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1 Title:

2 Genetic conditionality of strength by the ACE gene in women horse riders

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24 ABSTRACT

25 The sport performance relies on hard work, intensity, duration of workout and
26 undoubtedly on genetic background. These components fold on multicausal character of
27 sport achievements. One of the best known genes associated with sport performance is
28 angiotensin converting enzyme gene (ACE). In this study, we showed ACE distribution
29 in women horse riders and the correlation between physiological and genetic
30 background, that should be taken into account when deciding to train sports discipline.
31 Data from 40 healthy female adults were collected. Participants trained horse-riding for
32 at least 3 years and participated in sport competition at least at national level and control
33 group consist of out of training persons. We analysed BMI, %FAT, cardiorespiratory
34 efficiency (VO2max), Complex Reaction Time, body balance, force and Lower Limbs
35 Explosive Force between both calculated groups. Physiological data was calculated by
36 non-parametric Mann-Whitney test with Dunnett`s post hoc test. The DNA
37 amplification from saliva was performed by PCR method to analyze the ACE genotype
38 distribution. In our study, for the first time we showed that the women training dressage
39 have higher force and complex reaction time than the control group. It was correlated
40 with possession of the D allele in genotype that is associated with muscle strength,
41 efficiency and muscle power. There is no research relating to ACE polymorphism
42 among horse riders. This paper showed angiotensin converting enzyme gene
43 distribution polymorphism in female Polish professional horse riders. This article
44 confirms correlation between genotype, sports results and physiological parameters.

45

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47 ## 1. INTRODUCTION

48 Angiotensin converting enzyme (ACE) is well-known and one of the most
49 frequent under examined gene among sportsmen. An insertion/deletion (I/D)
50 polymorphism in the ACE gene occurrence is responsible for the control of blood
51 pressure in the body [1], as well as for predisposition to short-term or strength sports.
52 It's been long since known that sport success is the sum of genetic predisposition and
53 regular training. There is a lot of research of frequency of an insertion/deletion of
54 polymorphism among sportspeople training various olympic disciplines. Lack of
55 literature concerning I/D ACE polymorphism with horse-riders [1]. The aim of this
56 study was to show insertion/deletion of polymorphism in the ACE gene correlated with
57 horse-riders' physical condition and their professing sport discipline and enrich existing
58 literature about genetics of athletic performance. We also wanted to enrich the current
59 canon of knowledge about equestrianism.

60 Horse-riding is a highly demanding sport rank among olympic disciplines. It
61 split into 7 groups: dressage, show jumping, teaming, western riding, vaulting, long-
62 distance rail and three-day event. Regardless of trained discipline, horse-riding demands
63 high physical condition, force and strength. Riding is most prestigious and well known
64 discipline, but not everyone predispose to this sport [2,3].

65 All types of sports are marked by determined physical requirements. Sport qualification
66 depend on intensity, length of practice and genetic conditioning of individual facilities.
67 These compounds reflect on the multicasual nature of sport performance [4].

68 Sport efficiency involves interaction between musculoskeletal, cardiovascular,
69 respiratory and nervous systems. It constitute as one of the most complex properties that
70 predispose to determinated sport. Differences in efficiency can also be the result of

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71 anatomy (height, body composition), strength and force. Aerobic efficiency results from
72 the cardiovascular system's ability to provide and utilize oxygen for muscles. It is not
73 correlated with strength performance, which is related to aerobic threshold [5].
74 Muscle power is a muscle strength to quantitative force generation. The
75 connection between strength and muscle cramp rate is defined as muscle force. It has a
76 great value in sprint, jumps or weightlifting [6]. Cognitive and enviromental factors
77 (training, nutrition) and injury susceptibility are components of physical fitness. Body
78 response to physical activity partly depends on genetic factors and differs between
79 sports [7]. Physical condition (66%), height (80%) or body build are highly inherited
80 properties connected with force and strength among athletics. Strength and muscle
81 power phenotype research demonstrate 50% aerobic efficiency heritability and 30- 83%
82 strength and force heritability dependent upon type and muscle cramp [4].
83 Many genes and gene polymorphisms influence on body efficiency and manual
84 effort, such as agene converting angiotensin enzyme ACE [1], ACTN3 α-actinin-3 gene
85 [8], bradykinin β2BDKBR2 receptor gene [9,10], PPAR α receptor gene [11,12], GDF-
86 8 myostatin gene [13,14], APOE apolipoprotein E gene [15]. The ACE gene converting
87 angiotensin I enzyme, which inactivates bradykinin, is part of the renin- angiotensin
88 system responsible for controlling blood pressure in the body. The gene encoding ACE
89 had been localized on 17 chromosomes. Insertion/deletion (I/D) of polymorphism
90 impacts on the predisposition to short-term or strength sports. I/D of polymorphism
91 relies on the presence or absence of a 287 bp Alu repeat element in intron 16 of this
92 gene. The ACE I allele represents an insertion and is associated with lower serum and
93 tissue ACE activity while the D (deleted) allele is associated with higher serum and
94 tissue ACE activity. The ACE I/I genotype is associated with endurance performance

