Title

The influence of angiotensin converting enzyme and bradykinin receptor B2 gene variants on voluntary fluid intake and fluid balance in healthy men during moderate intensity exercise in the heat.

Authors

Yau, Adora M.W; Moss, Andrew D; James, Lewis John; Gilmore, William; Ashworth, Jason J; Evans, Gethin H.

Affiliations and email addresses

Adora M.W. Yau: School of Healthcare Science, Manchester Metropolitan University, M1 5GD, UK. A.Yau@mmu.ac.uk

Andrew D. Moss: Department of Exercise and Sport Science, Manchester Metropolitan University, CW1 5DU, UK. A.D.Moss@mmu.ac.uk

Lewis J. James: School of Sport, Exercise and Health Sciences, Loughborough University, LE11 3TU. L.James@lboro.ac.uk

William Gilmore: School of Healthcare Science, Manchester Metropolitan University, M1 5GD, UK. B.Gilmore@mmu.ac.uk

Jason J. Ashworth: School of Healthcare Science, Manchester Metropolitan University, M1 5GD, UK. J.Ashworth@mmu.ac.uk

Gethin H. Evans: School of Healthcare Science, Manchester Metropolitan University, M1 5GD, UK. gethin.evans@mmu.ac.uk

Corresponding author information

Dr. Gethin Evans: School of Healthcare Science, Manchester Metropolitan University, Manchester, M1 5GD, United Kingdom, Telephone: +44 161 247 1208, gethin.evans@mmu.ac.uk**Abstract**

Angiotensin converting enzyme (ACE) and *bradykinin receptor B2 (B_2R)* genetic variation may affect thirst due to effects on angiotensin II production and bradykinin activity respectively. To examine this, 45 healthy Caucasian men completed 60 minutes of cycle exercise at 62 ± 5% $_{2peak}$ in a room heated to 30.5 ± 0.3°C with *ad libitum* fluid intake. Blood samples were collected pre-, mid-, and immediately post-cycle. Fluid intake, body mass loss (BML), sweat loss (determined via changes in body mass and fluid intake) and thirst sensation were recorded. All participants were genotyped for the *ACE* insert fragment (*I*) and the B_2R insert sequence (*P*). Participants were homozygous for the wild type allele (*WW* or *MM*), heterozygous (*WI* or *MP*) or homozygous for the insert (*II* or *PP*). No differences between genotype groups were found in mean (± SD) voluntary fluid intake (*WW*: 613 ± 388, *WI*: 753 ± 385, *II*: 862 ± 421 mL, $P = 0.31$; *MM*: 599 ± 322, *MP*: 745 ± 374, *PP*: 870 ± 459 mL, $P = 0.20$), percentage BML or any other fluid balance variables for both the *ACE* and B_2R genes, respectively. Mean thirst perception in the B_2R *PP* group, however, was higher ($P < 0.05$) than both *MM* and *MP* at 30, 45 and 60 minutes. In conclusion, the results of this study suggest that voluntary fluid intake and fluid balance in healthy men performing 60 minutes of moderate intensity exercise in the heat are not predominantly influenced by *ACE* or B_2R genetic variation.

Key Words

Thirst; Angiotensin converting enzyme; Bradykinin receptor B2; Dipsogenic; Exercise; Fluid Balance

Introduction

It has long been observed that the majority of humans performing physical work or exercising do not voluntarily drink enough water to replace sweat losses despite adequate fluid supply (Pitts *et al*. 1944). This phenomenon has been termed 'voluntary dehydration' (Rothstein *et al*. 1947) and has been found to frequently occur in athletes (Maughan *et al*. 2004; Passe *et al*. 2007) and the general population even in favourable conditions for fluid intake. Based on these observations, it is apparent that relying on thirst mechanisms does not always guarantee sufficient total water intake (Armstrong and Grandjean 2007).

Intracranial and systemic administration of physiological doses of angiotensin II, a component of the renin-angiotensin aldosterone system (RAAS), has been shown to promote drinking in animals (Fitzsimons 1972) and humans (Fitzsimons 1998). Angiotensin II is produced from the cleavage of angiotensin I by angiotensin converting enzyme (ACE). A 287 base pair (bp) Alu repeat insert within intron 16 of the *ACE* gene has been identified as a common allelic variant (Rigat *et al*. 1990) unique to humans (Montgomery *et al*. 2002). The wild type allele (*W*) is significantly associated with both higher tissue (Danser *et al*. 1995), and serum (Rigat *et al*. 1990) ACE activity.

Another known function of ACE is the degradation of vasodilator kinins within the kallikrein kinin system, particularly bradykinin (Dzau *et al*. 1988). Bradykinin exerts its effects on bradykinin B2 receptors (B_2R) and has been shown to be a powerful dipsogen. Endogenous bradykinin (Cadnapaphornchai *et al*. 2004) and exogenous infusion of bradykinin (Fregly and Rowland 1991), during acute ACE inhibition has been observed to cause polydipsia and polyuria. Furthermore, the antagonism of bradykinin receptors reverses such effects (Cadnapaphornchai *et al*. 2004). An allelic variant containing a nine bp repeat

insertion (*P*) has been identified in exon 1 of the gene encoding for B_2R (Braun *et al*. 1996). The wild type allele (*M*) is significantly associated with higher gene transcriptional and receptor activity (Braun *et al*. 1996; Lung *et al*. 1997). It is possible, therefore, that variants in *ACE* and *B_2R* genes may affect thirst and fluid intake in humans. Understanding the potential role of these, and other, dipsogenic gene variants on thirst and fluid intake may explain some of the large differences in these variables that are often observed in athletes and recreational exercisers.

