An integrated comparative physiology and molecular approach pinpoints mediators of breath-hold capacity in dolphins

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Abstract

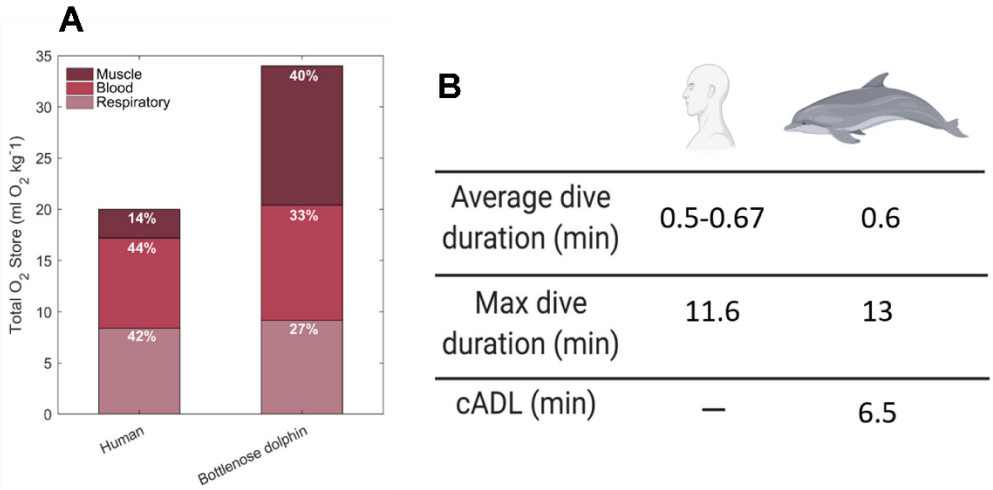
Ischemic events, such as ischemic heart disease and ischemic stroke, are the number one cause of death globally. Ischemia prevents blood, carrying essential nutrients and oxygen, from reaching tissues and organ systems, leading to cell and tissue death, and eventual organ failure. While humans are relatively intolerant to these ischemic events, other species, such as marine mammals, have evolved remarkable tolerance to chronic ischemia/reperfusion during diving. Here we capitalized on the unique adaptations of bottlenose dolphins (*Tursiops truncatus*) as a comparative model of ischemic stress and hypoxia tolerance to identify molecular features associated with breath-holding. Using RNA-Seq we observed time-dependent upregulation of the arachidonate 5-lipoxygenase (ALOX5) gene during breath-holding. Consistent with the RNA-Seq data, we also observed increased ALOX5 enzymatic activity in the serum of dolphins undergoing breath holds. ALOX5 has previously been shown to be activated during hypoxia in rodent models, and its metabolites, leukotrienes, induce vasoconstriction. The upregulation of ALOX5 occurred within the estimated aerobic dive limit of the species, suggesting that ALOX5 enzymatic activity may promote tolerance to ischemic stress through sustained vasoconstriction in dolphins during diving. These observations pinpoint a potential molecular mechanism by which dolphins, and perhaps other marine mammals, have adapted to the prolonged breath-holds associated with diving.

52 **Introduction**

53 Ischemic stress and hypoxia are associated with negative clinical outcomes in humans.
54 Maintenance of homeostatic function in mammalian tissues is directly dependent on a continuous
55 supply of oxygenated blood. Interruption of this blood supply, known as ischemia, results in
56 reduction in local oxygenation compared to normal physiologic levels, or hypoxia, and can lead
57 to inflammation and cell/tissue death (Bona et al., 1999; Choi, 1996; Eltzschig and Carmeliet,
58 2011; Gottlieb and Engler, 1999; Murdoch et al., 2005). Ischemia is the causative factor in
59 multiple clinical settings, and ischemic heart disease is the number one cause of death globally,
60 accounting for over 9 million deaths each year (Nowbar et al., 2019; World Health Organization,
61 2018).

62

63 Marine mammals have evolved tolerance to ischemic stress. While humans have little tolerance
64 for ischemic stress and hypoxia, a number of species have evolved unique physiologies that
65 allow them to seemingly thrive despite regular tissue-level ischemia and low-oxygen
66 environments. One group of animals that undergo repeated daily ischemic events is marine
67 mammals. During a dive, a marine mammal experiences a suite of cardiovascular changes that
68 aid in reducing aerobic metabolism (Irving et al., 1941; Scholander, 1940). As part of this
69 response, both heart rate (f_H) and stroke volume decrease, resulting in reduced cardiac output
70 (Fahlman et al., 2020b, 2019b). Increased peripheral resistance, through selective
71 vasoconstriction, helps assure that mean arterial blood pressure is maintained, at least in studies
72 on forced diving in seals (Blix et al., 1976; Zapol et al., 1979). Ultimately, this response
73 conserves oxygen in the blood and lungs for oxygen-sensitive tissues like the brain and the heart,
74 while the skeletal muscles rely on endogenous myoglobin-bound oxygen for aerobic metabolism



	Human	Bottlenose dolphin
Average dive duration (min)	0.5-0.67	0.6
Max dive duration (min)	11.6	13
cADL (min)	—	6.5

Figure 1. Dolphins as a model of ischemia. A. Dolphins and other cetaceans have increased oxygen stores that are reapportioned compared to humans. Oxygen store data were reported in Ponganis et al., 2011 (human) and Kooyman and Ponganis, 2018 (dolphin). B. The enhanced oxygen stores and diving capacity of dolphins makes them a unique model to study ischemic stress tolerance. Dive data and calculated aerobic dive limit (cADL) were reported by AIDA and Foster and Sheel, 2005 (human) and Fahlman et al., 2018 (dolphin).

(Davis and Kanatous, 1999; Fahlman et al., 2009). While these responses to submersion in water are largely conserved across all vertebrates, many of the physiological adaptations that support diving are exaggerated in marine mammals compared to other taxa (Kooyman and Ponganis, 1998; Panneton, 2013) (**Figure 1**). For example, maintenance of increased peripheral resistance does not appear to occur in human breath-hold divers, as mean arterial blood pressure increases with dive duration (Breskovic et al., 2011; Gooden, 1994; Taboni et al., 2019). These physiological differences highlight the tremendous potential to study marine mammals as model organisms for the investigation of adaptations to ischemic and hypoxic stress tolerance, and the cardiorespiratory plasticity that helps prevent hypertension (Blawas et al., 2021; Fahlman et al., 2019b, 2020b).

86 Marine mammals have evolved molecular adaptations to ischemic stress tolerance. Increasing
87 attention has been paid to the defenses marine mammals possess against the oxidant by-products
88 and inflammation associated with ischemic, hypoxia, and reperfusion at the molecular level
89 (Allen and Vázquez-Medina, 2019; Hindle, 2020; Zhu et al., 2018). Using phylogenetic and
90 evolutionary convergence approaches, several gene families have been identified to contribute to
91 the increased ischemic stress tolerance of marine mammals, including hypoxia-inducible factor 1
92 (HIF-1) (Bi et al., 2015; Johnson et al., 2005, 2004), genes relating to the glutathione system and
93 peroxiredoxins (Bagchi et al., 2018; Tift et al., 2014; Yim et al., 2014; Zhou et al., 2018), and
94 several genes linked to oxygen storage, particularly hemoglobin and myoglobin (Mirceta et al.,
95 2013; Nery et al., 2013; Tian et al., 2017, 2016). Yet, few studies have examined differential
96 gene expression in marine mammals under conditions of ischemia and hypoxia (i.e. diving
97 conditions).