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95 and higher exercise efficiency and represents competitive sportspeople and among
96 mountaineers. D/D genotype is associated with strength and power performance.
97 Differences between frequency of I/D ACE polymorphism was observed in population.
98 Abilities to physical effort is also modulated by polymorphism variants in other genes
99 involved in heart and skeletal muscle metabolism [1,4,16].

100 Most research is concentrated on ACE polymorphism among athletics. Lack of
101 literature concerning I/D polymorphism with horse riders incline us to extend the
102 knowledge about this sport group. The aim of our study was to show the correlation
103 between genetic and physical background at equestrianism.

104

105 ## 2. MATERIALS AND METHODS

106 ### *2.1. Subject and sample collection.*

107 The study was conducted in accordance with the ethical rules of the Helsinki
108 Declaration. Forty people were invited to participate in this study. Eligibility criteria
109 included healthy female adults between the ages of 15 to 35 years old. Participants
110 trained horse-riding (19 persons) for at least 3 years and participated in sport
111 competition at least at national level and control group consist of 21 out of training
112 persons. After explaining the purpose of the study and how to participate, a consent
113 form or permission slip, containing general information about the examined was
114 completed and signed by all participants. The survey was completely anonymous. This
115 study was approved by the [redacted] Bioethics Committee (Poland), under
116 protocol number 2018/01/04.

117 Briefly, participants expectorated at least 5 mL of unstimulated saliva into a
118 sterile, 20 mL polyethylene tube at least 30 minutes prior to eating, drinking, smoking

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119 or kissing to minimize contaminate. Saliva samples were collected from participants and
120 subjected to extract genomic DNA followed by PCR amplification and analysis for
121 ACE I/D polymorphism using specific primers. Collection tubes were maintained on ice
122 and the saliva was aliquoted into sterile 2 mL microcentrifuge tubes containing Saliva
123 DNA Preservative buffer according to manufacturer. Next, each participant's aliquoted
124 preserved samples were stored at room temperature for three days. The storage periods
125 for the saliva aimed to transport samples into laboratory for further analysis.

126 *2.2. DNA extraction*

127 The genomic DNA extraction protocols used are described below. For Saliva
128 DNA Collection, Preservation and Isolation Kit commercial kit (Cat. RU35700,
129 NorgenBiotek Corp., Thorold, Canada) according to the manufacturer's specific
130 instructions.

131 Half a milliliter of saliva was collected from each donor into a 2 mL tube (Eppendorf,
132 Hamburg, Germany) containing 0.5 mL Norgen's Saliva DNA Preservative and mixed
133 by shaking for 10 seconds. In that manner the saliva sample was preserved for storage,
134 shipping and processing. The kit's procedure was modified by the collection and
135 isolation of saliva DNA from 1 ml of preserved saliva samples, and overnight sample
136 incubation with proteinase K. Isolated DNA was suspended in ddH20. NorgenBiotek
137 commercial kits, the manufacturer's instructions were followed [17].

138 *2.3. Spectrophotometric analysis*

139 The concentrations of DNA from saliva samples were detected using a
140 NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).
141 A spectrophotometer at wavelengths of 230 nm, 260 nm and 280 nm was used to
142 quantify and analyse the condition of each extracted DNA sample from saliva. The

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143 concentration and purity of DNA was detected via the relative 260/280 nm and 260/230
144 nm absorbance ratios. To analyse 1 μL of each sample was required. The 260/280 nm
145 absorbance ratio between 1.6 and 2.0 for samples were considered pure. Ratio lower
146 than 1.6 testifies to higher protein contaminates [17,18].