To date, only one study has previously investigated the influence of these allelic variants on thirst and voluntary dehydration. Saunders *et al*. (2006) investigated the association of these allelic variants with weight changes in 428 Caucasian male ironman triathletes during competition. The authors concluded that the *B_2R PP* genotype was associated with greater weight loss during the ironman triathlon with a significant linear trend for the distribution of this genotype across weight loss groups. On the other hand, no association was found with *ACE* genotype.

The aim of the present study was to conduct a controlled laboratory study investigating the influence of *ACE* and *B_2R* allelic variations on thirst, voluntary fluid intake and fluid balance during moderate intensity exercise in the heat. It was hypothesized that individuals homozygous for the wild type *ACE* or *B_2R* allele would drink more fluid during exercise in the heat.

Materials and methods

Participants

Forty-five Caucasian men aged 18-45 years (mean ± SD, age 28 ± 8 y, height 178.3 ± 6.7 cm, body mass 78.93 ± 14.29 kg, body mass index 24.74 ± 3.59 $kg.m^{-2}$, and peak oxygen uptake 50.95 ± 10.10 $ml.kg^{-1}.min^{-1}$) volunteered to participate in the present investigation. All participants were healthy non-smokers, not concurrently taking medication, and not supplementing with creatine. Verbal and written explanations of the experimental procedures were given before participants provided written consent and completed a medical screening questionnaire. The study had prior approval from the Manchester Metropolitan University's Faculty of Science and Engineering ethical advisory committee, in accordance with the Declaration of Helsinki.

Preliminary trials

Each participant completed two preliminary trials, separated by a minimum of 48 hours, prior to their main experimental trial. In the first of these, peak oxygen uptake ($_{2peak}$) was assessed through the performance of a continuous, incremental cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) test to volitional fatigue in a thermoneutral environment (21.4 ± 1.0°C; 33 ± 7% relative humidity). Pulmonary gas-exchange (Cosmed $K4b^2$, Italy) and heart rate (HR) (Polar FS2c, Kempele, Finland) were continuously analysed and recorded using a breath-by-breath system. Rating of perceived exertion (RPE) (Borg, 1998) was obtained every 2 minutes. Utilising the rolling mean of 10 breaths and work rate, the work-rate to oxygen uptake ($_2$) relationship was determined. The highest mean value of the rolling 10 breath oxygen consumption values was accepted as $_{2peak}$. The desired work rate eliciting 55% of $_{2peak}$ was then calculated, with account taken for the mean response time for $_2$ during ramp exercise (i.e. two thirds of the ramp rate was deducted from the calculated work rate) (Whipp *et al*. 1981).

The second preliminary trial involved familiarising the participants with the experimental trial protocol. All procedures undertaken in the main trial, described in detail below, were performed in a room heated to 30°C, with the exception of body mass (BM) weighing and urine and blood sampling.

Experimental protocol

In the 24 h period preceding the experimental trial, participants refrained from strenuous exercise and abstained from alcohol and caffeine ingestion. In addition, participants were also asked to fast from 2100 hours the evening before the experimental trial, and to consume 500 mL of water approximately 90 minutes prior to arrival at the laboratory in an effort to ensure an adequate level of hydration.

All participants reported to the laboratory for their experimental trial between 0800 and 1000 hours. Upon arrival at the laboratory, participants were asked to completely empty their bladder into a container from which a 5 mL urine sample was retained for future analysis. Participants were then seated in an upright position for 15 minutes in an air conditioned environment maintained at approximately 20°C before a 9 mL blood sample was obtained by venous puncture of an antecubital vein. Mid- and post-exercise blood samples were taken with the participants seated on the cycle ergometer in an upright position in an attempt to reduce the influence posture has on circulating blood and plasma volumes (Hagan *et al*. 1978). During the 15 minute seating period, resting HR and thirst sensation (on adapted Borg scales) were recorded.

Following the pre-exercise blood sample, participants were weighed nude to the nearest 0.01 kg (Adam Equipment Co Ltd., GFK 150, Milton Keynes, UK) before initiation of the exercise protocol. Participants cycled at a constant work-rate equivalent to an initial load of 55% of $_{2peak}$ for 60 minutes on an electronically braked cycle ergometer (Lode

147 Excalibur Sport, Groningen, Netherlands), with a three to five minute rest interval at 30
148 minutes for blood sample collection. Breath-by-breath expired air samples, analysed for
149 oxygen uptake and HR, were collected for 3 minutes pre-exercise and at every 15 minutes of
150 cycling. Ratings of perceived exertion and thirst sensations were recorded pre-exercise then
151 every 15 minutes of cycling. Post-exercise nude BM was recorded after participants towel
152 dried.

153 During the 60 minute cycle, participants were provided with constant access to a
154 commercially available hypotonic sports drink containing 2% carbohydrate. The participants
155 were given a choice of two flavours and asked to consume *ad libitum*. Drinks were provided
156 in specialised drinks bottles (CamelBak Better Bottle™) kept in opaque insulating sleeves to
157 prevent participants from viewing the volume of fluid contained and aimed to maintain drinks
158 at regular room temperature. Drink bottles were also changed every 15 minutes. The volume
159 of fluid consumed was determined by weighing of drinks bottles. A 5 mL sample of the drink
160 was obtained for analysis. Total sweat loss was calculated through change in BM corrected
161 for fluid intake. For the purposes of this investigation, it was assumed that all mass loss
162 during exercise was due to sweating since respiratory water loss and mass loss due to
163 substrate exchange would have accounted for only a small component of total mass loss
164 (Maughan *et al*. 2007).

