From Blue to Pink: Resazurin as a High-Throughput Proxy for Metabolic Rate in Oysters

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Abstract

Metabolic rate assays are critical tools for assessing organismal stress and resilience, yet their widespread application in aquaculture and ecological monitoring is limited. Improving these assays is essential for hatchery managers, farmers, and scientists seeking to identify resilient stocks and monitor stress in shellfish populations. Resazurin, a redox-sensitive dye commonly used in cell viability assays, offers a promising, high-throughput assay for metabolic rate assessment, but its application at the whole-organism level remains under explored. This study evaluates the efficacy of a resazurin-based metabolic assay in oysters (*Crassostrea gigas* and *Crassostrea virginica*) through four experimental approaches: (1) adaptation of the resazurin assay to measure oyster metabolism, (2) examination of temperature effects on oyster metabolism, (3) characterization of acute thermal stress responses, (4) examination of genetic variability in metabolism, and (5) correlations between metabolism and predicted performance in a selective breeding case study. Our findings confirm that resazurin fluorescence is correlated with oxygen consumption, validating its use as a measure of metabolism. Thermal performance assays reveal expected metabolic responses to temperature, including identification of optima and tipping points where metabolic stimulation shifts to depression under temperature stress. Acute thermal stress experiments demonstrate that oysters exhibiting greater metabolic

40 depression are more likely to survive, supporting metabolism as a predictor of mortality. Further,
41 genetic variation in stress responses is detected as family-level variation in metabolism.
42 Metabolism of 50 families (*C. virginica*) selectively bred for performance in varying
43 environments was measured and significantly correlated with predicted performance. By
44 establishing resazurin as an additional reliable and scalable method for metabolic assessment,
45 this study lays the groundwork for its broader adoption in aquaculture and conservation.
46 Implementing this approach may provide a tool to enhance stock selection, improve hatchery
47 management practices, and support adaptive strategies in the face of climate variability and
48 increased environmental stress in coastal oceans.

Introduction

50

51   Metabolic assays are powerful tools for assessing physiological stress and resilience in
52 marine organisms, particularly as climate change and environmental stressors increasingly
53 threaten aquatic ecosystems. By quantifying energy expenditure, these assays provide critical
54 insight into organismal health, performance, and survival potential (Sokolova, 2013). In
55 shellfish, metabolic measurements are essential for understanding responses to environmental
56 fluctuations such as temperature extremes, hypoxia, and ocean acidification (Chen et al., 2022;
57 Lannig et al., 2010, 2006; Le Moullac et al., 2007; Méthé et al., 2020; Widdows et al., 1989).
58 There is a need to develop rapid, high-throughput metabolic assays for use by researchers and
59 aquaculture practitioners to enhance efforts to monitor stock health, identify resilient genotypes,
60 and optimize hatchery management.
61   Resazurin, a redox-sensitive dye, presents a promising assay for high-throughput
62 metabolic rate assessments. Widely used in cell viability and toxicity assays due to its simplicity,
63 sensitivity, and cost-effectiveness (Petiti et al., 2024), resazurin provides a fluorescent indicator
64 of metabolic activity. As the organism conducts metabolism, resazurin undergoes a stepwise
65 reduction from its blue, non-fluorescent, oxidized form to pink, fluorescent, resorufin by NADH
66 and reductases, producing a strong fluorescent response (Candeias et al., 1998; Chen et al., 2018;
67 O'Brien et al., 2000). This fluorometric shift provides a robust, quantitative measure of whole-
68 organism metabolic activity. Resazurin-based assays have been extensively applied across
69 diverse fields, including cytotoxicity screening (Fields and Lancaster, 1993; Hussain et al., 2011;
70 O'Brien et al., 2000; Pace and Burg, 2015; Petiti et al., 2024), microbial metabolism studies (Fai
71 and Grant, 2009; González-Pinzón et al., 2012; Van den Driessche et al., 2014; Zare et al., 2015),
72 and biomedical applications (Anoopkumar-Dukie et al., 2005; McMillian et al., 2002). In marine
73 biology, resazurin has been used to assess sperm viability in marine invertebrates and fish
74 (Hamoutene et al., 2000) and to measure metabolic activity in zebrafish larvae (Reid et al., 2018;
75 Renquist et al., 2013), demonstrating its potential as a non-lethal, reliable metabolic indicator.
76 One study applied resazurin to evaluate hemocyte viability in oysters exposed to toxins (Estrada
77 et al., 2021), but its use for whole-organism metabolic rate assessment in shellfish remains
78 largely unexplored.