147 *2.4. Electrophoretic analysis*

148 To determine the quality and condition of the extracted DNA, samples from
149 saliva were electrophoresed using a 2% agarose gel in Tris-borate-EDTA (TBE) buffer,
150 Tris base (100 mM), Boric acid (100 mM) and EDTA (2 mM). Standard molecular
151 weight GeneRuler DNA Ladder Mix (SM0333; Thermo Fisher Scientific) in the range
152 of 100 bp to 10,000 bp was loaded onto gel containing Midori Green Advance DNA
153 Stain (MG04; ABO, Gdańsk, Poland). 10 μl of each sample and negative control (10 μl
154 of ddH2O) with 2 μl loading buffer 6x DNA Gel Loading Dye (R0611, Thermo Fisher
155 Scientific) were loaded onto gel. Electrophoresis was driven using 100 V ~ 70 mA for
156 45 minutes at room temperature. Gels were visualized using UV light and photographed
157 with a digital camera Enduro Gel XL and Transiluminator Gel Vue GVM20 (Syngene,
158 Cambridge, UK).

159 *2.5. PCR*

160 Conventional PCR was used to investigate extracted DNA. Polymerase chain
161 reaction detection of the insertion/ deletion polymorphism of human angiotensin
162 converting enzyme gene (ACE). For the I/D of polymorphism, the following primers
163 were used to amplify the region of Alu insertion, with the Alu element's presence or
164 absence being detected by running the product on an agarose gel. The sequences of
165 primers used were sense oligo: 5'- CTGGAGACCACTCCCATCCTTTCT-3' (forward)
166 and anti-sense oligo 5'- GATGTGGCCATCACATTCGTCAGAT-3' (reverse).

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167 Cycling conditions were 95°C for 5 minutes; then for 40 cycles with denaturation at
168 95°C for 30 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 30
169 seconds and final extension at 72°C for 10 minutes by Eppendorf Mastercycler personal
170 (Eppendorf). Genotypes of all individuals resulted in an amplified fragment of 190 bp
171 for DD, 490 and 190 bp for ID, and 490 bp for II. The 20 μl reaction mix was composed
172 of 1x GoTaq G2 Green Master Mix (M7823; Promega, Fitchburg, Wisconsin, USA)
173 with dNTPs, $MgCl_2$ and Polymerase, 0.1 μM of each primers (upstream, downstream),
174 100 ng DNA template and nuclease- free water. As a negative control 2 μl nuclease-free
175 water was used. Reaction products were analysed in a 2% agarose gel using the same
176 procedures outlined above (excluding 6x Loading Dye).

177 *2.6. Physiological procedures - Body Mass Index (BMI)*

178 To calculate BMI body height and weight was measured in light clothing and
179 barefoot. Body height was measured with SECA (Hamburg, Germany) portable
180 stadiometer to the nearest 0.1 cm according to the protocol recommended by the
181 International Society for the Advancement of Kinanthropometry (ISAK)[19]. Body fat
182 percentage (%FAT) and weight were assessed by bioelectrical impedance using Tanita
183 Body Composition Analyser (TBF-300). BMI was calculated from the equation:
184 BMI =body mass (kg)·stature -1 (m^2).

185 *2.7. Static Balance test*

186 A postural sway was assessed with baropodometric platform (Sensor Medica –
187 FreeMedBase, Rome, Italy). Distribution of the ground reaction forces was used to
188 register Centre Of Pressure (COP) displacements with sampling frequency at the level
189 of 400 Hz. Subjects performed 3 consecutive trials of maximal voluntary forward (FL)
190 and then backward leaning (BL) (Limits Of Stability test - LOS test) proposed by Juras

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191 et al. [20]. Subjects stand barefoot on the force platform with feet in natural position,
192 with arms by their sides and palms directed to thighs. Subjects were instructed to look
193 straight ahead on fixation point placed 2 m away on the wall. The procedure started with
194 10 seconds of quiet standing and then after acoustic signal subjects executed the leaning
195 movement at their own pace until they reached their maximal range. After reaching
196 maximal leaning subjects maintained position to the end of trial (approximately 15
197 seconds), each trial lasted 30 seconds. Subjects were instructed to execute the leaning
198 only by movement in ankle joint, without raising their heels, or bending in their hip
199 joints or lumbar part of spine. For each trial of forward and backward leaning phase,
200 quantitative measurements of anterior - posterior sway amplitude (ΔX) were assessed to
201 assess limits of stability of each subject.