165

166 ***Biochemical analysis***

167 Drink and urine samples were stored at 4°C until analysis for osmolality by freezing point
168 depression (Gonotec Osmomat 030 Cryoscopic Osmometer; Berlin, Germany). EDTA blood
169 samples were analysed for haemoglobin concentration by the cyanmethaemoglobin method,
170 packed-cell volume by microcentrifugation). The haemoglobin and packed-cell volume
171 values were used to estimate percentage changes in blood, erythrocyte and plasma volumes,

as described by Dill and Costill (1974). Remaining whole blood samples were stored at -80°C until DNA extraction. Serum tube blood samples were centrifuged at 1500 *g* for 15 min at 4°C before serum was removed and kept at 4°C until the analysis of osmolality using the method described previously. All analyses were performed in duplicate, with the exception of the packed-cell volume measurements, which were made in triplicate.

Genotyping

Genomic DNA was extracted from 5 mL of EDTA whole blood using Qiagen FlexiGene DNA Kit (West Sussex, UK) according to the manufacturer's instructions. The participants were genotyped for the *W/I* allelic variants within intron 16 of the *ACE* gene, and the *M/P* allelic variants within exon 1 of the B_2R gene. For each gene to be assayed, primers unless stated, were designed using publicly available genomic sequences obtained through GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and the public domain primer design software Primer3 (http://frodo.wi.mit.edu/primer3/; Rozen and Skaletsky 2000). Target gene sequences to be amplified within whole gene sequences were identified from published literature. Primers were designed with Primer3 using the human mispriming library.

ACE genotype was ascertained using two separate PCR reactions. Firstly, the following forward (5'-GGGGACTCTGTAAGCCACTG-3') and reverse (5'-TCGGGTAAAACTGGAGGATG-3') primers were used to detect the presence of a *W* allele. The presence of a *W* allele resulted in an approximate 300 bp PCR product whilst the absence of a *W* allele resulted in no PCR products being formed. Due to the preferential amplification of the *W* allele observed with the above primers and furthermore owing to the preferential amplification of the *W* allele in heterozygous samples (Shanmugam *et al*. 1993), a second insertion specific PCR was performed on all samples using the following forward (5'-TGGGACCACAGCGCCCGCCACTAC-3') and reverse (5'-

197 TCGCCAGCCCTCCCATGCCCATAAC-3') primers as previously utilised by Settin *et al*.
198 (2009). The presence of an *I* allele resulted in an approximate 350 bp PCR product whilst for
199 samples homozygous for the *W* allele, no products were visualised. The PCR reactions were
200 carried out in a total volume of 20 µl containing 20 ng of DNA, 1 x NH_4, 1.5 mM $MgCl_2$, 25
201 µM each of dATP, dTTP, dCTP and dGTP, 250 nM of each primer, and 0.02 units of *Taq*
202 DNA polymerase. For the first *ACE* PCR reaction, the PCR conditions consisted of an initial
203 denaturing step at 95°C for 5 min; followed by 10 cycles of denaturing at 95°C for 30
204 seconds, annealing for 30 seconds at 58°C and extension for 45 seconds at 72°C; then 30
205 cycles of denaturing at 89°C for 20 seconds, annealing for 30 seconds at 58°C and extension
206 for 45 seconds at 72°C; and a final extension step at 72°C for 5 min. The *ACE* insertion
207 specific PCR conditions consisted of these identical steps except for an annealing temperature
208 of 59°C. The amplified fragments were resolved by electrophoresis on 2% agarose gel stained
209 with ethidium bromide and visualised under ultra violet (UV) light.
210 *B_2R* genotype was ascertained using the following forward (5'-CACT
211 CCAGCTCTGGCTTCTG-3') and reverse (5'-TTCAGTCGCTCCCTGGTACT-3') primers
212 to produce approximately 100 and/or 90 bp fragments. The PCR reactions were carried out in
213 volumes and concentrations as above. The PCR conditions were also as above except for an
214 annealing temperature of 55°C. The amplified fragments were subsequently resolved on 4.5%
215 microfagorose gel stained with ethidium bromide and visualised under UV light. All
216 fragments were sized using a molecular weight marker (Hyperladder^{TM} V, Bioline, UK).
217
218 ***Statistical analysis***
219 Differences in participant characteristics, environmental conditions during exercise,
220 and pre-exercise hydration status between genotype groups were examined using one-way
221 ANOVA. One-way ANOVA were also used to examine differences in percentage BML, fluid

intake, sweat loss, sweat rate, and drink osmolality between genotype groups. Significant *F*-tests were followed by multiple independent Student's *t*-tests. The false discovery rate procedure was applied to control for type 1 error. Pre and post BM data within groups was analysed using paired Student's *t*-test. Differences in $_2$ and subjective measures during the 60 minute submaximal exercise between genotype groups were determined using two-way mixed model ANOVA (time x group). Two-way mixed model ANOVA (time x group) were also used to examine differences in serum osmolality, blood volume, and plasma volume. Sphericity for repeated measures was assessed and where appropriate, Greenhouse-Geisser corrections were applied for epsilon <0.75, and the Huynh-Feldt correction adopted for less severe asphericity. Significant *F*-tests were followed with one-way ANOVA at time points and multiple independent Student's *t*-tests. The false discover rate procedure was applied to control for type 1 error. All data were analysed using SPSS for Windows version 14.0 (Chicago, IL). Statistical significance was accepted at the 5% level and results presented as mean ± standard deviation (SD).

