Physiology & Behavior 170 (2017) 141–150

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Circulating levels of endocannabinoids respond acutely to voluntary exercise, are altered in mice selectively bred for high voluntary wheel running, and differ between the sexes

Zoe Thompson [a], Donovan Argueta [b], Theodore Garland Jr. [c,*], Nicholas DiPatrizio [b]

[a] Neuroscience Graduate Program, University of California, Riverside, CA 92521, USA
[b] Division of Biomedical Sciences, School of Medicine, University of California, Riverside, CA 92521, USA
[c] Department of Biology, University of California, Riverside, CA 92521, USA

HIGHLIGHTS

- The endocannabinoid system is involved in regulation of voluntary locomotion.
- We studied 4 replicate lines of high runner (HR) mice bred for wheel exercise.
- Blood was collected during peak running on night 6 of wheel access.
- 2-AG and/or AEA levels differed between the sexes and between HR and control lines.
- Levels were also affected by wheel access (training effect) and acutely by activity.

ARTICLE INFO

Article history:
Received 23 August 2016
Received in revised form 7 November 2016
Accepted 22 November 2016
Available online 22 December 2016

Keywords:
Artificial selection
Locomotor activity
Reward
Sex differences
Training effect
Voluntary exercise

ABSTRACT

The endocannabinoid system serves many physiological roles, including in the regulation of energy balance, food reward, and voluntary locomotion. Signaling at the cannabinoid type 1 receptor has been specifically implicated in motivation for rodent voluntary exercise on wheels. We studied four replicate lines of high runner (HR) mice that have been selectively bred for 81 generations based on average number of wheel revolutions on days five and six of a six-day period of wheel access. Four additional replicate lines are bred without regard to wheel running, and serve as controls (C) for random genetic effects that may cause divergence among lines. On average, mice from HR lines voluntarily run on wheels three times more than C mice on a daily basis. We tested the general hypothesis that circulating levels of endocannabinoids (i.e., 2-arachidonoylglycerol [2-AG] and anandamide [AEA]) differ between HR and C mice in a sex-specific manner. Fifty male and 50 female mice were allowed access to wheels for six days, while another 50 males and 50 females were kept without access to wheels (half HR, half C for all groups). Blood was collected by cardiac puncture during the time of peak running on the sixth night of wheel access or no wheel access, and later analyzed for 2-AG and AEA content by ultra-performance liquid chromatography coupled to tandem mass spectrometry. We observed a significant three-way interaction among sex, linetype, and wheel access for 2-AG concentrations, with females generally having lower levels than males and wheel access lowering 2-AG levels in some but not all subgroups. The number of wheel revolutions in the minutes or hours immediately prior to sampling did not quantitatively predict plasma 2-AG levels within groups. We also observed a trend for a linetype-by-wheel access interaction for AEA levels, with wheel access lowering plasma concentrations of AEA in HR mice, while raising them in C mice. In addition, females tended to have higher AEA concentrations than males. For mice housed with wheels, the amount of running during the 30 min before sampling was a significant positive predictor of plasma AEA within groups, and HR mice had significantly lower levels of AEA than C mice. Our results suggest that voluntary exercise alters circulating levels of endocannabinoids, and further demonstrate that selective breeding for voluntary exercise is associated with evolutionary changes in the endocannabinoid system.

1. Introduction

The endocannabinoid system is involved in a variety of physiological processes, including regulation of motor behavior [10]. Motor behavior

* Corresponding author.
E-mail address: tgarland@ucr.edu (T. Garland).

http://dx.doi.org/10.1016/j.physbeh.2016.11.041
0031-9384/

is tremendously diverse, encompassing voluntary exercise, consummatory behaviors, spontaneous physical activity (SPA) of various types, including "fidgeting" [22], and performance during measures of forced-exercise capacity [6]. Studies of cannabinoid effects on motor behavior have utilized a variety of approaches, involving variation in testing apparatus, length of observation period, time of day, etc., and, not surprisingly, this has led to conflicting results. Depending on the apparatus and length of test, these studies may be gauging multiple aspects of motor behavior, including, in some cases, reactivity to a novel environment. Generally, systemic administration of cannabinoids leads to a decrease in activity. In male rats, for example, doses of Δ-9-tetrahydrocannabinol higher than 1 mg/kg caused a decrease in ambulation and rearing as measured in a five-minute novel open-field test [33]. However, another study, also usingΔ-9-tetrahydrocannabinol in male rats, reported a triphasic effect, where very low and very high doses (0.2 mg/kg and 2.5 mg/kg, respectively) reduced the number of photobeam breaks produced in either a horizontal or vertical direction, and moderate doses (1–2 mg/kg) increased the number of beam breaks as measured over 1 h in an activity chamber [56].

Cannabinoids activate the cannabinoid type-1 (CB_1) and type-2 receptor (CB_2), the former being found in high density in brain areas that control movement (basal ganglia, substantia nigra, etc., [65]), including those involved in both spontaneous physical activity and voluntary exercise [22]. Recent research suggests that the CB_1 receptor is specifically involved in voluntary exercise. For example, male CB_1 receptor knockout mice exhibit a 30–40% reduction in voluntary wheel running, but no change in number of horizontal squares crossed during 5 min in a large, dimly-illuminated, novel "activity cage" [12]. In a subsequent study, in which the CB_1 receptor was deleted only from brain GABAergic neurons, mice showed a 25–30% decrease in wheel running and no difference in habituated locomotor activity measured over five days in a cage with infrared sensors to detect horizontal beam breaks [13]. Previous studies of CB_1 knockout mice, however, have shown differences in other tests of locomotor activity. CB_1 knockout mice spend more time immobile in a test of catalepsy, and showed fewer beam breaks during an open-field test (with undescribed parameters) [71]. A separate experiment found that CB_1 knockout mice had a significant decrease of ambulatory movements, as measured in a dimly-lit box with photocells placed to measure both horizontal and vertical movements (in this experiment, animals were measured for 15 min on several different days after being habituated to the cage) [43].

Limited evidence suggests that the endocannabinoid system may specifically affect motivation for voluntary exercise. The two primary endocannabinoids in humans and other mammals are 2-arachidonylglycerol (2-AG) and anandamide (AEA). Both 2-AG and AEA can cross the blood-brain barrier, bind to the CB_1 receptor, and cause dopamine release in areas involved in reward signaling (such as the nucleus accumbens, reviewed in [19]). After rats were trained to press a door to unlock wheel access (as a reward), systemic administration of 2-AG reduced the number of times that obese rats would press the door to gain wheel access before giving up, and also reduced the revolutions run once the rats were in the wheel [both obese and lean individuals [59]]. In a similar study, rimonabant (a CB_1 antagonist/inverse agonist) caused a similar reduction in number of door-presses prior to giving up, but did not affect number of revolutions run once rats did gain wheel access [49]. In addition, signaling through the CB_1 receptor produces analgesia in both peripheral and central sites (reviewed in [10]). Analgesia might interact with motivation per se by reducing pain that could occur during exercise (e.g., see [17,40]).

The endocannabinoid system is also activated by exercise. Several studies have examined how circulating levels of both 2-AG and AEA respond to exercise. In general, exercise increases levels of AEA in the blood, but does not seem to affect levels of 2-AG [15,29,47,48,60]. Most previous studies of circulating endocannabinoid levels have involved forced exercise, which may cause "stress" relative to voluntary exercise, or confound the effects of stress with exercise [2,24]. Arguably, no (ethical) study of humans involves "forced" exercise, but an examination of the studies cited above shows that subjects were not allowed to choose the quantity, length of time, and/or the speed at which they exercised (but see [15]), which may cause stress in either the psychological or physical sense. Voluntary exercise has been examined in two animal studies. One small-scale study (n = 3 per group; sex not reported) measured mice after they were allowed wheel access for 3 h/day for 8 days, with samples taken after 30 min of running. Plasma levels of both endocannabinoids tended to be lower in the running mice as compared with mice housed with locked wheels, although the differences were non-significant [5]. One additional study of rats used voluntary exercise, and although they did not measure circulating

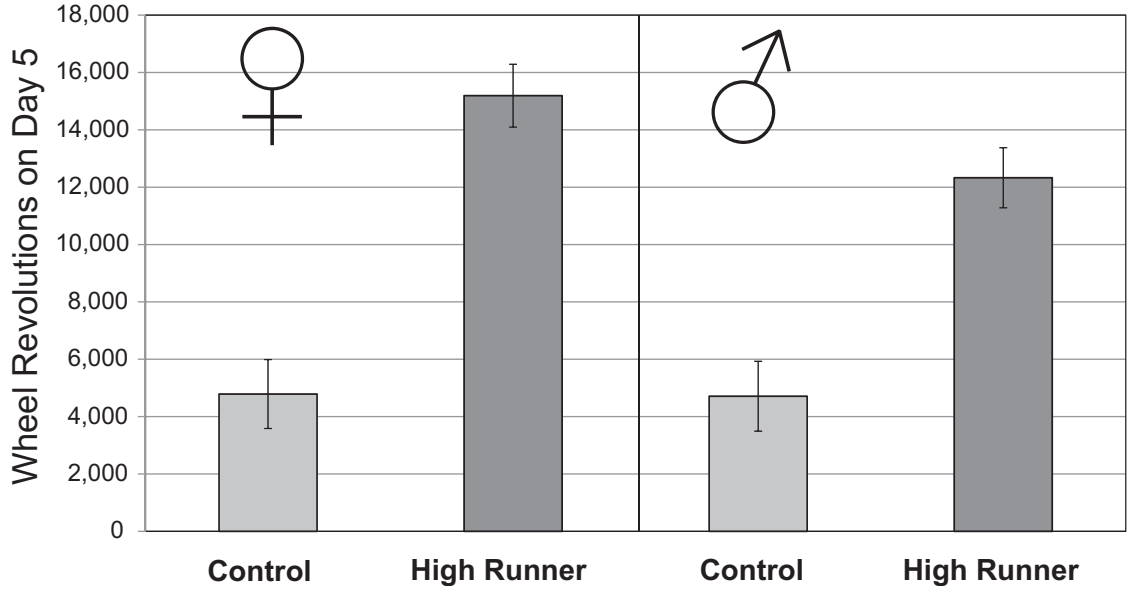


Fig. 1. Wheel running on day five of experiment. Values are LS means ± standard error from SAS Proc Mixed. N = 90.

endocannabinoids, they did find an increase in AEA in the hippocampus after 8 days of wheel access [30].

The endocannabinoid system may also be altered in response to selective breeding for voluntary exercise behavior. An ongoing artificial selection experiment (currently in generation 81) has produced four replicate high-runner (HR) lines while also maintaining four non-selected control (C) lines [23,62,68]. In the routine breeding protocol, mice are allowed access to wheels for six days as young adults, and mice in the HR lines are selected to breed based on the average number of wheel revolutions on days five and six. Mice in the control lines are bred without regard to how much they run. Mice from the HR lines run about three times as much on a daily basis as compared with C mice, which they accomplish primarily by running faster [4,21,25]. In addition, female mice run more than male mice, which they also accomplish by running faster [38]. When given a systemic injection of a CB_1 receptor antagonist (SR141716; rimonabant), HR female mice decreased their running more than C female mice over the next hour, which was accomplished by a decrease in total revolutions, average speed and maximum speed, but not amount of time spent running [34]. Male HR and C mice did not show a differential response. In contrast, when given a systemic injection of a CB_1 receptor agonist (WIN 55,212-2), both female and male HR mice showed a differential decrease in running (compared to C mice; [35]. For 2 h after injection, female HR mice had a reduction in total revolutions, average speed, and maximum speed. In the second hour they also decreased their time spent running. Male HR mice showed decreased total revolutions, average speed and maximum speed during the entire 2 h after injection. As a CB_1 receptor agonist and antagonist both decreased running in HR mice in different ways in a sex-specific manner, it is difficult to infer from these studies alone precisely how the endocannabinoid system has changed in response to selective breeding.

One possible cause of differential responses to pharmacological manipulation could be differences in circulating levels of endocannabinoids (alternatively, tissue-specific receptor densities or receptor sensitivity might have evolved). In addition, circulating concentrations of endocannabinoids might show different quantitative responses to exercise, either between the sexes or potentially between HR and control lines of mice. The purpose of the present study was, therefore, to measure circulating endocannabinoid levels in mice given access to wheels, as compared with those housed in standard cages. We studied both male and female HR and C mice given access to wheels for six days (as used in the regular selection protocol), or housed without wheels for six days, and sampled during the time of peak running. We expected that circulating levels of endocannabinoids (2-AG and AEA) would differ between HR and C mice, possibly in a sex-specific manner. We also predicted that the amount of wheel running might be a quantitative predictor of circulating endocannabinoid levels. We also tested the relationship between the plasma concentrations of 2-AG and AEA at the level of individual variation within groups as well as among the average values for the eight subgroups (combinations of sex, linetype, and wheel access).

2. Materials & procedures

2.1. Ethical approval

All experimental procedures were approved by the UC Riverside Institutional Animal Care and Use Committee.

2.2. Experimental animals

Mice were taken from a long-term artificial selection experiment on voluntary wheel running that was started in 1993 [62]. Originally, outbred Hsd:ICR mice (*Mus domesticus*) were obtained from Harlan Sprague Dawley (Indianapolis, Indiana, USA). These mice were randomly split into eight closed lines, with four designated to become "high runner" (HR) and four designated as "control" (C) lines. All mice are allowed access to wheels for six days and HR mice are chosen to breed based on their average number of wheel revolutions on days five and six. Control mice are bred without regard to wheel running. HR mice now run on average three times as much as C mice do, primarily by running faster.

