1   # Title: mRNA expression explains metabolic and thermal

2   # physiology

3   **Short Title:** mRNA expression explains physiology

4   **Authors:** Melissa Drown[1*], Douglas Crawford[1] and Margie Oleksiak[1]

5   **Affiliations:**

6   1. University of Miami Rosenstiel School of Marine and Atmospheric Science, 6400

7   Rickenbacker Causeway, Miami, FL

8

9   *Corresponding author

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12   adaptation, co-expression network analysis

Abstract

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14   Quantifying mRNA expression, which is heritable and physiologically inducible, reveals

15   biologically important networks and pathways underlying complex traits. Here, we quantify

16   mRNA expression in *Fundulus heroclitus*, a small teleost fish, among three populations

17   acclimated to 12°C and 28°C and relate it to variation in six, complex, physiological traits

18   (whole animal metabolism (WAM), critical thermal maximum (CT_{max}), and four substrate

19   specific cardiac metabolic rates (CaM)). Although 366 heart mRNAs and 528 brain mRNAs had

20   significant differential expression between the two acclimation temperatures, none of the mRNA

21   acclimation responses were shared across all three populations in any tissue. Yet, within an

22   acclimation temperature across all three populations, weighted gene co-expression network

23   analyses show that mRNA expression patterns explained WAM, CT_{max}, and CaM trait variation.

24   These analyses revealed 9 significant heart MEs (first principal component of module

25   expression) and 4 significant brain MEs. Heart MEs explained variation in WAM, CT_{max}, and

26   two of the four substrate specific cardiac metabolic rates at 12°C, and CT_{max} at 28C. In contrast,

27   brain MEs explained CT_{max} and WAM at 28°C but not at 12°C. Combining MEs as multiple

28   correlations, 82% of variation in WAM at 12°C was explained by four heart MEs, 80% of

29   variation in fatty-acid CaM at 12°C was explained by three heart MEs, and 72% of variation in

30   CT_{max} at 28°C was explained by three brain MEs. These MEs were enriched for Kyoto

31   Encyclopedia of Genes and Genomes (KEGG) terms related to specific metabolic pathways,

32   suggesting that they represent biologically relevant pathways. Together these data suggest that

33   mRNA co-expression explains complex traits; moreover, physiological traits are more reliant on

34   heart expression at 12°C and brain expression at 28°C.

Author Summary

Despite an abundance of genomic data, the molecular and genetic underpinnings of complex traits remain poorly understood. To better understand the molecular basis of complex traits, we used heart and brain mRNA expression to explain complex traits- physiological responses to temperature- in individuals collected from three saltmarsh fish (*Fundulus heteroclitus*) populations acclimated to 12°C and 28°C. We found that while physiological traits did not differ among populations, the mRNAs important for acclimation responses were >88% unique to a single population and differed between heart and brain tissues. We also found tissue specific co-expressed mRNAs that explain up to 82% of complex traits including whole animal metabolism, upper thermal tolerance, and substrate specific cardiac metabolism measured at 12°C or 28°C acclimation conditions. Notably, sets of co-expressed mRNAs related to these traits are enriched for molecular pathways affecting metabolism, giving insight into the molecular underpinnings of these traits.

Introduction

Many genotype to phenotype mapping approaches such as expression quantitative trait loci (eQTL) mapping, co-expression network analysis, and functional genomics investigations (i.e., systems genetics) combine data across biological organization levels among inbred lines and leverage significant genomic resources (*e.g.,* well annotated genomes and transcriptomes, inbred lines, gene editing) (1). In several model organisms, these approaches have improved our ability to dissect complex trait architecture. For example, in fruit flies (*Drosophila*) variation in starvation resistance and startle response is explained by mRNA expression and polymorphic loci (2). In *Caenorhabditis elegans*, 199 recombinant inbred lines recently were used to identify

57   36 loci related to metabolism (3). In *Arabidopsis*, both segregating and isogenic lines were used

58   to uncover complex genetic architecture of growth and morphology related traits (4).

59   In comparison to inbred lines, non-model organisms have more polymorphic alleles and

60   greater heterozygosity, and thus non-model organism studies can provide a more nuanced

61   understanding of how natural genetic variation impacts complex phenotypes (5, 6). Furthermore,

62   non-model organism studies have become even more relevant with increased genomic resource

63   availability and development of high-throughput approaches to measure behavioral and

64   physiological traits under realistic ecological conditions (7-10).

65   Despite these recent advances, the genomic basis of well-studied traits, like metabolism,

66   remain poorly understood, especially among a diversity of species. This may be largely due to

67   the highly polygenetic nature of complex traits like metabolism that results in smaller average

68   effect sizes, thus making it more challenging to detect nucleotide variation associated with

69   physiological trait variation. Additionally, identifying genotype-to-phenotype associations is

70   even more challenging when the genetic architecture is both polygenic and redundant because

71   redundancy allows many small effect genetic polymorphisms associated with phenotypic

72   variation to differ among environments, individuals, and populations (11-13). Although

73   elucidating genotype-to-phenotype relationships is challenging for complex redundant polygenic

74   traits, this knowledge is critical to understand evolutionary processes contributing to phenotypic

75   variation.

76   One approach to the challenge of relating genotype to phenotype is to use mRNA

77   expression, which provides three clear advantages. First, mRNA expression more readily

78   explains trait variation than nucleotide variation (2, 6) because nucleotide variation is typically

79   only binary while mRNA expression results from a combination of genetic polymorphisms.

80   These genetic polymorphisms collectively effect mRNA expression to provide a continuous trait

81   distribution, and relating this polygenic continuous trait to more complex physiological traits is

82   statistically stronger than relating binary nucleotide variation to complex traits. Second,

83   quantifying mRNA expression is likely to provide more insight into the genetic architecture of

84   physiological traits than genome wide association studies (GWAS) alone because mRNA

85   expression provides information for both heritable and plastic responses (6, 14-19). That is,

86   mRNAs capture multiple genetic effects including gene by environment interactions (GxE) in a

87   single measure, which is essential when genetic architecture is context dependent (11, 13).

88   Finally, a greater mechanistic understanding of complex traits is likely using mRNAs because

89   mRNAs are more often defined genes associated with biochemical or physiological pathways

90   (*e.g.,* through KEGG or Gene Ontology terms or molecular investigation). For example, mRNA

91   expression variation across the tree of life explains phenotypes, including toxin response in yeast

92   (20), flower induction in plants (21), diabetes (22), schizophrenia (23), cardiac metabolism (6),

93   and many others (14, 24, 25). Overall, an improved mechanistic understanding of traits enables

94   us to parse redundancy across biological organization levels and elucidate the evolutionary

95   processes and genetic architectures contributing to phenotypic variation.

96   Here, we used mRNA expression patterns to identify molecular mechanisms underlying

97   physiological phenotypes in the small saltmarsh teleost fish, *Fundulus heteroclitus,* captured

98   from three wild populations and acclimated to 12°C and 28°C. To identify molecular

99   mechanisms underlying physiological phenotypes, we quantified heart and brain mRNA

100   expression among 86 *F. heteroclitus* individuals for which we had 6 measured physiological

101   traits: whole animal metabolic rate (WAM), critical thermal maximum (CT_{max}), and four

102   substrate specific cardiac metabolic rates (CaM substrates: glucose [Glu], fatty acids [FA],

103   lactate+ketones+ethanol [LKA], and endogenous [END]) (26). These traits are heritable and

104   related to fitness, and CaM traits have been previously explained by mRNA expression variation

105   (6, 27-31). In these fish, trait-specific acclimation responses to 12°C and 28°C differed.

106   Specifically, acclimation responses eliminated temperature effects in CaM such that metabolic

107   rates were the same when assayed at 12°C and 28°C, reduced the temperature effect in WAM to

108   a small (1.2x) increase at higher temperatures (28°C), and enhanced CT_{max} by 6°C at higher

109   temperatures (28°C) (26). Importantly, within an acclimation temperature individuals showed

110   high trait variation with CVs (standard deviation/mean) for metabolic traits ranging from 22% to

111   55% (26). Presented here, we show that among these individuals, acclimation induced

112   differential expression of 366 heart mRNAs and 528 brain mRNAs across all three populations,

113   yet few differentially expressed mRNAs (one or less) were shared across all three populations.

114   Within each acclimation temperature, co-expressed mRNA modules were significantly

115   associated with WAM, CT_{max}, and CaM. Using Kyoto Encyclopedia of Genes and Genomes

116   (KEGG) and gene ontology (GO) enrichment, we identify biologically relevant networks among

117   co-expressed mRNA modules that explain these traits. These data link a simpler molecular

118   phenotype (mRNA expression) to complex trait variation to enhance our understanding of

119   biological pathways that affect these traits and may be important for evolutionary adaptation.

Results

121   *F. heteroclitus* used in this study were collected from three populations along the central

122   coast of New Jersey, USA near the Oyster Creek Nuclear Generating Station (OCNGS), which

123   produces a thermal effluent that locally heats the water. Three populations were sampled: 1)

124   north reference (N.Ref; 39°52'28.000 N, 74°08'19.000 W), 2) south reference (S.Ref;

125   39°47'04.000 N, 74°11'07.000 W) and 3) a central site located between the southern and

126   northern reference that is within the OCNGS thermal effluent (TE; 39°48'33.000 N,

127   74°10'51.000 W). The TE population used here differs by 4°C in habitat temperature from the

128   two references populations (average summer high tide temperature 28°C N.Ref and S.Ref, and

129   32°C for TE) but is otherwise ecologically similar (26) with evidence that the TE population has

130   locally adapted to life near the OCNGS (32). Prior to mRNA analyses for this study, individuals

131   were acclimated to both 12°C and 28°C, and whole animal metabolic (WAM) and thermal

132   tolerance traits (CTmax) were measured at both temperatures in all individuals. Next, substrate

133   specific cardiac traits (CaM) were measured at either 12°C or 28°C (10). Finally, heart and brain

134   samples were collected at the time of CaM measurements and prepared for mRNA sequencing.

135   In total 219 individual hearts and brains were collected and used for mRNA sequencing.

Sequencing analysis

137   mRNA expression was quantified by counting 3' end reads from two Illumina HiSeq3000

138   lanes that yielded 10,535 mRNAs among hearts and 10,932 mRNAs among brains after filtering

139   for 1.5 million reads per sample among hearts, 1 million reads per sample among brains, and at

140   least 30 counts in 10% of individuals per mRNA with each tissue. In hearts this yielded an

141   average of 8,224.7 reads per transcript and in brains an average of 6,578.5 reads per transcript.

142   Sequencing statistics and sample sizes are summarized in Table 1. Using all heart and brain

143   samples we examined mRNA expression variation using the top 500 most variable mRNAs for

144   principal component analysis (PCA). While 86% of variance on PC1 clearly split 86 heart and

145   brain samples into 2 distinct clusters, 25 samples (12 hearts, 13 brains) were clustered with the

146   wrong tissue and were excluded (Fig. S1). The remaining 86 samples were used for all further

147   tissue specific analyses. Using only these 86 individuals provides sufficient variation among

148   individuals to examine the relationships between mRNA expression and physiology, although

149   our analyses may be conservative because we removed 25 samples with ambiguous tissue

150   expression.

Differential expression analysis

152   Differential expression patterns among populations and acclimation temperatures were

153   identified using DESeq2 (33). First, to examine population and temperature specific expression

154   we used model design: (~Population + Acclimation-Temperature + Population*Acclimation-

155   Temperature). This analysis revealed significant Population*Acclimation-Temperature

156   interactions, suggesting acclimation temperature specific mRNA expression patterns among

157   populations. Because of the significant interactions, we analyzed individuals acclimated to 12°C

158   or 28°C separately with model design: ~Population to identify differentially expressed mRNAs

159   among populations within an acclimation temperature. Similar to other species, there were

160   significant differentially expressed mRNAs between acclimation temperatures, reflecting

161   changes in response to environmental temperature ((17, 34, 35), Table S1). Across all 3

162   populations, hearts had 366 mRNAs (3.5% of total) that were significantly different between the

163   two acclimation temperatures (FDR <0.05) with equal up and down regulated for 12°C *versus*

164   28°C (183 up and 183 down). For brains, 528 mRNAs (4.8% of total) were significantly

165   differently expressed between the two acclimation temperatures (FDR <0.05) with ~2.5-fold

166   more down regulated at 28°C relative to 12°C (148 up and 380 down).

167   While all three populations showed acclimation effects for heart and brain mRNAs, the

168   affected mRNAs were not shared among all populations (Fig 1). For any single population,

169   acclimation to 12°C and 28°C had significant mRNAs that were unique to each population

170   (Table S1). For the 366 significant heart acclimation mRNAs, 94-98% were unique to a single

171   population, and no acclimation heart mRNAs were shared across all three populations (Fig. 1A,

172   C, Table S1). Similarly, for the 528 significant brain acclimation mRNAs, 88-96% were unique

173   to one population (Fig. 1B, D, Table S1), and only one acclimated brain mRNA was shared

174   across all populations. These heart and brain population specific mRNA acclimation responses

175   were significant (chi-squared heart $p=1.64x10^{-10,}$ brain $p=2x10^{-16}$). Additionally, acclimation

176   significant mRNAs were unique to either heart or brain with no shared (0 mRNAs) acclimation

177   response between tissues. This reflected different expression patterns between tissues, previously

178   identified with PCA analysis (Fig S1).

179   At each acclimation temperature, populations also had significant expression differences

180   (Fig. 2, Table S2). Hearts at 12°C and 28°C had 158 or 153 differentially expressed mRNAs

181   among populations, respectively (Table S2). These represent 1.50% or 1.45% of all expressed

182   heart mRNAs at 12°C and 28°C, respectively; brains had 242 or 330 differentially expressed

183   mRNAs among populations at 12°C and 28°C, respectively. These represent 2.21% or 3.02% of

184   all expressed brain mRNAs at 12°C and 28°C, respectively. None of the population effects were

185   significant across all three populations (Fig. S3) for any acclimation temperature or tissue.