202 *2.8. Lower limbs explosive force, anaerobic power and complex reaction time (RT)*
203 *assessment*

204 Optojump Next System (Microgate, Bolzano, Italy) was used for lower limbs
205 explosive force, anaerobic power and complex reaction time (RT) assessment.
206 Optojump is an optical measurement system which consist of two parallel bars- one
207 receiver and one transmitter unit. The transmitter bar contains light emitting diodes
208 which communicate continuously with those on the receiving bar. The system detects
209 any interruptions in communication between the bars and calculates their duration
210 measuring thereby flight and contact times during the performance of a series of jumps
211 with an accuracy of 0.001 of a second. Jump height was then estimated as $9.81 \cdot$flight
212 time$^2 \cdot 8^{-1}$ [21]. Bars were placed approximately 1 m apart and parallel to each other.
213 Lower limbs visual and acoustic complex reaction time test involve performance of a
214 jump starting from squad jump position. It was not specified whether the stimulus were

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215 to be visual or acoustic. The subject must react to both stimuli. The test result was the
216 average response time of three jumps. Evaluation of lower limbs explosive force
217 involve Counter Movement Jump (CMJ). Subjects perform a single jump starting from
218 an upright position with hands on hips and with counter movement. Subjects were
219 instructed to stand straight up for 1 - 2 seconds and jump as high as it is possible. Single
220 jump started with straight legs and performed a natural flexion before take off. Analysis
221 of anaerobic power involves performance of 15 seconds of jumps, subjects were
222 instructed to perform as many highest jumps as possible during a 15 seconds period.
223 Test result was the average jump power.

224 *2.9. Cardiorespiratory efficiency test*

225 Cardiorespiratory fitness was assessed one week apart with aerobic capacity
226 field test - 20 m shuttle run published by Leger et al. [22]. Subjects run as long as
227 possible with continuous movement back and forth between two lines 20 m apart, while
228 keeping the pace with audio signals. The initial speed was 8.5 $km \cdot h^{-1}$ and was increased
229 by 0.5 $km \cdot h^{-1}$ each minute (each stage lasted approximately 1 min). Participants were
230 instructed to run in a straight line, to pivot on completing a shuttle, and to pace
231 themselves in accordance with the audio signals. The subjects were encouraged to keep
232 running as long as possible. The test was finished when the participant failed to reach
233 the end lines concurrent with the audio signals on two consecutive occasions or when
234 the subject stopped because of fatigue. All participants received a comprehensive
235 instruction about the test after which they also practiced the test. They also were
236 instructed to abstain from intense exercises 48 h prior to the test. Tests were carried out
237 under standardized conditions on an outdoor court with hard surface.

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238 The maximal shuttle run speed was used to estimate subjects VO_{2max} using Léger and
239 Gadoury equation for adults: VO_{2max} =-32.678 + 6.592 V, were "V" =last completed
240 stage speed in $km \cdot h^{-1}$ [23].
241

242 3. RESULTS

243 *3.1. Molecular analysis*

244 Performed PCR amplification of DNA from saliva samples showed extensive
245 variability of genotypes in examined people. Insertion allele was identified at size 490
246 kb and Deletion allele at 190 kb in agarose electrophoresis (Fig.1.).
247 Please insert figure 1 about here
248 Fig.1. Photography of PCR product showing the ACE genotype distribution among
249 examined people. Lane: 1, 4, 9, 10 - heterozygous I/D; 2, 5 - homozygous I/I; 3, 6, 7, 8 -
250 homozygous D/D; M - DNA ladder.
251 ACE genotype frequency distribution measured by Pearson`s Chi-square (χ^2)
252 test showed not statistically significant differences between control (CTR) and horse
253 riders (HS) groups. The Chi-square statistic was 1.9973. The p-value was 0.368376. The
254 result was not significant at $0.001 > p < 0.05$. 38% of control group had predisposition to
255 high-performance long-distance sports (I/I genotype), 62% to short-term effort and
256 requiring more muscle strength, efficiency and muscle power (I/D and D/D genotype).
257 Among horse riders sport predispositions were 37% and 63% (respectively) (Fig.2.).
258 Please insert figure 2 about here
259 Fig.2. Angiotensin converting enzyme (ACE) genotype frequency distribution measured
260 by Pearson`s Chi-square (χ2) (A) and measured as a percentage (B) in control (CTR)