For the present study, 50 male and 50 female mice from generation 74 (half HR and half C) were allowed access to wheels for six days, while another 50 males and 50 females (also half HR and half C) were kept without access to wheels. Wheel revolutions for mice with wheels were recorded for 23 h per day, and home-cage activity was recorded for all mice. On the sixth day of the study, animals were anesthetized with isoflurane and blood samples were taken by cardiac puncture. Animals were on a reversed photoperiod, with lights off from 7 am to 7 pm, so that sampling could occur during the time of peak wheel running, which starts approximately 2 h after lights are turned off [24,25,41,42]. Sampling occurred from ~9 am to 1 pm (from 2 h after lights off to 6 h after lights off). The age range at the time of sampling was 71–91 days old.

Mini-muscle status was determined for each mouse by dissection and weighing of the triceps surae muscles at the end of the experiment. The "mini-muscle" phenotype is caused by a recessive allele that, when homozygous, reduces triceps surae and total hindlimb muscle mass by ~50% [20]. However, the mass-specific aerobic capacity of the muscles

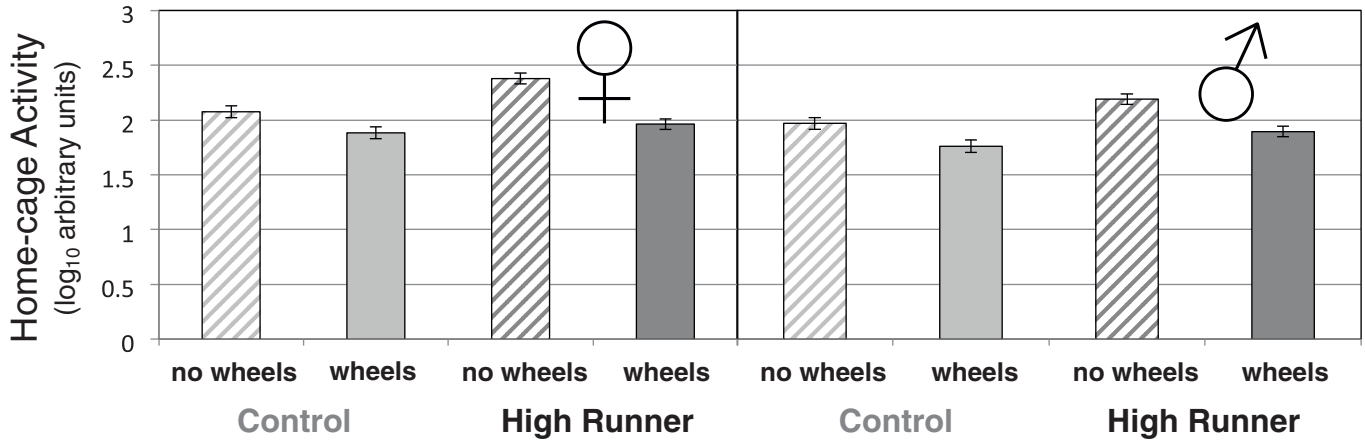


Fig. 2. HCA on day 5 of experiment. See Table 1 for statistical results. Mice with wheel access always had reduced HCA ($p = 0.0001$), this reduction was greater in HR than in C mice (linetype by wheel access interaction, $p = 0.0558$), females always had higher HCA than males ($p = 0.0047$), and HR mice always had higher HCA than C mice ($p = 0.007$). Values are LS means (log_{10} transformed) ± standard error from SAS Proc Mixed. N = 190.

Table 1
Three-way analysis of covariance of home-cage activity on day 5 (log_{10} transformed) (N = 190). See Fig. 2 for graphical representation of adjusted group means.

Effect	d.f.	F	P
Sex	1,6	19.22	0.0047
Linetype	1,6	16.08	0.0070
Wheel access	1,6	73.52	0.0001
Sex * linetype	1,6	0.07	0.8043
Sex * wheel access	1,6	0.81	0.4029
Linetype * wheel access	1,6	5.60	0.0558
Sex * linetype * wheel access	1,6	1.49	0.2681
Mini-muscle	1,155	5.72	0.0180
Age	1,155	0.53	0.4695
HCA sensor sensitivity	1,155	0.62	0.4306

is approximately doubled [31]. Mini-muscle individuals tend to run faster, but for fewer minutes per day, as compared with unaffected individuals [20,27,31,36,63,64]. In addition, mini-muscle individuals have larger internal organs than normal-muscled mice (liver, kidneys, and heart ventricles: [20,63]). As the mini-muscle phenotype clearly has pleiotropic effects, mini-muscle status was used as a cofactor in all analyses.

2.3. Wheel running

Mice were housed individually during the experimental period and half were provided with cages with a hole for wheel access. The wheels used were the same as in the regular selection protocol [62] – Wahman-type activity wheels with a circumference of 1.12 m. Wheel revolutions are recorded automatically for 23 h per day (1 h is used to download data and check mice), and a measure of wheel freeness was used as a covariate in all analyses of wheel running. We did not choose to provide locked wheels for the mice without wheel access, as we have shown that when HR mice are housed with locked wheels, they climb more than do mice from the non-selected Control lines [39], so locked wheels, unfortunately, provide more than just "environmental enrichment."

2.4. Home-cage activity

Home-cage activity (HCA) was measured using passive infrared sensors that detect motion. These sensors give readings 3 times per second – either 0 (no movement) or 1 (movement). Readings are then averaged over a one-minute interval and reported using arbitrary units. We used a similar setup and calibration procedure as previously reported [1,7]. HCA was measured for 23 h per day and all analyses were performed using a measure of sensor sensitivity as a covariate [1,7].

2.5. Measurement of plasma 2-AG and AEA

Isoflurane was used to anesthetize animals prior to tissue harvest. Blood was collected by cardiac puncture and stored in EDTA-lined tubes on ice, and then plasma was obtained by centrifugation (1500 g for 10 min, maintained at 4 °C). All samples were stored at −80 °C until processing. One hundred microliters (μL) of plasma was used for extraction of lipids in one milliliter (mL) of methanol containing the following internal standards: [$^2H_5$]-2-AG and [$^2H_4$]-AEA (Cayman Chemical, Ann Arbor, MI, USA). Lipids were extracted with chloroform (2.0 mL) and washed with 0.9% saline (0.9 mL). Organic phases were collected and fractionated by open-bed silica gel column chromatography as previously described [11]. Eluted fractions were dried under N_2 and reconstituted in 0.1 mL of methanol:chloroform (9:1) for liquid chromatography/tandem mass spectrometry (LC/MS/MS) analyses.

Lipids were analyzed using a Waters Acquity I-Class Ultra Performance Liquid Chromatography system coupled to a Waters TQS-micro Triple Quadrupole Mass Spectrometer. Lipids were separated using an Acquity UPLC BEH C_{18} column (50 × 2.1 mm; i.d. 1.7 μm), eluted by a gradient of methanol (0.25% acetic acid, 5 millimolar [mM] ammonium acetate) in water (0.25% acetic acid, 5 mM ammonium acetate) (from 80 to 100% methanol in 2.5 min, 100% 2.5–3.0 min, 100–80% 3.0–3.1 min) at a flow rate of 0.4 mL/min. Column temperature was kept at 40 °C and samples were maintained in the sample manager at 10 °C. Argon was used as collision gas. 2-AG, [$^2H_5$] 2-AG, AEA, and [$^2H_4$]-AEA were identified in the positive ionization mode, based on their retention times and MS^2 properties, using authentic standards (Cayman Chemical) as references. Multiple reaction monitoring was used to acquire full-scan tandem MS spectra of selected ions. Extracted ion chromatograms were used to quantify 2-AG (m/z = 379.3 > 287.3), AEA (m/z = 348.3 > 62.04), and [$^2H_5$] 2-AG (m/z = 384.3 > 93.4) and [$^2H_4$]-AEA (m/z = 352.3 > 66.11), which were used as internal standards.

2.6. Statistical analyses

Following numerous previous studies of these eight lines of mice [50,62], plasma endocannabinoid concentrations were analyzed by nested analysis of covariance (ANCOVA), with line nested within linetype (HR vs C) as a random effect, and with covariates of age and time of day that plasma sampling occurred (SAS Procedure Mixed).

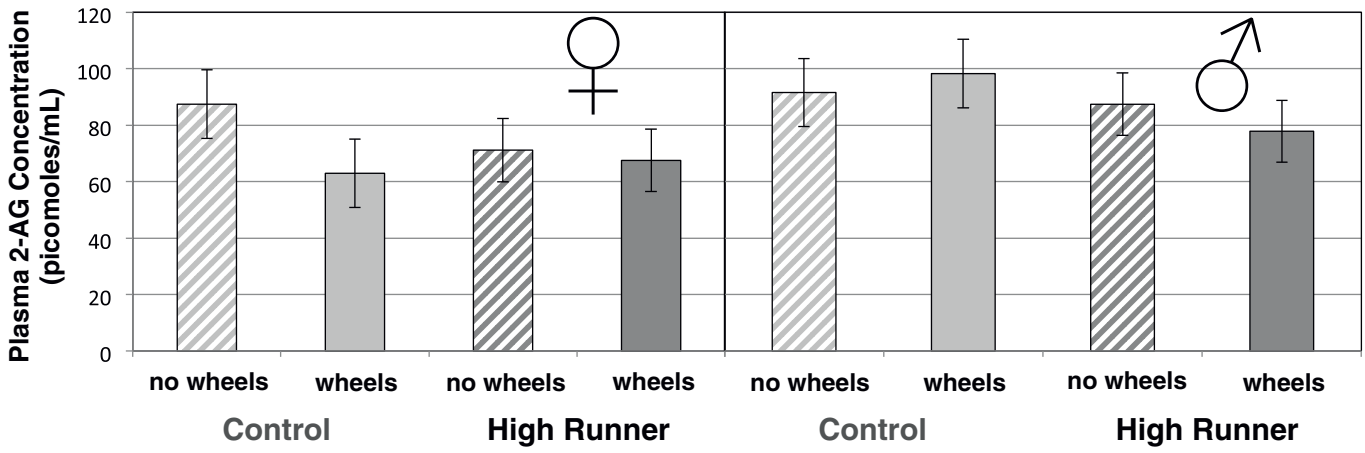


Fig. 3. Levels of 2-AG in mouse plasma collected during peak activity on the 6th night of wheel running. See Table 2 for statistical results. The three-way interaction among sex, linetype, and wheel access was statistically significant (p = 0.0408), with females also having lower levels than males (p = 0.0265). Values are LS means ± standard error from SAS Proc Mixed. N = 189.

For the individuals with wheel access, we repeated the foregoing analyses with amount of wheel running (revolutions/unit time) as a covariate. Although we expected a possible quantitative relationship with the amount of exercise prior to sampling, we could not predict the precise nature of this relationship. Therefore, we computed the number of wheel revolutions in each minute before plasma sampling, from 1 to 10 min before, and then in 10-min bins from 10 to 120 min before sampling. We fitted models using each of these alternative covariates and examined the restricted maximum log-likelihood (REML) values from each analysis to determine which time period of summed running effort provided the best fit. For wheel running, the number of revolutions in the previous 30 min before plasma sampling provided the best fit and was used in all analyses with wheel running as a covariate. For consistency, we also used the amount of home-cage activity in the previous 30 min as a covariate in certain analyses.

Dependent variables were transformed when needed to improve the normality of residuals. Residuals that were >3 standard deviations above or below the mean were excluded from analyses. Main effects were considered statistically significant when $p \leq 0.05$. Interactions of main effects were considered significant when $p \leq 0.10$ because the power to detect interactions is generally substantially lower than for detecting main effects in ANOVAs [66,67]. Least squares means and associated standard errors from SAS Procedure Mixed are presented in figures and were inspected to determine the directions of main effects and interactions. In addition, for some pairwise comparisons of subgroup means, we refer to differences of least squares means from Proc Mixed, unadjusted for multiple comparisons.

3. Results

3.1. Wheel running

Day five of the wheel access period was analyzed, as mice were removed and blood collected in the middle of night six (Fig. 1). As expected, HR mice ran more than C mice ($p = 0.0004$). Neither the effect of sex ($p = 0.1431$) nor the sex by linetype interaction ($p = 0.1576$) was significant. Wheel freeness, mini-muscle status, and age were not statistically significant predictors of wheel running (results not shown).

3.2. Home-cage activity

Data from day five of the experiment were analyzed, as day six was interrupted when mice were removed from cages for collection of blood. Values from each minute were summed over 23 h, then $\log_{10}$ transformed to improve normality of the residuals. In the three-way analysis of covariance (Fig. 2, Table 1), mice with wheel access always had reduced HCA ($p = 0.0001$), this reduction was greater in HR than in C mice (wheel access by linetype interaction, $p = 0.0558$), females always had higher HCA than males ($p = 0.0047$), and HR mice always had higher HCA than C mice ($p = 0.007$). In addition, mice with the mini-muscle trait had lower HCA than those that did not ($p = 0.018$). Age and a measure of sensor sensitivity were not significant predictors of home-cage activity. We also tested body mass as an additional covariate, but it had no statistical effect ($p > 0.50$) and caused little change in the significance levels of the other factors and covariates (results not shown).