186   Importantly, there is an adaptive hypothesis: that the mRNAs in the anthropogenically

187   warmed population, TE, are uniquely different, where TE is significantly different from both

188   northern and southern reference populations with no significant differences between the

189   references (17, 36). For heart mRNAs at 12°C there are 10 mRNAs (6.33% of significant

190   mRNAs), and at 28°C there are 3 mRNAs (1.96%) where the TE population is uniquely different

191   from both references (Fig S3, Table S2). For brain mRNAs at 12°C there are 11 mRNAs (4.55%

192   of all significant mRNAs), and at 28°C there are 27 mRNAs (8.18%) where the TE population is

193   uniquely different from both reference populations. While the overall frequency of differentially

194   expressed genes is small (1.45% to 3.02% *vs.* total 10K mRNAs), the pattern where the TE

195   population is different from both northern and southern reference populations but the two

196   reference populations are not different is indicative of adaptation.

Variation in mRNA expression

198   In addition to differential expression analysis, we were interested in the degree of mRNA

199   expression variance. Previously, we found that variation in WAM, CT_{max}, and substrate specific

200   CaM was greater at 12°C than at 28°C. To determine if this was also true for mRNA expression,

201   we quantified each mRNA's coefficient of variation (CV, standard deviation/mean*100%) at

202   12°C and 28°C. For both heart and brain tissue there was greater average CV across all mRNAs

203   at 12°C than at 28°C (T-test, heart p= 9.587e-05, brain p= 0.02014), similar to our findings of

204   greater variation in physiological traits measured at 12°C.

Weighted gene co-expression network analysis

206   We used weighted gene co-expression network analysis (WGCNA, (37)) to detect co-

207   expressed mRNA clusters. WGCNA approaches group mRNAs with similar expression patterns

208   into independent modules. Expression patterns for all mRNAs within a module were then

209   summarized into principal components called module eigengenes (MEs, Table 2 for heart

210   mRNAs and Table 4 for brain mRNAs), and these MEs were correlated to each of the six

211   physiological traits (Table 3 shows significant heart MEs, and Table 5 shows significant brain

212   MEs). Each ME has a "hub-MM", the mRNA with the highest correlation to the ME, with MM

213   being the correlation coefficient (Tables 2 and 4).

214   MEs were correlated to the body mass residuals for the six traits (WAM, CTmax, and the

215   four substrate specific CaM). These analyses were done across all three populations because

216   populations did not have any significant differences among traits. Each of the ME-trait

217   correlations had a "hub-GS" – the mRNA in the module with the highest correlation to the trait,

218   with GS (gene specific) being the correlation coefficient for this single mRNA. Both heart and

219   brain WGCNA analysis used a minimum module size of 30 mRNAs per module and combined

220   modules with correlation >75% (see methods). To verify that trait *versus* ME correlations were

221   not driven by spurious outliers, we used a jack-knife approach to subsample 90% of individuals

222   and repeat ME-trait correlations 100 times. Correlations that were significant in at least 70 out of

223   100 repeated correlations in the same direction (positive or negative correlation coefficient) were

224   retained for further analysis (see methods).

225   Heart WGCNA

226   For heart mRNAs we found 39 co-expression modules with 90 to 554 mRNAs in each

227   module (Table 2), and these heart MEs (first principal component of co-expressed mRNAs) were

228   correlated to six physiological traits at each acclimation temperature. There were 12 significant

229   ME-trait correlations: 9 heart MEs with 5 temperature specific traits (FDR <0.05, Table 3, Fig 3

230   and 4). Traits correlated with at least one of these 9 heart MEs included: at 12°C WAM, CT_{max},

231   FA CaM, heart mass, LKA CaM, and at 28°C, CTmax (Table 3, Fig. 3, 4). Two of these modules

232   (ME4_heart, ME5_heart) were correlated with both WAM at 12°C and FA CaM at 12°C, and

233   one module (ME6_heart) was correlated with both LKA CaM at 12°C and heart mass at 12°C.

234   WAM at 12°C had the most significant ME correlations (4 total), followed by FA CaM at 12°C

235   (3 total) and LKA CaM at 12°C (2 total). The other three traits were each correlated with a single

236   module (Table 3). On average, a single heart module explained 55% of variance for one trait

237   with a minimum of 48.5% (ME1_heart with CT_{max} 12°C) and a maximum of 65% (ME6_heart

238   with LKA 12°C).

239   For traits that were significantly correlated with more than one ME, a multiple correlation

240   coefficient was calculated. For WAM at 12°C, the four significant MEs together had a multiple

241   correlation coefficient of 82%, the three significant MEs for FA CaM at 12°C had a multiple

242   correlation coefficient of 79.5%, and the two significant MEs for LKA CaM at 12°C had a

243   multiple correlation coefficient of 75.5%. All modules correlated with FA CaM at 12°C and

244   CT_{max} at 12°C had positive correlation coefficients while all other significant trait *versus* ME

245   correlations in hearts had negative correlation coefficients.

246   Brain WGCNA

247   Brain mRNAs had 42 total co-expressed modules with 142 to 393 mRNAs per module

248   (Table 4). There were 6 significant ME-trait correlations (FDR <0.05) that included 4 unique

249   modules and 4 temperature specific traits (Table 5, Fig. 5): at 12°C, body mass and at 28°C,

250   CT_{max}, WAM, and body mass. The trait with the most significant correlations was CT_{max} at 28°C

251   (3 significant ME's), and two of these were also significant with body mass at 28°C

252   (ME3_brain) or WAM at 28°C (ME4_brain). On average, the correlation coefficient for a brain

253   ME was 62% with a minimum of 56.8% (ME3_brain with CT_{max} 28°C) and a maximum of

254   70.2% (ME4_brain with WAM 28°C). For CT_{max} at 28°C, which was correlated with three MEs,

255   the multiple correlation coefficient was 71.7%. All correlations between traits and brain MEs

256   were negative except for body mass at 28°C.

257   KEGG and GO Enrichment

258   Critical thermal maximum enriched terms

259   MEs were tested for KEGG and GO term enrichment using the complete set of tissue

260   specific mRNAs as the gene universal or reference set (10,535 for heart, 10,932 for brain; Tables

261   S3 and S4). For CT_{max}, all 5 MEs were significantly enriched for KEGG pathways (ME1_heart,

262   ME2_heart, ME2_brain, ME3_brain, and ME4_brain) and included MAPK signaling, mTOR

263   signaling, glyoxylate and dicarboxylate metabolism, insulin signaling pathway, glutathione

264   metabolism, metabolic pathways, carbon metabolism, and tryptophan metabolism. Enriched GO

265   terms included regulation of organ growth and cellular stress response in heart and AMP-

266   activated protein kinase (AMPK) activity in brain. Notably, MAPK has been linked to adaptive

267   cold tolerance (38, 39) and lipid metabolism (40). Additionally, mTOR is involved with energy

268   homeostasis, has been linked to growth and longevity, and may be sensitive to temperature

269   variation (41-43). Finally, AMPK induces cellular ATP production in mammals and is important

270   for thermal stress response in ectotherms (44-46). Furthermore, AMPK phosphorylation in Coho

271   salmon and rainbow trout hearts has been correlated with exposure above optimum temperatures

272   (47), suggesting a role of AMPK in fish thermal response. ME for CT_{max} at 28°C contained

273   substantial overlap in enriched KEGG pathways related to metabolism with 5 out of 8 terms

274   enriched in both heart and brain modules. So, although there were different mRNAs in heart and

275   brain modules correlated with CT_{max} at 28°C, the KEGG terms related to metabolism were

276   shared (5 out of 8), suggesting that different mRNAs in heart and brain belonged to similar

277   pathways impacting CT_{max} at 28°C.

278   Metabolic rate enriched terms

279   Modules significantly correlated with WAM were enriched for KEGG pathways including

280   oxidative phosphorylation (heart only), glutathione metabolism (brain only), and metabolic

281   pathways (both heart and brain). In addition, ME3_heart was correlated with FA CaM at 12°C

282   and enriched for KEGG terms including metabolic pathways, forkhead protein (FoxO) signaling,

283   and metabolism of NADH derivatives (nicotinate and nicotinamide). Notably, FoxO proteins,

284   especially FoxO1, are involved in energy homeostasis and may aid in the switch from

285   carbohydrate (glycolytic) to fatty acid metabolites (48). One module, ME5_heart, was

286   significantly correlated with WAM at 12°C and FA CaM at 12°C and contained several KEGG

287   pathways directly related to fatty acid metabolism as well as known transcription factors like

288   PPAR that impact metabolic homeostasis by controlling expression of many metabolism related

289   genes (49). Previously, partial correlation coefficients between FA CaM at 12°C and WAM at

290   12°C were negatively correlated (26), and similarly ME_5 had opposite correlation coefficients

291   for these two traits (Table 3, Fig. 3, 4). This correlation between traits and their correlations to

292   MEs also occur for CT_{max} and WAM at 28°C (26) and ME4_brain (enriched for glutathione

293   metabolism and metabolic pathways). This emphasizes the biological relevance of the MEs in

294   that the same MEs are associated with traits that have significant partial correlations.

Discussion

The role of mRNA expression in acclimation and evolution

297   For mRNA expression in both tissues, we found significant interactions between

298   acclimation and population effects: the expression of several hundred mRNAs differed between

299   acclimation temperatures, but these were not shared among all three populations (Fig. 1); also,

300   the thermal effluent site (TE) had adaptive patterns different from the two reference sites that

301   alter mRNA expression, but these differed between acclimation temperatures (Fig, S3).

302   Previously (26), in these same individuals, acclimation response to 12°C and 28°C affected six

303   physiological traits (WAM, CT_{max}, and the four substrate specific CaM). For CT_{max}, there was an

304   expected enhancement: higher CT_{max} in individuals experiencing warmer environments. For

305   metabolic rates (WAM and CaM), acclimation to 12°C and 28°C mitigated the effect of

306   temperature (26). Specifically, without physiological acclimation there is an expected ~3-fold

307   increase in metabolic rates with the 16°C increase in acclimation and assay temperature (*i.e.,*

308   with a doubling for every 10°C) (50). Yet, WAM had only ~1.2-fold increase (34) from 12°C to

309   28°C, and CaM had no significant increase between temperatures (26). Presented here, across all

310   three populations, acclimation produced significant differential mRNA expression (FDR <0.05)

311   in hearts (366 mRNAs) and brains (528 mRNAs, (Table S1)). These mRNA expression changes

312   associated with acclimation responses are similar to prior studies among ectotherms where

313   transcriptomic response to temperature acclimation enhances thermal performance (34, 35, 39,

314   51). For example, in three-spine stickleback and other fishes, metabolic enzyme expression and

315   mitochondrial volume density increase in response to cold acclimation can compensate for

316   reduced enzyme catalytic rate with decreased temperature (34, 52, 53). Similarly, in eastern

317   oysters (*Crassostrea virginica*), among a suite of environmental factors (temperature, pH,

318   salinity, dissolved oxygen, etc.) temperature was the most important transcriptomic variation

319   predictor with thermal stress increasing oxidative phosphorylation transcript expression (54).

320   Even Antarctic fish, which are adapted to extreme cold, show plasticity in metabolic transcripts

321   with temperature acclimation that impacts whole animal performance (53, 55).

322   While quantitative gene expression changes are common with acclimation (affecting both

323   mRNA and proteins (39, 56)), what was surprising was that mRNA acclimation responses were

324   different among populations—for heart mRNAs, no significant acclimation responses were

325   shared among all three populations, and for brains only 1 mRNA was shared among the three

326   populations. Further, 88-98% of significant acclimation responsive mRNAs are unique to each

327   population. In contrast, the six physiological traits' acclimation responses were not different

328   among populations. All populations were subjected to a common environment for a long time (~

329   1 year or nearly 30-50% of a fish's expected life span) with acclimation to the 12°C and 28C.

330   Thus, the difference in acclimation mRNA response among populations was not due to short-

331   term physiological effects and may be due to genetic polymorphisms driving acclimation

332   responses but could also arise by irreversible developmental effects or trans-generational effects.

333   Regardless of the genetic mechanisms responsible for the divergent mRNA acclimation

334   responses among populations, these data suggest that multiple different mRNA expression

335   patterns drive acclimation responses. This conclusion is similar to CaM measurements in Maine

336   and Georgia populations, where the mRNAs that explain substrate specific metabolism varied

337   among groups of individuals (6). The observations that plasticity in the six physiological traits

338   between temperatures are similar among populations, yet mRNA acclimation responses differ

339   among population, suggest that multiple redundant molecular mechanisms drive temperature

340   compensation.

341   There is a single difference among populations for the six physiological traits: endogenous

342   CaM at 28°C. Yet, populations had significant differences in mRNA expression specific for each

343   temperature, and none of the population significant mRNAs were shared at 12°C or 28°C (Fig.

344   S3, Table S2). One pattern, where the anthropogenically warmed TE population was

345   significantly different from both northern and southern reference populations (not heated by

346   thermal effluent from nuclear power plant), is indicative of adaptation (17, 32, 36). What differs

347   here is that we examined mRNA expression that can be affected by DNA polymorphisms and

348   also influenced by environment (i.e., GxE). Thus, the difference among populations in mRNA

349   expression are dependent on the thermal environment, and if adaptive, suggest that the different

350   genetic polymorphisms are responsible for adaptive divergence at different temperatures. This

351   conclusion is similar to comparison within and among species: adaptive divergence in mRNA

352   expression is dependent on the thermal environment experienced by individuals (17). For the TE

353   population, Dayan *et al.,* conclude that there was adaptive divergence based on evolutionarily

354   significant DNA polymorphisms (32). We would extend this to suggest that populations have

355   evolved different mRNA expression patterns that are dependent on the thermal environment but

356   that, nevertheless, produce similar physiological phenotypes.

Biological relevance of co-expressed mRNAs

358   WGCNA identified co-expressed mRNA modules, MEs, highly correlated with WAM,

359   CT_{max}, FA CaM, LKA CaM, and body and heart mass, depending on the acclimation temperature

360   (Fig, 3, 4, and 5). The average ME-trait correlation was 0.55 for heart and 0.62 for brains (Table

361   3 and 5). These MEs, containing 90-554 mRNAs each, contained few (0-10) mRNAs with

362   significant expression differences among populations. WGCNA has been previously used to

363   identify mRNA expression networks important for various pathologies including cardiovascular

364   disease (57, 58), cancers (59-63), and diabetes (58, 64), among others. In non-human organisms,

365   WGCNA has been used to characterize response to the environment, including heat stress in

366   turbot (65), carotenoid metabolism in apricot fruit (66), and disease response in corals (67).