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261 and horse riders (HR) group. Insertion/ Insertion genotype (I/I), Insertion/ Deletion
262 genotype (I/D), Deletion/ Deletion genotype (D/D).
263
264 *3.2. Physiological analysis*
265 Due to the small number of male riders for further analysis we took a group that
266 included only women. We also analysed, BMI, %FAT, cardiorespiratory efficiency
267 (VO_{2max}), Complex Reaction Time (CRT), body balance, force and Lower Limbs
268 Explosive Force (LLEF) between both calculated groups. Physiological data was
269 calculated by non-parametric Mann-Whitney test (one-way ANOVA) with Dunnett`s
270 post hoc test. No statistically significant differences were observed between control and
271 horse riders groups in BMI (0.1917), %FAT (0.8927), VO_{2max} (0.4519), CRT (0.1320),
272 balance in posterior (0.1503) and anterior (0.7702) groups and LLEF (0.3971). Only
273 differences at $p < 0.01$ level between force in analysed groups were observed. The p-
274 value was 0.0032.
275 BMI in all was normal and ranged from 18.2 - 25.7 and 17.1 - 23.8 in control
276 and horse riders group respectively. %FAT in control group was between 13.1 - 30.5%
277 and in examined group was between 10.1 - 31.6%. The higher VO_{2max} (49.72 ml·kg-
278 1·min^{-1}) was observed in control group and the lower was 36,54 ml·kg-1·min^{-1}. Horse
279 riders were 49.72 ml·kg^{-1}·min^{-1} and 33.24 ml·kg^{-1}·min^{-1} respectively. Maximum 245.78
280 mm X axis length (anterior) and 175.66 mm of Y axis length (posterior) and minimum
281 105.05 mm and 70.97 mm (posterior) were observed in non-training persons. Second
282 group were maximum 223.66 mm and 152.39 mm and minimum 99.68 mm and 69.83
283 respectively. Complex reaction time oscillated between 0.61 - 0.92 seconds and 0.52 -
284 1.01 seconds in controls and horse riders group respectively. Lower limbs explosive was

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285 between 18.5 - 38.0 cm (CTR) and 19.4 - 34.6 cm (HR). CTR force ranged from 28.97
286 to 42.2 $W \cdot kg^{-1}$ and HR 25.47 - 61.36 $W \cdot kg^{-1}$.
287 Moreover, we have classified horse riders into 2 groups. The first one consisted
288 of athletes training jumps, the second were training dressage. We compared every of
289 each groups to control with one-way ANOVA and Dunnett's a post hoc test. Significant
290 differences were observed only between second study group in force ($p < 0.01$) and CRT
291 ($p < 0.05$) (Fig.3.).
292 Please insert figure 3 about here
293 Fig.3. Force and Complex Reaction Time distribution in horse riders training dressage;
294 ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, no indication - no statistical significance (one-way
295 ANOVA and Dunnett's a post hoc test). Data presented as mean ± SEM; **$P < 0.01$, by
296 non-parametric Mann-Whitney test.
297

298 ## 4. DISCUSSION

299 Polymorphism in angiotensin converting enzyme gene is one of the most
300 frequent examined factors related to sports predispositions. ACE gene is also associated
301 with differences with blood pleasure [24] and fertility [1,25], cardiovascular diseases
302 [1,26–28], neurological diseases like Parkinson`s disease [1,29] or Alzheimer disease
303 [1,30,31]. Since years, many of scientists study the role of Insertion/ Deletion
304 polymorphism and their correlation between genetic and physiological backgrounds in
305 sportspeople. Most research was concentrated on polymorphism in angiotensin
306 converting enzyme (ACE) among athletes [32–36], swimmers [1,37,38], football
307 players [39], volleyball players [24,40] or ball games players [41]. There is no research
308 relating to ACE polymorphism among horse riders.