Results

Subject characteristics and environmental conditions

The genotype distributions for *ACE* and B_2R of the participants in this study were in Hardy-Weinberg equilibrium. Participant characteristics and exercise conditions grouped according to genotype are presented in Table 1 (*ACE*) and Table 2 (B_2R). Genotype groups for both variants were not different in age, height, body mass, BMI, $_{2peak,}$ average exercise intensity or pre-exercise urine osmolality ($P > 0.05$). Six participants were classed as dehydrated at the start of exercise with pre-exercise urine osmolalities of >900 mOsm.kg^{-1}. There were no

differences in the environmental exercise conditions between the *ACE* genotypes. However, for B_2R, ambient temperature for the *MP* genotype group was significantly higher than the *PP* group ($P < 0.05$). Relative humidity was also significantly higher for *PP* compared to *MM* and *MP* ($P < 0.05$). Data is presented in Tables 1 and 2.

Mean exercise intensity and HR significantly increased over time ($P < 0.05$) but no interaction effect or main effect of genotype was observed in any of these variables ($P > 0.05$) for both variants.

Fluid intake and fluid balance

ACE

Both the *WW* and *WI* genotype groups exhibited a significant decrease in body mass at the end of the 60 minute cycle. The *II* group also tended to a significant decrease in body mass ($P = 0.07$). There were no differences in mean percentage BML, fluid intake, and total sweat loss (Table 1).

B_2R

A significant decrease in body mass occurred in all three genotype groups at the end of 60 minute cycle ($P < 0.05$). There were no differences in mean percentage BML, fluid intake, and total sweat loss between genotypes

Blood variables

ACE

Plasma volume significantly decreased by 9.1 ± 5.5, 6.0 ± 7.8 and 7.8 ± 6.6% from pre to post for *WW*, *WI,* and *II*, respectively. No interaction effect ($P = 0.61$) or main effect of

genotype ($P = 0.91$) was found. Serum osmolality (Figure 1a) significantly increased over time ($P < 0.05$) but no interaction effect ($P > 0.05$) or main effect of genotype ($P > 0.05$) was present.

B_2R

Plasma volume significantly decreased by 6.0 ± 8.4, 6.8 ± 6.0 and 9.0 ± 6.7% from pre to post for *MM*, *MP,* and *PP*, respectively. No interaction effect ($P = 0.69$) or main effect of genotype ($P = 0.51$) was found. Serum osmolality (Figure 1b) significantly increased over time ($P < 0.05$) but no interaction effect ($P > 0.05$) or main effect of genotype ($P > 0.05$) was present.

Subjective measures

ACE

Ratings of perceived exertion and thirst (Figure 2a) significantly increased over time ($P < 0.05$). However, no interaction effects ($P = 0.92$, $P = 0.81$) or main effects of genotype ($P = 0.57$, $P = 0.83$), were found for these subjective measures, respectively.

B_2R

Ratings of perceived exertion and thirst (Figure 2b) significantly increased over time ($P < 0.05$). No interaction effect ($P = 0.22$) or main effect of genotype ($P = 0.20$) were found for RPE. Both an interaction effect and main effect of genotype ($P < 0.05$) was found for thirst, however. Thirst was significantly higher ($P < 0.05$) in the *PP* genotype group compared to both *MM* and *MP* in the latter half of the 60 minute cycle at 27-30 min, 42-45 min, and 57-60 min.

297 **Discussion**

298 The primary finding of the present investigation was that both *ACE* and *B_2R* allelic variation
299 did not significantly influence voluntary fluid intake or fluid balance during moderate
300 intensity exercise in the heat. All six genotype groups exhibited indications of the voluntary
301 dehydration phenomena however, with five of the groups manifesting decreases in body mass
302 from pre-exercise that reached significance. Furthermore, the pattern of results surprisingly
303 appeared the reverse of that expected. Participants with the *ACE II* genotype and participants
304 with the *B_2R PP* genotype did not exhibit the greatest level of voluntary dehydration but
305 rather exhibited the intermediate and lowest level of dehydration, respectively.
306
307 With regards to *ACE*, the findings of the present study lend support to those of Saunders *et al*.
308 (2006) who reported no significant association of *ACE* genotype and weight change in
309 competing Caucasian male ironman triathletes. The present study was the first controlled
310 laboratory study to be conducted that confirms this previous finding to the wider population
311 of healthy Caucasian males partaking in a period of moderate intensity exercise in the heat.
312
313 It is acknowledged that a limitation of this study is sample size and that more participants
314 should be investigated or added to the dataset for firm conclusions to be drawn. However, it
315 is worth noting that previous studies such as de Souza *et al*. (2013), Folland *et al*. (2000),
316 Santana *et al*. (2011) and Williams *et al*. (2011) have found positive *ACE* results/associations
317 with sample sizes smaller than in the present study (range n = 27 to n = 41).
318
319 Given the influences of *ACE* genotype on inter-individual variation in circulating ACE
320 concentrations (Day *et al*. 2007; Rigat *et al*. 1990), it was theorised that *ACE* allelic variation
321 may have a resulting effect on angiotensin II concentrations and thus thirst and fluid intake

during exercise. The results of the present study suggest that this may not be the case. Instead, other rate limiting steps in the production of angiotensin II or its activity such as angiotensin I concentration or angiotensin II receptor activity may be responsible for inter-individual differences in thirst, voluntary fluid intake and voluntary dehydration.