367   Although few studies, to our knowledge, have validated correlations using jack-knife

368   subsampling to ensure that the correlations were consistent among most individuals and not

369   driven by a few outliers, these studies similarly identified potentially meaningful correlations

370   between traits and co-expressed mRNAs. Importantly, in this study, the correlation patterns

371   between MEs and physiological traits are similar to the correlations among physiological traits.

372   For example at 12°C, FA CaM and WAM were negatively correlated (26) and similarly

373   ME5_heart was significantly correlated with opposite signs with these two traits (*i.e.,* positively

374   correlated with FA CaM but negatively correlated with WAM, Table 3, Fig. 3, 4). Additionally,

375   MEs correlated to WAM and CaM were enriched in KEGG metabolic pathways and GO terms

376   related to metabolism. These data indicate that modules represent independent, biologically

377   important mRNA networks.

378   The biological importance of co-expressed mRNA networks is also supported by their

379   relation to metabolic processes. Eleven of the 13 significant heart or brain MEs were

380   significantly enriched for at least one KEGG term, and 6 were significantly enriched for at least

381   one GO term. KEGG terms mapped to biologically relevant KEGG pathways including

382   metabolic pathways, mechanistic target of rapamycin (mTOR) signaling, mitogen activated

383   protein kinase (MAPK) signaling, insulin signaling, and metabolism and biosynthesis of various

384   macromolecules including glycogen, NADH precursors, amino sugars, and fatty acids (Table S3,

385   S4). Importantly, 8 out of 11 modules with significantly enriched KEGG terms mapped to at

386   least one metabolism related KEGG pathway. Yet, we also found several enriched KEGG

387   pathways and GO terms that were uniquely enriched in only one or few modules and seemingly

388   unrelated to the correlated trait(s) (*e.g.,* cellular senescence). This could indicate a limited

389   understanding of the complexity and interconnectedness among biological pathways and how

390   different pathways affect a diversity of traits, mRNAs that are minimally annotated and missing

391   relevant pathway involvement, or that mRNA expression impacts biological processes, that

392   indirectly impact the traits we have measured. The concept that there is a limited understanding

393   of the interactions among pathways is supported by mitochondrial respiration studies,

394   specifically concerning the oxidative phosphorylation pathway (OxPhos) (68). When examining

395   the selectively important nuclear genes effecting OxPhos, none of the genes were among the 97

396   proteins in the OxPhos pathway; instead, they were in diverse pathways, some of which made

397   sense (*e.g,* ADP transport- where ADP is a substrate for OxPhos) (68). Thus, the few MEs

398   associated with unexpected pathways may indicate a complexity in physiological traits where

399   many pathways and the genes in these pathways affect trait variation.

400   Previously, data from our laboratory demonstrated that natural variation in substrate

401   specific cardiac metabolism in *F. heteroclitus* could be explained by cardiac mRNA expression

402   using microarray data (6). Similar to the WGCNA approach presented here, the first principal

403   component of mRNA expression from different metabolic pathways (oxidative phosphorylation,

404   TCA cycle, glycolysis) explained substrate specific CaM among individuals with different

405   pathways of mRNAs explaining substrate specific metabolisms in different individuals. Here, we

406   found that mRNA expression explained a similar proportion of substrate specific CaM as

407   previously reported (~80%) using three MEs to explain a single trait (FA CaM at 12°C).

408   Few, if any, studies have examined the correlation of co-expressed mRNA with CT_{max}

409   (although see (69)). Our analyses found that 341 heart mRNAs in two co-expressed modules and

410   682 brain mRNAs in three co-expressed modules were associated with CT_{max} at 12°C or 28°C,

411   with different MEs at each temperature. Futhermore, heart and brain significant ME for CT_{max} at

412   28°C share enriched KEGG pathways, yet do not share any mRNAs, suggesting that different

413   mRNAs affect a common set of pathways that impact CTmax. These data suggest that CTmax is

414   polygenic and relies on different mRNAs in different tissues at different temperatures. There is

415   prior evidence suggesting that CT_{max} is polygenic: a GBS study covering ~ 0.1% of the genome

416   found up to 47 single nucleotide polymorphisms (SNPs) that explained 43.4% of variation in

417   CT_{max} among *F. heteroclitus* individuals collected from Georgia, New Jersey, and New

418   Hampshire, USA (70). Here, a greater proportion of CT_{max} was explained with mRNA

419   expression, up to 71.7% with 3 brain MEs. This increase in explained CT_{max} variance by mRNA

420   expression is likely due to the combined heritable and physiologically inducible nature of mRNA

421   expression. Few (0-10) of the mRNAs in MEs were differentially expressed between 12°C and

422   28°C, and thus MEs that explained CT_{max} variation within each of acclimation temperatures are

423   not due to acclimation effects on mRNA expression. Instead, the CT_{max} variation within each

424   acclimation temperature appears to be due to individual variation in mRNA expression, which

425   may be explained by nucleotide variation driving differential expression.

426   Whole animal metabolism, WAM, is a fundamental physiological process that defines how

427   animals live, niche space, evolutionary transition, and the human condition (71-75). There is

428   significant literature investigating metabolic rate variation (*e.g.* (31, 76, 77)); however, the

429   relationship between metabolic rate and mRNA expression remains poorly understood. This may

430   be due to the complex nature of whole animal metabolism, which is a sum of tissue specific

431   metabolic demands and a balance between growth, maintenance, and energy storage. Yet, we

432   find 82% of 12°C WAM variation related to four heart MEs with 736 mRNAs and 50% of 28°C

433   WAM variation related to one brain ME with 142 mRNAs. These data indicate that a large

434   proportion of WAM can be explained by mRNAs within a common pathway impacting cardiac

435   metabolic processes and thus provides insight into the physiological relationship between

436   cardiorespiratory performance and overall metabolism (78-81).

437   These WGCNA analyses suggest that many mRNAs in several biochemical pathways

438   define the physiological state among individuals. Yet, the careful reader will note two substantial

439   complexities: 1) heart MEs explain the variation in many physiological traits at 12°C but few at

440   28°C, and brain MEs explain the variation in many physiological traits at 28°C but few at 12°C

441   and 2) mRNAs within MEs are enriched for many diverse and unexpected pathways as discussed

442   above.

443   The difference between tissue specific MEs and their association with physiological traits

444   is related to CaM, WAM, and CT_{max} having higher inter-individual variation at 12°C than 28°C,

445   and similarly there is greater mRNA expression variation at 12°C than at 28°C. Thus, the more

446   frequent explanation of physiological traits by 12°C mRNA expression may simply result from

447   greater statistical power due to the greater variance in both physiological traits and mRNA. Yet,

448   in brains, mRNAs explain WAM and CT_{max} at 28°C. While we can only speculate, these data

449   suggest that at the higher temperature brain mRNA expression is more important than cardiac

450   mRNA expression. There is evidence that acclimatory response to temperature in brain is greater

451   than in hearts (more acclimatory mRNAs and greater decreased mitochondrial function in brain

452   when compared to heart tissue (82)). This is similar to our data: brains at 28°C have more

453   acclimation responsive mRNAs than hearts, and more population divergence than brains at 12°C

454   or hearts at either temperature. Together these data suggest that the variation in WAM and

455   CTmax are more dependent on brain specific expression at 28°C.

456   Many of MEs are associated with KEGG pathways that impinge on metabolic processes.

457   Physiological processes are within 9 heart MEs and 4 brain MEs (Tables 2 and 4), and these ME

458   each contain 90-554 mRNAs. Each of these MEs is significantly enriched for multiple KEGG

459   and GO pathways (Tables S3 and S4), including pathways not typically thought to be directly

460   involved in metabolism or thermal tolerance. Similar to nuclear genes that impact *Fundulus*

461   mitochondrial respiration (68), these data suggest that many metabolically distant genes affect

462   physiological variation. This is important because too often publications have "just so stories" (a

463   la (83, 84)) that only focus on a few preconceived genes to explain functional physiological

464   variation (85). While it is understandable to highlight a prior expectation, doing so limits our

465   understanding of how genotypes effect phenotypes.

466   In summary, the data provided here build on previous findings that inter-individual mRNA

467   expression variation is biologically important by identifying that 1) mRNAs important for

468   acclimation are population specific, 2) divergence among geographically close populations does

469   not include acclimation responsive mRNAs, and 3) mRNA expression associated with

470   physiological traits is tissue specific and dependent on the thermal environment. We highlight

471   that biologically important mRNA networks are related to 48-82% of variation in whole animal

472   metabolism thermal tolerance, or substrate specific cardiac metabolism and are different at

473   different thermal environments. This suggests that mRNA variation among individuals within

474   and among populations is important for explaining complex trait variation and, surprisingly, that

475   while similar pathways can be important at different temperatures, the tissues where they are

476   expressed differ: heart mRNA expression explains variation in more traits at 12°C, and brain

477   mRNA explains variation in more traits at 28°C.

478   ## Methods

479   ### Animal care and use

480   Fish were collected in live traps in September 2018 at three sites in central New Jersey,

481   USA near the Oyster Creek Nuclear generating station (OCNGS). Sites included one north

482   reference (N.Ref; 39°52'28.000 N, 74°08'19.000 W), one south reference (S.Ref; 39°47'04.000

483   N, 74°11'07.000 W), and a central site located within the thermal effluent of the OCNGS (TE;

484   39°48'33.000 N, 74°10'51.000 W). All fish were transferred live to the University of Miami, FL

485   where they were kept in accordance with the University of Miami Institutional Animal Care and

486   Use Committee (IACUC) guidelines.

487   Individuals from all three populations were common gardened to 20°C for three months

488   (12:12 light dark cycle) and kept at 20°C in a common recirculating seawater system (15ppt) at

489   12 hours light:12 hours dark, then subjected to *pseudo*-winter for 6 weeks at 8°C (8:16 light dark

490   cycle). Following the *pseudo-winter*, half of the fish from each population were acclimated to

491   12°C and the other half to 28°C (16:8 light dark cycle) for four weeks prior to determination of

492   WAM and CT_{max}. Following this acclimation, fish originally acclimated to 12°C were acclimated

493   to 28°C and *vice versa* for at least four weeks, and WAM and CT_{max} were measured at the new

494   acclimation temperature. After a minimum one-week recovery period post- CT_{max}, fish were

495   sacrificed, substrate specific CaM was measured at the second acclimation temperature, and

496   mRNA was isolated. Thus, WAM and CT_{max} were measured in all individuals at 12°C and 28°C,

497   but CaM and mRNA were sampled from half the individuals acclimated at 12°C and the other

498   half at 28°C.

499

500   Quantifying metabolic and thermal tolerance traits

501   Individuals acclimated to 12°C and 28°C for at least 4 weeks were measured for whole

502   animal metabolic rate (overnight intermittent flow respirometry) with a minimum of 20 replicate

503   metabolic rate measures per individual used to determine the standard metabolic rate (SMR) in

504   mgO2 hr^{-1} (7). Critical thermal maximum (CT_{max}) was measured in a 10-gallon tank that was

505   slowly heated at a rate of 0.3°C min^{-1} as in (86) and was defined as the point when fish lost

506   equilibrium in the water column for 5 consecutive seconds. Finally, substrate specific cardiac

507   metabolic rate (CaM, substrates: 5mM glucose, fatty acids – 1 mM Palmitic acid conjugated to

508   fatty-acid-free bovine serum albumin, lactate+ketones+ethanol – 5 mM lactate, 5 mM

509   hydroxybutyrate, 5 mM ethyl acetoacetate, 0.1% ethanol, endogenous – substrate free Ringers

510   media) was measured using micro-respirometry. Heart ventricles were dissected out and splaying

511   in Ringers media before being transferred to a custom 1mL chamber system (10). Each heart was

512   measured for glucose (GLU), then fatty acids (FA), followed by lactate+ketones+ethanol (LKA),

513   and endogenous (END) cardiac metabolic rate. All substrates except GLU used non-reversible

514   glycolytic enzyme inhibitors, so the order of substrates did not differ among hearts. Each heart

515   was measured for 6 minutes per substrate with the last 3 minutes used to calculated oxygen

516   consumption in $pmolO_2\ sec^{-1}$. All respirometry measurements used Presens fiber optic oxygen

517   sensors with flow through cells (WAM) or sensor spots (CaM). Both WAM and CaM were

518   measured at both acclimation temperatures (12°C and 28°C) for the same individuals, and CaM

519   was measured at a single acclimation temperature. For additional methods and analysis of

520   physiological data see (26).

521   mRNA library preparation and sequencing

522   Tissues for mRNA expression were stored in chaotropic buffer at the time of CaM

523   measurement and captured gene expression variation due to long term temperature acclimation

524   rather than heat shock or acute temperature response (*e.g.,* resulting from higher temperatures

525   during CT_{max} measurements) because CT_{max} measurements were performed at least a week prior

526   to tissue isolation. We extracted total RNA from homogenized heart and brain tissues using

527   phenol-chloroform isoamyl alcohol isolation and treated RNA samples with DNAse to remove

528   genomic DNA. For each sample we started with 50ng of RNA and captured the 3' mRNA ends

529   using an NVdT primer with a poly-A tail for first strand cDNA synthesis (Table S5). This primer

530   contained a unique barcode for each sample (1-96), which allowed all samples in a single plate to

531   be pooled for the remaining library preparation steps. Nick translation was used to make double

532   stranded cDNA that was digested with an in-house purified Tn5 transposase (as in (87)) loaded

533   with partial adapter sequences to generate fragments of double stranded cDNA ranging from

534   ~300-800bp (Table S6). Libraries were amplified for 17 PCR cycles using primers

535   complimentary to the inserted partial adapter sequence and a plate level barcode to fully

536   multiplex samples.