79   Despite its promise, adapting and testing resazurin for metabolic rate assays has not yet
80 occurred in shellfish and requires testing of factors such as incubation time, stress exposure
81 profiles, and comparison with established methods. By addressing these technical considerations,
82 resazurin-based assays could offer a scalable, complementary approach for evaluating metabolic
83 responses in shellfish, with applications in both research and aquaculture to predict and evaluate
84 stress resilience and performance. Therefore, the objective of this study was to develop and
85 validate a resazurin-based metabolic assay for oysters (*Crassostrea gigas* and *Crassostrea*
86 *virginica*), focusing on its utility for assessing metabolic resilience to environmental stress.
87   We had five objectives in this study: (1) Adaptation of the resazurin assay to measure
88 whole organism oyster metabolism. In this objective, we expected resazurin fluorescence
89 measurements of metabolism to positively correlate with oxygen consumption. Further, we
90 expected resazurin fluorescence to increase with oyster size and to measure metabolism in live
91 oysters higher than that of empty shell and blank samples. (2) Examination of temperature
92 effects on oyster metabolism. In this approach, we hypothesized that temperature would affect
93 metabolism with metabolism peaking at thermal optima and decreasing at high temperatures. (3)
94 Characterization of acute thermal stress responses. We expected to identify clear signals of acute
95 stress in metabolism with metabolism corresponding to mortality. (4) Examination of genetic
96 variability in metabolic responses. We hypothesized that families would exhibit distinct
97 metabolic responses to thermal stress. (5) Conduct a case study to identify relationships between
98 metabolism and performance. We expected that metabolism would correlate with predicted
99 performance breeding values in selectively bred *C. virginica* families.
100   By establishing a high-throughput metabolic assay for shellfish that is informative for
101 stress resilience and performance assessments, this study provides a new application of a
102 practical tool for monitoring shellfish health, identifying stress-resilient lineages, and improving
103 aquaculture management in a changing climate.

Materials and Methods

105
106   We conducted a series of experiments in this study to adapt the resazurin assay for use in
107 oysters and test protocols to evaluate oyster stress response and performance. First, we describe
108 the general protocol for resazurin metabolic assays and then provide methodologies for each
109 objective individually.

A. Resazurin standard protocol

111
112   We have included a general resazurin assay protocol for use in shellfish in
113 **Supplementary Information Appendix A.** All experiments used a standardized resazurin
114 solution. The resazurin assay protocol is based on previously published protocols in zebrafish
115 (Reid et al., 2018; Renquist et al., 2013). A concentrated resazurin stock solution was prepared