It may be that the RAAS plays little role in the regulation of fluid intake and balance during exercise in the heat. Greenleaf *et al*. (1983) have suggested the RAAS is influenced by reductions in total body water and plasma volume is the predominant stimulus to thirst during exercise in the heat. Maresh *et al*. (2004), on the other hand, suggest that the RAAS has a minor role whilst the AVP system has a dominant role in regulating thirst during exercise heat challenge. The lack of influence of *ACE* genotype on thirst and voluntary fluid intake found in the present study appears to support the conclusion of Maresh *et al.* (2004).

The greater role and importance of the AVP system in regulating thirst and fluid balance during exercise in the heat is supported by serum osmolality data presented in this investigation. In healthy humans, AVP is released in response to small increases in blood plasma osmolality. Mid-exercise serum osmolality in the present study increased a significant amount over time to elicit enhanced AVP secretion and stimulated thirst response. It is also worth noting that only three participants exhibited a serum osmolality above 295 $mOsm.kg^{-1}$ at any point of measurement, suggesting that serum osmolality was generally well regulated within the normal range of 280-295 $mOsm.kg^{-1}$ when free access to fluid was available.

The B_2R results of the present study are in disagreement to the findings of Saunders *et al*. (2006) who found a significant linear trend for the distribution of the *PP* genotype across three percentage weight loss groups. The lack of genotype influence obtained in the present

study compared to Saunders *et al*. (2006) is likely due to the difference in study population and exercise stimulus. With a few exceptions, the group of healthy participants who volunteered for the present study were active recreational exercisers who were relatively fit. The 60 minutes of moderate intensity exercise at 30°C and approximately 30% relative humidity would have therefore been no doubt challenging and hard, but comparably less physiologically challenging than an ultra-endurance event lasting 10-15 h in a hot and humid climate. It may therefore only be in such extreme situations that an influence of B_2R variation is observed.

Despite the absence of a significant influence of genotype for voluntary fluid intake and fluid balance in the present study, subjective perception of thirst was significantly higher in the *PP* genotype group compared to the others in the latter half of the 60 minute cycle. Since serum osmolality was regulated within normal range, there was no significant difference in serum osmolality and no significant differences in blood or plasma volume decrease between genotypes, it is unlikely that the excess thirst experienced by this group was either osmotic or hypovolemic thirst due to a greater physiological need for water. This finding supports the perception that thirst in humans is not solely influenced by physiological need but other factors such as habit and psychology may also play a role. The results of the other subjective measures are suggestive towards this.

Sweat loss during this study (approximately 1.1 L/h) was relatively low compared to other studies. Byrne *et al*. (2006) observed a sweat rate of 1.47 ± 0.34 L/h in runners participating in a half marathon in environmental conditions of 25.6 - 27.3 °C and a relative humidity of 75 – 90%. Similarly, Lee *et al*. (2010) observed a sweat rate of 1.45 ± 0.32 °C during a run at 26.4 °C and 81% humidity and O'Neal *et al*. (2014) observed a sweat rate of 1.35 ± 0.4 L/h

during a run at 20 ± 3 °C and 54 ± 14% humidity. This difference could be attributed to the relatively low humidity in the present study. Rate of fluid intake amounted to 0.37 ± 0.26 L/h and 0.075 ± 0.062 L/h in the studies by Byrne *et al*. (2006) and Lee *et al*. (2010) which are much lower than in the present study (approximately 0.74 L/h). A likely reason for this difference in ingestion rate is the mode of exercise. Cycling allows for greater ingestion due to easier access to fluid and also less likely to suffer from gastrointestinal comfort compared to running. Furthermore, fluid intake is highly variable between individuals and subject to the effect of a number of variables including palatability of fluid provided (Minehan *et al*. 2002). A greater volume of fluid is ingested during exercise if the drink is considered to be acceptable in comparison to less acceptable or to water (Passe *et al*. 2000). Similarly, greater fluid intake during exercise is observed with ingestion of a carbohydrate electrolyte solution compared to water and other solutions that are rated less palatable (Passe *et al*. 2004; Rivera-Brown *et al*. 1999). Some of the difference in fluid ingestion volume may therefore be attributed to greater subjective acceptability and palatability of the flavoured 2% carbohydrate drink provided in this present study compared to the water and 6% carbohydrate electrolyte solutions provided in the studies by Byrne *et al.* (2004) and Lee *et al*. (2010).

In conclusion, the results of the present study suggests that *ACE* and B_2R allelic variation do not play a major role in governing voluntary fluid intake and fluid balance in normal healthy males partaking in a 60 minute period of moderate intensity exercise in the heat. It appears that other factors such as subjective feeling and subjective perception of the exercise environment may be more important in determining thirst and fluid intake in exercise of this nature and in this population. Additional or a larger number of participants should be investigated whilst future work should also examine the responses of hormones and enzymes involved in body fluid regulation in order to elucidate the underlying mechanism involved and attempt to explain the observed individual differences in thirst, fluid intake and fluid balance. **References**

Armstrong, L.E. and Grandjean, A.C. 2007. Hydration and health promotion. J. Am. Coll. Nutr. **26**(5 Suppl): 529S. PMID:17921460.

Borg, G. 1998. Borg's Perceived Exertion and Pain Scales. Human Kinetics, Leeds.

Braun, A., Kammerer, S., Maier, E., Bohme, E. and Roscher, A.A. 1996. Polymorphisms in the gene for the human B-2-bradykinin receptor: new tools in assessing a genetic risk for bradykinin-associated diseases. Immunopharmacology. **33**(1-3): 32-35. doi: 10.1016/0162-3109(96)00079-3. PMID: 8856111.