3.3. Plasma 2-AG concentrations

Females had lower levels of 2-AG than males in all four experimental groups, and the main effect of sex was significant (Table 2: $p = 0.0265$). Fig. 3 illustrates the significant three-way interaction among sex, linetype, and wheel access ($p = 0.0408$). Levels of 2-AG are lower in mice with wheel access, except for C males. However, when examining the pairwise comparisons, 2-AG levels were significantly lower for mice with wheel access only for the C female group ($p = 0.0301$). The main effect of linetype was not significant ($p = 0.4604$).

Table 2
Three-way analysis of covariance of plasma concentration of 2-AG (N = 189). See Fig. 3 for graphical representation of adjusted group means.

Effect	d.f.	F	P
Sex	1,6	8.56	0.0265
Linetype	1,6	0.62	0.4604
Wheel access	1,6	2.42	0.1711
Sex * wheel access	1,6	3.04	0.1316
Sex * linetype	1,6	0.33	0.5883
Linetype * wheel access	1,6	0.05	0.8244
Sex * linetype * wheel access	1,6	6.75	0.0408
Mini-muscle	1,147	0.71	0.4021
Age	1,147	11.34	0.0010
Time of day	1,147	8.23	0.0047

Separate analyses of mice with (N = 92) and without (N = 94) wheels (not including amount of physical activity as covariates) indicated that females had significantly lower levels of 2-AG only when they had wheel access ($p = 0.0169$ for mice with wheels, $p = 0.1235$ for mice without wheels).

Mini-muscle status was not a significant factor for any analyses, but both time of day that plasma sampling occurred and age at time of plasma sampling were significant in the overall analysis and the analysis of mice without wheel access. Only time of day was significant for the analysis of mice with wheels. Plasma 2-AG concentrations tended to be higher later in the sampling period, which ranged from ~9 am to 1 pm. Age was positively correlated with 2-AG levels (age range was 71–91 days old).

3.4. Plasma 2-AG concentrations with wheel running as a covariate

The first analysis was restricted to mice housed with wheel access. The amount of wheel running (transformed to the 0.4 power to reduce positive skew) in the previous 30 min (before the cardiac puncture and plasma sample) was added as a covariate to the statistical models indicated above. Amount of prior running was not a significant predictor of 2-AG levels, nor was the amount of home-cage activity during this period (also transformed to the 0.4 power), but females still had significantly lower 2-AG concentrations than males ($p = 0.0274$), with no difference between HR and Control lines (Table 3). Age was still a significant positive predictor, but time and mini-muscle status were not.

To test whether acute physical activity had a different effect than the 5 days of wheel access, we reran the analysis with the amount of wheel running and home-cage activity in the previous 30 min as covariates, but this time also including the mice housed without wheels and assigning values of zero for their wheel running (as in [7]). In this analysis (N = 186), neither measure of physical activity was a significant predictor of 2-AG concentrations (both $p > 0.5$), and the effects of sex ($p = 0.0252$) and the 3-way interaction ($p = 0.0544$) remained similar to those reported in Table 2.

Table 3
Plasma concentration of 2-AG with wheel running as a covariate (N = 92).

Effect	d.f.	F	P
Sex	1,6	8.39	0.0274
Linetype	1,6	0.24	0.6446
Sex * linetype	1,6	2.41	0.1713
Mini-muscle	1,64	3.77	0.0567
Age	1,64	19.12	<0.0001
Time of day	1,64	3.30	0.0742
Running in previous 30 min	1,64	0.57	0.4518
HCA in previous 30 min	1,64	1.40	0.2405

Table 4
Three-way analysis of covariance of plasma concentration of AEA (N = 185). See Fig. 4 for graphical representation of adjusted group means.

Effect	d.f.	F	P
Sex	1,6	5.36	0.0599
Linetype	1,6	2.29	0.1809
Wheel access	1,6	0.92	0.3741
Sex * wheel access	1,6	0.04	0.8550
Sex * linetype	1,6	0.12	0.7451
Linetype * wheel access	1,6	5.20	0.0628
Sex * linetype * wheel access	1,6	0.86	0.3886
Mini-muscle	1,143	1.35	0.2476
Age	1,143	0.00	0.9484
Time of day	1,143	82.97	<0.0001

3.5. Plasma AEA concentrations

Females tended to have higher levels of AEA than males ($p = 0.0599$, Fig. 4, Table 4). A linetype by wheel access interaction ($p = 0.0628$) indicated that wheel access tended to increase levels of AEA in C mice (and decrease levels in HR males).

Separate analyses of mice with and without wheels revealed a trend for females to have higher levels only when they were housed without wheels ($p = 0.0683$ without wheels, $p = 0.1637$ with wheels). In addition, there was a trend for HR mice to have lower levels when housed with wheels ($p = 0.0883$ with wheels, $p = 0.8950$ without wheels).

Mini-muscle status and age at time of plasma sampling were not significant factors for any analyses. Time of day that plasma sampling occurred was significant for all three analyses (overall, mice with wheels, mice without wheels), indicating reduced values later in the sampling period.

3.6. Plasma AEA concentrations with wheel running as a covariate

The amount of wheel running (raised to the 0.4 power) was a highly significant positive predictor ($p = 0.0043$) of the amount of plasma AEA (Table 5, Fig. 5), home-cage activity was a negative predictor (also raised to the 0.4 power, $p = 0.0383$), and HR mice had significantly lower plasma AEA concentrations than C mice ($p = 0.0235$), with no difference between the sexes. Both age (positive) at the time of plasma sampling and time of day that sampling occurred (negative) were also significant predictors of plasma AEA.

For the analysis including mice housed without wheel access (assigning them values of zero revolutions, as also done for plasma 2-AG concentrations - see Section 3.4 above), the amount of wheel running was still a significant positive predictor of AEA concentrations ($p = 0.0044$), home-cage activity became non-significant ($p = 0.1954$, negative effect), the effects of sex ($p = 0.0548$) and wheel access ($p = 0.0503$) were marginally non-significant, and the wheel access by linetype interaction was significant ($p = 0.0110$). In this analysis, wheel access lowered AEA levels in all mice, but much more so in HR lines than in Control lines (Table 6, Fig. 6).

Table 5
Plasma concentration of AEA with wheel running as a covariate (N = 89; see also Fig. 5)..

Effect	d.f.	F	P
Sex	1,6	2.97	0.1354
Linetype	1,6	9.11	0.0235
Sex * linetype	1,6	0.33	0.5859
Mini	1,61	0.02	0.9029
Age	1,61	4.89	0.0307
Time of day	1,61	33.95	<0.0001
Running in previous 30 min	1,61	8.79	0.0043
HCA in previous 30 min	1,61	4.49	0.0383

Table 6
Analysis of covariance of plasma concentration of AEA with physical activity covariates (N = 183). See Fig. 6 for graph of adjusted group means.

Effect	d.f.	F	P
Sex	1,6	5.66	0.0548
Linetype	1,6	3.93	0.0946
Wheel access	1,6	5.97	0.0503
Sex * wheel access	1,6	0.01	0.9080
Sex * linetype	1,6	0.02	0.8906
Linetype * wheel access	1,6	13.17	0.0110
Sex * linetype * wheel access	1,6	0.09	0.7796
Mini-muscle	1,139	1.08	0.3011
Age	1,139	1.01	0.3175
Time of Day	1,139	72.65	<0.0001
Running in previous 30 min	1,139	8.38	0.0044
HCA in previous 30 min	1,139	1.69	0.1954

3.7. Relationship between plasma 2-AG and AEA concentrations

We examined the relationship between circulating levels of these two endocannabinoids in several ways, as also shown in the online Supplementary data. Overall, these analyses indicate that 2-AG and AEA concentrations tend to be positively related.

First, considering the raw values for all 191 mice, the Pearson correlation was 0.175, 2-tailed $p = 0.0155$. Second, we analyzed the correlation within each of the eight subgroups (N = 23–24 per group), and found values ranging from −0.07 to +0.54, with only one of the eight correlations differing significantly from zero by a 2-tailed test (Control females housed with wheel access r = 0.535, N = 24, $p = 0.007$). The mean value of the eight correlations was 0.207 with a standard error of 0.0815 (Supplementary data), suggesting an overall positive correlation, on average. Third, we computed simple mean values for each of the eight subgroups (linetype by sex by wheel access) and found no statistically significant correlation (r = −0.199, $p = 0.636$).

Fourth, we used the model presented in Table 2 for 2-AG, and added concentrations of AEA as an additional covariate. In this model, AEA levels were a highly significant positive predictor of 2-AG levels ($p < 0.0001$). We then performed the reciprocal analysis, adding 2-AG levels as an additional independent variable for the model presented in Table 4, and found the same thing: 2-AG levels were a highly significant positive predictor of AEA levels ($p < 0.0001$).

Fifth, we repeated this procedure for the model shown in Table 3 for 2-AG, and found that AEA levels were again a highly significant positive predictor of 2-AG ($p < 0.0001$). We did the same for the model in Table 5 for AEA, and again found that 2-AG levels were a positive predictor ($p = 0.0003$).

Sixth, we compared residuals from the models shown in Tables 2 and 4, and found the following correlation: r = 0.347, N = 184, $p = 0.000001$. Seventh, we compared residuals from Tables 3 and 5, and found r = 0.430, N = 89, $p = 0.000026$.

4. Discussion

Much remains to be understood regarding the role of the endocannabinoid system in exercise behavior and physiology. In the present study, we used selectively bred lines of mice to test four specific hypotheses regarding circulating levels of endocannabinoids, i.e., that they would (i) differ between the sexes, (ii) be affected by selective breeding for high levels of voluntary exercise on wheels, (iii) change following six days of wheel access, and (iv) be affected by the acute amount of wheel running or home-cage activity immediately prior to sampling. We measured plasma concentrations of two endocannabinoids, 2-AG and AEA, during the time of normal peak wheel running. Our results indicate that circulating levels of 2-AG and AEA differ (i) between sexes, (ii) between selectively bred HR and non-selected C lines of mice, (iii) are affected by six days of wheel access (training effect), and (iv) are affected

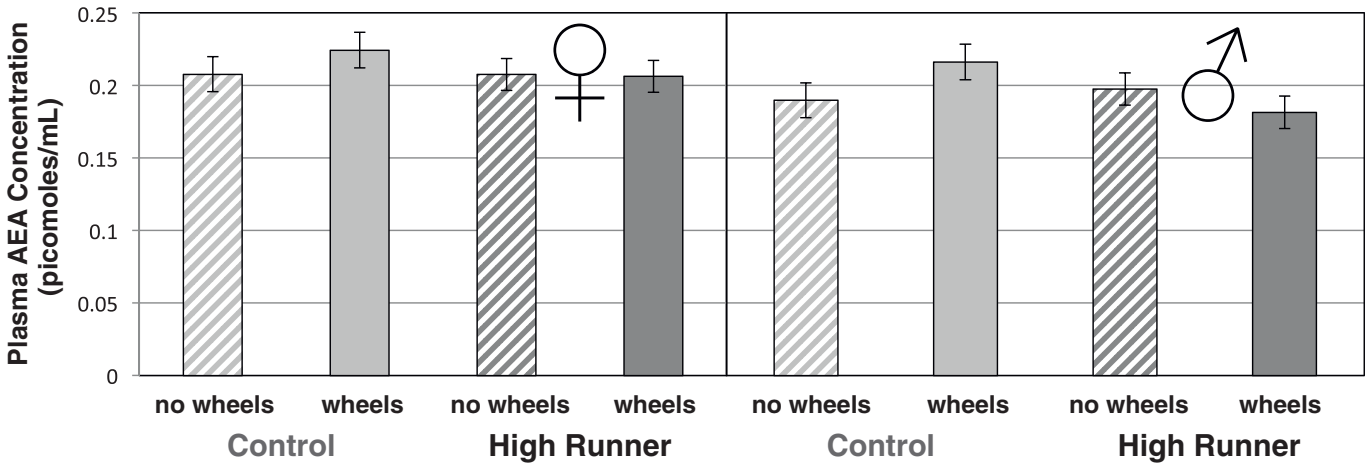


Fig. 4. Levels of AEA in mouse plasma collected during peak activity on the 6th night of wheel running. See Table 4 for statistical results, which indicated a linetype by wheel access interaction ($p = 0.0628$). Values are LS means ± standard error from SAS Proc Mixed. N = 185.

acutely by physical activity. Furthermore, some of these effects differ between 2-AG and AEA. Finally, we tested the relationship between the plasma concentrations of 2-AG and AEA at the level of individual variation within groups as well as among the average values for the eight subgroups, and found evidence that the two endocannabinoids tend to covary positively.

4.1. Physical activity

As expected from numerous previous studies (e.g., [7,25,42,50,53]), HR mice of both sexes ran much more than Control mice on day five of wheel access (Fig. 1). Also, as reported previously for HR and Control mice [1,7], housing with wheel access reduced the amount of home-cage activity measured simultaneously (Fig. 2 and Table 1). Irrespective of housing condition, HR mice had higher HCA than did Control mice, again consistent with previous reports that studied mice only when housed without wheel access [41,42,52]. In addition, irrespective of housing condition, females of both linetypes had higher HCA than males, a difference not reported previously when mice were housed and tested without wheels, using a different apparatus [42]. Finally, mice with the mini-muscle phenotype had lower HCA than those that did not (Table 1), an effect not observed previously [7].

