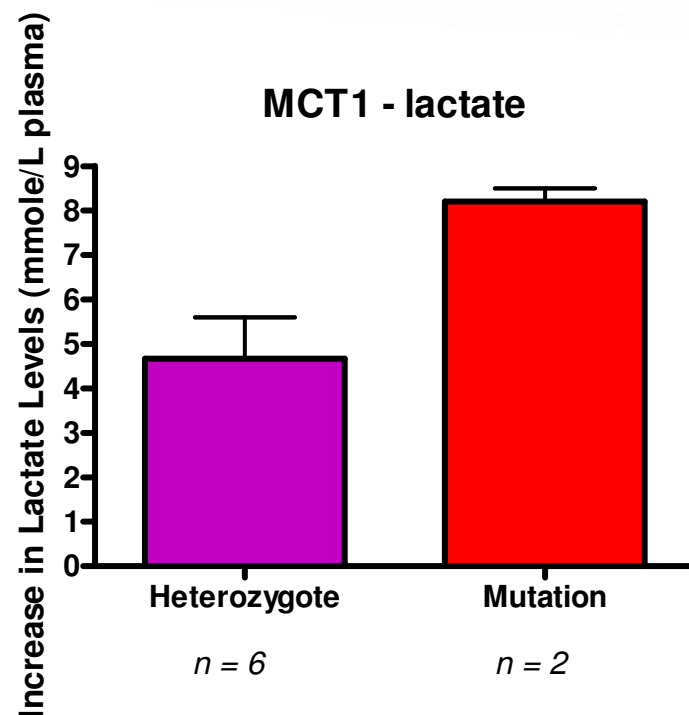
MCT1 HRM (A → T)



A and T T_m = 82.19 & 83.79°C
T base T_m = 83.83°C
No A base samples

Anaerobic Glycolytic Performance

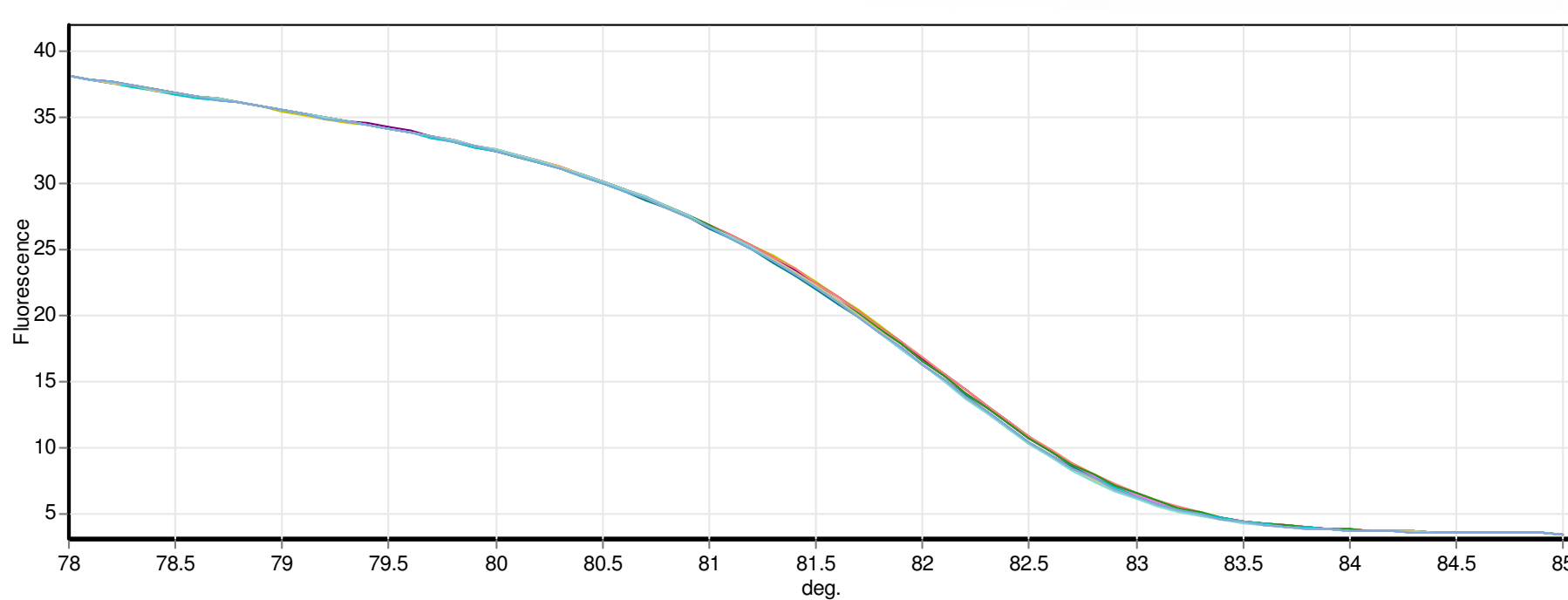
MCT1 (A1470T) polymorphism



- There was an observable difference between genotype and lactate levels for this group of rugby players.
- Players with the mutation accumulated higher amounts of lactate compared with heterozygotes.