Byrne, C., Lee, J.K., Chew, S.A.N., Lim, C.L. and Tan, E.Y.M. 2006. Continuous thermoregulatory responses to mass-participation distance running in the heat. Med. Sci. Sports Exerc. **38**(5): 803-810. doi: 10.1249/01.mss.0000218134.74238.6a. PMID: 16672830.

414 Cadnapaphornchai, M.A., Rogachev, B., Summer, S.N., Chen, Y.C., Gera, L., Stewart, J.M.,
415 *et al*. 2004. Evidence for bradykinin as a stimulator of thirst. Am. J. Physiol – Renal. **286**(5):
416 F875-F880. doi: 10.1152/ajprenal.00243.2003. PMID: 15075183.
417
418 Danser, A.H.J., Schalekamp, M., Bax, W.A., Vandenbrink, A.M., Saxena, P.R., Riegger,
419 G.A.J., *et al.* 1995. Angiotensin-converting enzyme in the human heart - effect of the deletion
420 insertion polymorphism. Circulation. **92**(6): 1387-1388. doi: 10.1161/01.CIR.92.6.1387.
421 PMID: 7664416.
422
423 Day, S.H., Gohlke, P., Dhamrait, S.S. and Williams, A.G. 2007. No correlation between
424 circulating ACE activity and VO2max or mechanical efficiency in women. Eur. J. Appl.
425 Physiol. **99**(1): 11-18. doi: 10.1007/s00421-006-0309-3. PMID: 17006710.
426
427 de Souza, J.C., Tibana, R.A., de Souza, N.M.F., de Souza, V.C., Karnikowski, M.G.O.,
428 Prestes, J., *et al*. 2013. Association of cardiovascular response to an acute training session
429 with the ACE gene polymorphism in sedentary women: a randomized trial. BMC
430 Cardiovascular Disorders. **13**: 3. doi: 10.1186/1471-2261-13-3. PMID: 23305118.
431
432 Dill, D.B. and Costill, D.L. 1974. Calculation of percentage changes in volumes of blood,
433 plasma, and red cells in dehydration. J. Appl. Physiol. **37**(2): 247-248. PMID: 4850854.
434
435 Dzau, V.J., Bernstein, K., Celermajer, D., Cohen, J., Dahlof, B., Deanfield, J., *et al*. 2001. The
436 relevance of tissue angiotensin-converting enzyme: manifestations in mechanistic and
437 endpoint data. Am. J. Cardiol. **88**(9A): 1L-20L. doi: 10.1016/S0002-9149(01)01878-1.
438 PMID: 11694220.

Fitzsimons, J.T. 1972. Thirst. Physiol. Rev. **52**(2): 468-561. PMID: 4336576.

Fitzsimons, J.T. 1998. Angiotensin, thirst, and sodium appetite. Physiol. Rev. **78**(3): 583-686. PMID: 9674690.

Folland, J., Leach, B., Little, T., Hawker, K., Myerson, S., Montgomery, H., *et al*. 2000. Angiotensin-converting enzyme genotype affects the response of human skeletal muscle to functional overload. Exp. Physiol. **85**(5): 575-579. doi: 10.1017/S0958067000020571. PMID: 11038409

Fregly, M.J. and Rowland, N.E. 1991. Bradykinin-induced dipsogenesis in captopril-treated rats. Brain. Res. Bull. **26**(1): 169-172. doi: 10.1016/0361-9230(91)90204-W. PMID: 2015514.

Greenleaf, J.E., Brock, P.J., Keil, L.C. and Morse, J.T. 1983. Drinking and water-balance during exercise and heat acclimation. J. Appl. Physiol. **54**(2): 414-419. PMID: 6833039.

Hagan, R.D., Diaz, F.J. and Horvath, S.M. 1978. Plasma volume changes with movement to supine and standing positions. J. Appl. Physiol. **45**(3): 414-418. PMID: 701126.

Lee, J.K., Nio, A.Q.X, Lim, C.L., Teo, E.Y.N. and Byrne, C. 2010. Thermoregulation, pacing and fluid balance during mass participation distance running in a warm and humid environment. Eur. J. Appl. Physiol. **109**(5): 887-898. doi: 10.1007/s00421-010-1405-y. PMID: 20237797.

Lung, C.C., Chan, E.K.L. and Zuraw, B.L. 1997. Analysis of an exon 1 polymorphism of the B2 bradykinin receptor gene and its transcript in normal subjects and patients with C1 inhibitor deficiency. J. Allergy Clin. Immun. **99**(1): 134-146. doi: 10.1016/S0091-6749(97)81054-8. PMID: 9003221.

Maresh, C.M., Gabaree-Boulant, C.L., Armstrong, L.E., Judelson, D.A., Hoffman, J.R., Castellani, J.W., *et al*. 2004. Effect of hydration status on thirst, drinking, and related hormonal responses during low-intensity exercise in the heat. J. Appl. Physiol. **97**(1): 39-44. doi: 10.1152/japplphysiol.00956.2003. PMID: 14990557.

Maughan, R.J., Shirreffs, S.M. and Watson, P. 2007. Exercise, heat, hydration and the brain. J. Am. Coll. Nutr. **26**(5): 604S-612S. PMID: 17921473.

Maughan, R.J., Merson, S.J., Broad, N.P. and Shirreffs, S.M. 2004. Fluid and electrolyte intake and loss in elite soccer players during training. Int. J. Sport Nutr. Exerc. Metab. **14**(3): 333-346. PMID: 15256693.