98 Here, we investigate the dynamic molecular changes that occur during an apnea in
99 bottlenose dolphins using genomic analysis of peripheral blood mononuclear cells (PBMCs) and
100 serum sampled at regular intervals during breath-holds. We couple these analyses with
101 previously-published f_H measurements from the same dolphins to understand how the timing of
102 molecular changes relates to the physiologic dive response (Blawas et al., 2021; Fahlman et al.,
103 2019b, 2020b). Our integrated analyses pinpoint a gene regulatory network centered around the
104 arachidonate 5-lipoxygenase (ALOX5) gene and its downstream metabolites, leukotrienes, as
105 differentially activated during breath-holding. This activation of ALOX5 is consistent with
106 cardiovascular control through reduction in f_H and peripheral vasoconstriction to efficiently
107 manage O_2 use during diving. Based on our collective results we propose a model in which the

108 ALOX5 pathway is upregulated during extended breath-holds as a mechanism to sustain

109 vasoconstriction and maintain oxygen stores for critical organs while diving.

110

111 **Materials and Methods**

112 Data collection and animal information. Four adult male bottlenose dolphins (*Tursiops*

113 *truncatus*) housed at Dolphin Quest Oahu (Honolulu, HI, USA) with an average (± S.D.) age of

114 22.8±9.9 years (range = 11 – 35 years) and body mass of 198.1±42.9 kg (range = 147.0 – 251.7

115 kg, **Table 1**) participated in this study. All data were collected under voluntary participation and

116 the animals could end a trial at any time. Routine veterinary assessments include venous blood

117 sampling, and the dolphins that participated in this study had previously been desensitized to the

118 blood sampling protocol. The study protocols were accepted by Dolphin Quest and the Animal

119 Care and Welfare Committee at the Oceanogràfic (OCE-17-16, amendments OCE-29-18 and

120 OCE-3-19i).

Table 1. Animal ID, age (years), body mass (kg), and included analyses for all dolphins in the study.

Animal ID	Age (years)	Body Mass (kg)	RNA-Seq	Lipoxygenase assay
6JK5	24	200.9	x	x
9FL3	35	251.7	x	
9ON6	21	192.8	x	x
83H1	11	147.0		x
Mean±S.D.	22.8±9.9	198.1±42.9		

121

122 Experimental trials. Serum was isolated from whole blood samples at baseline, 3 minutes, and 4

123 ½ - 5 minutes of breath-holding on fasted dolphins at Dolphin Quest, Oahu, March 2018 and

124 May 2019. All trials were performed in the morning, when the animals were fasted with at least

125 12 hours having passed since the last meal on the previous day to minimize the potential
126 confounding effect of nutritional state. To ensure that the samples were collected during resting
127 behavior each breath-hold was proceeded by 2 minutes of rest or slow swimming at the surface.
128 A trial was initiated when the dolphin rolled into dorsal recumbency with its blowhole
129 submerged and continued for approximately 5 minutes. The breath-hold ended when the animal
130 rolled into ventral recumbency and took a breath. Prior to this study the animals had previously
131 participated in breath-hold experiments of durations up to 5 minutes (Fahlman et al., 2019a,
132 2020b).
133
134 Blood collection and processing. Whole blood was collected from tail flukes at baseline (0-30
135 seconds into the breath-hold) and during breath-holding for 3 minutes and 4 ½ (2018) or 5
136 (2019) minutes while the animal was in dorsal recumbency with its blowhole submerged (**Figure**
137 **2A**). Blood was collected into PAXgene tubes and RNA-Seq was performed subsequent to
138 shipping, red blood cell lysis, RNA extraction (**Figure 2A**). All samples were shipped the same
139 day via overnight courier to Duke University for downstream processing. For RNA extraction,
140 tubes were equilibrated to room temperature for 2 hours to achieve complete lysis of blood cells.
141 Subsequently, tubes were centrifuged at 4,000 x g for 10 minutes. Pellets were resuspended in 4
142 mL of RNase-free water and RNA was extracted according to the PAXgene Blood RNA kit
143 (PreAnalytiX #762164). Prior to library prep, RNA quality was evaluated on a Bioanalyzer 2100
144 (Agilent). Stranded mRNA-seq libraries were prepared using the Nugen Universal Plus mRNA-
145 seq Library preparation kit with Globin AnyDeplete (Tecan #9147-A01). Libraires were
146 sequenced at 150bp paired-end on one lane of an Illumina NovaSeq 6000 instrument S-Prime
147 flow cell. Library preparation and sequencing was performed in conjunction with the Duke

148 University Sequencing and Genomic Technologies Shared Resource. Samples collected in 2018
149 were used to conduct RNA-Seq analysis and samples collected in 2019 were used for the
150 lipoxygenase assays.
151
152 RNA-Seq data analysis. RNA-seq data was processed using the TrimGalore toolkit (Krueger,
153 2020) which employs Cutadapt (Martin, 2011) to trim low-quality bases and Illumina sequencing
154 adapters from the 3' end of the reads. Only reads that were 20nt or longer after trimming were
155 kept for further analysis. Reads were mapped to the turTru1v92 version of the dolphin genome
156 and transcriptome (Kersey et al., 2012) using the STAR RNA-seq alignment tool (Dobin et al.,
157 2013). Reads were kept for subsequent analysis if they mapped to a single genomic location.
158 Gene counts were compiled using the HTSeq tool (Anders et al., 2015). Only genes that had at
159 least 10 reads in any given library were used in subsequent analysis. Normalization and
160 differential expression across the time points were carried out using the DESeq2 (Love et al.,
161 2014) Bioconductor (Huber et al., 2015) package with the R statistical programming
162 environment (R Core Team, 2020). The false discovery rate was calculated to control for
163 multiple hypothesis testing. To identify relevant molecular features of dolphin breath-holding we
164 first analyzed the RNA-Seq data from all individuals at baseline using gene set enrichment
165 analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005). GSEA is a standard pathway
166 analysis tool that calculates enrichment scores for annotated pathways based on the rank order of
167 genes present in the data for each pathway. Pathways with genes that are more up- or down-
168 regulated are more likely to be enriched in a data set than pathways whose genes are randomly
169 distributed throughout the data. Pathway enrichments in dolphin PBMCs at baseline, with genes

170 ranked on total expression value, were compared with human whole blood pathway enrichments
171 from the Genotype-Tissue Expression (GTEx) project.
172
173 Construction of gene regulatory networks. Gene expression networks were created using
174 GeneMANIA (Franz et al., 2018), implemented within the Cytoscape platform (Shannon et al.,
175 2003). For time-dependent gene network construction all nodes with 0 or 1 connection were
176 trimmed out of the networks. Two additional non-coding RNA genes were eliminated (RF00016
177 and RF00026). To quantify network connectivity, all genes in the network were individually
178 ranked by the following network parameters: degree, clustering coefficient, closeness,
179 betweenness, neighborhood connectivity, and stress. These rankings were summed to generate a
180 sum rank score for each gene. Pathway enrichments were performed in STRING using the
181 trimmed network of 123 genes. Human whole blood transcriptomics data used for the analyses
182 described in this manuscript were obtained from the Genotype-Tissue Expression (GTEx)
183 Program Portal (https://gtexportal.org/home/) accessed on 9/20/2020.
184
185 Lipoxygenase assays. Briefly, 5 ml of blood was collected directly into BD Vacutainer® SST™
186 Tubes (SST) using a 21 g, ¾ in. winged infusion set with a BD Vacutainer adapter and holder.
187 Tubes were gently inverted 5 times to activate clotting reagent and allowed to clot at room
188 temperature for 30 minutes in an upright position. Tubes were centrifuged at 1,500 x g for 15
189 minutes to separate serum fractions, and serum was transferred to 15 ml conical tubes, frozen on
190 dry ice, and shipped to Duke University for downstream analyses. Sera were stored at -80°C
191 until use. Lipoxygenase activity was quantified from 1 μg of total protein using a Fluorometric
192 Lipoxygenase Activity Assay Kit (BioVision Inc; cat. #K978).