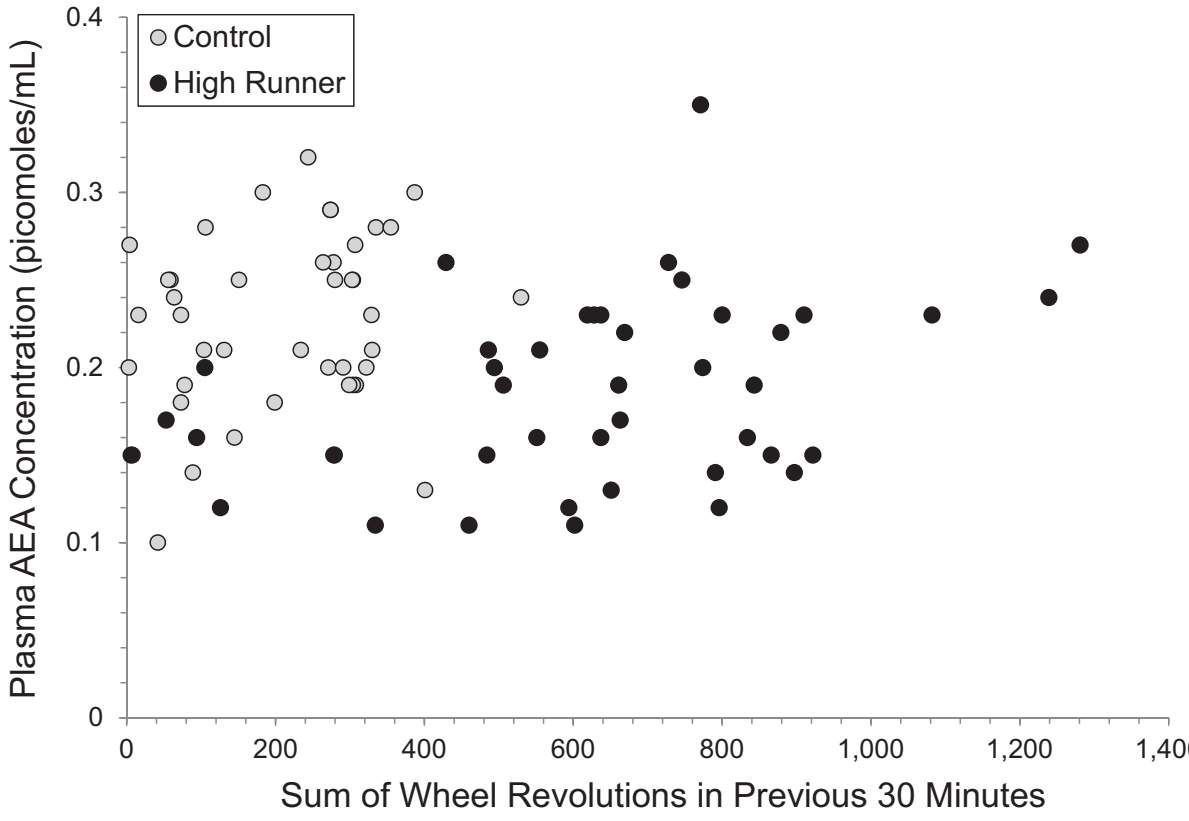


Fig. 5. Plasma AEA concentration from mice with wheel access as a function of the number of wheel revolutions in the 30 min prior to plasma sampling (N = 89). The number of wheel revolutions (transformed to the 0.4 power for statistical analyses [Table 5], but shown here as raw values) was a significant positive predictor of AEA values ($p = 0.0043$), home-cage was a negative predictor ($p = 0.0383$), and HR mice had lower levels than C mice after adjusting for these relationships (Table 5). The interactions between linetype and amount of wheel running or home-cage activity were not statistically significant (results not shown and these terms not included in final statistical model).

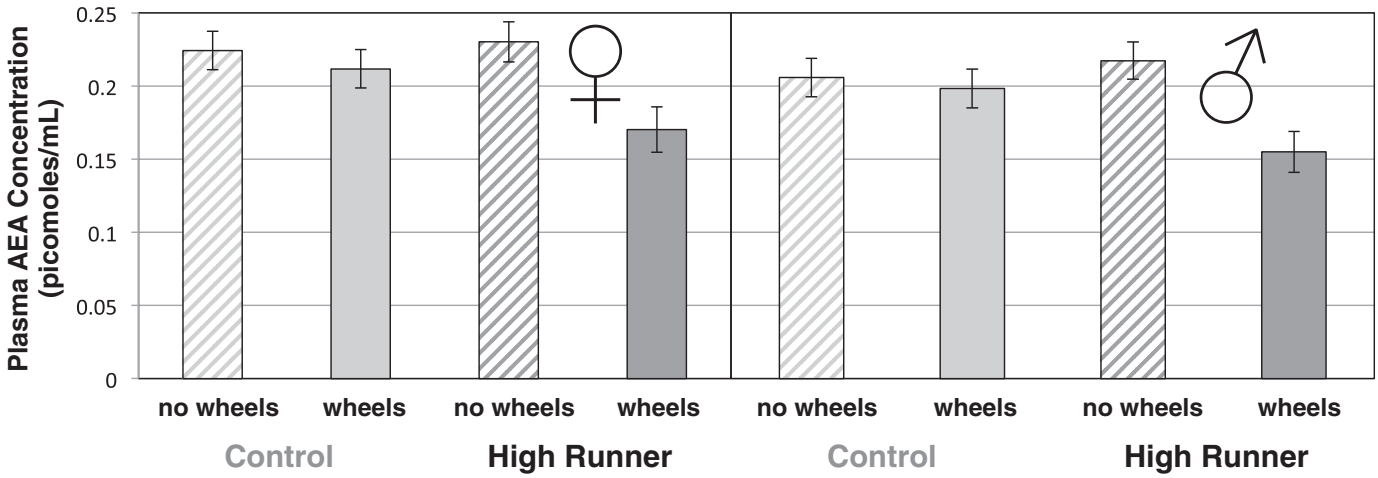


Fig. 6. Levels of AEA in mouse plasma collected during peak activity on the 6th night of wheel running, with amount of wheel running and home-cage activity used as covariates (see text), and including mice without wheels in the analysis by assigning them zero for wheel revolutions run. Values are LS means ± standard error from SAS Proc Mixed, based on analyses presented in Table 6. N = 183.

4.2. Training effects on plasma endocannabinoid levels

To our knowledge, the effects of several days of training (physical conditioning) on plasma endocannabinoid levels have not previously been reported for both male and female mice. Our combined analyses of all groups indicated a three-way interaction among sex, linetype, and wheel access for 2-AG levels (Table 2). However, it is important to note that our sampling design included 2–4 h of acute exercise prior to blood sampling, in addition to the five prior days of wheel access, which could confound training effects with acute exercise effects. Hence, we also conducted analyses of 2-AG levels that included the acute amounts of wheel running and home-cage activity as covariates, and found that they showed the same three-way interaction (see Results Section 3.4). Thus, our results show that, with respect to circulating 2-AG concentrations, even when taking differences in acute exercise into account, the endocannabinoid system responds to exercise differently based on sex and genetic background, and these differences may be a result of the exercise training undergone by the animal.

For plasma AEA levels, we found an interaction between linetype and wheel access, with wheel access lowering AEA levels for HR mice but raising them for C mice (Fig. 4, Table 4). Analyses that included the acute amounts of wheel running and home-cage activity as covariates indicated a stronger two-way interaction and a trend for an overall decrease in AEA levels for mice housed with wheel access (Table 6, Fig. 6). Interestingly, however, the effect of acute wheel running was highly significant and positive within groups (Fig. 6). Thus, our results show that, for AEA, the effects of voluntary exercise on the endocannabinoid system differ acutely versus chronically.

Physical conditioning in response to aerobic exercise has been studied extensively in both rodents and humans, although typically over the course of weeks rather than days [28,55,57,61]. Only two previous studies of rodents have examined endocannabinoids in relation to voluntary exercise that lasts for days, and these support our general finding that the endocannabinoid system can "train" in response to physical activity. Eight days of wheel access for male rats was associated with increased AEA levels in the hippocampus, but not the prefrontal cortex, and no effects on 2-AG levels in either brain region [30]. In addition, CB_1 receptor density was increased in the hippocampus. However, the authors did not examine plasma levels of endocannabinoids. An additional small study measured endocannabinoid levels in mice after they were allowed to run for 3 h per day for eight days, and compared them to mice who were in locked wheels for the same amount of time [5]. They did not find any significant differences in plasma endocannabinoid levels, although AEA was decreased in the hippocampus of running mice. However, since these animals also experienced acute exercise right before measurements were done, there is no way to tell if these differences are due to the acute or chronic effects of exercise.

Aside from training effects per se, several studies have now shown that AEA levels are raised after acute exercise [15,29,47,48,60]. The same studies generally suggest that 2-AG levels are not raised after acute exercise [29], although three studies have reported a non-significant trend for raised 2-AG levels in humans and dogs [15,47,60]. Finally, dogs and ferrets did not show significant increases in plasma 2-AG levels following treadmill exercise [47]. Thus, it appears that species of mammals may differ in the extent to which exercise acutely alters circulating 2-AG levels. Alternatively, some of the apparent species differences could be explained by methodological differences related to the exercise intensity imposed on, or chosen by, the running subjects. For example, Raichlen et al. [48] measured four different levels of exercise intensity in humans and found that AEA levels only increased for the two middle intensities. In addition, the amount of training experienced by the individual may affect how the endocannabinoid system reacts to acute exercise. Although plasma AEA was positively correlated with the amount of wheel running for both C and HR mice, there was a significant effect of linetype, with HR mice having lower levels of AEA even though they run more. This finding may also indicate that selective breeding for voluntary exercise has caused associated evolutionary changes in how the endocannabinoid system responds to exercise.

4.3. Sex differences in plasma endocannabinoid levels

We found that female mice have lower levels of 2-AG than males, especially when exercising, and tend to have higher levels of AEA (Figs. 3–5). Sex differences are not unexpected, given previous studies on these mice that show sex differences in the response of wheel running to CB_1 agonists and antagonists [34,35]. Aside from those studies on the HR and C lines of mice, most previous studies of plasma endocannabinoids have used only males [15,29,60]. Two studies of humans included both males and females, but did not test for sex differences [47,48]. Studies in rats show that females, especially adolescents, are more vulnerable than males to disruption of CB_1 signaling by repeated exposure to THC [3],

and studies of humans indicate that females are more sensitive than males to the effects of cannabis [8].

4.4. Time-of-day effects

Plasma 2-AG concentrations tended to be higher later in the sampling period, which ranged over approximately four hours during the early part of the dark phase, the time when mice are normally most active. This pattern is similar to one seen in a study of human subjects, where their levels of 2-AG also increased during the morning and peaked in midafternoon [26]. For plasma AEA, time of day was significant for all three analyses (overall, mice with wheels, mice without wheels), indicating reduced values later in the sampling period. This pattern is similar to a study of AEA in the cerebrospinal fluid of rats, which found that it decreased during the dark phase [45]. Thus, although our study was not intended to sample over a time period long enough to address circadian patterns per se, the time-related variation that we observed is consistent with previous reports for other species.

4.5. Conclusions & future directions

Overall, our results demonstrate that voluntary physical exercise affects circulating endocannabinoid levels differently, depending on sex, recent activity, and genetic background. More specifically, we found that acute voluntary exercise was associated with plasma AEA concentrations in a way similar to the effects of forced exercise reported in previous studies, in that the amount of wheel running done by a rodent before plasma sampling was a positive predictor of the level of AEA found in the blood [47,48]. We also found differences in circulating 2-AG and AEA levels between the sexes and between lines of mice bred for high levels of voluntary exercise, when compared to their non-selected control lines. Furthermore, the effects of five days of wheel access on endocannabinoid levels varied between the sexes and/or between HR and C mice.

Although not considered in the present study, receptor density or sensitivity may also have evolved in our mice. Broad-scale (macroevolutionary) patterns of endocannabinoid receptor evolution have been discussed elsewhere [14], but we do not know of studies that have considered microevolutionary variation in CB receptors (e.g., among closely related species). Although microevolutionary studies are lacking, laboratory mice and rats can show changes in CB receptor gene expression and sensitivity over the course of several days. For example, female mice given wheel access for 10 days had increased CB_1 receptor gene expression in the hippocampus as compared with sedentary controls [69]. In another study, male rats that had wheel access for eight days had an increase in CB_1 receptor binding site density in the hippocampus [30].

In future studies, we will further examine acute and chronic effects of voluntary exercise on the endocannabinoid system, including possible changes in receptor densities in various target organs (brain, muscle, gut). Interestingly, although HR mice run much more than C mice, they had lower circulating levels of AEA when the acute effect of wheel running was taken into account (Fig. 6). This finding suggests that the HR endocannabinoid system is differentially regulated, although it is unknown at what level changes have occurred (receptor density, number of converting or recycling enzymes, etc.). Therefore, future studies will also explore the mechanisms and genetics underlying the evolved endocannabinoid system of these unique, high-activity lines of mice, and how this system may interact with neurotransmitter and endocrine systems that are also known to have evolved in the HR mice [24,41,51].

Conflict of interest statement

The authors declare there is no conflict of interest.

Acknowledgements

This work was supported by NSF grant IOS-1121273 to Theodore Garland, Jr. and NIH grant DA034009 to Nicholas DiPatrizio. We thank Jaspreet Kaur for technical assistance. We thank Layla Hiramatsu and three anonymous reviewers for their helpful comments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.physbeh.2016.11.041.