116   by dissolving 1.0 g of resazurin sodium salt (Cat. R7017 Sigma-Aldrich, Saint Louis, MO) in 20
117   mL (4.76% w/v) of deionized (DI) water with 20 µL dimethyl sulfoxide (DMSO; 0.10% v/v).
118   The concentrated stock solution was used to generate a working solution for assays and stored in
119   the dark at 4°C until use. Fresh working solution (111 µg/mL resazurin) was prepared prior to
120   each experiment by mixing 0.22% (v/v) resazurin stock solution, 0.1% DMSO (v/v), and 1.0%
121   (v/v) antibiotic antimycotic 100x Penn/Strep/Fungizone solution (Cat. SV30079.01, Cytiva,
122   Marlborough, MA) in filtered seawater or DI water adjusted to 25 ppt using Instant Ocean salts
123   (Instant Ocean, Blacksburg, VA). For example, a 500 mL working solution included 494 mL
124   seawater, 1.11 mL resazurin stock solution, 500 µL DMSO, and 5 mL antibiotic solution. The
125   working solution was either used immediately or stored at 4 °C in the dark and used within 1
126   week.
127   Oysters were then photographed to measure shell length (mm) in ImageJ (Rueden et al.,
128   2017) for size normalization and then allocated to individual size appropriate containers for
129   incubations. For example, small seed (3-8 mm) were added into 96-well plates with larger seed
130   (6-12 mm) added to 48 or 24-well plates. Larger oysters (8-20 mm) can be added to 12 or 6-well
131   plates with increasing sizes (>20 mm) added to individual plastic cups (20-40 mL). Containers
132   were then filled with the prepared resazurin solution with empty containers serving as resazurin
133   blank samples.
134   Samples were read on a plate reader in fluorescence mode with emission of 530 nm and
135   excitation of 590 nm set to collect endpoint measurements reading from the top of the sample.
136   For samples in multi-well plates, plates were placed directly on the plate reader for readings
137   while oysters in larger cups were measured by taking a 200 µL subsample and adding to a 96-
138   well plate for readings. Following the initial measurement, oysters were exposed to designated
139   temperature conditions controlled by benchtop incubators with readings collected every
140   subsequent hour for 4-6 h. Specific equipment and sample frequency are described for each
141   objective below. See an example of resazurin measurements in 96-well plates in **Fig 1**.
142   Fluorescence measurements were calculated as fold change by normalizing to the initial
143   fluorescence value and then corrected for background changes in resazurin solution fluorescence
144   by subtracting mean fluorescence values of blanks for each replicate plate or group. Corrected
145   fluorescence values were then normalized to size by dividing by shell length to generate size-
146   normalized fold change in fluorescence. Hereafter, size-normalized fold change in fluorescence
147   is referred to as "metabolism". Metabolism was quantified as metabolism over time and
148   evaluated by calculating "total metabolic activity" as Area Under the Curve (AUC) using
149   trapezoidal integration in the *pracma* package (Borchers, 2023). The effect of experimental
150   variables on metabolism and total metabolic activity was then tested using analysis of variance
151   (ANOVA) or linear mixed effect models in the *lme4* package (Bates et al., 2014) with post hoc
152   comparisons using estimated marginal means tests in the *emmeans* package (Lenth, 2018). All
153   analyses were conducted in R Statistical Programming v4.4.1 (R Core Team, 2022). Specific
154   statistical methods are described below for each objective.

B. Objective 1: Adaptation of resazurin assay to measure whole organism oyster metabolism

(1) Correlations between resazurin and oxygen measurements

We conducted oxygen consumption and resazurin assays on seed to examine the relationship between the two methods for measuring metabolism on the same individual oyster following the standard resazurin protocol described above. We selected n=95 oyster seed (4-7 mm) obtained from Pacific Hybreed (Port Orchard, WA) and held in tanks supplied with water adjusted to 25 psu using Instant Ocean salts (Instant Ocean, Blacksburg, VA) at the University of Washington. Oxygen measurements were conducted first on all individuals using a 24-well Loligo (Viborg, Denmark) glass plate (1.7 mL well volume) with PreSens (Regensburg, Germany) sensor spots (PSt5-2334-01) in the MicroResp software (v1.2.1.0). Microplate respirometry measurements have been reported in shellfish previously (Gurr et al., 2025, 2024). Wells were loaded with air saturated 0.2 µm filtered seawater (FSW, 25 psu) and sealed with plate sealing strips. Each run contained n=20 oysters per plate with n=4 blank wells containing only FSW. Measurements were conducted at 27°C with oxygen concentration (mmol per L) measured every 15 s for 50 min to 1 h. Five plates were run on the same day to measure all individuals. Oxygen consumption rates were extracted from oxygen measurements using localized linear regressions in the *LoLinR* package (Olito et al., 2017) using the percentile rank method with alpha set to 0.4 in R. Oxygen consumption rates were corrected by subtracting blank well values and dividing by shell length to produce μmol O_2 mm^{-1} min^{-1}.

Resazurin measurements were then conducted on the same set of individuals the following day in 48-well plates (n=3; 1 mL well volume) with n=40 oysters and n=8 blanks per plate. Measurements were conducted at 27°C over a 5 h period with hourly measurements using a BioTek Synergy HTX multi-mode plate reader (Agilent, Santa Clara, CA) in fluorescence mode with emission at 530 nm (528/20 nm filter) and excitation at 590 nm (590/20 nm filter) with associated software (Agilent Gen5 Software). Metabolism (fold change in fluorescence mm^{-1}) during the first hour of incubation was used to directly compare metabolism via resazurin with oxygen consumption. We then compared oxygen (cube-root transformed) and resazurin measurements (log-transformed) using a Pearson correlation. There was no observed mortality.