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309 In this study, we examined angiotensin converting enzyme gene distribution
310 polymorphism in female Polish professional horse riders (jumps and dressage
311 discipline) for the first time. ACE I/I and I/D genotype dominated among examined
312 sportswomen. Moreover, we compared the genomic and physiological results and
313 referred them to control group. D/D and I/I was the most common in control group.
314 Our hypothesis assumed correlation between genotype, sports results and physiological
315 parameters.
316 We demonstrated that female horse riders especially training dressage have
317 higher force and complex reaction time than sedentary people. These physiological
318 results correlated with possession of D allele in genotype which is associated with
319 muscle strength, efficiency and muscle power.
320 Genetic background of sport predisposition is relatively easy to verify by
321 molecular biology technics. ACE polymorphisms in combination with physiological
322 tests results, should be taken into account when deciding to train sports discipline in
323 order to achieve success in sport. Nowadays, some medical facilities offer a genetic
324 testing detecting possible polymorphisms within genes: ACTN3, ACE, HIF1A and
325 EPOR what correlate with physiological predispositions to sport.
326
327 5. CONCLUSION
328 Our hypothesis assumed correlation between genotype, sports results and
329 physiological parameters. We demonstrated that female HR especially training dressage
330 have higher anaerobic power and CRT than CTR. These physiological results correlated
331 with possession of D allele in genotype which is associated with muscle strength,
332 efficiency and muscle power. In conclusion, ACE polymorphisms combine

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333 physiological tests results, should be taken into account when deciding to train sports
334 discipline to achieve success in sport.
335
336
337 Acknowledgements

338 The authors would like to thank [redacted]
339 [redacted] for the help in performing physiological tests, [redacted]
340 [redacted] for statistical help.
341 This work was supported by the [redacted] from the founds of Student
342 Research Society of Diagnostics in Sport and Health Training.
343
344 Disclosure statement
345 The authors declare no conflict of interest.
346
347 Funding

348 This work was supported by the University of Rzeszów from the founds of
349 Student Research Society of Diagnostics in Sport and Health Training.
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482 Figure Legends

483

484 Fig.1. Photography of PCR product showing the ACE genotype distribution among
485 examined people. Lane: 1, 4, 9, 10 - heterozygous I/D; 2, 5 - homozygous I/I; 3, 6, 7, 8 -
486 homozygous D/D; M - DNA ladder

487

488 Fig.2. Angiotensin converting enzyme (ACE) genotype frequency distribution measured
489 by Pearson`s Chi-square ($\chi 2$) (A) and measured as a percentage (B) in control (CTR)
490 and horse riders (HR) group. Insertion/ Insertion genotype (I/I), Insertion/ Deletion
491 genotype (I/D), Deletion/ Deletion genotype (D/D).

492

493 Fig.3. Force and Complex Reaction Time distribution in horse riders training dressage;
494 ***p <0.001, **p <0.01, *p <0.05, no indication - no statistical significance (one-way
495 ANOVA and Dunnett's a post hoc test). Data presented as mean ± SEM; **P <0.01, by
496 non-parametric Mann-Whitney test.