References

[1] W. Acosta, T.H. Meek, H. Schutz, E.M. Dlugosz, K.T. Vu, T. Garland Jr., Effects of early-onset voluntary exercise on adult physical activity and associated phenotypes in mice, Physiol. Behav. 149 (2015) 279–286.
[2] D.A. Brown, M.S. Johnson, C.J. Armstrong, J.M. Lynch, N.M. Caruso, L.B. Ehlers, M. Fleshner, R.L. Spencer, R.L. Moore, Short-term treadmill running in the rat: what kind of stressor is it? J. Appl. Physiol. 103 (2007) 1979–1985.
[3] J.J. Burston, J.L. Wiley, A.A. Craig, D.E. Selley, L.J. Sim-Selley, Regional enhancement of cannabinoid CB1 receptor desensitization in female adolescent rats following repeated Δ9-tetrahydrocannabinol exposure, Br. J. Pharmacol. 161 (2010) 103–112.
[4] V. Careau, M.E. Wolak, P.A. Carter, T. Garland Jr., Limits to behavioral evolution: the quantitative genetics of a complex trait under directional selection, Evolution 67 (2013) 3102–3119.
[5] F. Chaouloff, S. Dubreucq, I. Matias, G. Marsicano, Physical activity feel-good effect, Routledge Handbook of Physical Activity and Mental Health, Routledge, London 2012, pp. 71–87.
[6] G.C. Claghorn, I.A.T. Fonseca, Z. Thompson, C. Barber, T. Garland Jr., Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running, Physiol. Behav. 161 (2016) 145–154.
[7] L.E. Copes, H. Schutz, E.M. Dlugosz, W. Acosta, M.A. Chappell, T. Garland Jr., Effects of voluntary exercise on spontaneous physical activity and food consumption in mice: results from an artificial selection experiment, Physiol. Behav. 149 (2015) 86–94.
[8] R.M. Craft, J.A. Marusich, J.L. Wiley, Sex differences in cannabinoid pharmacology: a reflection of differences in the endocannabinoid system? Life Sci. 92 (2013) 476–481.
[10] A. Dietrich, W.F. McDaniel, Endocannabinoids and exercise, Br. J. Sports Med. 38 (2004) 536–541.
[11] N.V. DiPatrizio, G. Astarita, G. Schwartz, X. Li, D. Piomelli, Endocannabinoid signal in the gut controls dietary fat intake, Proc. Natl. Acad. Sci. 108 (2011) 12904–12908.
[12] S. Dubreucq, M. Koehl, D.N. Abrous, G. Marsicano, F. Chaouloff, CB1 receptor deficiency decreases wheel-running activity: consequences on emotional behaviours and hippocampal neurogenesis, Exp. Neurol. 224 (2010) 106–113.
[13] S. Dubreucq, A. Durand, I. Matias, G. Bénard, E. Richard, E. Soria-Gomez, C. Glangetas, L. Groc, A. Wadleigh, F. Massa, et al., Ventral tegmental area cannabinoid type-1 receptors control voluntary exercise performance, Biol. Psychiatry 73 (2013) 895–903.
[14] M.R. Elphick, M. Egertová, Cannabinoid receptor genetics and evolution, in: P.H. Reggio (Ed.), The Cannabinoid Receptors, Humana Press, Totowa, NJ 2009, pp. 123–149.
[15] M. Feuerecker, D. Hauer, R. Toth, F. Demetz, J. Hölzl, M. Thiel, I. Kaufmann, G. Schelling, A. Chouker, Effects of exercise stress on the endocannabinoid system in humans under field conditions, Eur. J. Appl. Physiol. 112 (2012) 2777–2781.
[17] J. Fuss, J. Steinle, L. Bindila, M.K. Auer, H. Kirchherr, B. Lutz, P. Gass, A runner's high depends on cannabinoid receptors in mice, Proc. Natl. Acad. Sci. 112 (2015) 13105–13108.
[19] E.L. Gardner, Endocannabinoid signaling system and brain reward: emphasis on dopamine, Pharmacol. Biochem. Behav. 81 (2005) 263–284.
[20] T. Garland Jr., M.T. Morgan, J.G. Swallow, J.S. Rhodes, I. Girard, J.G. Belter, P.A. Carter, Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels, Evolution 56 (2002) 1267–1275.
[21] T. Garland Jr., S.A. Kelly, J.L. Malisch, E.M. Kolb, R.M. Hannon, B.K. Keeney, S.L. Van Cleave, K.M. Middleton, How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels, Proc. R. Soc. B Biol. Sci. 278 (2011) 574–581.
[22] T. Garland Jr., H. Schutz, M.A. Chappell, B.K. Keeney, T.H. Meek, L.E. Copes, W. Acosta, C. Drenowatz, R.C. Maciel, G. Van Dijk, et al., The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives, J. Exp. Biol. 214 (2011) 206–229.
[23] T. Garland Jr., M. Zhao, W. Saltzman, Hormones and the evolution of complex traits: insights from artificial selection on behavior, Integr. Comp. Biol. 57 (2017) (in press).
[24] I. Girard, T. Garland Jr., Plasma corticosterone response to acute and chronic voluntary exercise in female house mice, J. Appl. Physiol. 92 (2002) 1553–1561.
[25] I. Girard, M.W. McAleer, J.S. Rhodes, T. Garland Jr., Selection for high voluntary wheel-running increases speed and intermittency in house mice (*Mus domesticus*), J. Exp. Biol. 204 (2001) 4311–4320.
[26] E.C. Hanlon, E. Tasali, R. Leproult, K.L. Stuhr, E. Doncheck, H. De Wit, C.J. Hillard, E. Van Cauter, Circadian rhythm of circulating levels of the endocannabinoid 2-arachidonoylglycerol, J. Clin. Endocrinol. Metab. 100 (2014) 220–226.

[27] R.M. Hannon, S.A. Kelly, K.M. Middleton, E.M. Kolb, D. Pomp, T. Garland Jr., Phenotypic effects of the "mini-muscle" allele in a large HR × C57BL/6J mouse backcross, J. Hered. 99 (2008) 349–354.
[28] R.P. Harpur, The rat as a model for physical fitness studies, Comp. Biochem. Physiol. A Physiol. 66 (1980) 553–574.
[29] E. Heyman, F.-X. Gamelin, M. Goekint, F. Piscitelli, B. Roelands, E. Leclair, V. Di Marzo, R. Meeusen, Intense exercise increases circulating endocannabinoid and BDNF levels in humans—possible implications for reward and depression, Psychoneuroendocrinology 37 (2012) 844–851.
[30] M.N. Hill, A.K. Titterness, A.C. Morrish, E.J. Carrier, T.T.-Y. Lee, J. Gil-Mohapel, B.B. Gorzalka, C.J. Hillard, B.R. Christie, Endogenous cannabinoid signaling is required for voluntary exercise-induced enhancement of progenitor cell proliferation in the hippocampus, Hippocampus 20 (2010) 513–523.
[31] P. Houle-Leroy, H. Guderley, J.G. Swallow, T. Garland Jr., Artificial selection for high activity favors mighty mini-muscles in house mice, Am. J. Phys. Regul. Integr. Comp. Phys. 284 (2003) R433–R443.
[33] T.U. Järbe, M.E. Andrzejewski, N.V. DiPatrizio, Interactions between the CB1 receptor agonist Δ9-THC and the CB1 receptor antagonist SR-141716 in rats: open-field revisited, Pharmacol. Biochem. Behav. 73 (2002) 911–919.
[34] B.K. Keeney, D.A. Raichlen, T.H. Meek, R.S. Wijeratne, K.M. Middleton, G.L. Gerdeman, T. Garland Jr., Differential response to a selective cannabinoid receptor antagonist (SR141716: rimonabant) in female mice from lines selectively bred for high voluntary wheel-running behaviour, Behav. Pharmacol. 19 (2008) 812–820.
[35] B.K. Keeney, T.H. Meek, K.M. Middleton, L.F. Holness, T. Garland Jr., Sex differences in cannabinoid receptor-1 (CB1) pharmacology in mice selectively bred for high voluntary wheel-running behavior, Pharmacol. Biochem. Behav. 101 (2012) 528–537.
[36] S.A. Kelly, P.P. Czech, J.T. Wight, K.M. Blank, T. Garland Jr., Experimental evolution and phenotypic plasticity of hindlimb bones in high-activity house mice, J. Morphol. 267 (2006) 360–374.
[38] P. Koteja, T. Garland Jr., Response to R. Eikelboom, Anim. Behav. 61 (2001) F25–F26.
[39] P. Koteja, T. Garland Jr., J.K. Sax, J.G. Swallow, P.A. Carter, Behaviour of house mice artificially selected for high levels of voluntary wheel running, Anim. Behav. 58 (1999) 1307–1318.
[40] G. Li, J.S. Rhodes, I. Girard, S.C. Gammie, T. Garland Jr., Opioid-mediated pain sensitivity in mice bred for high voluntary wheel running, Physiol. Behav. 83 (2004) 515–524.
[41] J.L. Malisch, C.W. Breuner, F.R. Gomes, M.A. Chappell, T. Garland Jr., Circadian pattern of total and free corticosterone concentrations, corticosteroid-binding globulin, and physical activity in mice selectively bred for high voluntary wheel-running behavior, Gen. Comp. Endocrinol. 156 (2008) 210–217.
[42] J.L. Malisch, C.W. Breuner, E.M. Kolb, H. Wada, R.M. Hannon, M.A. Chappell, K.M. Middleton, T. Garland Jr., Behavioral despair and home-cage activity in mice with chronically elevated baseline corticosterone concentrations, Behav. Genet. 39 (2009) 192–201.
[43] M. Martin, C. Ledent, M. Parmentier, R. Maldonado, O. Valverde, Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice, Eur. J. Neurosci. 12 (2000) 4038–4046.
[45] E. Murillo-Rodriguez, F. Désarnaud, O. Prospéro-García, Diurnal variation of arachidonoylethanolamine, palmitoylethanolamide and oleoylethanolamide in the brain of the rat, Life Sci. 79 (2006) 30–37.
[47] D.A. Raichlen, A.D. Foster, G.L. Gerdeman, A. Seillier, A. Giuffrida, Wired to run: exercise-induced endocannabinoid signaling in humans and cursorial mammals with implications for the "runner's high", J. Exp. Biol. 215 (2012) 1331–1336.
[48] D.A. Raichlen, A.D. Foster, A. Seillier, A. Giuffrida, G.L. Gerdeman, Exercise-induced endocannabinoid signaling is modulated by intensity, Eur. J. Appl. Physiol. 113 (2013) 869–875.
[49] E.B. Rasmussen, C. Hillman, Naloxone and rimonabant reduce the reinforcing properties of exercise in rats, Exp. Clin. Psychopharmacol. 19 (2011) 389.
[50] J.S. Rhodes, P. Koteja, J.G. Swallow, P.A. Carter, T. Garland Jr., Body temperatures of house mice artificially selected for high voluntary wheel-running behavior: repeatability and effect of genetic selection, J. Therm. Biol. 25 (2000) 391–400.
[51] J.S. Rhodes, G. Hosack, I. Girard, A. Kelley, G. Mitchell, T. Garland, Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior, Psychopharmacology 158 (2001) 120–131.
[52] J.S. Rhodes, G.R. Hosack, I. Girard, A.E. Kelley, G.S. Mitchell, T. Garland Jr., Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior, Psychopharmacology 158 (2001) 120–131.
[53] J.S. Rhodes, T. Garland Jr., S.C. Gammie, Patterns of brain activity associated with variation in voluntary wheel-running behavior, Behav. Neurosci. 117 (2003) 1243.
[55] B. Saltin, P.D. Gollnick, Skeletal muscle adaptability: significance for metabolism and performance, Compr. Physiol. (1983) 555–631.
[56] M.C. Sañudo-Peña, J. Romero, G.E. Seale, J.J. Fernandez-Ruiz, J.M. Walker, Activational role of cannabinoids on movement, Eur. J. Pharmacol. 391 (2000) 269–274.
[57] T.D. Scribbans, S. Vecsey, P.B. Hankinson, W.S. Foster, B.J. Gurd, The effect of training intensity on VO2max in young healthy adults: a meta-regression and meta-analysis, Int. J. Exerc. Sci. 9 (2016) 230.
[59] S.L. Smith, E.B. Rasmussen, Effects of 2-AG on the reinforcing properties of wheel activity in obese and lean Zucker rats, Behav. Pharmacol. 21 (2010) 292–300.
[60] P.B. Sparling, A. Giuffrida, D. Piomelli, L. Rosskopf, A. Dietrich, Exercise activates the endocannabinoid system, Neuroreport 14 (2003) 2209–2211.
[61] K.I. Stanford, L.J. Goodyear, Exercise regulation of adipose tissue, Adipocytes 5 (2016) 153–162.
[62] J.G. Swallow, P.A. Carter, T. Garland Jr., Artificial selection for increased wheel-running behavior in house mice, Behav. Genet. 28 (1998) 227–237.
[63] J.G. Swallow, J.S. Rhodes, T. Garland Jr., Phenotypic and evolutionary plasticity of organ masses in response to voluntary exercise in house mice, Integr. Comp. Biol. 45 (2005) 426–437.
[64] D.A. Syme, K. Evashuk, B. Grintuch, E.L. Rezende, T. Garland Jr., Contractile abilities of normal and "mini" triceps surae muscles from mice (*Mus domesticus*) selectively bred for high voluntary wheel running, J. Appl. Physiol. 99 (2005) 1308–1316.
[65] K. Tsou, S. Brown, M.C. Sanudo-Pena, K. Mackie, J.M. Walker, Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system, Neuroscience 83 (1998) 393–411.
[66] D. Wahlsten, Insensitivity of the analysis of variance to heredity-environment interaction, Behav. Brain Sci. 13 (1990) 109–120.
[67] D. Wahlsten, Sample size to detect a planned contrast and a one degree-of-freedom interaction effect, Psychol. Bull. 110 (1991) 587–595.
[68] I.J. Wallace, T. Garland Jr., Mobility as an emergent property of biological organization: insights from experimental evolution, Evol. Anthropol. 25 (2016) 98–104.
[69] S.A. Wolf, A. Bick-Sander, K. Fabel, P. Leal-Galicia, S. Tauber, G. Ramirez-Rodriguez, A. Müller, A. Melnik, T.P. Waltinger, O. Ullrich, et al., Cannabinoid receptor CB1 mediates baseline and activity-induced survival of new neurons in adult hippocampal neurogenesis, Cell Commun. Signal 8 (2010).
[71] A. Zimmer, A.M. Zimmer, A.G. Hohmann, M. Herkenham, T.I. Bonner, Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice, Proc. Natl. Acad. Sci. 96 (1999) 5780–5785.