537   mRNA data processing and analysis

538   A total of 219 libraries (110 individuals, 2 tissues per individual, 1 individual only heart

539   was collected) were pooled and sequenced on 2 lanes of Illumina HiSeq4000 (dual end 150bp

540   reads) at the Genewiz LCC facility, South Plainfield NJ, USA. Raw reads were trimmed with

541   BBDuk (from BBMap v. 38.87) to remove adapter sequences, aligned with STAR (v. 2.7.5) to

542   the *Fundulus heteroclitus* genome, and counted with Featurecounts (v. 1.4.6-p5, parameters: -T 4

543   -s 2 -t gene -g gene_id).

544   The raw counts table was imported into R Studio (v. 1.4.1106) and all counts were

545   normalized for library size using the median ratio method (88) with the "estimateSizeFactors"

546   function in DESeq2 (33). Samples with a minimum of 1.5 million reads for hearts or 1 million

547   reads for brains were retained and filtered to keep only mRNAs with at least 30 counts in 10% of

548   individuals. Principal component analysis (PCA) using "plotPCA" function from the DESeq2

549   package was used to examine variation among all samples. Among all samples, the 500 most

550   variable mRNAs were used for PCA. In this analysis, 12 hearts and 13 brains were removed as

551   outliers because they differed in expression from other same-tissue samples (*i.e.,* some hearts had

552   "brain-like" expression patterns and *vice versa,* Fig. S1). This reduced variation within a tissue

553   and reduced sample size; however, the individuals removed were not from a single acclimation

554   temperature or population so likely had little overall impact on further analyses. In a separate

555   tissue specific principal component analysis, clustering of samples by biological effects

556   including sex, habitat temperature, population, acclimation temperature, and date of tissue

557   collection (possible batch effect) was examined. Batch effects did not split individuals along any

558   of the principal components examined (PC1-PC4) for heart or brain. In this separate analysis,

559   heart PC1 accounted for 18%, heart PC2 for 7%, brain PC1 for 11%, and brain PC2 for 7% of

560   the variation among individuals. No biologically relevant clustering was detected among the first

561   4 principal components for either tissue (chi-squared test p>0.05, Fig. S2A, B). To determine the

562   degree of variation in mRNA expression among groups within a tissue, the coefficient of

563   variation (CV, standard deviation/mean) for each expressed mRNA was calculated and the

564   average CV compared among groups.

565   Differential expression analysis

566   DESeq2 (33) package in R was used for differential expression analysis separately for

567   heart and brain. To identify differentially expressed mRNAs between acclimation temperatures

568   within populations, the DESeq model used was: ~Population + Acclimation_Temperature +

569   Population*Acclimation_Temperature. Additionally, due to significant interaction between

570   population and acclimation temperature, a separate analysis was used to find differentially

571   expressed mRNAs among populations within an acclimation temperature; individuals measured

572   for CaM only at 12°C or 28°C were used with DESeq model: ~Population. Multiple test

573   correction across all comparisons made within a model used the Benjamin Hochberg false

574   discovery rate with a significance threshold of 0.05.

575   Weighted gene co-expression network analysis

576   To identify sets of co-expressed mRNAs, weighted gene co-expression network analysis

577   (WGCNA, (37)) was completed for heart and brain separately. Network calculation, used to

578   group mRNAs into co-expressed modules, for heart and brain, used soft thresholding to generate

579   a scale free network with high similarity (soft thresholding power set to 5 in heart, 4 in brain)

580   before calculating the topological overlap measure (TOM) and using dynamicTree with

581   minimum module size of 30 and threshold for module merging of 0.75. The first principal

582   component of each independent module (below the threshold for module merging), known as the

583   module eigengene (ME), was then correlated with temperature specific quantitative traits using

584   signed Pearson's correlation. Multiple test correction across all correlations made for a single

585   trait used the Benjamin Hochberg false discovery rate with a significance threshold of 0.05. To

586   remove significant correlations potentially driven by outliers, a jack-knife approach was used to

587   subsample 90% of individuals and repeat the signed Pearson correlation analysis 100 times.

588   Correlations that were significant in >70 out of 100 subsamples in the same direction were robust

589   to outliers and reported as significant. A multivariate correlation coefficient was calculated for

590   traits significantly correlated with more than one module by correlating the fitted values from a

591   linear model with formula: trait~ME1+ME2..ME# with trait data. This multivariate correlation

592   coefficient represents the ability of the MEs together to accurately predict the trait. For

593   significant modules, the mRNAs with the highest module membership (MM, correlation between

594   mRNA expression and module eigengene) and the highest gene significance for a given trait

595   within a module (GS, correlation between mRNA expression and a given quantitative trait) were

596   also identified.

597   Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment

598   To identify biologically important networks within WGCNA modules that were

599   significantly correlated with at least 1 trait, we used Kyoto Encyclopedia of Genes and Genomes

600   (KEGG) pathway and gene ontology (GO) enrichment analyses. First, the genome was mapped

601   to KEGG and GO terms using eggNOG mapper (89) with default parameters. The KEGG and

602 GO terms were then matched to the set of expressed mRNAs for heart and brain. The list of

603 mRNAs in each module was compared to the set of expressed mRNAs (set as the reference or

604 gene universe) in each tissue for enrichment analysis in R using the clusterProfiler package

605 "enricher" function for KEGG terms (90). To map enriched KEGG terms to KEGG pathways,

606 the KEGG Mapper online tool was used with annotations from the closest relative, zebrafish

607 (*Danio rerio*) (91, 92). Cytoscape BiNGO was used for GO enrichment using the set of

608 expressed mRNAs as the reference to examined enrichment of biological process, molecular

609 function, and cellular component GO terms (93). Significant KEGG and GO terms are reported

610 with FDR p-value threshold of 0.05.

Data Availability

612 All sequence data is available in NCBI SRA:

613 https://dataview.ncbi.nlm.nih.gov/object/PRJNA796010?reviewer=f1u5dbo46558lklv8m2cknap

614 87 (public DOI available upon publication). All physiological data is available in Dryad:

615 https://doi.org/10.5061/dryad.0gb5mkm0w . Code for processing of raw sequence files and all

616 data analysis and visualization conducted in R is available in Github:

617 https://github.com/mxd1288/OCNJ_F18_RNA.git .

Author Contributions

Acknowledgements

627 Tables and Figures

Table 1: Sequencing statistics. Sequencing statistics and sample size distribution among tissues, acclimation temperatures, and populations.

| Heart Tissue	Sequencing Statistics		Acclimation Temperature	Population	Sample Size
Total N	41		12°C	N.Ref	4
Total mRNAs	10,535			TE	6
Average reads per mRNA	8,224.70			S.Ref	9
Minimum reads per individual	1.5 million		28°C	N.Ref	4
Average reads per individual	2.17 million			TE	8
				S.Ref	10
Brain Tissue	Sequencing Statistics		Acclimation Temperature	Population	Sample Size
Total N	45		12°C	N.Ref	9
Total mRNAs	10,932			TE	11
Average reads per mRNA	6578.5			S.Ref	8
Minimum reads per individual	1 million		28°C	N.Ref	5
Average reads per individual	1.74 million			TE	6
				S.Ref	6

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Table 2: Heart Significant Modules. Columns include: Module = identifier for module eigengene (ME, first principal component of module), KEGG Pathway = top KEGG pathway determined by number of KEGG terms, Module Size = number of mRNAs in the module, Hub MM = mRNA with highest correlation with module eigengene, MM = correlation of hub mRNA with module eigengene, Positive MM = proportion of mRNAs with positive MM in the module, KEGG Terms in Pathway = enriched KEGG terms in the listed KEGG pathway. *Indicates modules where more than one KEGG Pathway had the same number of enriched KEGG terms, in which case the most informative KEGG pathway was selected.

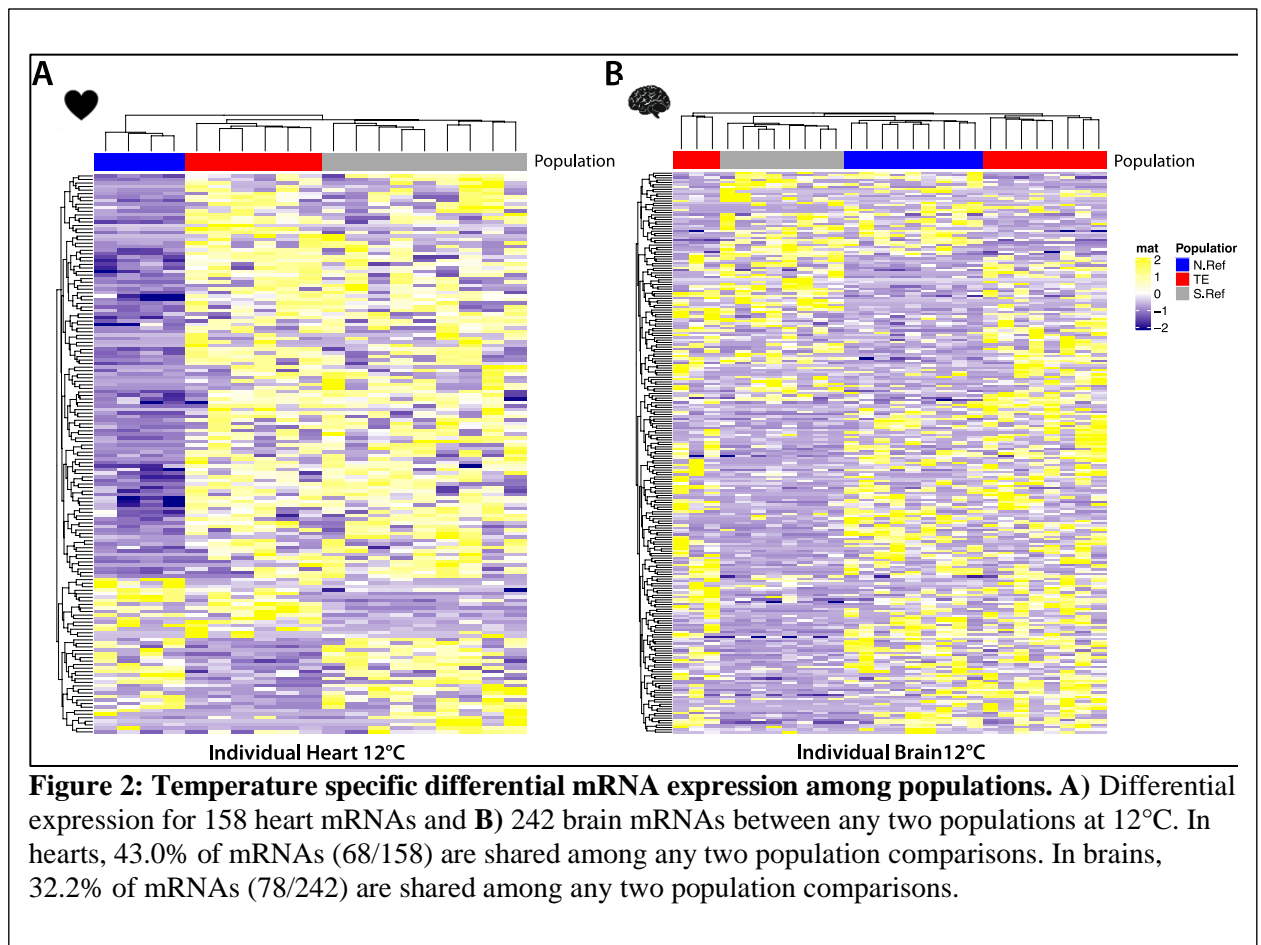| Module	KEGG Pathway	Module Size	Hub MM	MM	Positive MM	KEGG Terms in Pathway
ME1_heart	MAPK signaling pathway*	194	SAC3D1	0.51	74.70%	K04459 K20216
ME2_heart	Endocytosis*	147	NCKAP5L	0.52	31.30%	K12471
ME3_heart	FoxO signaling pathway*	281	CLMPB	0.53	23.80%	K11411
ME4_heart	RNA degradation	168	TRAFD1	0.49	32.10%	K03681
ME5_heart	Metabolic pathways	336	LOC105935504	0.39	30.90%	K00166 K00232 K03844 K05546
ME6_heart	NA	554	WNT5B	0.65	34.10%	NA
ME7_heart	NA	454	MOSPD2	0.57	30.60%	NA
ME8_heart	Oxidative phosphorylation*	142	TXLNA	0.45	32.40%	K0393
ME9_heart	Ribosome*	90	YIF1B	0.54	66.70%	K02899