Minehan, M.R., Riley, M.D. and Burke, L.M. 2002. Effect of flavour and awareness of kilojoule content of drinks on preference and fluid balance in team sports. Int. J. Sport Nutr. Exerc. Metab. **12**(1): 81-92. PMID: 11993625.

Montgomery, H., Humphries, S. and Danilov, S. 2002. Is genotype or phenotype the better tool for investigating the role of ACE in human cardiovascular disease? Eur. Heart J. **23**(14): 1083-1086. doi: 10.1053/euhj.2001.2980. PMID: 12090745.

O'Neal, E.K., Caufield, C.R., Lowe, J.B., Stevenson, M.C., Davis, B.A. and Thigpen, A.K. 2014. 24-h fluid kinetics, and perception of sweat losses following a 1-h run in a temperate environment. Nutrients **6**: 37-49. doi: 10.3390/nu6010037. PMID: 24451307.

Passe, D., Horn, M. and Murray, R. 2000. Impact of beverage acceptability on fluid intake during exercise. Appetite **35**(3): 219-229. doi: 10.1006/appe.2000.0352. PMID: 11073704.

Passe, D., Horn, M., Stofan, J. and Murray, R. 2004. Palatability and voluntary intake of sports beverages, diluted orange juice and water during exercise. Int. J. Sport Nutr. Exerc. Metab. **14**(3): 266-278. PMID: 15256688.

Passe, D., Horn, M., Stofan, J., Horswill, C. and Murray, R. 2007. Voluntary dehydration in runner despite favourable conditions for fluid intake. Int. J. Sport Nutr. Exerc. Metab. **17**(3): 284-295. PMID: 17693689.

Pitts, G.C., Johnson, R.E. and Consolazio, F.C. 1944. Work in the heat as affected by intake of water, salt and glucose. Am. J. Physiol. **142**(2): 253-259.

Rigat, B., Hubert, C., Alhencgelas, F., Cambien, F., Corvol, P. and Soubrier, F. 1990. An insertion deletion polymorphism in the angiotensin i-converting enzyme gene accounting for half the variance of serum enzyme levels. J. Clin. Invest. **86**(4): 1343-1346. doi: 10.1172/JCI114844. PMID: 1976655.

513 Rivera-Brown, A.M., Gutierrez, R., Gutierezz, J.C., Frontera, W.R. and Bar-Or, O. 1999.
514 Drink composition, voluntary drinking and fluid balance in exercising, trained, heat-
515 acclimatized boys. J. Appl. Physiol. **86**(1): 78-84. PMID: 9887116.
516
517 Rothstein, A., Adolph, E.F. and Wills, J.H. 1947. Voluntary dehydration. *In* Physiology of
518 man in the desert. *Edited by* E.F. Adolph. Interscience Publishers, New York. pp. 254-270.
519
520 Rozen, S. and Skaletsky, H.J. 2000. Primer3 on the WWW for general users and for biologist
521 programmers. *In* Bioinformatics Methods and Protocols: Methods in Molecular Biology.
522 *Edited by* S. Krawetz, and S. Misener, Humana Press, Totowa, NJ. pp. 365-386.
523
524 Santana, H.A.P., Moreira, S.R., Neto, W.B., Silva, C.B., Sales, M.M., Oliveira, V.N., *et al*.
525 2011. The higher exercise intensity and the presence of the allele I of ACE gene elicit a
526 higher post-exercise blood pressure reduction and nitric oxide release in elderly women: an
527 experimental study. BMC Cardiovascular Disorders, **11**: 71. doi: 10.1186/1471-2261-11-71.
528 PMID: 22136292.
529
530 Saunders, C.J., de Milander, L., Hew-Butler, T., Xenophontos, S.L., Cariolou, M.A.,
531 Anastassiades, L.C., *et al*. 2006. Dipsogenic genes associated with weight changes during
532 Ironman Triathlons. Hum. Mol. Genet. **15**(6): 2980-2987. doi: 10.1093/hmg/ddl240. PMID:
533 16950802.
534
535 Settin, A., Elbaz, R., Abbas, A., Abd-Al-Samad, A. and Noaman, A. 2009. Angiotensin-
536 converting enzyme gene insertion/deletion polymorphism in Egyptian patients with

myocardial infarction. J. Renin Angiotensin Aldosterone Syst. **10**(2): 96-100. doi: 10.1177/1470320309105198. PMID: 19502257.

Shanmugam, V., Sell, K.W. and Saha, B.K. 1993. Mistyping ace heterozygotes. PCR Meth. Appl. **3**(2): 120-121. doi: 10.1101/gr.3.2.120. PMID: 8268786.

Whipp, B.J., Davis, J.A., Torres, F. and Wasserman, K. 1981. A test to determine parameters of aerobic function during exercise. J. Appl. Physiol. **50**(1): 217-221. PMID: 6782055.

Williams, A.D., Anderson, M.J., Selig, S., Carey, M.F., Febbraio, M.A., Hayes, A., *et al*. 2011. Differential response to resistance training in CHF according to ACE genotype. Int. J. Cardiol. **149**(3): 330-334. doi: 10.1016/j.ijcard.2010.02.012. PMID: 20189663.

549 **Tables**

550

551 Table 1: Participant characteristics, environmental conditions during exercise, and fluid
552 balance grouped according to angiotensin converting enzyme (*ACE)* genotype. *WW*;
553 homozygous wild type, *WI*; heterozygous, *II*; homozygous insertion. Values are mean ± SD.