(2) Fluorescence signals from shells compared to live animals

In order to confirm resazurin signals are due to live animal metabolism and not an artifact of microbial communities on shells, we examined the difference in metabolism of live oysters (15-20 mm; n=15), empty shells (15-20 mm; n=15), and blank wells (n=6) of resazurin solution using 12-well plates at 35°C hourly for 4 h using a BioTek Synergy HTX multi-mode plate reader (Agilent, Santa Clara, CA). There was no observed mortality during the trials. We then examined metabolism before and after size normalization to compare fluorescence between

194 blanks, empty shells, and live oysters. Differences were tested using one-way ANOVA tests with
195 sample type as the main effect.

196 *(3) Relationship between oyster size and metabolism*

197

198 We evaluated the relationship between total metabolism, calculated as AUC (prior to size
199 normalization), and oyster size by combining metabolism measurements and shell length (mm)
200 data obtained from objectives 3 and 4 (described below) because these experiments were
201 conducted at the same site (University of Washington) with a similar size class (approx. 15-35
202 mm shell length) at 18°C. The relationship between oyster size and metabolism was evaluated
203 using a Spearman correlation test as well as a linear model (LM; square-root transformed AUC).

204 **C. Objective 2: Examination of temperature effects on oyster metabolism**
205

206 *(1) Thermal response in small seed (3-8 mm)*

207

208 We examined the effect of temperature on metabolism measured using the resazurin
209 fluorescence assay by exposing small seed to a range of temperature conditions in two tests of
210 small seed (3-8 mm) and larger seed (5-15 mm). First, we exposed small seed (3-8 mm shell
211 length) to 20, 36, 38, 40, and 42°C for 4 h. Seed were added to individual wells of 96-well plates
212 filled with 280 µL of resazurin working solution. Oyster seed were obtained from the Point
213 Whitney Jamestown S'Klallam Shellfish Hatchery from two separate broodstock spawning
214 groups and held at 14°C in two tanks filled with water adjusted to 25 psu using Instant Ocean
215 salts (Instant Ocean, Blacksburg, VA) at the University of Washington. Prior to measurements,
216 seed were photographed to measure shell length (mm) in ImageJ (Rueden et al., 2017).
217 Fluorescence was measured by placing the plate containing oysters directly on a plate reader
218 (BioTek FLx800; Agilent, Santa Clara, CA) in fluorescence mode with emission of 530 nm
219 (530/20 nm filter) and excitation of 590 nm (590/20 nm filter) with associated software (BioTek
220 Gen5). Seed were then exposed to control (18-20°C) or high temperature conditions (36°C,
221 38°C, 40°C, or 42°C) in a benchtop incubator (25 L, Vevor). The control and one elevated
222 temperature were run each day with elevated temperatures run in random order (n=1 plate per
223 temperature). Each plate contained n=84 seed and n=12 blank samples and were measured
224 hourly for 4 h. After the final measurement, we assessed survival of all seed by rinsing in DI
225 water and examining under a dissecting microscope. Seed were considered alive if the shell was
226 closed or moved in response to probing or were considered dead if the shell was open and did not
227 respond to probing.
228 Metabolism was calculated as described in the standard protocol. The effect of
229 temperature on metabolism (size-normalized fluorescence; cube-root transformed) over time was
230 tested using a linear mixed effect model (LMM) with hour and temperature as main effects,
231 sample nested within plate within date, tank, and broodstock group as random intercepts in the

232 *lme4* package (Bates et al., 2014) in R Statistical Programming v4.4.1 (R Core Team, 2022).
233 Total metabolism over the trial was calculated as AUC and analyzed using linear mixed effect
234 models with temperature as the main effect and tank and broodstock group as random effects.
235 We then examined differences in metabolism between seed that were found to either be alive or
236 dead at the end of the trials (at 42°C only as the other treatments did not show mortality) using a
237 linear mixed effect model with time point and final mortality status as main effects with tank and
238 broodstock group as random intercepts. Mortality effects on AUC were analyzed using linear
239 mixed effect models with final mortality status as main effect with tank and broodstock group as
240 random intercepts. For all models, significance was assessed using Type III Analysis of Variance
241 tests with Satterthwaite's approximation in the *lmerTest* package (Kuznetsova et al., 2015). Post
242 hoc comparisons were evaluated using estimated marginal means tests in the *emmeans* package
243 (Lenth, 2018). Temperature sensitivity (Q_{10}) values were calculated as group means between
244 20°C and 36°C as well as between 36°C and 42°C using the following equation: (AUC at
245 temperature 2 / AUC at temperature 1)^(10 / (temperature 2 - temperature 1)) (Casas et al.,
246 2018).