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Table 3: Heart Significant Module Trait Correlations. Significant heart ME correlations with FDR p<0.05. Columns include: Trait = traits significantly correlated with a given ME (critical thermal maximum: CT_{max}, whole animal metabolic rate: WAM, cardiac metabolic rate: CaM with substrates fatty acids = FA, lactate+ketones+ethanol = LKA), Module = identifier for module eigengene (ME, first principal component of module), Correl coef = Pearson's signed correlation coefficient for trait and ME, FDR P-value = multiple test corrected p-value for trait *versus* module correlation, Hub GS = mRNA with highest gene significance for the trait in the module, GS = gene significance, correlation between top module mRNA and trait, Positive GS = proportion of mRNAs in the module that are positively correlated with trait.
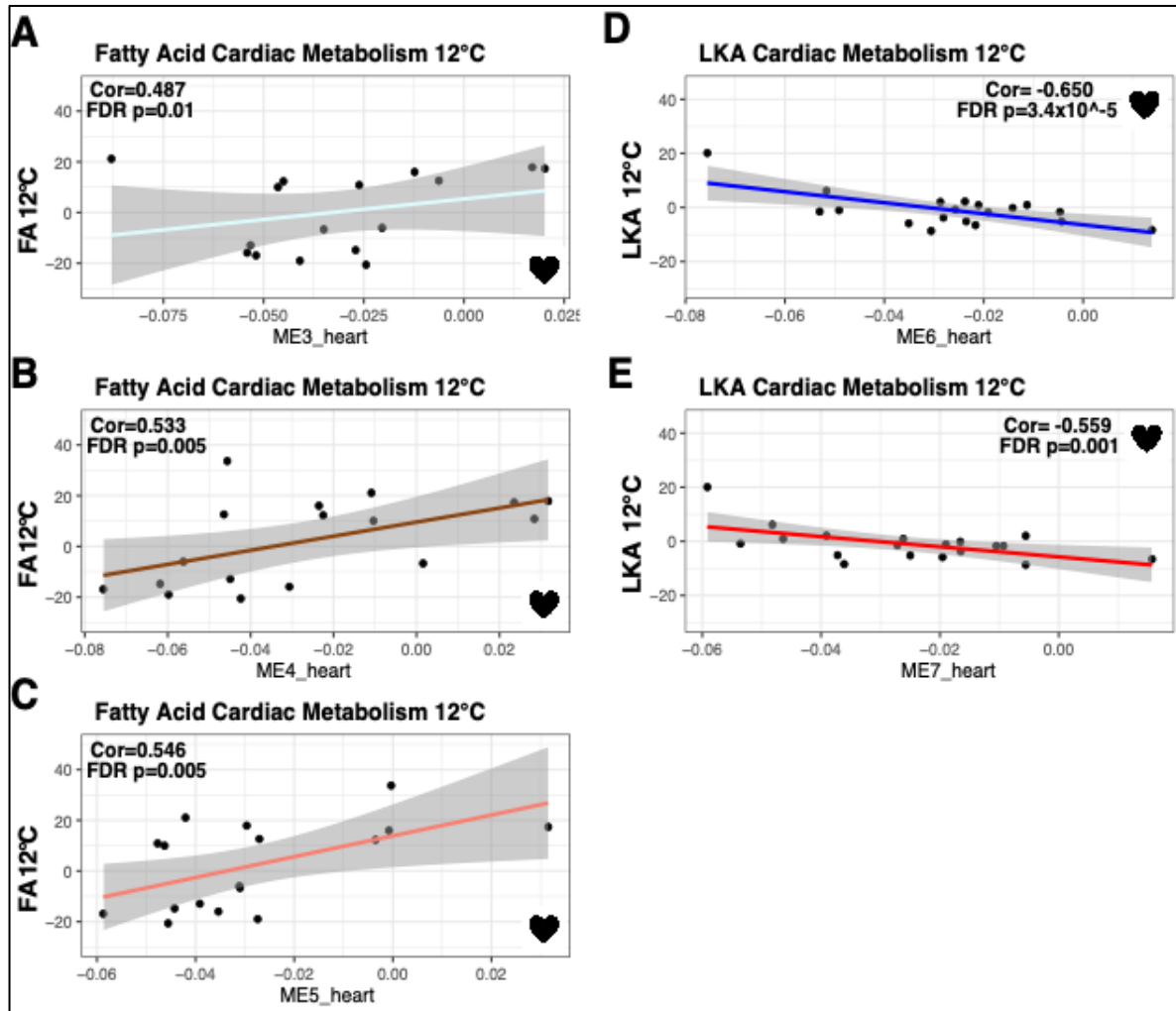
Trait	Module	Correl coef	FDR P-value	Hub GS	GS	Positive GS
CTMax 12°C	ME1_heart	0.49	2.10E-02	GPM6AA	-0.51	54.60%
CTMax 28°C	ME2_heart	-0.53	9.50E-03	NCKAP5L	-0.50	35.30%
FA 12°C	ME3_heart	0.50	1.02E-02	PDCD4A	0.55	65.80%
FA 12°C	ME4_heart	0.53	4.82E-03	RSRC1	-0.58	13.10%
FA 12°C	ME5_heart	0.55	4.82E-03	RPL5A	0.62	81.80%
heart mass 12°C	ME6_heart	-0.57	1.80E-03	WRAP53	0.72	48.20%
LKA 12°C	ME6_heart	-0.65	3.39E-05	LOC118563371	0.80	46.90%
LKA 12°C	ME7_heart	-0.56	1.29E-03	LOC105934375	0.80	44.70%
WAM 12°C	ME8_heart	-0.56	1.20E-03	LOC118563115	0.70	36.60%
WAM 12°C	ME9_heart	-0.55	1.20E-03	LRRC58B	-0.56	10.00%
WAM 12°C	ME4_heart	-0.54	1.74E-03	LOC118563570	0.61	65.50%
WAM 12°C	ME5_heart	-0.56	1.20E-03	HPRT1L	0.66	40.50%
	AVERAGE	0.55				

632

633

Table 4: Brain Significant Modules. Columns include: Module = identifier for module eigengene (ME, first principal component of module), KEGG Pathway = top KEGG pathway determined by number of KEGG terms, Module Size = number of mRNAs in the module, Hub MM = mRNA with highest correlation with module eigengene, MM = correlation of hub mRNA with module eigengene, Positive MM = proportion of mRNAs with positive MM in the module, KEGG Terms in Pathway = enriched KEGG terms in the listed KEGG pathway.

Module	KEGG Pathway	Module Size	Hub MM	MM	Positive MM	KEGG Terms in Pathway
ME1_brain	Metabolic pathways	393	IDH3B	0.47	26.40%	K00323 K01443 K10106 K19006
ME2_brain	Tight junction	198	LOC105921153	0.52	28.20%	K07198 K08018 K08020
ME3_brain	Lysosome	342	LRCH3	0.58	25.10%	K01134 K08568
ME4_brain	RNA degradation*	142	SI:CH211-244C8.4	0.36	21.10%	K03681

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Table 5: Brain Significant Module Trait Correlations. Significant brain *versus* ME correlations with FDR p<0.05. Columns include: Trait = traits significantly correlated with a given ME (critical thermal maximum: CT_{max}, whole animal metabolic rate: WAM), Module = identifier for module eigengene (ME, first principal component of module), Correl coef = Pearson's signed correlation coefficient for trait and ME, FDR P-value = multiple test corrected p-value for trait *versus* module correlation, Hub GS = mRNA with highest gene significance for the trait in the module, GS = gene significance, correlation between top module mRNA and trait, Positive GS = proportion of mRNAs in the module that are positively correlated with trait.

Trait	Module	Correl coef	FDR P-value	Hub GS	GS	Positive GS
body mass 12°C	ME1_brain	-0.60	5.75E-04	MAP3K5	0.61	44.80%
body mass 28°C	ME3_brain	0.58	1.46E-03	LOC105919139	0.63	68.70%
CT_{max} 28°C	ME2_brain	-0.60	2.01E-04	CIAO2B	0.36	44.90%
CT_{max} 28°C	ME3_brain	-0.57	4.83E-04	RAD17	0.64	83.90%
CT_{max} 28°C	ME4_brain	-0.67	2.38E-05	APOC1	-0.65	48.60%
WAM 28°C	ME4_brain	-0.70	3.15E-06	APOC1	-0.68	50.00%
	AVERAGE	0.62				

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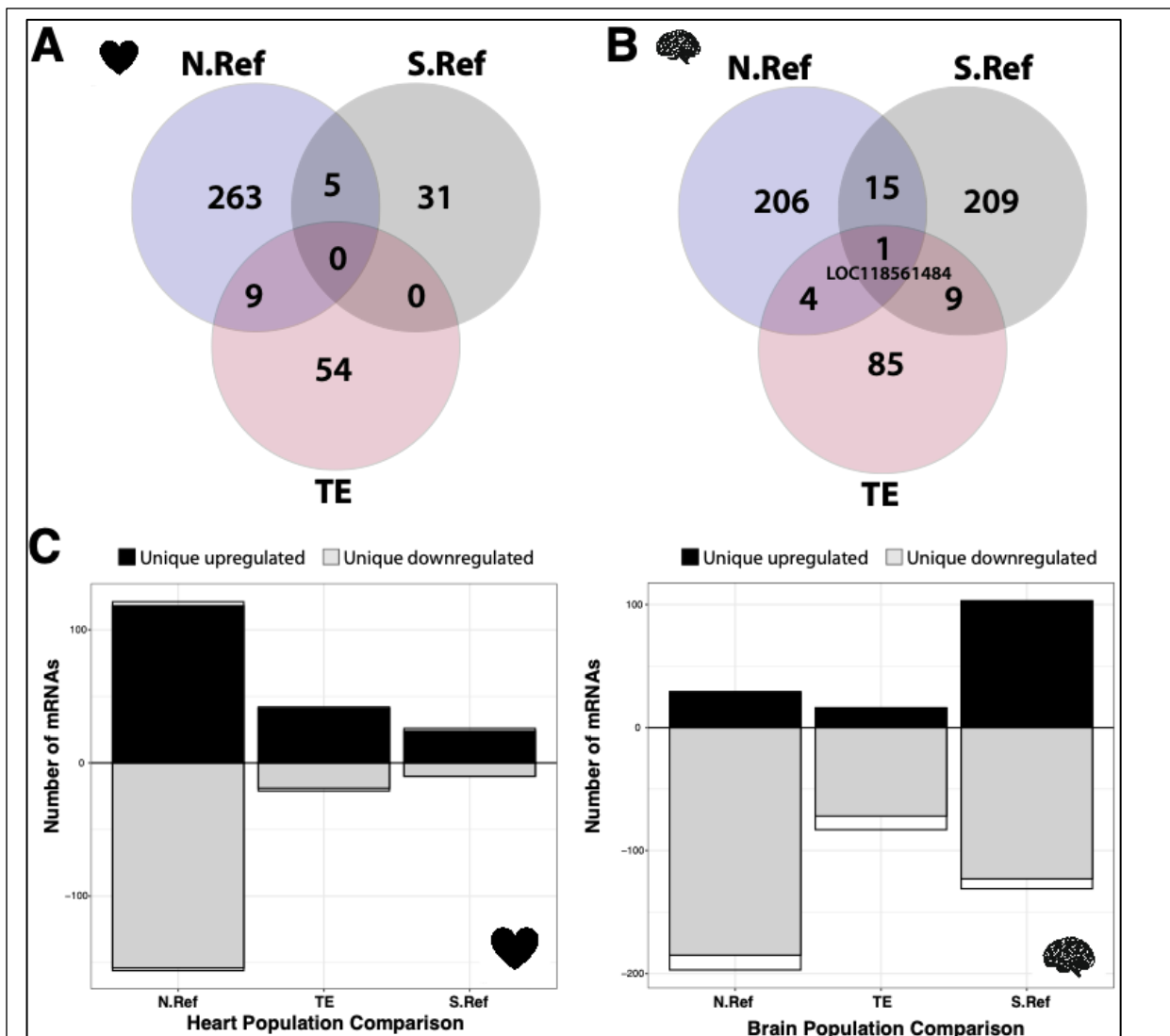
Figure 1: Population and tissue specific transcriptomic response to acclimation temperature. Number of differentially expressed mRNAs (DEGs) within each population between 12°C and 28°C acclimated hearts (**A and C**) and 12°C and 28°C acclimated brains (**B and D**). For both heart and brain, populations had many unique DEGs (**C and D**, upregulated=black, downregulated=grey) that were differentially expressed between acclimation temperatures and few shared DEGs (**C and D**, shared=white), with only 1 DEG shared among all three populations for brain (LOC118561484 in brains). Population and number of DEGs are not independent, Chi-Squared test, heart p=1.64x10^{-10}, brain p=2x10^{-16}.

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Figure 2: Temperature specific differential mRNA expression among populations. A) Differential expression for 158 heart mRNAs and **B)** 242 brain mRNAs between any two populations at 12°C. In hearts, 43.0% of mRNAs (68/158) are shared among any two population comparisons. In brains, 32.2% of mRNAs (78/242) are shared among any two population comparisons.

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Figure 3: Significant cardiac metabolism-heart module correlations from weighted gene co-expression network analysis. Significant correlation of fatty acid cardiac metabolic rate at 12°C (N=16) with ME3_heart (**A**), ME4_heart (**B**), and ME5_heart (**C**). Significant correlation of lactate, ketone, and alcohol (LKA) cardiac metabolic rate at 12°C (N=19) with ME6_heart (**D**) and ME7_heart (**E**). Pearson correlation coefficients (Cor) and FDR p-values are displayed for each significant correlation.

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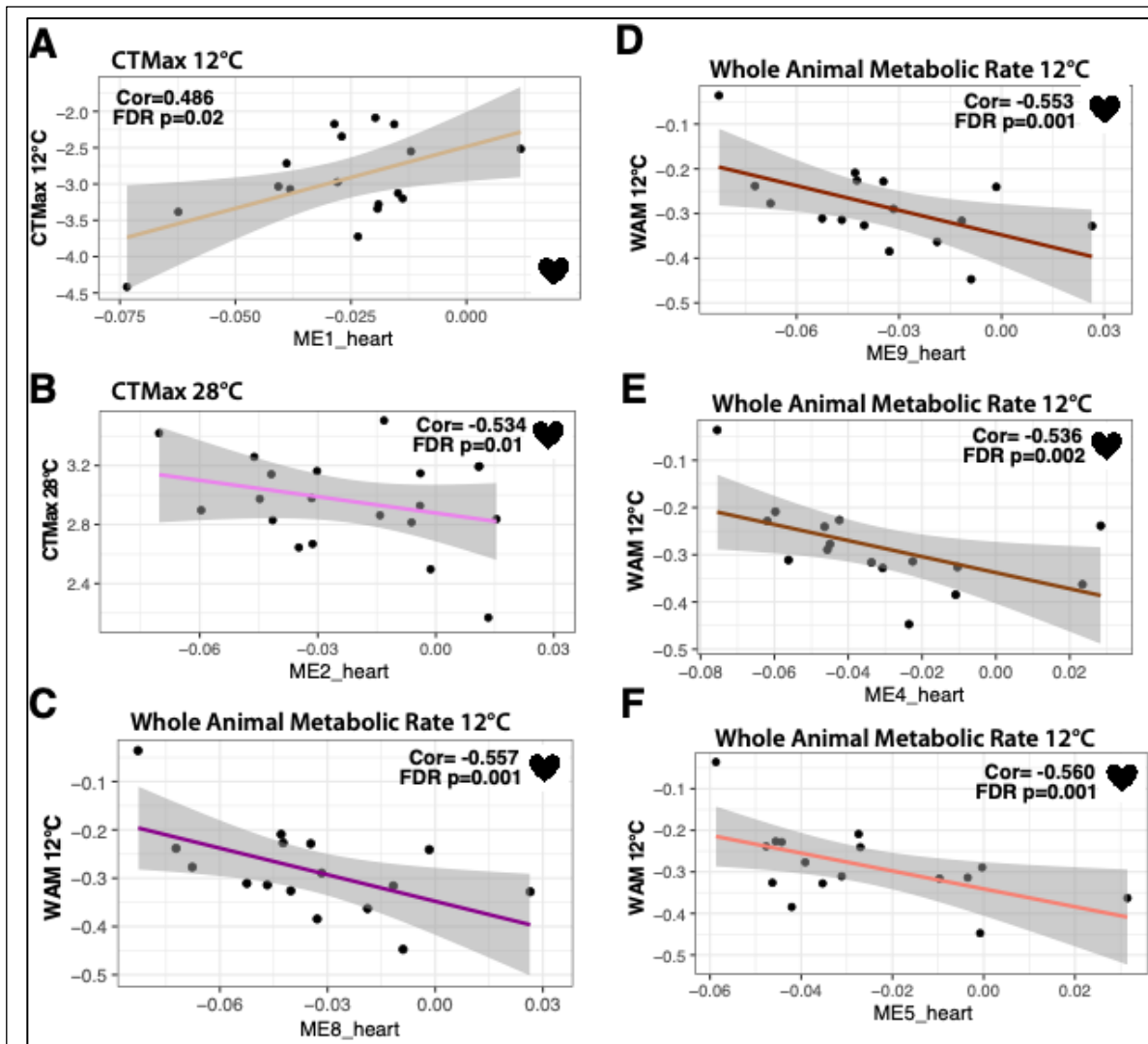
Figure 4: Significant whole animal trait-heart module correlations from weighted gene co-expression network analysis. Significant correlation of critical thermal maximum at 12°C (N=17) with ME1_heart (**A**), critical thermal maximum at 28°C (N=19) with ME2_heart (**B**), whole animal metabolic rate at 12°C (N=16) with ME8_heart (**C**), ME9_heart (**D**) ME4_heart (**E**), and ME5_heart (**F**). Pearson correlation coefficients (Cor) and FDR p-values are displayed for each significant correlation.