Online Supplemental Material for Thompson et al. 2017.:

Circulating levels of endocannabinoids respond acutely to voluntary exercise, are altered in mice selectively bred for high voluntary wheel running, and differ between the sexes. *Physiology & Behavior*.

From file = Correlations_AG_AEA_within_groups_1.xlsx

R = the within group Pearson correlation coefficient for each of the eight subgroups.

P = the 2-tailed significance level for the correlation coefficient.

Means and standard errors for each group for both endocannabinoids are also shown.

Linetype	Sex	Exercise	R	N	P	Mean PMAEA	SE	Mean PMAG	SE
Control	Female	Sedentary	0.350663	23	0.100899	0.203478	0.008379	88.559130	6.568864
		Wheels	0.535030	24	0.007062	0.214583	0.012910	68.208333	6.304621
	Male	Sedentary	-0.070912	24	0.741954	0.188333	0.010012	100.020417	6.760471
		Wheels	0.296920	24	0.158846	0.218750	0.010840	91.834583	7.159051
High Runner	Female	Sedentary	0.185547	24	0.385373	0.197083	0.010674	79.320000	8.909360
		Wheels	-0.174127	24	0.415789	0.202083	0.011468	71.742083	6.219043
	Male	Sedentary	0.212498	24	0.318815	0.185833	0.008229	93.040417	6.627705
		Wheels	0.320810	24	0.126396	0.170417	0.010776	78.942500	8.060669

*** Descriptive statistics for table above and correlation of raw group means.**

```
GET DATA /TYPE=XLSX  /FILE='E:\SELECT\g75\EC\PPT_and_Manuscript\Correlations_AG_AEA_within_groups_1.xlsx'
  /SHEET=name 'Sheet1'
  /CELLRANGE=full
  /READNAMES=on
  /ASSUMEDSTRWIDTH=32767.
EXECUTE.
DATASET CLOSE DataSet10.
DESCRIPTIVES VARIABLES = R N P PMAEA pmaeaSE PMAG pmasSE
  /STATISTICS=MEAN STDDEV MIN MAX SEMEAN.
```

Descriptive Statistics

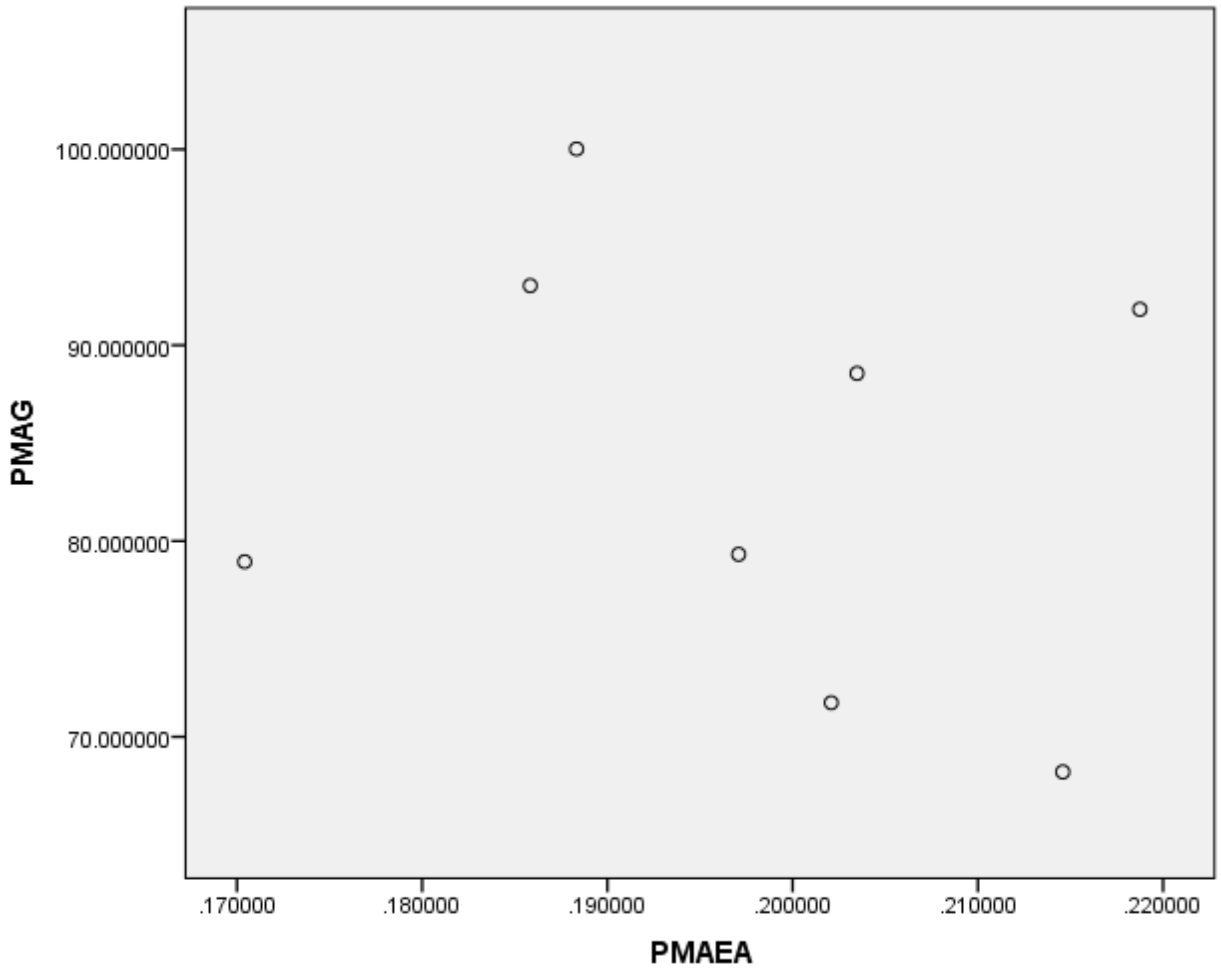
	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
R	8	-.174127	.535030	**.20705363**	**.081532680**	.230609245
N	8	23	24	23.88	.125	.354
P	8	.007062	.741954	.28189175	.083373771	.235816634
PMAEA	8	.170417	.218750	.19757008	.005596429	.015829092
pmaeaSE	8	.008229	.012910	.01041105	.000547658	.001549010
PMAG	8	68.208333	100.020417	83.95843292	3.936208763	11.133279635
pmasSE	8	6.219043	8.909360	7.07622290	.333402232	.943003918
Valid N (listwise)	8					

```
CORRELATIONS
  /VARIABLES=PMAG PMAEA
  /PRINT=TWOTAIL NOSIG
  /STATISTICS DESCRIPTIVES
  /MISSING=PAIRWISE.
```

Correlations

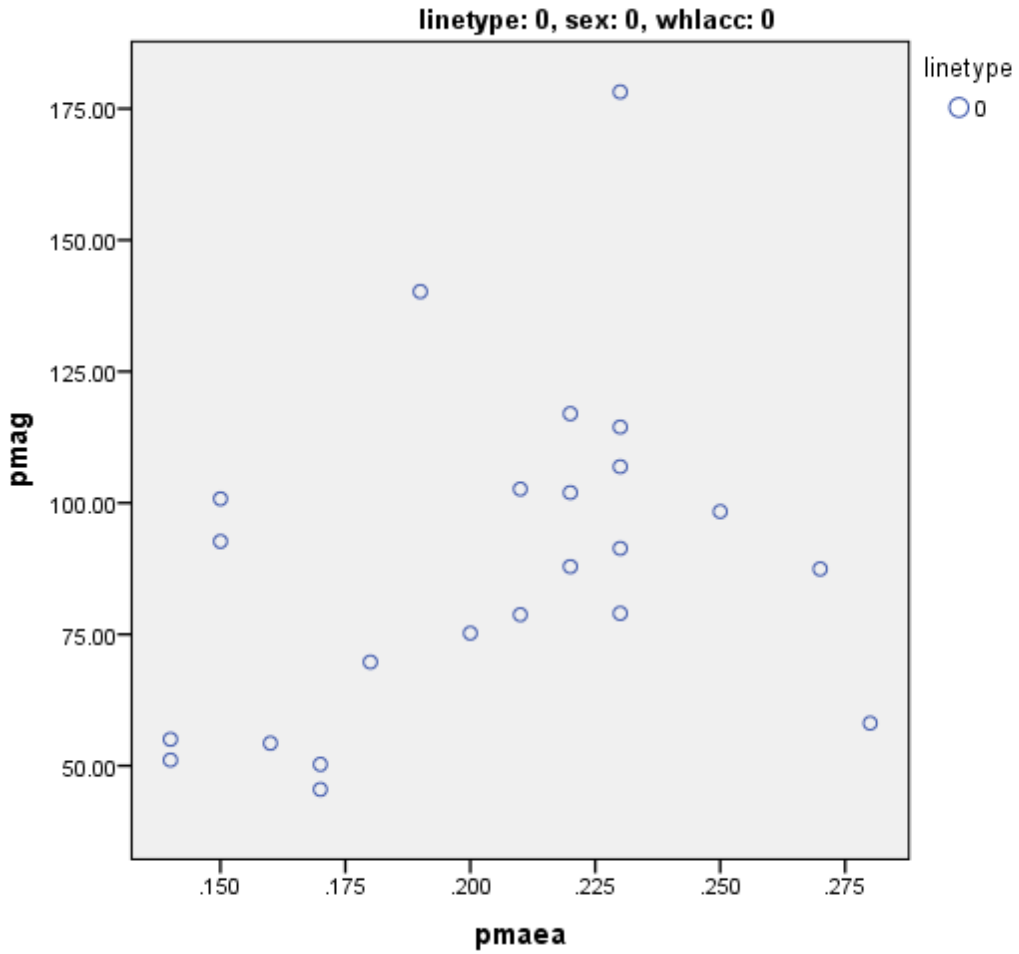
		PMAG	PMAEA
PMAG	Pearson Correlation	1	**-.199**
	Sig. (2-tailed)		.636
	N	8	8
PMAEA	Pearson Correlation	-.199	1
	Sig. (2-tailed)	.636	
	N	8	8

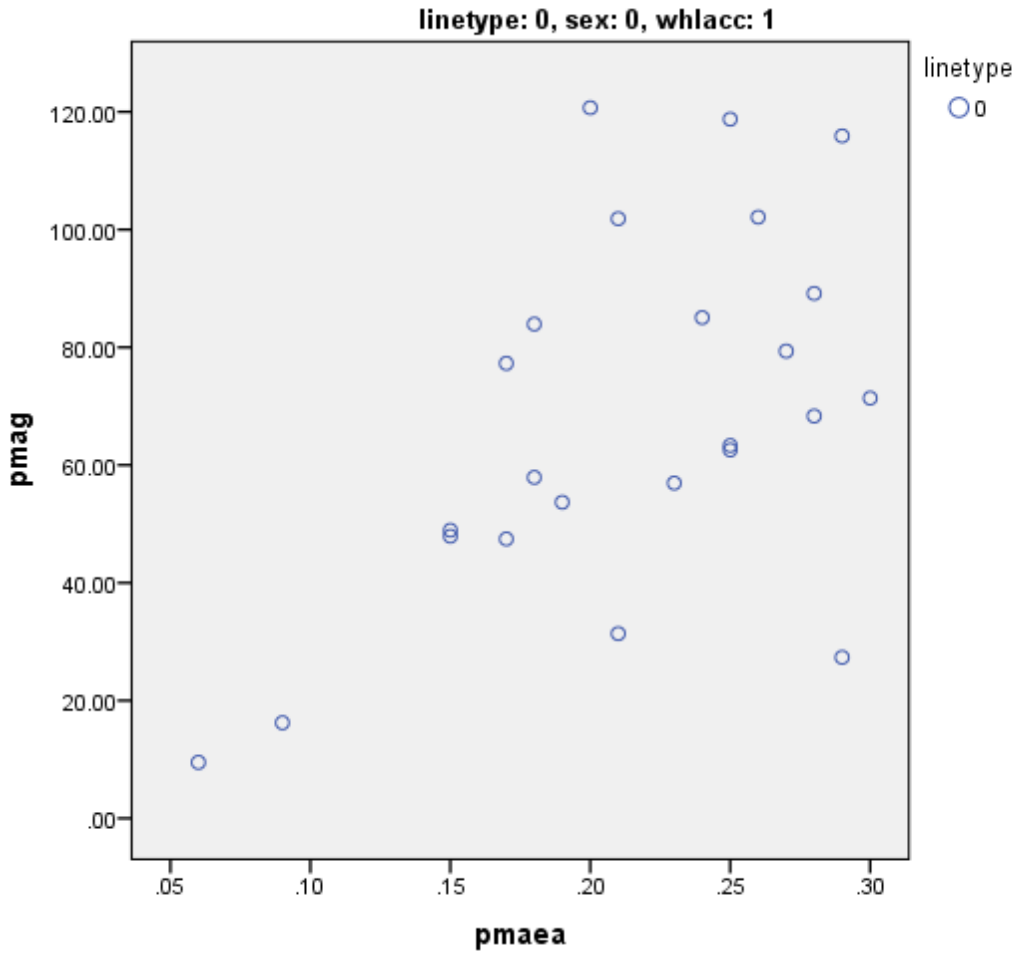
```
* Scatterplot for group means.
* r = -0.199, P = 0.636.

GRAPH
  /SCATTERPLOT(BIVAR)=PMAEA WITH PMAG
  /MISSING=LISTWISE.
```