247   *(2) Thermal response in medium seed (5-15 mm)*
248
249   Oyster metabolism was measured across a range of temperatures to generate a thermal
250 response curve for seed (5-15 mm) at the USDA Pacific Shellfish Research Unit in Newport,
251 OR. Six temperatures were tested: 21°C, 27°C, 32°C, 37°C, 42°C and 45°C. Pediveliger oyster
252 larvae were obtained from Whiskey Creek Hatchery (Tillamook, OR) and set with epinephrine at
253 the Hatfield Marine Science Center (Newport, OR) to produce seed for the temperature trials.
254 Seed were held in ambient sea water (12-16°C, 28-32 psu) at the Hatfield Marine Science Center
255 until resazurin assays were conducted. For the experiments, oyster seed were placed individually
256 into 12-well tissue culture plates (CellTreat) and photographed to measure size in ImageJ
257 (Rueden et al., 2017) before adding 4 mL of resazurin working solution to each well. After
258 resazurin working solution was added, 300 µL were sampled immediately from each well for an
259 initial fluorescence measurement on a 96-well plate with fluorescence read on a Biotek Synergy
260 LX plate reader (Agilent, Santa Clara, CA) with an emission of 528 nm (528/20 nm filter) and
261 excitation of 590 nm (590/20 nm) with the Gen 5 software (Agilent, Santa Clara, CA). Well
262 plates with seed and resazurin were then placed in a benchtop incubator set to one of the six
263 temperatures with subsequent samplings and fluorescence readings of 300 µL of resazurin
264 working solution every hour for 5 h with n=5 blanks per trial. Trials were performed over a
265 series of 6 days with one temperature run each day in random order. Mortality was not assessed
266 in this experiment.
267   Data were analyzed as described above. The effect of temperature on metabolism was
268 tested with two-way ANOVA models with temperature and time point as the main effects. The
269 effect of temperature on AUC was tested by using a one-way ANOVA model with temperature

270   as the main effect. Q_{10} values were calculated as group means between each pair of temperatures
271   (e.g., 21°C to 27°C, 27°C to 32°C, and so on) as described above.

272   **D. Objective 3: Characterization of acute thermal stress responses**

273

274   We examined oyster metabolic response to acute temperature stress by exposing seed
275   (15-35 mm) to either control (18°C; n=20 oysters per trial) or high (42°C; n=20 oysters per trial)
276   temperatures for 4 h. Oyster seed were obtained from the Point Whitney Jamestown S'Klallam
277   Shellfish Hatchery and held at 14°C in tanks filled with water adjusted to 25 psu using Instant
278   Ocean salts (Instant Ocean, Blacksburg, VA) at the University of Washington. Seed were
279   obtained from a separate experiment in which seed were exposed 2 months prior to weekly 1 h
280   exposures to temperature (25°C), fresh water, or immune (Poly(I:C) exposure) sublethal stressors
281   (n=2 replicate bags of oysters per treatment). Treatment effects were not significant on
282   metabolism and are therefore included as a random effect but are not evaluated here directly (see
283   Results). Seed were imaged for shell length (mm) and placed in plastic cups with lids filled with
284   20 mL of resazurin working solution made as described above. Fluorescence measurements were
285   taken by sampling 200 μL of the resazurin solution from each cup, placing in a 96-well plate,
286   and reading fluorescence on a plate reader (PerkinElmer Victor 3, Shelton, CT) in fluorescence
287   mode with emission of 528 nm (528/20 nm filter) and excitation of 590 nm (590/20 nm filter)
288   with associated software (PerkinElmer Victor X Software). Due to the larger size of seed, we
289   were able to visually assess the survival of each oyster every hour while seed remained in plastic
290   cups. Seed were considered alive if the shell was closed or moved in response to probing or were
291   considered dead if the shell was open and did not respond to probing. Trials (n=10) were
292   repeated over ten days (N=506 oysters) with n=6 blanks per trial.
293   Metabolism was calculated as described above. We analyzed the effect of temperature on
294   oyster metabolism that survived the trial and the effect of mortality status on the metabolism
295   between those that either lived or died during the trial at 42°C. For each analysis, metabolism
296   over time was tested using a linear mixed effect model with hour and temperature or mortality as
297   main effects, sample nested within bag and date for repeated measures, and prior stress treatment
298   as random intercepts in the *lme4* package (Bates et al., 2014). Effects of prior stress treatment
299   were evaluated using ANOVA-like tests for random effects in the *lmerTest* package (Kuznetsova
300   et al., 2015). We also analyzed the effect of temperature or mortality on AUC with bag and date
301   as random intercepts. For all models, significance was assessed using Type III Analysis of
302   Variance tests with Satterthwaite's approximation in the *lmerTest* package (Kuznetsova et al.,
303   2015) and post hoc tests were conducted using estimated marginal means tests in the *emmeans*
304   package (Lenth, 2018). Survival was analyzed using a logistic regression (GLMM; *glmer*
305   function; family=binomial, link=logit) in the *lme4* package (Bates et al., 2014) with binary
306   mortality as the response with time and temperature as main effects and prior stress treatment,
307   bag, and sample (for repeated measures) as random intercepts. Significance was assessed using
308   Type II Wald chi-square ANOVA tests in the *car* package (Fox and Weisberg, 2018).