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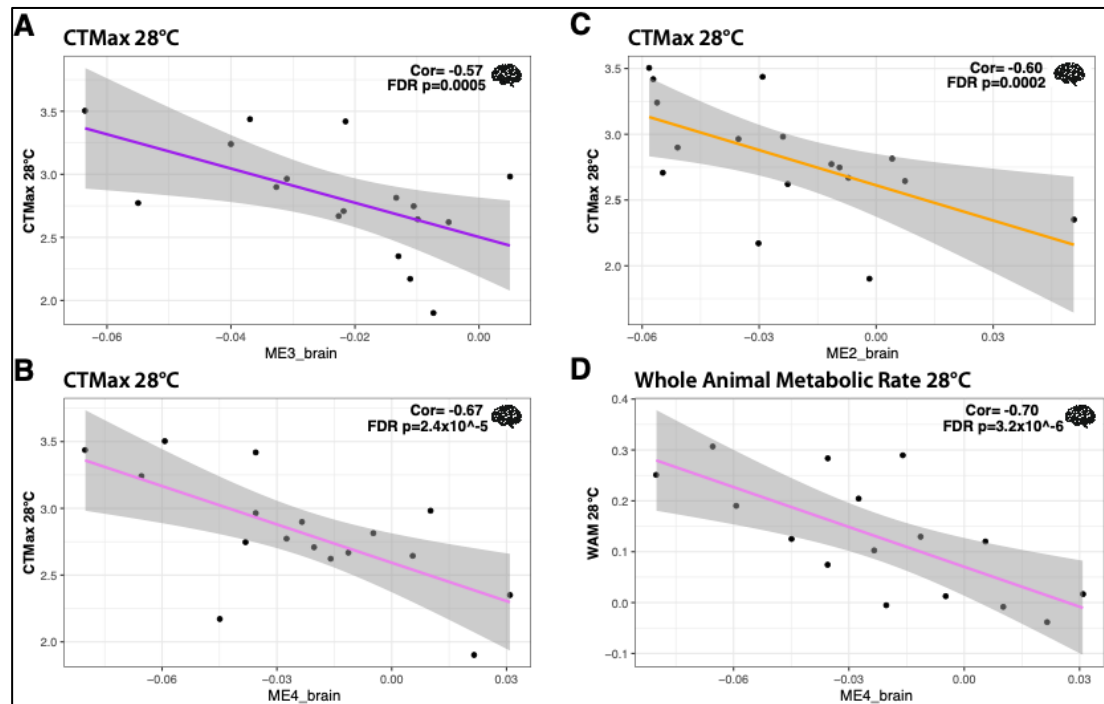
Figure 5: Significant whole animal trait-brain module correlations from weighted gene co-expression network analysis. Significant correlation of critical thermal maximum at 28°C (N=17) with ME3_brain (**A**), ME4_brain (**B**), and ME2_brain (**C**). Significant correlation of whole animal metabolic rate at 28°C (N=16) with ME4_brain (**D**). Pearson correlation coefficients (Cor) and FDR p-values are displayed for each significant correlation.

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642 Supplemental Figures
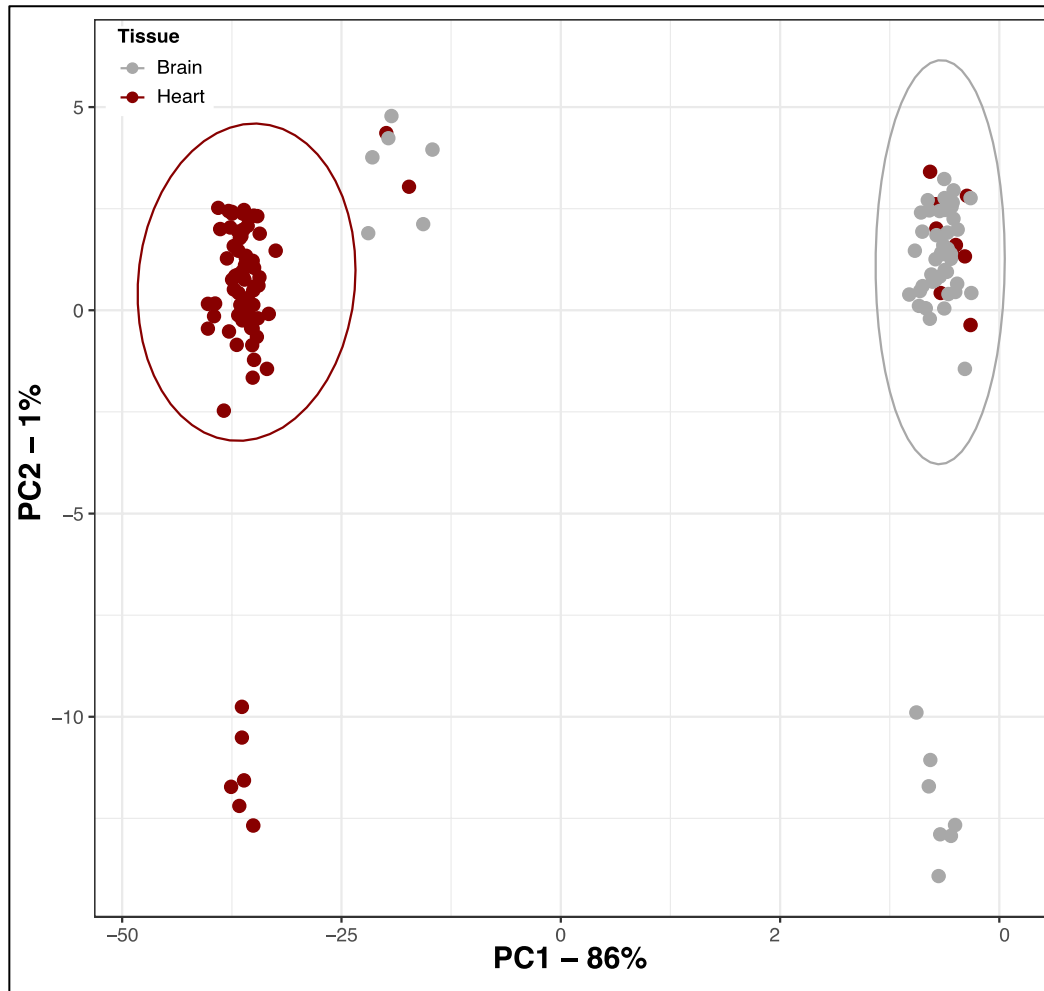
Figure S1: Principal component analysis of all samples. Principal component 1 split heart and brain tissue and explained 86% of variance. Individuals who did not clearly group with the appropriate tissue were removed as outliers.

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Figure S2: Tissue specific principal component analysis. Heart (N=41, A) first two principal components explain 19% and 7% of variance. Brain (N=45, B) first two principal components explain 11% and 7% of variance. Triangles are 28°C acclimated individuals, circles are 12°C. Individuals from the north reference (N.Ref) are blue, south reference (S.Ref) are purple, and thermal effluent population (TE) are red.

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Figure S3: Differentially expressed mRNAs among populations within tissue and acclimation temperature. A) Heart at 12°C, **B)** brain at 12°C, **C)** heart at 28°C, D) brain at 28°C. Populations are north reference (N.Ref), south reference (S.Ref), and thermal effluent (TE).

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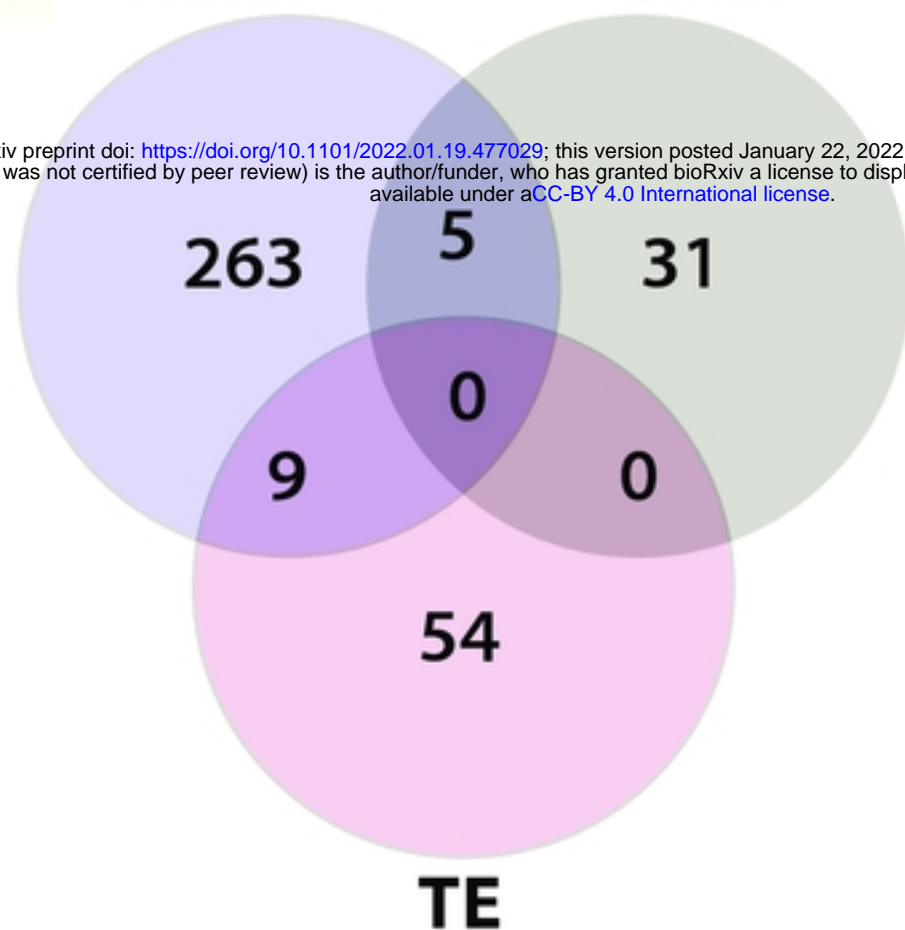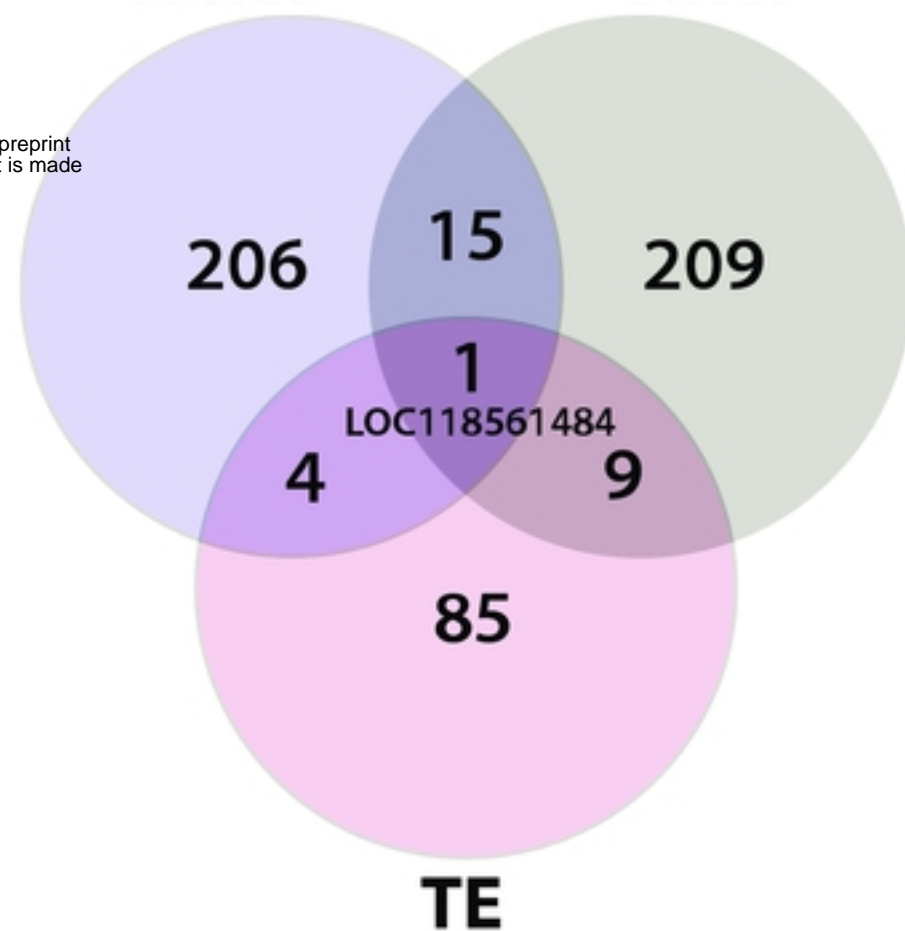
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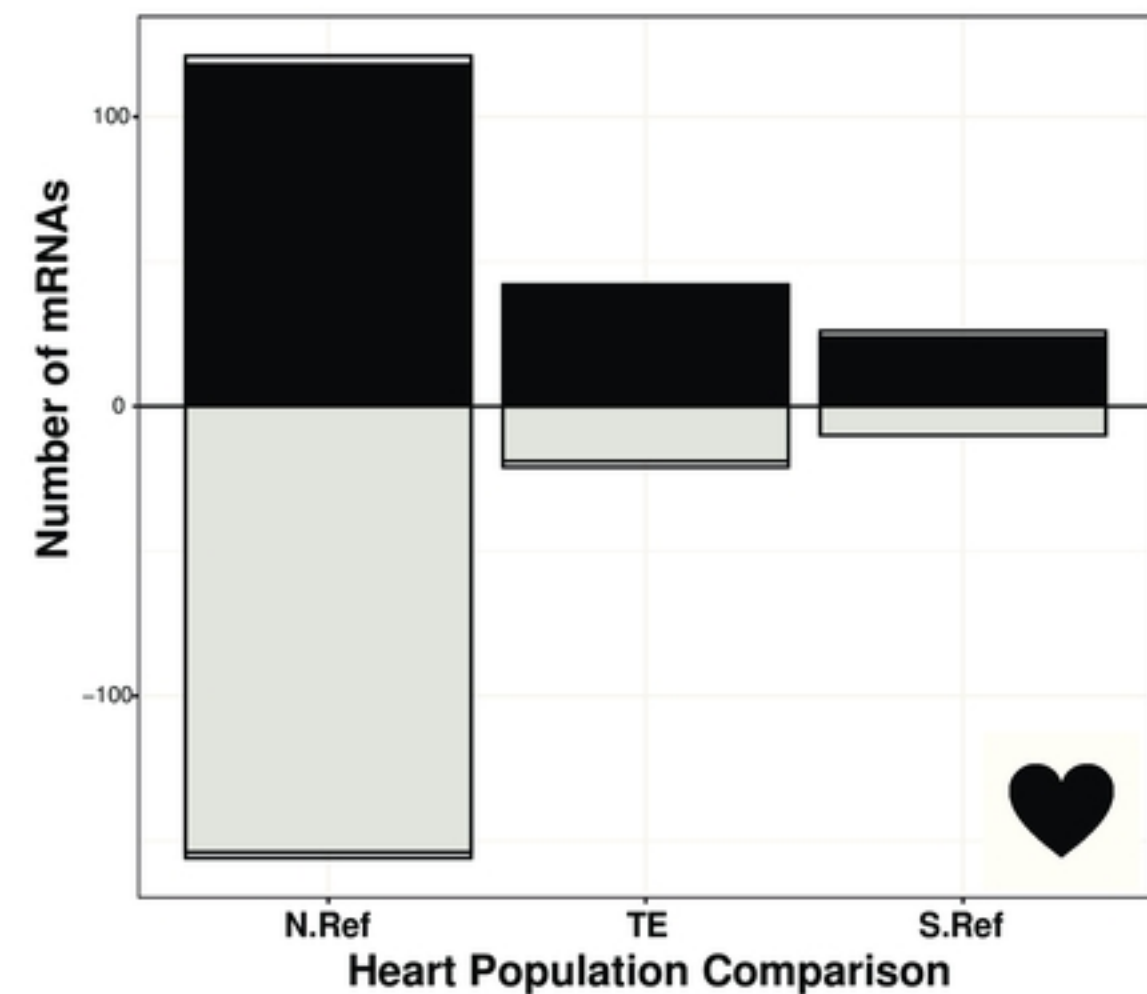873   heteroclitus). Journal of Experimental Biology. 2017;220(8):1459-71.