497

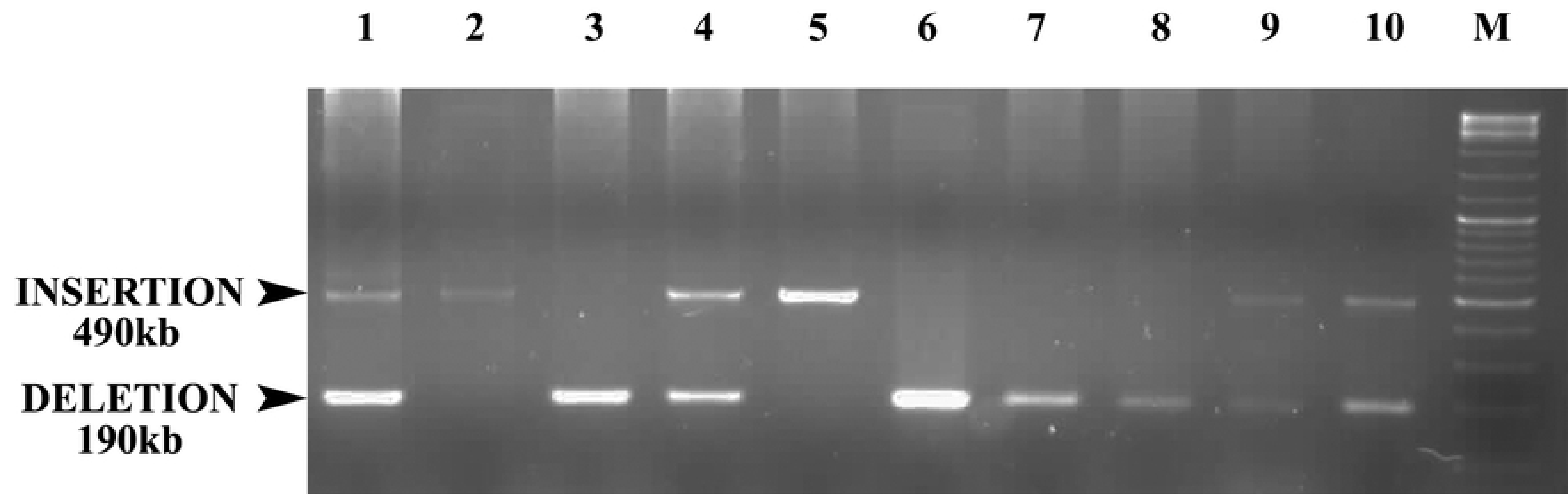

Figure 1

GROUP	D/D	I/I	I/D	Total
CTR	8	8	5	21
HR	4	7	8	19
All Groups	12	15	13	40

A

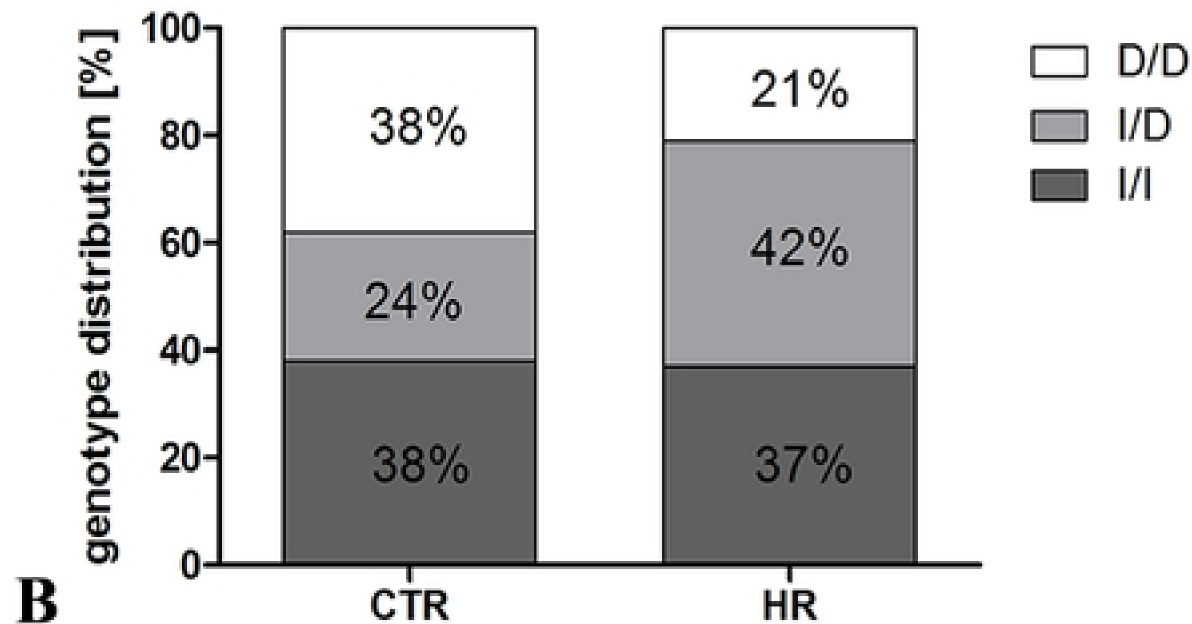

Figure 2

FORCE

**

Counter Movement Jump [W/kg]

80
60
40
20
0

CTR DRESSAGE

CRT

*

Complex Reaction Time [s]

1.1
1.0
0.9
0.8
0.7
0.6
0.5

CTR DRESSAGE

Figure 3