Profession Football players average blood lactate levels = 10 mmol/L
Source: Smith et al. (West Sussex Institute of Higher Education)

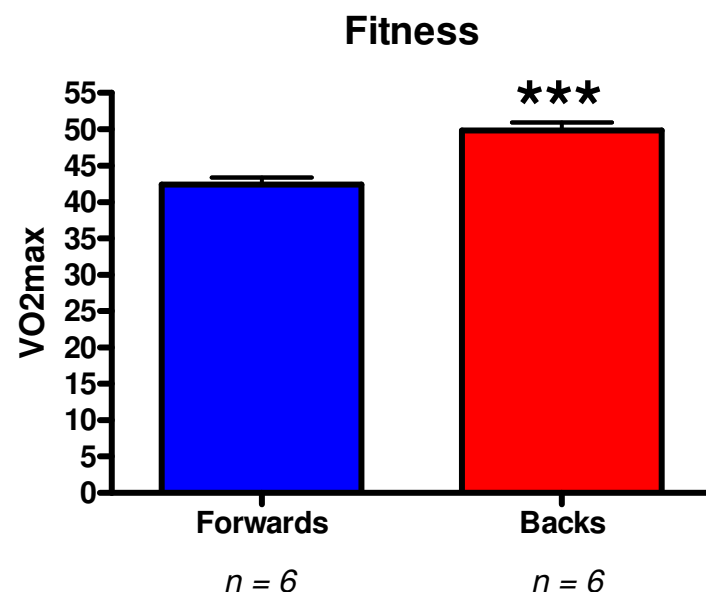
HIF1 HRM (X to Y)