193 **Results**

194 RNA-Seq from dolphins at baseline pinpoints enriched gene regulatory networks. All samples
195 produced between 30 and 40 million reads, with no time-dependent changes in read counts
196 across samples (**Supplementary Figure 1A**). Principle components analysis and hierarchical
197 clustering of all samples (three individual dolphins x three time points) revealed both individual-
198 and within-individual time-dependent grouping of the data (**Supplementary Figure 1B, C**).
199 GSEA identified multiple pathways enriched in dolphin PBMCs at baseline when ranked by total
200 expression, including hedgehog signaling and several pathways relevant to blood cell
201 metabolism, including heme metabolism, coagulation, IL6/JAK/STAT3 activation, apical
202 junctions, and allograft rejection (**Figure 2B, C**). GSEA also identified enrichment of pathways
203 related to apical junctions, interferon alpha response, estrogen response, complement activity,
204 and heme metabolism in RNA-Seq data from GTEx human whole blood transcriptomes (**Figure**
205 **2D**). Comparison of dolphin baseline RNA-Seq data ranked by total expression with the top 100
206 and 500 most highly-expressed genes in human whole blood showed significant enrichment
207 (FDR<0.0001) (**Figure 2E**). Together these analyses suggest that significant overlap exists in
208 mRNA expression at both the gene-level and pathway-level between dolphin and human blood.
209

210 Breath-holding induces upregulation of multiple regulatory pathways. We next reasoned that
211 patterns of step-wise increases in mRNA expression may pinpoint molecular responses to breath-
212 holding common across individuals. We constructed gene regulatory networks for 136 genes
213 with step-wise increases in mRNA expression from baseline to 3 minutes and again from 3
214 minutes to 4 ½ minutes (**Figure 3A**). We performed network analysis to identify genes that are
215 upregulated and have the most network interactions. To do this we analyzed the time-dependent

216 gene regulatory network for the following parameters: degree, clustering coefficient, closeness,

217 betweenness, neighborhood connectivity, and stress. We then plotted the sum rank score of these

218 network parameters with gene expression for each gene in the network. These analyses

219 pinpointed arachidonate 5-lipoxygenase (ALOX5) as among the most connected genes with a

220 time-dependent increase in expression (**Figure 3B, C**). Additional genes, including EPX,

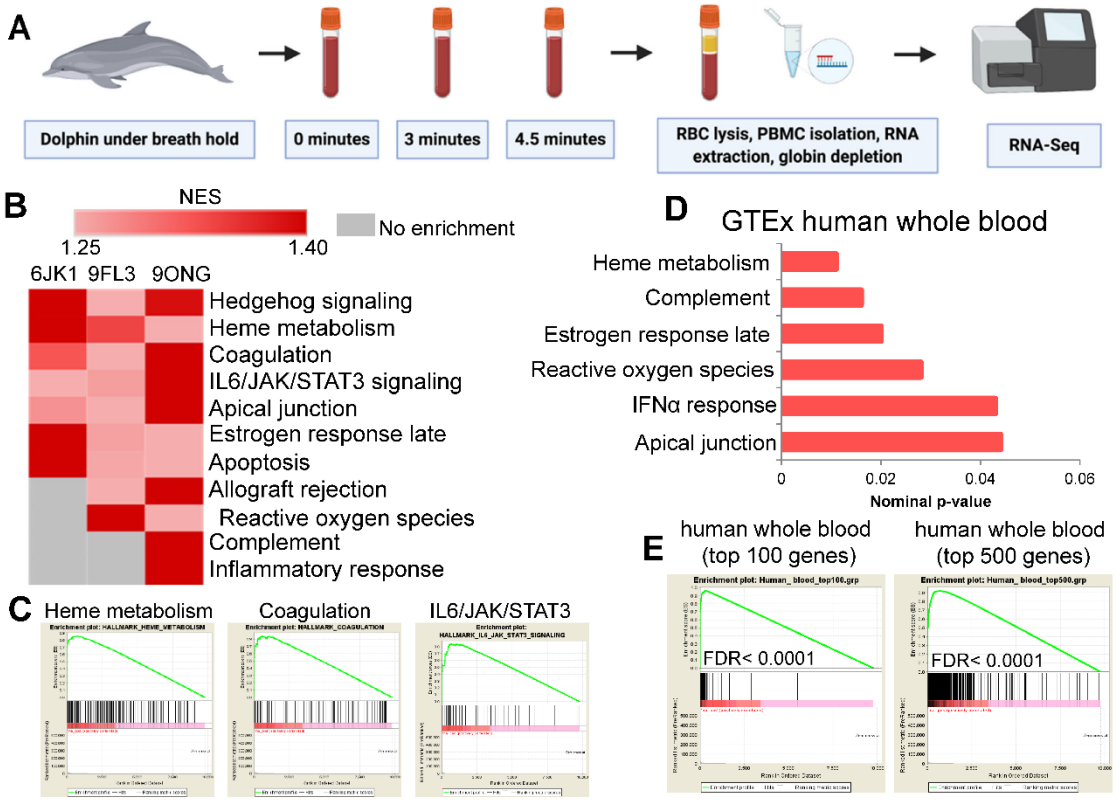


Figure 2. RNA-Seq from dolphin peripheral blood mononuclear cells reveals enrichment of pathways similar to humans. A. Whole blood from dolphins undergoing fasted breath-holds at baseline (0-30 seconds), 3 minutes, and 4 ½ minutes was collected from tail flukes and stored in PAXgene tubes for RNA extraction of peripheral blood mononuclear cells and RNA-Seq. B. Gene set enrichment analysis of baseline RNA-Seq data ranked by total expression pinpoints highly expressed relevant pathways. C. Enrichment plots for heme metabolism, coagulation, and IL6/JAK/STAT3 signaling from baseline dolphin RNA-Seq data. D. GSEA-based pathway enrichment from GTEx human whole blood RNA-Seq data ranked by total expression. E. GSEA enrichment plots comparing dolphin RNA-Seq data ranked by total expression with top 100 and top 500 expressed genes in human whole blood.