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Drown_2022_mRNA_Fig1

A ♥
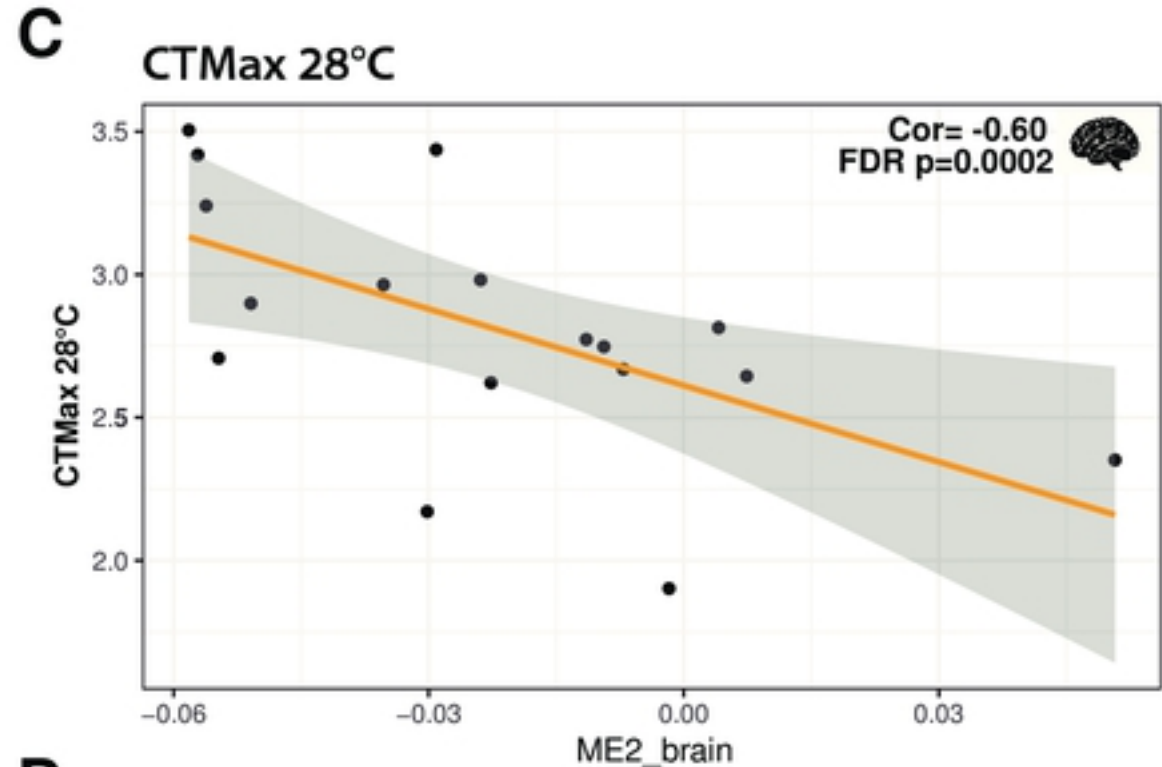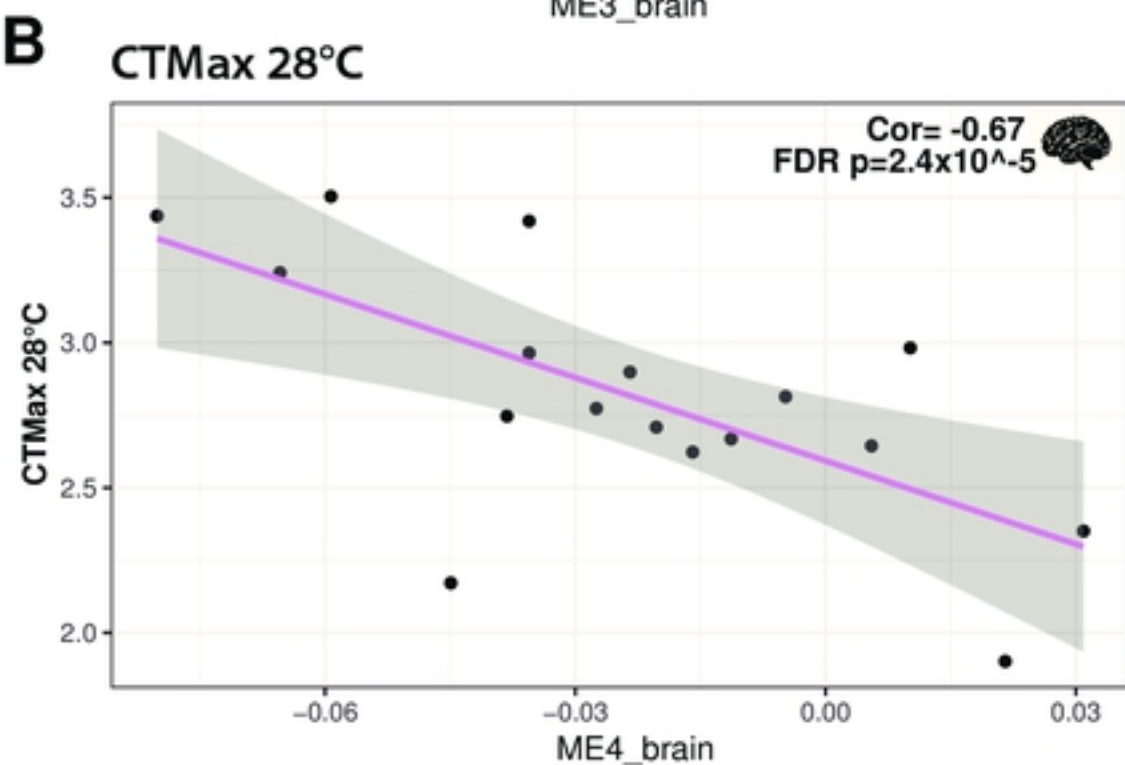
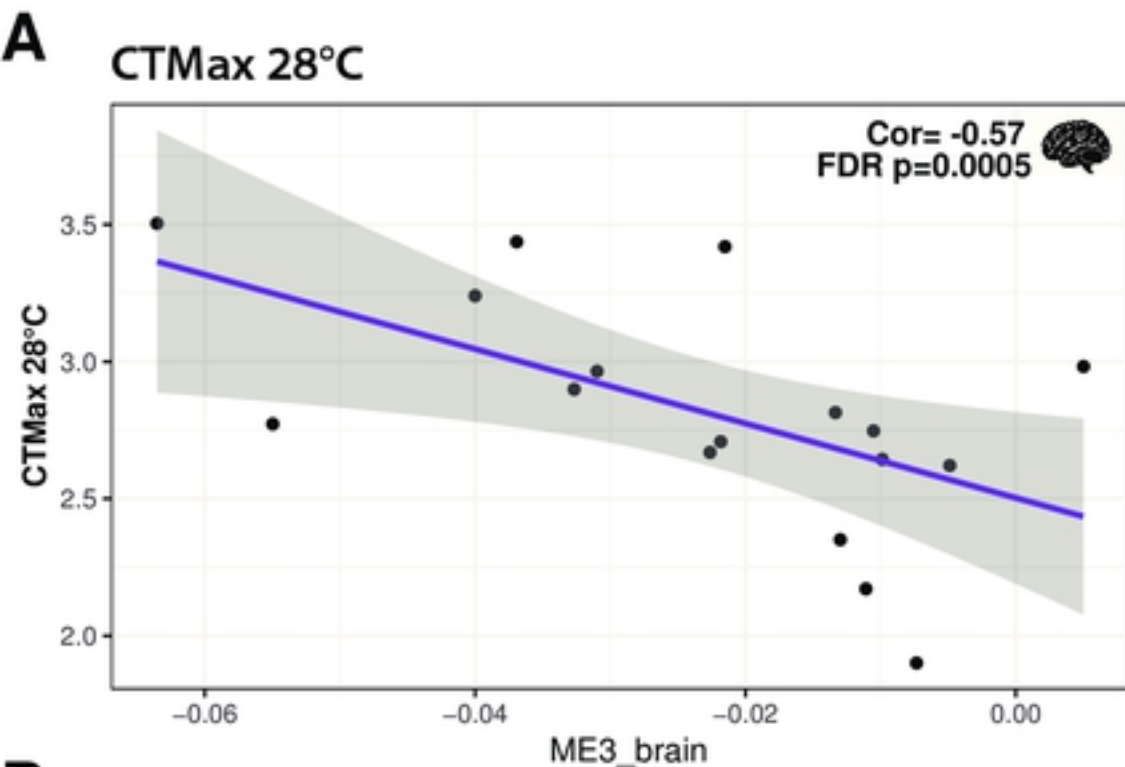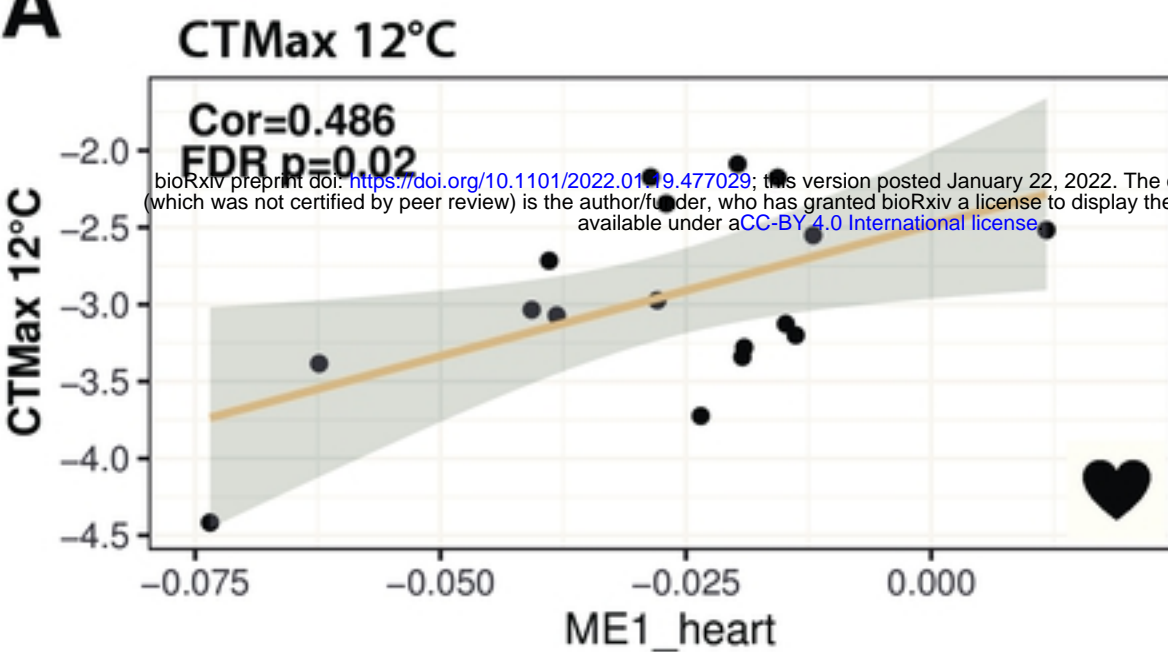**B** 🧠