X base Tm = 82.07°C
No Y base samples

Aerobic Performance

HIF1 (P582S) polymorphism



*** P < 0.001

VO2max Ratings

Age: 20-29

36.5-42.4ml/kg/min = Fair

42.5-46.4ml/kg/min = Good

46.5-52.4ml/kg/min = Excellent

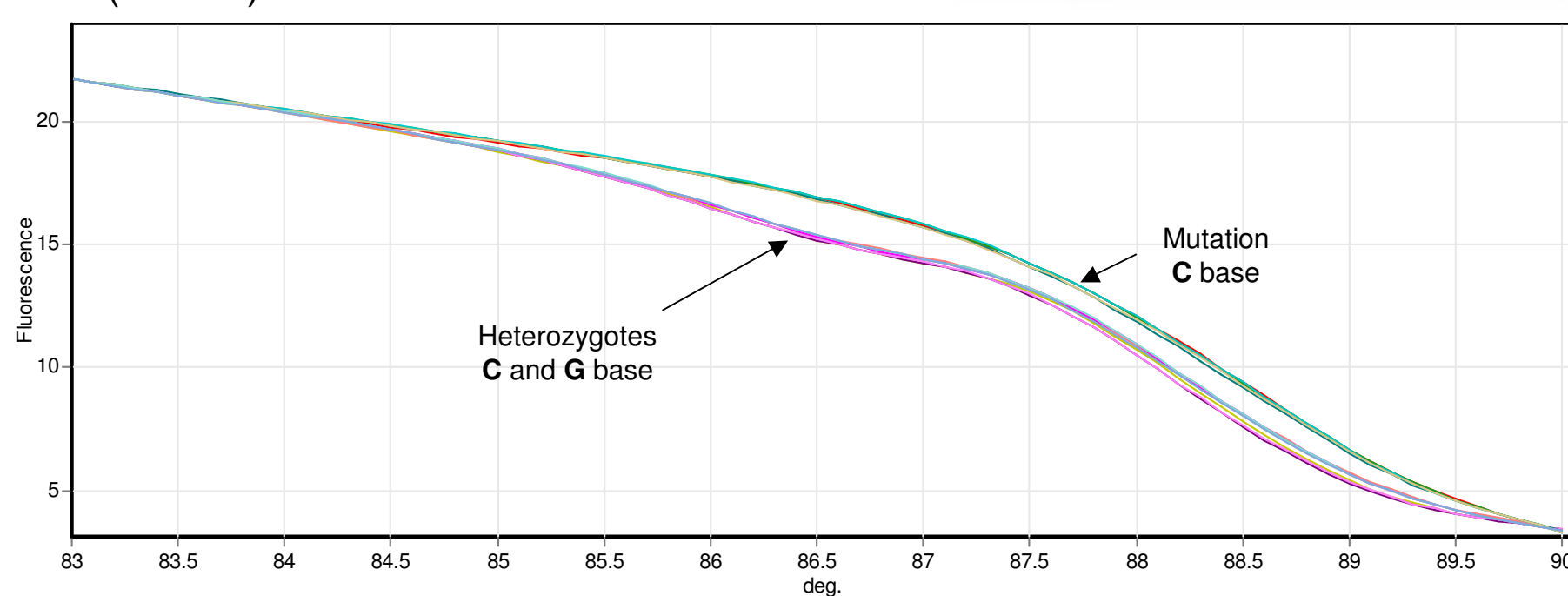
> 52.4 = Superior

All players were wild type

- Backs were more aerobically fit compared to the forwards.
- The HIF (P582S) polymorphism played no part in aerobic fitness for this group of rugby players.
- Fitness differences could be attributed to training and the nature of the position played.

Professional Footballers average = 55 ml/kg/min
Endurance runner average = > 75 ml/kg/min

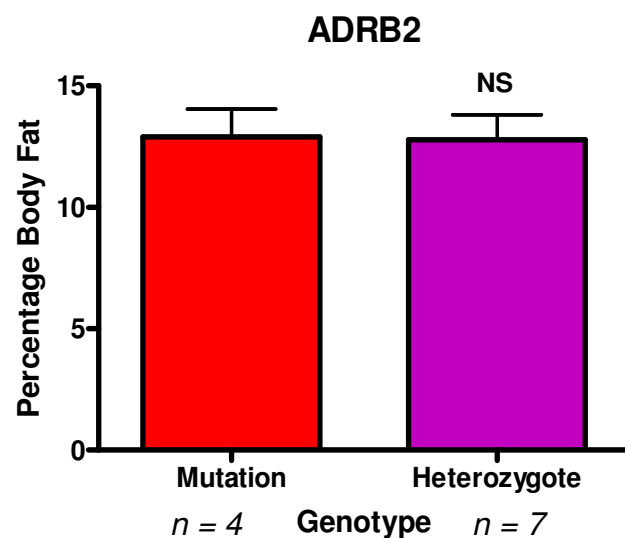
ADRB2 HRM (G → C)



G and C T_m = 88.22 & 86.07°C
C base T_m = 88.57°C
No G base samples

Body Composition

ADRB2 (Gln27Glu) polymorphism



NS = not significant

- There was no significant difference between genotypes for body fat percentage.
- The ADRB2 (Gln27Glu) polymorphism played no role in determining body composition for this group of rugby players.
- Body composition may be attributed directly to diet and the exercise regime of individual players.

Discussion

ACTN3 – Power and Fatigue

- Power did not relate to the ACTN3 genotype for this group of amateur rugby players.
- This could be due to the body composition of the wild type players, most of which weighed over 100kg.
- Perhaps a test for strength such as weight lifting would have been a more appropriate test for power.
- The relationship with fatigue indicated that perhaps fast twitch muscle contraction levels were reducing more rapidly in players with the mutation, thereby they were unable to sustain a sprint for a longer period. We know that the ACTN3 gene produces alpha actinin 3, a key component to fast twitch muscle contraction.
- Both players that contained the 577R genotype played in the backs, a position more suited to endurance than power.

MCT1 – Anaerobic glycolysis

- There was relationship between the MCT1 genotype and lactate levels.
- Players with the variation in both genes accumulated more lactate following anaerobic exercise.
- This could be due to poorer lactate transport associated with this genotype.
- Players might compensate for this by improving their aerobic fitness levels, which can reduce lactate. Both players had fitness levels that were considered to be in the mid level range (good).

HIF1 – Aerobic fitness

- Although no player had the variation in the HIF1 gene, some were measured to be very aerobically fit.
- This could be attributed to their training program that includes significant amounts of aerobic exercise.
- As predicted the backs were more aerobically fit, which is important for that position.

ADRB2 – Body Fat

- Body composition was equivalent between genotypes for this group of players.
- This could be due to the amount of exercise undertaken by the players during training (two nights a week).
- Note that the mutation is considered not to influence body type in subjects that participate in adequate amounts of exercise.

Conclusions

- Although the cohort of players was small, genotype analysis using HRM demonstrated some associations with sporting phenotypes such as fatigue.
- This information will be used to develop training regimes that will help improve the players ability to reduce fatigue. For example improve their level of fitness through more aerobic exercise.
- HRM also demonstrated that it could be used as a cost effective and user friendly method of genotyping.
- The football team in this study did go on to the semi final of their competition, narrowly missing out on the win by 4 points.

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