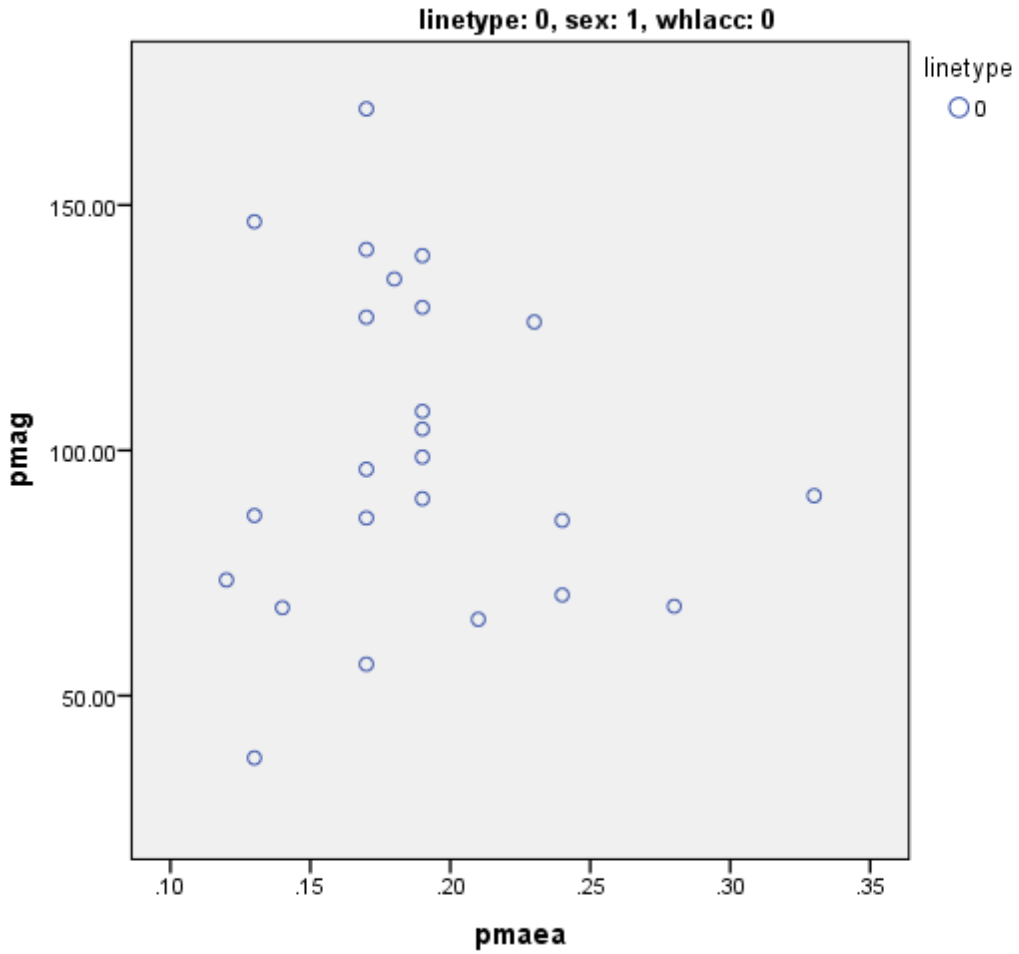

100.000000
90.000000
80.000000
70.000000
PMAG
.170000
.180000
.190000
.200000
.210000
.220000
PMAEA

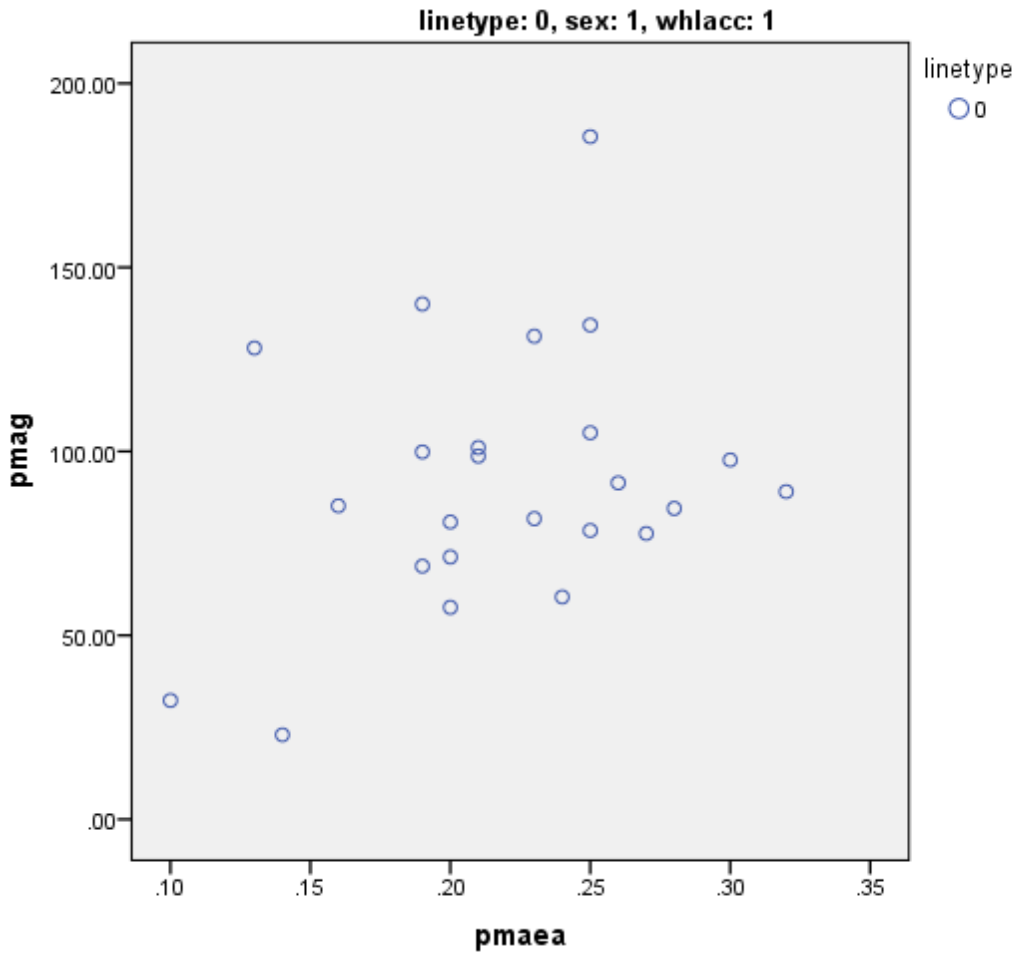
*** Analysis of correlation of raw individual values within each of the eight subgroups.**
*** linetype 0 = Control, 1 = High Runner lines.**
*** sex 0 = female, 1 = male.**
*** wheel access 0 = no, 1 = yes for 6 days.**

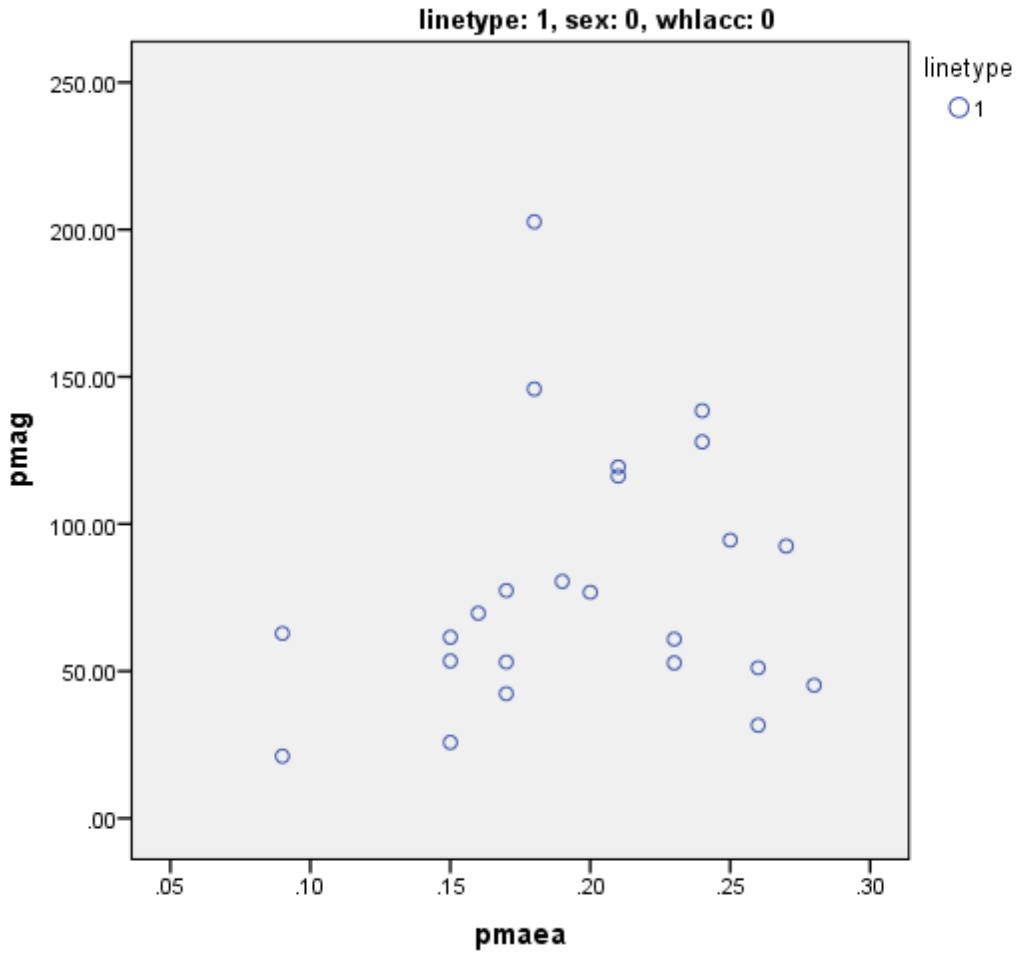
```
GET FILE='E:\SELECT\g75\g75ALL2_with_EC_RUN16_HCA16_Buffer.sav'.
SORT CASES BY linetype sex whlacc.
SPLIT FILE
  SEPARATE BY linetype sex whlacc.

GRAPH
  /SCATTERPLOT(BIVAR) = PMAEA WITH PMAG BY linetype BY mouseid (IDENTIFY)
  /MISSING=LISTWISE .
```

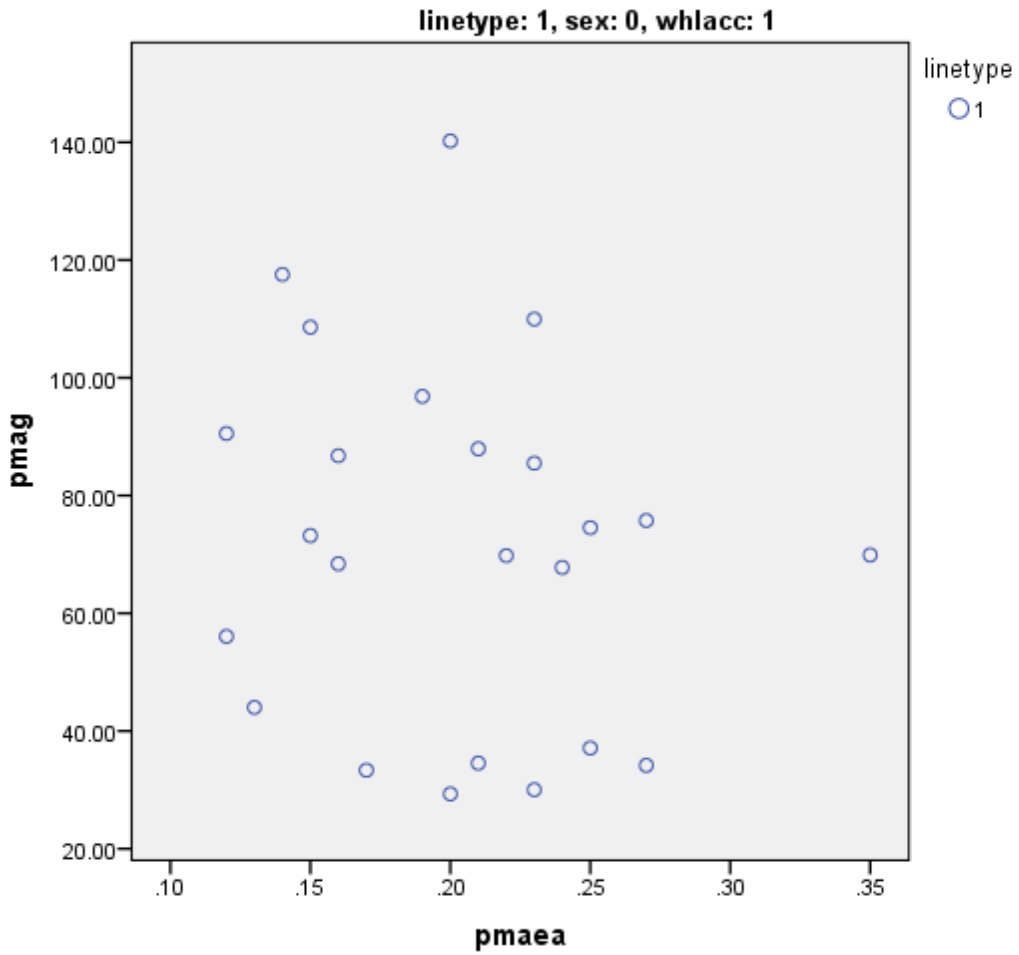

linetype: 0, sex: 0, whlacc: 0
linetype
0
175.00
150.00
125.00
100.00
75.00
50.00
pmag
.150
.175
.200
.225
.250
.275
pmaea