554

Variable	*WW*	*WI*	*II*	*P*-value
n	12	21	12	-
Age (yr)	30 ± 9	27 ± 8	28 ± 7	0.50
Height (cm)	177.2 ± 5.9	178.6 ± 6.1	178.8 ± 8.7	0.82
Body Mass (kg)	76.73 ± 14.84	82.79 ± 15.15	74.39 ± 11.10	0.22
BMI ($kg.m^{-2}$)	24.31 ± 3.54	25.87 ± 3.94	23.19 ± 2.38	0.11
$_{2peak}$ ($mL.min^{-1}.kg^{-1}$)	49.98 ± 12.83	48.44 ± 8.50	56.30 ± 8.24	0.09
Temperature (°C)	30.4 ± 0.3	30.5 ± 0.2	30.5 ± 0.3	0.50
Humidity (%)	30 ± 7	30 ± 6	27 ± 8	0.48
% $_{2peak}$	63.0 ± 3.2	61.7 ± 5.1	59.7 ± 4.1	0.19
Urine osmolality ($mOsmol.kg^{-1}$)	459 ± 298	502 ± 291	472 ± 311	0.92
Drink osmolality ($mOsmol.kg^{-1}$)	116 ± 4	116 ± 4	115 ± 3	0.70
Body mass loss (Kg)	0.40 ± 0.48	0.30 ± 0.37	0.40 ± 0.68	0.79
Body mass loss (%)	0.51 ± 0.63	0.35 ± 0.48	0.46 ± 0.90	0.78
Sweat loss (L)	1.013 ± 0.257	1.048 ± 0.254	1.257 ± 0.674	0.28
Fluid intake (L)	0.613 ± 0.388	0.753 ± 0.385	0.862 ± 0.421	0.31

555

556 Table 2: Participant characteristics, environmental conditions during exercise, and fluid
557 balance grouped according to bradykinin receptor B2 (*B_2R)* genotype. *MM*; homozygous wild
558 type, *MP*; heterozygous, *PP*; homozygous insertion. Values are mean ± SD.
559

Variable	*MM*	*MP*	*PP*	*P*-value
n	13	17	15	-
Age (yr)	31 ± 9	26 ± 8	27 ± 8	0.36
Height (cm)	177.9 ± 5.7	180.0 ± 7.5	177.4 ± 6.6	0.42
Body Mass (kg)	77.87 ± 15.58	78.89 ± 15.78	79.89 ± 12.13	0.94
BMI ($kg.m^{-2}$)	24.78 ± 4.43	24.21 ± 3.66	25.30 ± 2.79	0.70
$_{2peak}$ ($mL.min^{-1}.kg^{-1}$)	49.45 ± 10.12	50.64 ± 11.68	52.59 ± 8.49	0.71
Temperature (°C)	30.5 ± 0.3	30.6 ± 0.2*	30.3 ± 0.2	<0.05
Humidity (%)	27 ± 6	27 ± 6	33 ± 6†	<0.05
% $_{2peak}$	61.3 ± 4.6	62.1 ± 5.2	61.0 ± 3.8	0.78
Urine osmolality ($mOsmol.kg^{-1}$)	477 ± 288	436 ± 270	537 ± 329	0.63
Drink osmolality ($mOsmol.kg^{-1}$)	117 ± 5	116 ± 4	116 ± 3	0.57
Body mass loss (Kg)	0.39 ± 0.36	0.34 ± 0.50	0.33 ± 0.58	0.95
Body mass loss (%)	0.50 ± 0.49	0.41 ± 0.64	0.37 ± 0.77	0.87
Sweat loss (L)	0.986 ± 0.254	1.089 ± 0.401	1.195 ± 0.521	0.41
Fluid intake (L)	0.599 ± 0.322	0.745 ± 0.374	0.870 ± 0.459	0.20

560 *****Significantly higher than *PP;* †Significantly higher than *MM* and *MP*
561

562 **Figure captions**

563

564 Fig. 1: (a) Serum osmolality pre, mid and immediately post 60 min of cycling for each
565 *angiotensin converting enzyme (ACE)* genotype group. *WW*, homozygous wild type; *WI*,
566 heterozygous; *II*, homozygous insertion. (b) Serum osmolality pre, mid and immediately post
567 60 min of cycling for each *bradykinin receptor B2* (B_2R) genotype group. *MM*, homozygous
568 wild type; *MP*, heterozygous; *PP*, homozygous insertion. Values are mean ± SD. *Significant
569 increase from baseline pre-exercise for *ACE WW* and *WI* genotype groups and all *B2R*
570 genotypes ($P < 0.05$). #Significant increase from baseline pre-exercise for *MM* genotype
571 group ($P < 0.05$).

572

573 Fig. 2: (a) Subjective feeling of thirst at baseline, pre-exercise and 15 min intervals
574 throughout 60 min of cycling for each *angiotensin converting enzyme (ACE)* genotype group.
575 *WW*, homozygous wild type; *WI*, heterozygous; *II*, homozygous insertion. (b) Subjective
576 feeling of thirst at baseline, pre-exercise and 15 min intervals throughout 60 min of cycling
577 for each *bradykinin receptor B2* (B_2R) genotype group. *MM*, homozygous wild type; *MP*,
578 heterozygous; *PP*, homozygous insertion. Values are mean ± SD. *Significant difference
579 between *PP* and *MM* and *MP* ($P < 0.05$).

580

581

582

583

584

585

586

587

588