221 PTGDR2, SIX5, DCN, ADAMTS12, and GLRX2 demonstrated upregulation and/or high
222 network connectivity (**Figure 3B, C**). We used GeneMANIA to infer transcription factor and
223 microRNA targets from this time-dependent network. The gene regulatory network produced
224 from these genes displayed enrichment in targets from several transcription factor families,
225 including GATA and the small, mothers against decapentaplegic (SMAD) families (**Figure 3D**),
226 both of which have been implicated in hematopoietic development and regulation (Blank and
227 Karlsson, 2011; Lentjes et al., 2016). Network inference also pinpointed enrichment of targets of
228 multiple microRNAs, including the miR148A/B/152 family, miR492, miR186, miR518A-2, the

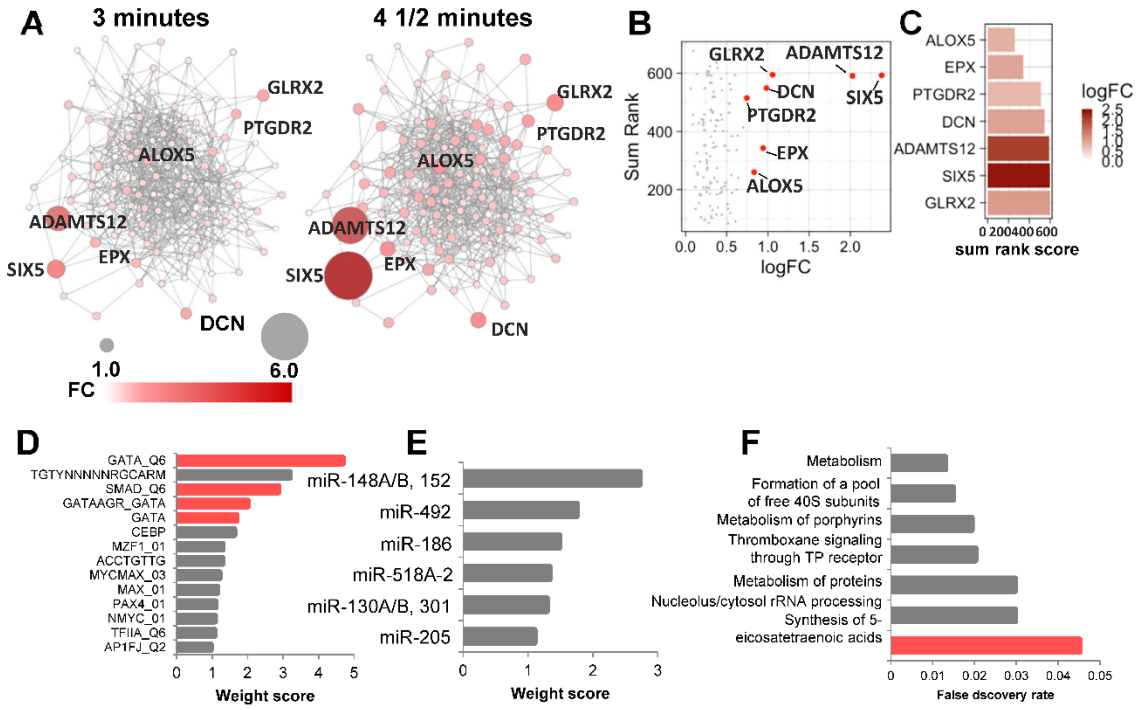


Figure 3. Time-dependent upregulation of gene regulatory pathways during dolphin breath holding. A. Gene regulatory network formed by the time-dependent increases in mRNAs from baseline to 3 minutes and 4 ½ minutes. Fold changes for each gene over time are indicated by darker red and larger nodes. B. Network analysis of genes within a co-expression network with increased expression over time. C. Top genes with increased expression sorted by their network analysis parameters. D. GeneMANIA-based transcription factor inference pinpoints GATA and SMAD transcription factor targets within the time-dependent network. E. MicroRNA enrichment inference based on the time-dependent network. F. Functional pathway enrichments for the time-dependent gene regulatory network.

229 miR130A/B/301 family, and miR205 (**Figure 3E**). Consistent with the identification of ALOX5
230 as a core network node, the network was functionally enriched the synthesis of 5-
231 eicosatetraenoic acid pathway, which is an initial step in the production of arachidonic acid by
232 ALOX5 (**Figure 3F**).
233
234 Arachidonate 5-Lipoxygenase (ALOX5) and subsequent lipoxygenase activity enhanced in
235 breath-holding dolphins. Based on the network analyses we focused on ALOX5 for further
236 validation. At the mRNA level, ALOX5 was one of just two genes, along with IL5RA, that was
237 significantly upregulated in all three individuals during breath-holding (**Figure 4A, B**).
238 Lipoxygenase assays from serum of three individual dolphins collected in 2019 revealed time-
239 dependent increases in lipoxygenase activity during breath-holding in all three individuals,
240 consistent with the RNA-Seq analyses (**Figure 4C**). Comparison of the timing of these molecular
241 changes with previously-published f_H measurements from the same dolphins demonstrated that
242 changes in gene expression and enzymatic activity were coincident with the expected timing of
243 bradycardia based on the heart rate data (**Figure 4D**). Overlay of the RNA-Seq data for ALOX5
244 expression with the heart rate data shows the upregulation of ALOX5 is concomitant with lower
245 heart rate (**Figure 4E**).

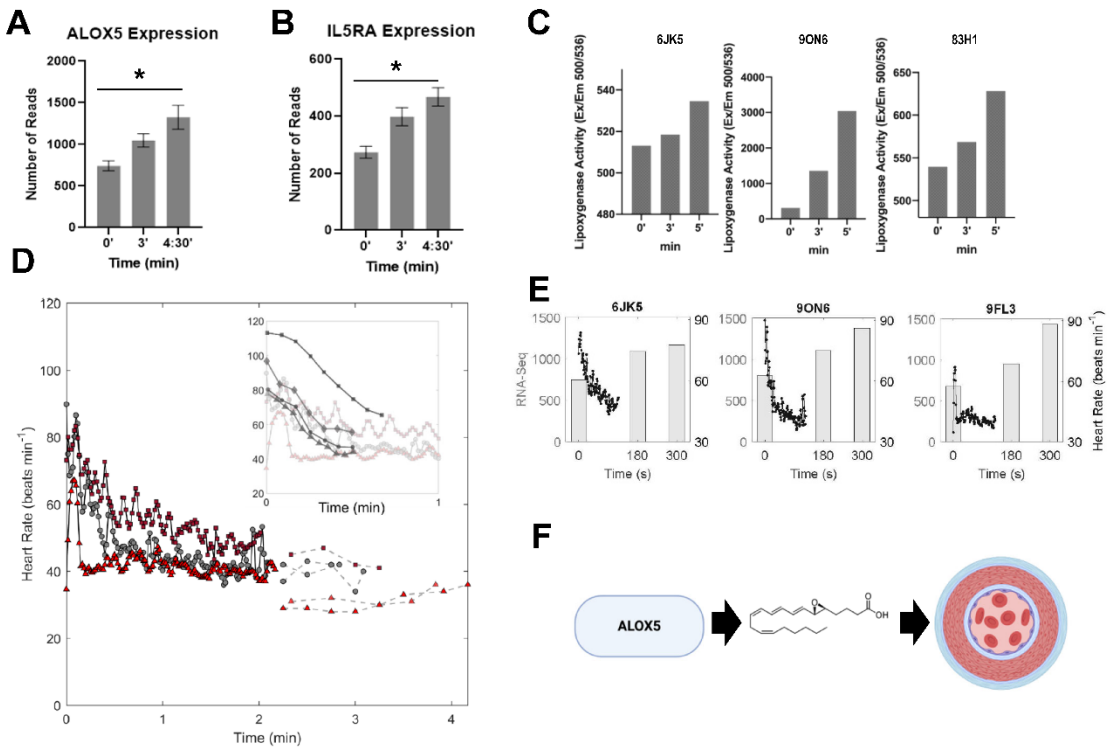


Figure 4. Dolphins induce ALOX5 activity during breath-holding. A. ALOX5 and B. IL5RA mRNA expression is significantly increased over time during breath-holding. C. Individual dolphin lipoxygenase activity in whole blood collected at an independent sampling date. D. Physiological measurements of heart rate for three individual dolphins (black lines from ECG data previously published in Blawas et al., 2020, dashed lines from echocardiogram data previously published in Fahlman et al., 2020) over time. Inset shows heart rate for humans performing breath-holds with facial immersion in water (dark gray in inset) overlaid on dolphin heart rate. Human heart rate traces were digitally extracted from Arnold, 1985; Andersson et al., 2004; and Shattock and Tipton, 2012. E. Overlay of heart rate data with ALOX activity in three individual dolphins. F. Hypothesized mechanism through which ALOX5 improves dive performance and mitigates ischemic stress tolerance.