Population

mat
2
1
0
-1
-2

Population
N.Ref
TE
S.Ref
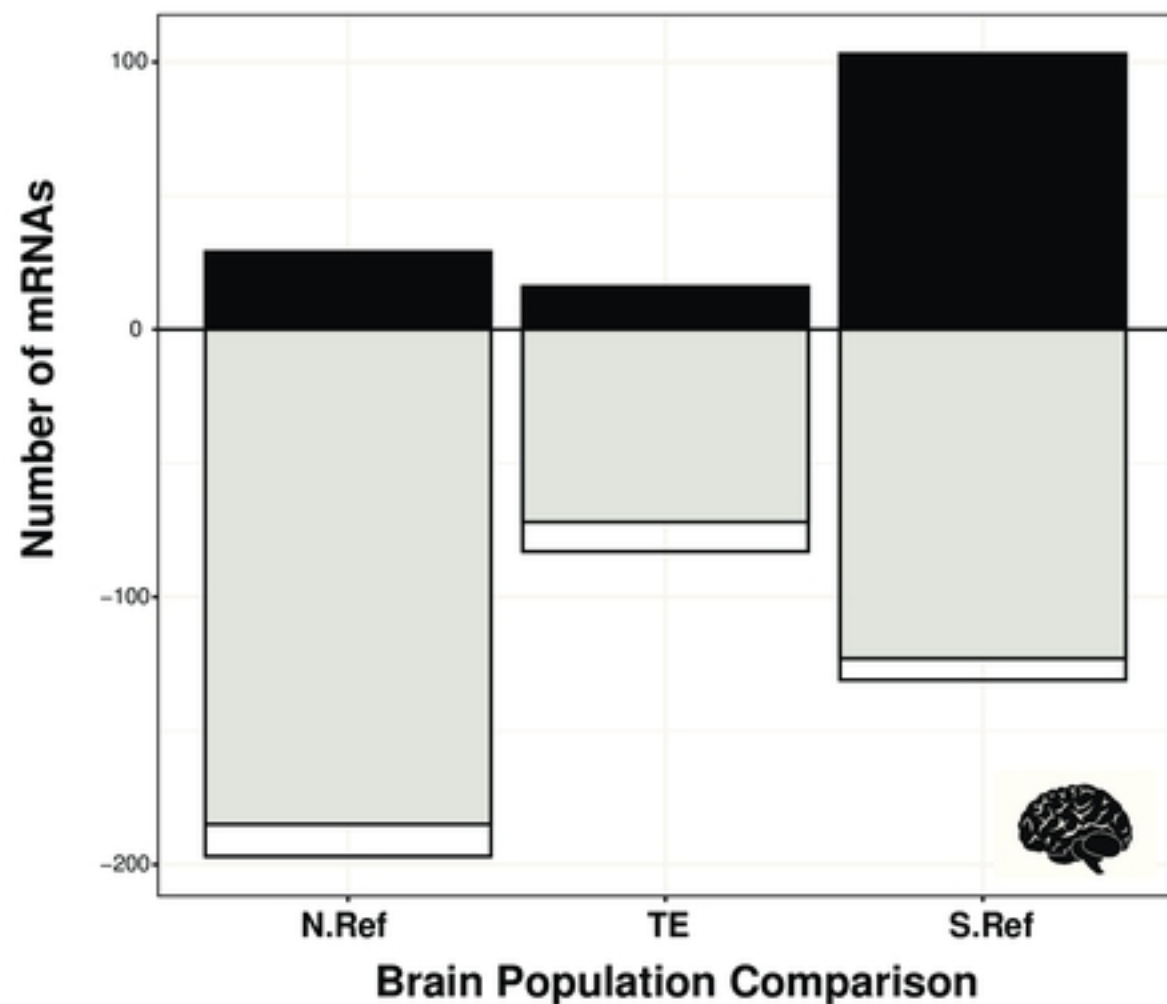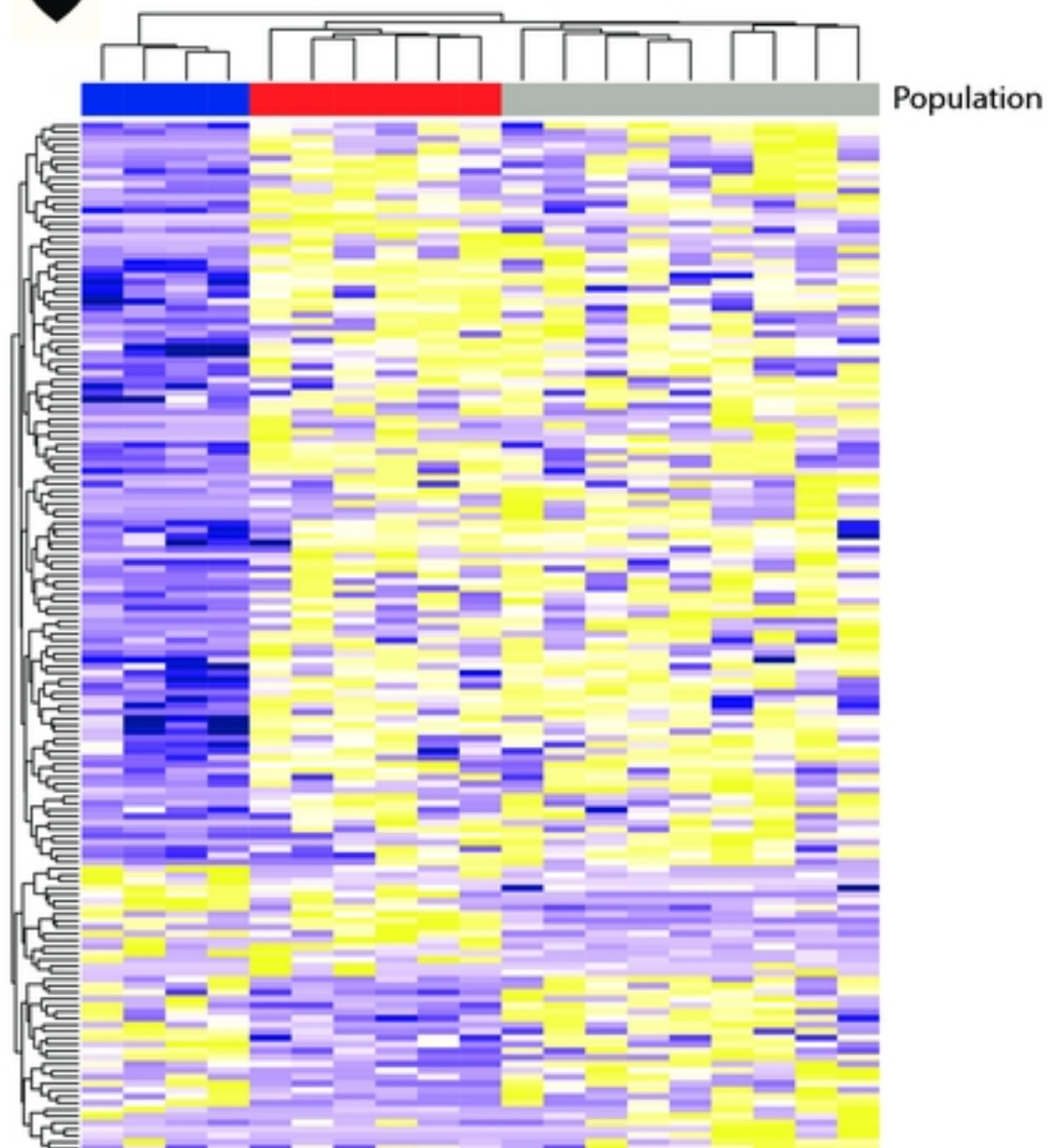
Individual Heart 12°C
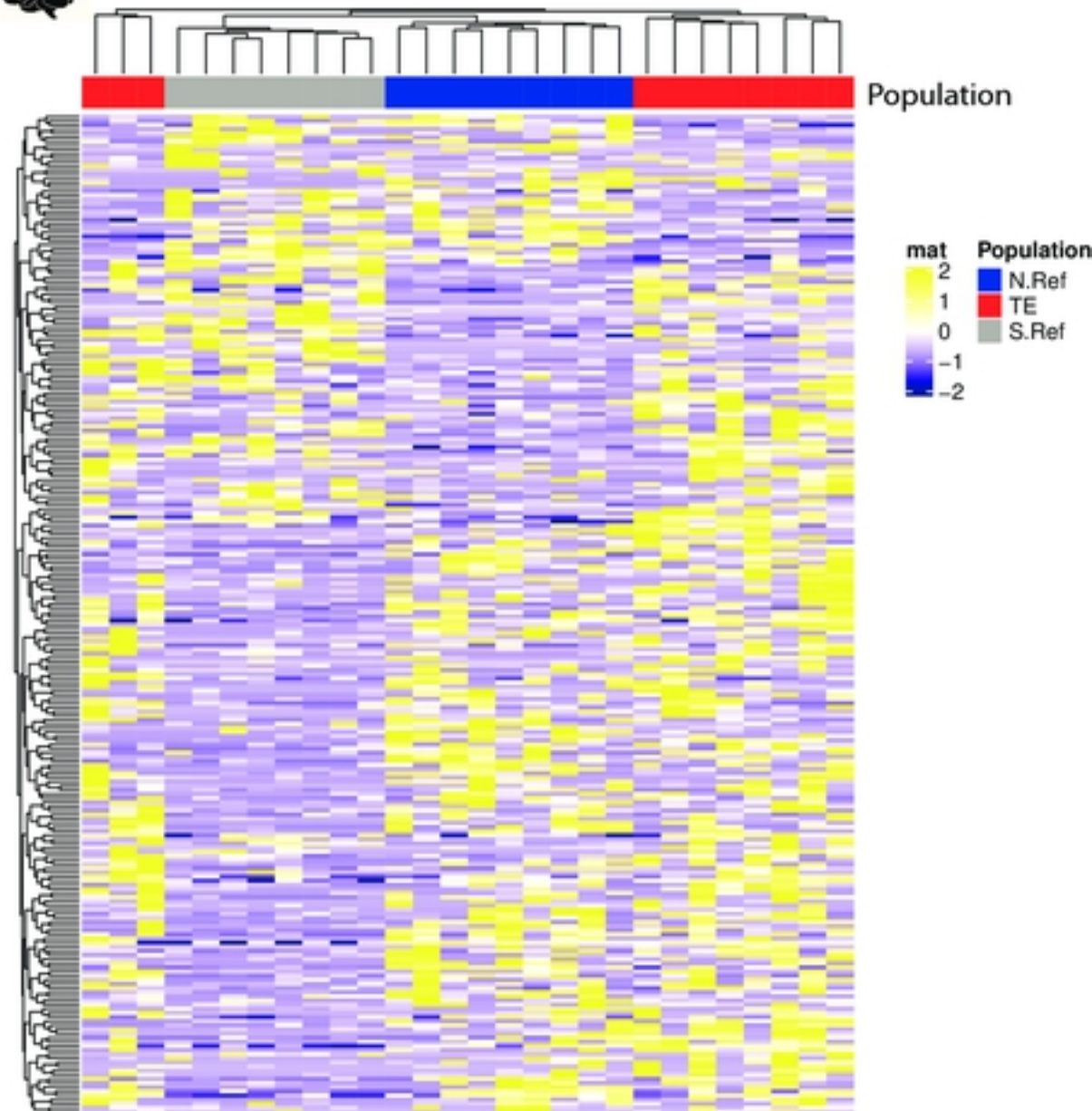
Individual Brain12°C

Drown_2022_mRNA_Fig2

A CTMax 28°C

Cor= -0.57
FDR p=0.0005

CTMax 28°C (y-axis), ME3_brain (x-axis)

B CTMax 28°C

Cor= -0.67
FDR p=2.4x10^-5

CTMax 28°C (y-axis), ME4_brain (x-axis)

C CTMax 28°C

Cor= -0.60
FDR p=0.0002

CTMax 28°C (y-axis), ME2_brain (x-axis)

D Whole Animal Metabolic Rate 28°C

Cor= -0.70
FDR p=3.2x10^-6

WAM 28°C (y-axis), ME4_brain (x-axis)

Drown_2022_mRNA_Fig5

Drown_2022_mRNA_Fig4

A

Fatty Acid Cardiac Metabolism 12°C

Cor=0.487
FDR p=0.01

FA 12°C

ME3_heart

B

Fatty Acid Cardiac Metabolism 12°C

Cor=0.533
FDR p=0.005

FA 12°C

ME4_heart

C

Fatty Acid Cardiac Metabolism 12°C

Cor=0.546
FDR p=0.005

FA 12°C

ME5_heart

D

LKA Cardiac Metabolism 12°C

Cor= -0.650
FDR p=3.4x10^-5

LKA 12°C

ME6_heart

E

LKA Cardiac Metabolism 12°C

Cor= -0.559
FDR p=0.001

LKA 12°C

ME7_heart

Drown_2022_mRNA_Fig3