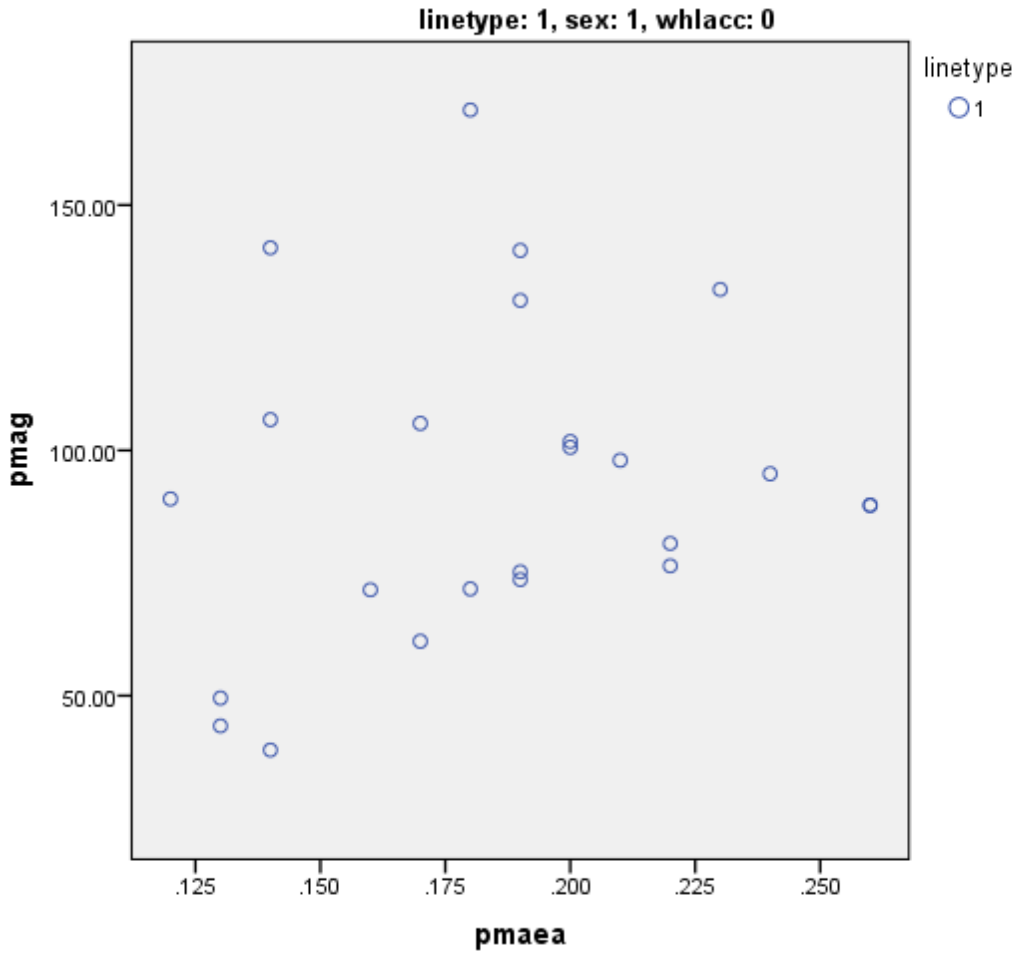

linetype: 0, sex: 0, whlacc: 1
linetype
0
pmag
120.00
100.00
80.00
60.00
40.00
20.00
.00
.05
.10
.15
.20
.25
.30
pmaea

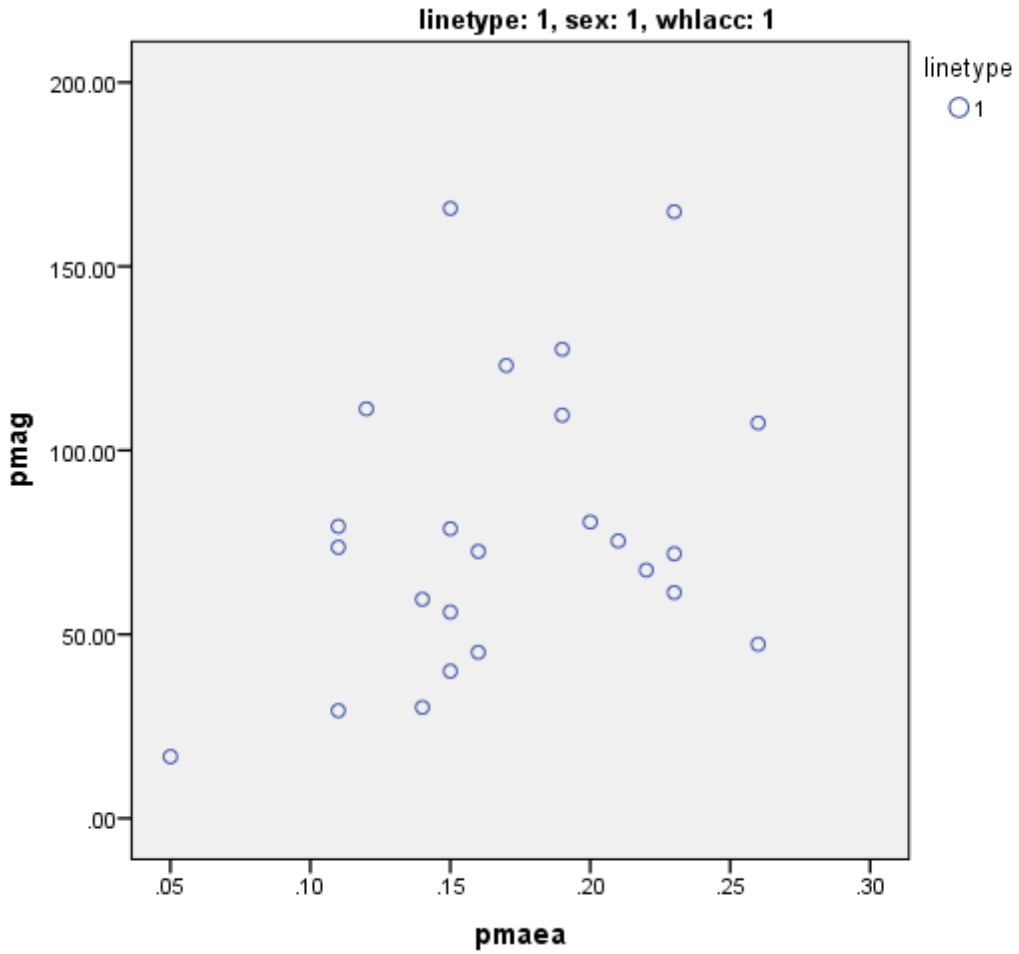

linetype: 0, sex: 1, whlacc: 0
linetype
0
150.00
100.00
50.00
pmag
.10
.15
.20
.25
.30
.35
pmaea


linetype: 0, sex: 1, whlacc: 1
linetype
0
200.00
150.00
100.00
50.00
.00
pmag
.10
.15
.20
.25
.30
.35
pmaea


linetype: 1, sex: 0, whlacc: 0
linetype
1
250.00
200.00
150.00
100.00
50.00
.00
pmag
.05
.10
.15
.20
.25
.30
pmaea


linetype: 1, sex: 0, whlacc: 1
linetype
1
140.00
120.00
100.00
80.00
60.00
40.00
20.00
pmag
.10
.15
.20
.25
.30
.35
pmaea


linetype: 1, sex: 1, whlacc: 0
linetype
1
150.00
100.00
50.00
pmag
.125
.150
.175
.200
.225
.250
pmaea


linetype: 1, sex: 1, whlacc: 1
linetype
1
200.00
150.00
100.00
50.00
.00
pmag
.05
.10
.15
.20
.25
.30
pmaea

```
CORRELATIONS
  /VARIABLES=pmag pmaea
  /PRINT=TWOTAIL NOSIG
  /STATISTICS DESCRIPTIVES
  /MISSING=PAIRWISE.
```

linetype = 0, sex = 0, whlacc = 0

Descriptive Statistics[a]

	Mean	Std. Deviation	N
pmag	88.5591	31.50316	23
pmaea	.2035	.04018	23

a. linetype = 0, sex = 0, whlacc = 0

Correlations[a]

		pmag	pmaea
pmag	Pearson Correlation	1	.351
	Sig. (2-tailed)		.101
	N	23	23
pmaea	Pearson Correlation	.351	1
	Sig. (2-tailed)	.101	
	N	23	23

a. linetype = 0, sex = 0, whlacc = 0

linetype = 0, sex = 0, whlacc = 1

Descriptive Statistics[a]

	Mean	Std. Deviation	N
pmag	68.2083	30.88621	24
pmaea	.2146	.06324	24

a. linetype = 0, sex = 0, whlacc = 1

Correlations[a]

		pmag	pmaea
pmag	Pearson Correlation	1	.535**
	Sig. (2-tailed)		.007
	N	24	24
pmaea	Pearson Correlation	.535**	1
	Sig. (2-tailed)	.007	
	N	24	24

**. Correlation is significant at the 0.01 level (2-tailed).

a. linetype = 0, sex = 0, whlacc = 1

linetype = 0, sex = 1, whlacc = 0

Descriptive Statistics[a]

	Mean	Std. Deviation	N
pmag	100.0204	33.11941	24
pmaea	.1883	.04905	24

a. linetype = 0, sex = 1, whlacc = 0

Correlations[a]

		pmag	pmaea
pmag	Pearson Correlation	1	-.071
	Sig. (2-tailed)		.742
	N	24	24
pmaea	Pearson Correlation	-.071	1
	Sig. (2-tailed)	.742	
	N	24	24

a. linetype = 0, sex = 1, whlacc = 0

linetype = 0, sex = 1, whlacc = 1

Descriptive Statistics[a]

	Mean	Std. Deviation	N
pmag	91.8346	35.07204	24
pmaea	.2188	.05310	24

a. linetype = 0, sex = 1, whlacc = 1

Correlations[a]

		pmag	pmaea
pmag	Pearson Correlation	1	.297
	Sig. (2-tailed)		.159
	N	24	24
pmaea	Pearson Correlation	.297	1
	Sig. (2-tailed)	.159	
	N	24	24

a. linetype = 0, sex = 1, whlacc = 1

linetype = 1, sex = 0, whlacc = 0

Descriptive Statistics[a]

	Mean	Std. Deviation	N
pmag	79.3200	43.64677	24
pmaea	.1971	.05229	24

a. linetype = 1, sex = 0, whlacc = 0

Correlations[a]

		pmag	pmaea
pmag	Pearson Correlation	1	.186
	Sig. (2-tailed)		.385
	N	24	24
pmaea	Pearson Correlation	.186	1
	Sig. (2-tailed)	.385	
	N	24	24

a. linetype = 1, sex = 0, whlacc = 0

linetype = 1, sex = 0, whlacc = 1

Descriptive Statistics[a]

	Mean	Std. Deviation	N
pmag	71.7421	30.46696	24
pmaea	.2021	.05618	24

a. linetype = 1, sex = 0, whlacc = 1

Correlations[a]

		pmag	pmaea
pmag	Pearson Correlation	1	-.174
	Sig. (2-tailed)		.416
	N	24	24
pmaea	Pearson Correlation	-.174	1
	Sig. (2-tailed)	.416	
	N	24	24

a. linetype = 1, sex = 0, whlacc = 1

linetype = 1, sex = 1, whlacc = 0

Descriptive Statistics[a]

	Mean	Std. Deviation	N
pmag	93.0404	32.46899	24
pmaea	.1858	.04032	24

a. linetype = 1, sex = 1, whlacc = 0

Correlations[a]

		pmag	pmaea
pmag	Pearson Correlation	1	.212
	Sig. (2-tailed)		.319
	N	24	24
pmaea	Pearson Correlation	.212	1
	Sig. (2-tailed)	.319	
	N	24	24

a. linetype = 1, sex = 1, whlacc = 0

linetype = 1, sex = 1, whlacc = 1

Descriptive Statistics[a]

	Mean	Std. Deviation	N
pmag	78.9425	39.48905	24
pmaea	.1704	.05279	24

a. linetype = 1, sex = 1, whlacc = 1

Correlations[a]

		pmag	pmaea
pmag	Pearson Correlation	1	.321
	Sig. (2-tailed)		.126
	N	24	24
pmaea	Pearson Correlation	.321	1
	Sig. (2-tailed)	.126	
	N	24	24

a. linetype = 1, sex = 1, whlacc = 1