Discussion

Dolphins and other cetaceans have evolved exquisite physiological adaptations to deal with the challenges of a fully aquatic lifestyle including having a hydrodynamic shape to reduce drag (Fish, 1993), counter-current heat exchangers for thermoregulation (Favilla and Costa, 2020;

251 Pabst et al., 1999), and cardiorespiratory plasticity for exquisite management of circulation and
252 respiratory gases (Blawas et al., 2021; Fahlman et al., 2020b, 2020a, 2019b; Noren et al., 2012).
253 The well-known dive response, a suite of adaptations that support reduced aerobic metabolism
254 during diving, involves apnea, bradycardia, and peripheral vasoconstriction that assures
255 maintained mean arterial blood pressure as blood flow to peripheral tissues is reduced and allows
256 regulation of perfusion to conserve oxygen-rich blood for the brain and heart. To maintain a
257 constant mean arterial blood pressure and prevent hypertension, these adaptations must work in
258 concert to ensure efficient autoregulation; however, extended dives also result in frequent events
259 of ischemia and hypoxia (Fahlman et al., 2019a; McKnight et al., 2019; Ridgway et al., 1969).
260 While these cardiorespiratory adaptations have been studied from the perspective of the changes
261 in f_H associated with diving, we are not aware of any study that has measured blood pressure in
262 voluntarily diving cetaceans. Thus, little is known about whether dolphins are able to maintain
263 constant mean arterial blood pressure throughout the breath-hold. In addition, knowledge of the
264 molecular adaptations that contribute to enhanced tolerance to hypoxia and ischemic stress, and
265 that prevent reperfusion injury during and following a dive, is rudimentary at best. To address
266 this lack of understanding, we combined an integrated genomics and systems-level analysis of
267 breath-hold responses at the molecular level with existing physiological measurements to define
268 the molecular responses to breath-holding in dolphins.
269 While this study is limited by a small sample size and relatively short breath-hold
270 durations, our analyses identified candidate genes and pathways with time-dependent changes in
271 expression throughout the breath-holds that were validated in functional studies using
272 independently-collected samples and assays. Consequently, these results provide evidence for
273 fine-scale cardiovascular control in bottlenose dolphins at the genetic level, and suggest that

274 dolphins may manage blood pressure changes during diving using both autonomic and molecular
275 pathways to regulate peripheral vasomotor control. Notably, these molecular changes occurred
276 within the calculated aerobic dive limit (cADL) of bottlenose dolphins - the duration of a dive
277 that can be sustained without requiring anaerobic respiration at the cellular level which has been
278 estimated to be 6.5 minutes (Fahlman et al., 2018). This suggests that changes in gene
279 expression may operate on a short enough time-scale that they could play a role in driving the
280 physiological changes that are observed during the breath-hold and recovery. It is also worth
281 considering the possibility that changes in gene expression could occur to support specific
282 physiological responses to diving during a dive, and that this gene expression differs when the
283 animal is at the surface. Future studies will be focused on using novel technologies, such as
284 GRO-Seq (Lopes et al., 2017) and others to measure nascent mRNAs, as well as measuring later
285 time points to understand the changes that occur upon recovery from breath-holds.
286 To provide physiological context for these molecular alterations on the time scales
287 observed, we compared molecular changes to changes in previously published f_H patterns in the
288 same individual dolphins during submerged breath-holds (Blawas et al., 2021; Fahlman et al.,
289 2020b). If we assume that the appearance of vasoconstriction is coincident with bradycardia, our
290 data provide evidence of an increase in the expression of a gene, ALOX5, known to promote
291 vasoconstriction coincident with the onset of vasoconstriction. Vasoconstriction, or a narrowing
292 of the blood vessels, has been suggested as a mechanism by which marine mammals during
293 forced dives have been observed to optimize the use of onboard oxygen stores in the blood and
294 muscle (Davis and Kanatous, 1999; Scholander and Grinnell, 1942; Zapol et al., 1979). Given
295 the long assumed link between vasoconstriction and bradycardia in marine mammals, the rapid
296 bradycardia we observed suggests that vasoconstriction was occurring in the dolphins in this

297 study during breath-holds (Hochachka, 1981; Van Citters et al., 1965). We found that changes in
298 gene expression occurred in all animals during the 5-minute breath-hold trials and that the same
299 gene families that were upregulated in the dolphins during breath-holds help manage
300 vasoconstriction in mice (Ichinose et al., 2001) and humans (Friedman et al., 1984).
301 Our integrated approach reveals possible molecular underpinnings that may support and
302 act synergistically with the cardiac response to breath-holding in bottlenose dolphins.
303 Specifically, we identified a suite of candidate genes that may support peripheral
304 vasoconstriction and provide defense against ischemic and hypoxic stress in dolphins, including
305 the GATA and SMAD transcription factors, several microRNAs, a disintegrin and
306 metalloproteinase with thrombospondin motifs 12 (ADAMTS12), mitochondrial glutaredoxin-2
307 (Glrx2), and ALOX5. Interestingly, many of these factors play known roles in regulating
308 hypoxia, hematopoiesis, and ischemic stress responses. For example, the GATA transcription
309 factor family is an important modulator of hematopoietic development of T lymphocytes, mast
310 cells, and erythrocytes (Lentjes et al., 2016). Likewise, the SMAD family regulates
311 hematopoietic stem cells (Blank and Karlsson, 2011). Of the microRNAs identified from our
312 analysis of target enrichments, nearly all have been shown to be protective against ischemia-
313 induced cell death, including miR148A (Zheng et al., 2018), miR492 (Guo et al., 2020), miR186
314 (Bostjancic et al., 2009; Li et al., 2013; Wang et al., 2018), miR130 (Lu et al., 2015), and
315 miR205 (Chen et al., 2019). At the protein-coding gene level, ADAMTS12 genetic variation is
316 associated with pediatric stroke (Witten et al., 2020), GLRX2 is implicated in neuroprotection
317 during hypoxia and ischemia (Romero et al., 2015), and ALOX5 is known to be induced by
318 hypoxia (Porter et al., 2014) and mediates the production of leukotrienes, which induce
319 bronchoconstriction and vasoconstriction (Poeckel and Funk, 2010). In addition, both ALOX5

320 and IL5RA have been identified as susceptibility genes associated with asthma and asthmatic
321 inflammation in humans (Cheong et al., 2005; Mougey et al., 2013), and a monoclonal antibody
322 to the IL5RA ligand, IL5, is FDA-approved for the treatment of severe eosinophilic asthma
323 (Fala, 2016; Pavord et al., 2012). Given the intricate connection between molecular control and
324 physiologic function to manage ischemia, hypoxia, and inflammatory responses in humans and
325 rodent models, (Bartels et al., 2013) it is intriguing to speculate as to how dolphins and other
326 marine mammals may uncouple or leverage these interconnected processes for improved
327 tolerance to ischemic/hypoxic stress without the pathological consequences associated with
328 hyper-stimulation of these processes.

329 These results demonstrate that the ALOX5 pathway is upregulated in bottlenose dolphins
330 during breath-holds and offer a potential mechanism for maintaining elevated peripheral
331 resistance through vasoconstriction, which helps manage blood distribution and the available
332 oxygen for critical organs. We suggest that the upregulation of ALOX5 could support a genetic
333 response that is secondary to the autonomic response during diving to prolong vasoconstriction
334 and maintain mean arterial blood pressure during extended periods of submersion (**Figure 4F**).
335 Interestingly, the changes we observed occurred within the cADL of the species, indicating that
336 fluctuations in gene expression could be occurring during regular dives. These fast-acting
337 changes in gene expression that support vasoconstriction provide evidence for fine-scale control
338 of perfusion in dolphins and an ability to maintain constant blood pressure, as is observed during
339 forced dives of pinnipeds (Blix et al., 1976; Zapol et al., 1979). Additionally, the data show that
340 during the breath-holds a large suite of candidate genes are upregulated that may support an
341 increased tolerance to the hypoxia and ischemic conditions that are expected to arise in some
342 peripheral tissues during diving. By interpreting these molecular data in the context of the

343 physiological changes known to occur in bottlenose dolphins during a breath-hold, we have
344 identified several genes that are upregulated during apnea in dolphins and may be an additional
345 mechanism to reinforce vasoconstriction while also providing defense against the hypoxia and
346 ischemia resulting from this response.

347 By examining molecular data through a physiological lens, these data connect the cellular
348 and tissue-level responses of dolphins to apnea to understand whether the bottlenose dolphin
349 may be genetically tuned to dive and withstand the hypoxia and the potential implications of this
350 to translational medicine. Our results uncover potential candidates at the intersection of ischemia,
351 hypoxia, and vasoconstriction that may contribute to the exquisite adaptation of dolphins and
352 other marine mammals to life in the ocean.

353

354 **Abbreviations List**

355 cADL, calculated aerobic dive limit

356 ALOX5, Arachidonate 5-Lipoxygenase

357 GSEA, Gene Set Enrichment Analysis

358 f_H, heart rate

359 IL5RA, Interleukin 5 receptor, alpha

360 PBMC, peripheral blood mononuclear cells

361

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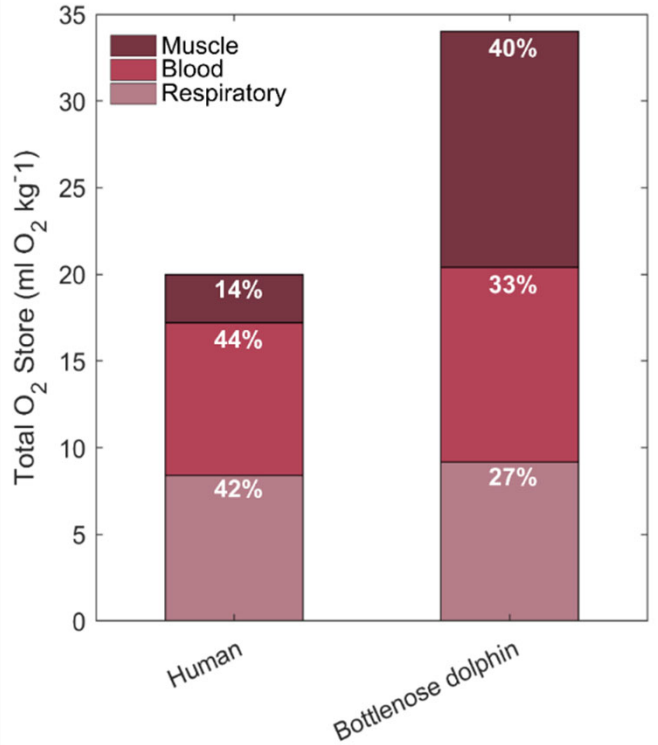
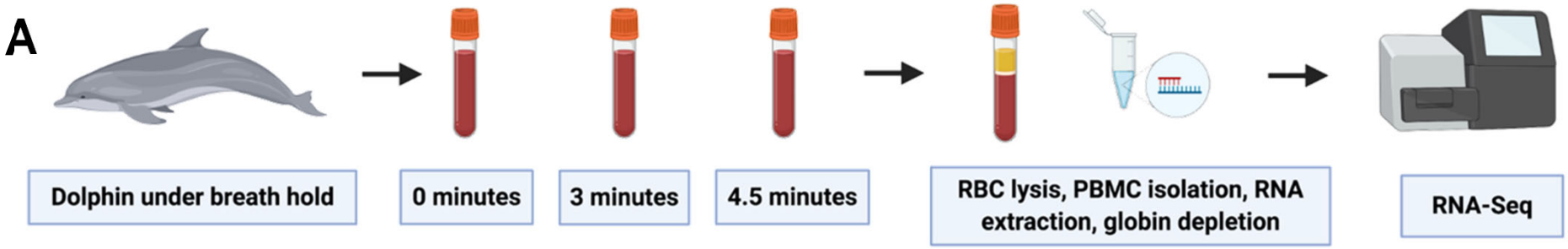
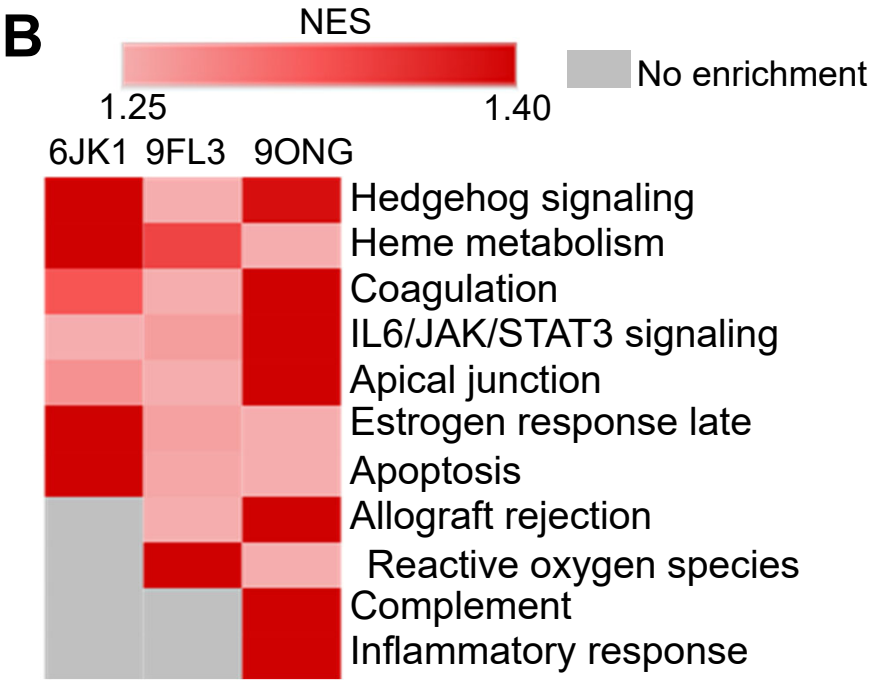
A

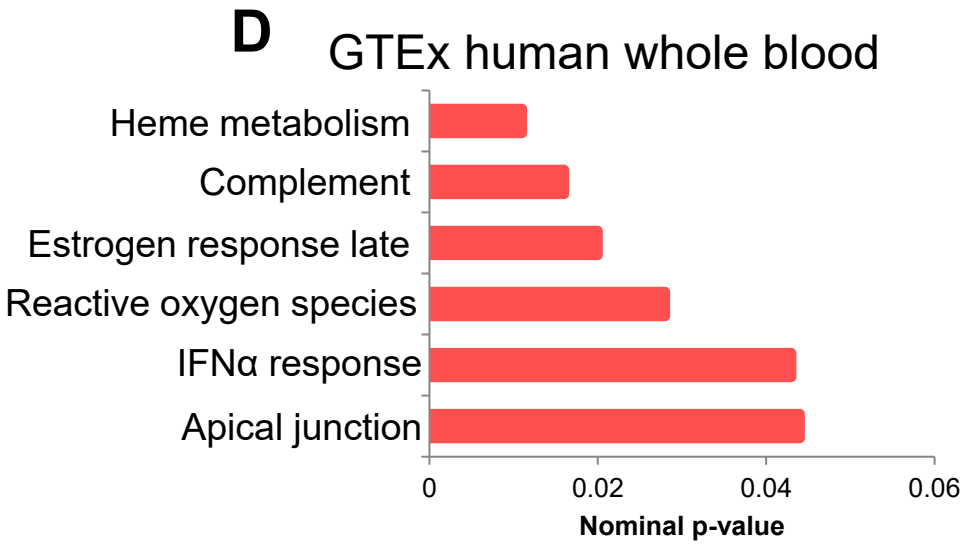


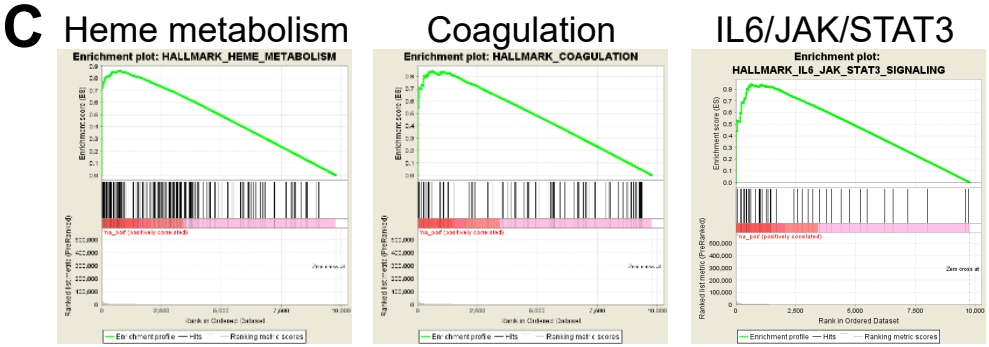
B

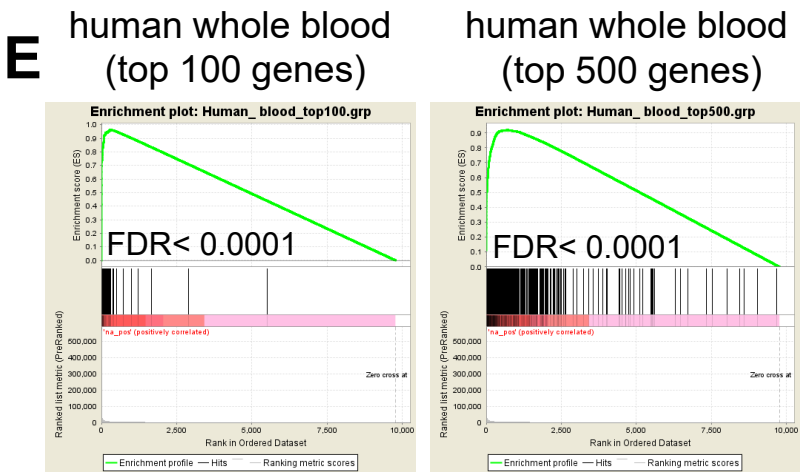
Average dive duration (min)	0.5-0.67	0.6
Max dive duration (min)	11.6	13
cADL (min)	—	6.5

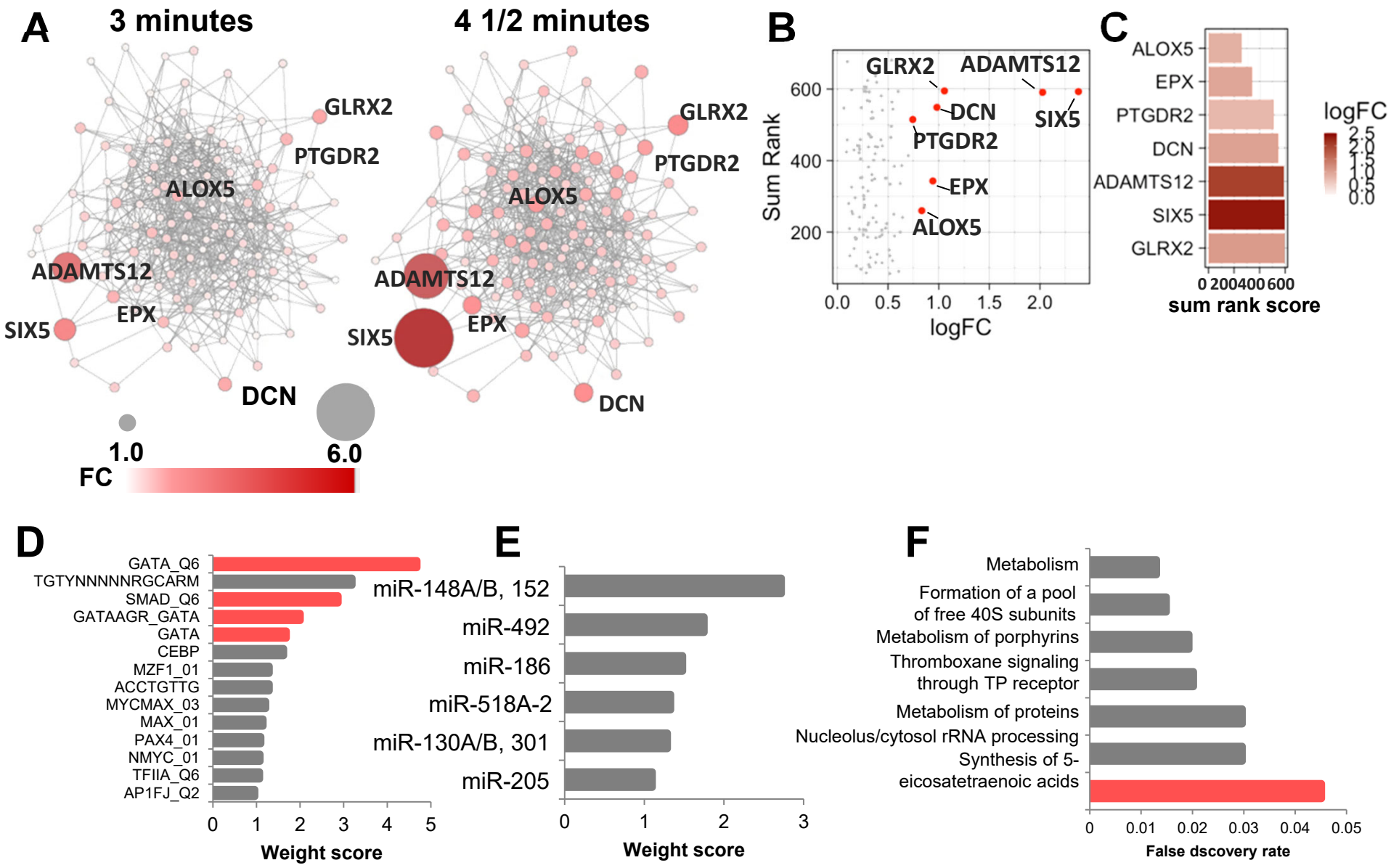

A
Dolphin under breath hold
0 minutes
3 minutes
4.5 minutes
RBC lysis, PBMC isolation, RNA extraction, globin depletion
RNA-Seq

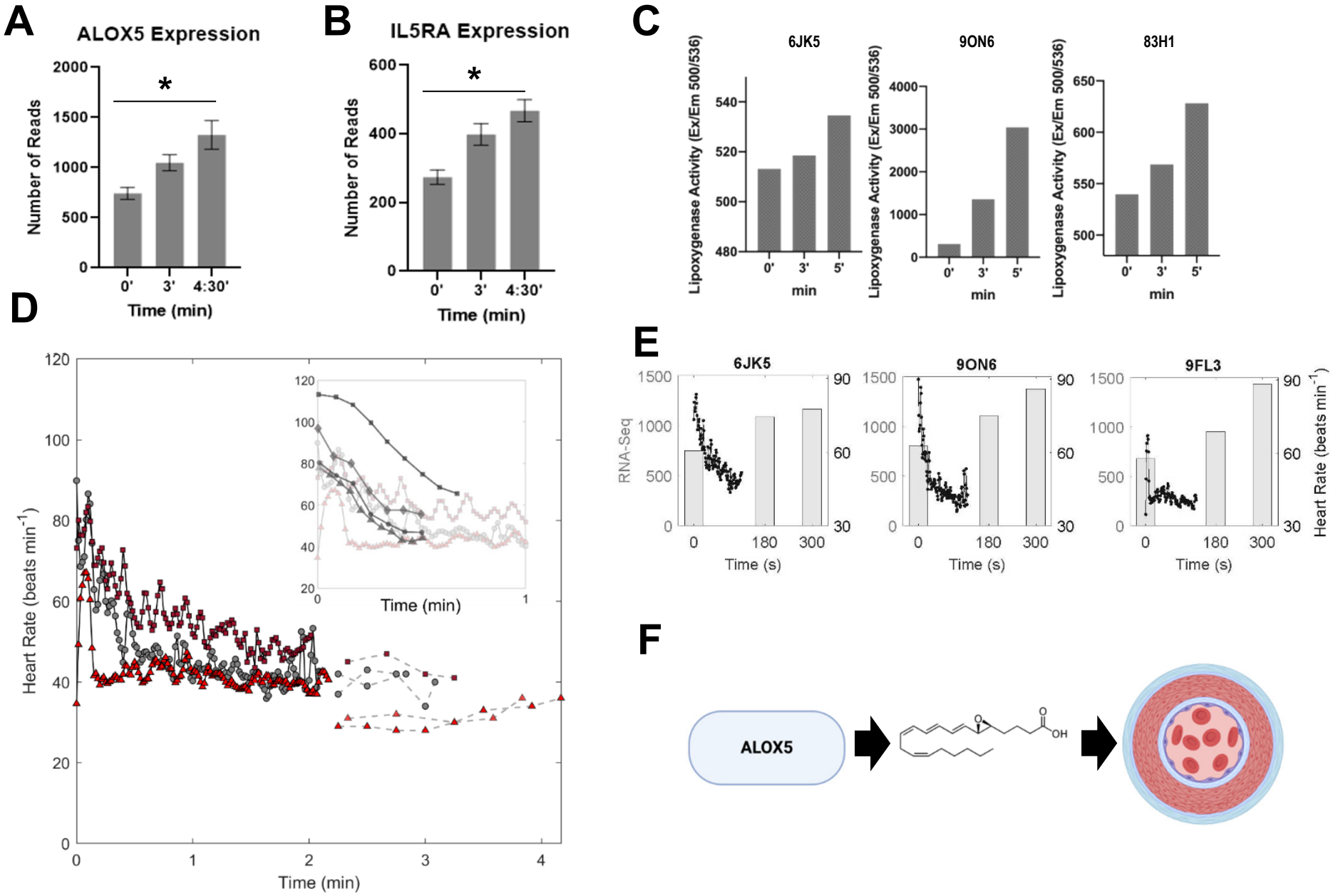

B
NES
1.25
1.40
No enrichment
6JK1
9FL3
9ONG
Hedgehog signaling
Heme metabolism
Coagulation
IL6/JAK/STAT3 signaling
Apical junction
Estrogen response late
Apoptosis
Allograft rejection
Reactive oxygen species
Complement
Inflammatory response


D
GTEx human whole blood
Heme metabolism
Complement
Estrogen response late
Reactive oxygen species
IFNα response
Apical junction
0
0.02
0.04
0.06
Nominal p-value


C
Heme metabolism
Coagulation
IL6/JAK/STAT3
Enrichment plot: HALLMARK_HEME_METABOLISM
Enrichment plot: HALLMARK_COAGULATION
Enrichment plot: HALLMARK_IL6_JAK_STAT3_SIGNALING


E
human whole blood (top 100 genes)
human whole blood (top 500 genes)
Enrichment plot: Human_ blood_top100.grp
Enrichment plot: Human_ blood_top500.grp
FDR< 0.0001
FDR< 0.0001


A
3 minutes
4 1/2 minutes
GLRX2
PTGDR2
ALOX5
ADAMTS12
EPX
SIX5
DCN
1.0
6.0
FC
B
GLRX2
ADAMTS12
DCN
SIX5
PTGDR2
EPX
ALOX5
Sum Rank
600
400
200
0.0
0.5
1.0
1.5
2.0
logFC
C
ALOX5
EPX
PTGDR2
DCN
ADAMTS12
SIX5
GLRX2
0 200400 600
sum rank score
logFC
2.5
2.0
1.5
1.0
0.5
0.0
D
GATA_Q6
TGTYNNNNNRGCARM
SMAD_Q6
GATAAGR_GATA
GATA
CEBP
MZF1_01
ACCTGTTG
MYCMAX_03
MAX_01
PAX4_01
NMYC_01
TFIIA_Q6
AP1FJ_Q2
0
1
2
3
4
5
Weight score
E
miR-148A/B, 152
miR-492
miR-186
miR-518A-2
miR-130A/B, 301
miR-205
0
1
2
3
Weight score
F
Metabolism
Formation of a pool of free 40S subunits
Metabolism of porphyrins
Thromboxane signaling through TP receptor
Metabolism of proteins
Nucleolus/cytosol rRNA processing
Synthesis of 5-eicosatetraenoic acids
0
0.01
0.02
0.03
0.04
0.05
False dscovery rate


A
ALOX5 Expression
Number of Reads
Time (min)
0'
3'
4:30'
B
IL5RA Expression
Number of Reads
Time (min)
C
6JK5
9ON6
83H1
Lipoxygenase Activity (Ex/Em 500/536)
min
D
Heart Rate (beats min-1)
Time (min)
E
6JK5
9ON6
9FL3
RNA-Seq
Heart Rate (beats min-1)
Time (s)
F
ALOX5
OH

Animal ID	Age (years)	Body Mass (kg)	RNA-Seq	Lipoxygenase assay
6JK5	24	200.9	x	x
9FL3	35	251.7	x	
9ON6	21	192.8	x	x
83H1	11	147.0		x
Mean±S.D.	22.8±9.9	198.1±42.9		