309   Finally, we evaluated whether metabolism at a particular time point predicted mortality at
310 the subsequent time point. We assessed the relationship between metabolism and subsequent
311 mortality using a logistic regression model (GLMM; *glmer* function; family = binomial, link =
312 logit) in the *lme4* package (Bates et al., 2014). Mortality at the subsequent time point was
313 included as the response variable with scaled fluorescence and temperature as main effects. Prior
314 stress treatment and sample nested within bag and date were included as random intercepts.
315 Model significance was evaluated using a Type II Wald chi-square ANOVA test in the *lmerTest*
316 package (Kuznetsova et al., 2015). Predicted probability of mortality was then plotted against
317 metabolism with confidence intervals generated using the *pROC* package (Robin et al., 2011).

318   **E. Objective 4: Examination of genetic variability in metabolic responses**
319
320   To examine genetic variation in metabolism between oyster families, we obtained oyster
321 seed (13-25 mm) from the United States Department of Agriculture Pacific Shellfish Research
322 Unit Pacific Oyster Genome Selection program (Newport, OR) from five families produced
323 during the 2024 spawning season. Oysters were held in heath trays at ambient conditions at the
324 Jamestown S'Klallam Shellfish Hatchery (Brinnon, WA) until April 2025 at which point they
325 were transported to the University of Washington. Oysters were then held at 14°C in tanks filled
326 with water adjusted to 25 psu using Instant Ocean salts (Instant Ocean, Blacksburg, VA) at the
327 University of Washington.
328   Resazurin solutions were prepared as described above and added to oysters placed in 20
329 mL plastic cups. Fluorescence readings were taken by sampling 200 µL of the resazurin solution
330 and reading on 96-well plates on a BioTek Synergy HTX multi-mode plate reader (Agilent,
331 Santa Clara, CA) in fluorescence mode with emission at 530 nm (528/20 nm filter) and
332 excitation at 590 nm (590/20 nm filter) with associated software (Agilent Gen5 Software).
333 Following initial measurement, all oysters were placed in an incubator set to the control
334 temperature (18°C) and samples were collected every hour for 3 h. After the 3 h sample was
335 taken, the incubator was set to 40°C and oysters were returned to the incubator with samples
336 taken every hour for 3 h. The total incubation time was 6 h with the first half at control
337 temperature and the second half at high temperature. This approach allows for tracking
338 individual oyster metabolic response to acute temperature stress. Temperatures in oyster cups
339 were monitored using a digital thermometer (Traceable Excursion-Trac, San Francisco, CA).
340 Following the final measurement at 6 h, oysters were assessed for mortality as described above
341 with analysis conducted only for oysters that survived. Each trial included n=5 oysters per family
342 and n=5 blank samples with trials (n=6) conducted over 6 days (N=150 oysters, n=30 per
343 family).
344   Metabolism calculations were performed for each individual during control and elevated
345 temperature phases. The effect of family and temperature on metabolism (cube-root transformed)
346 over time was tested using a linear mixed effect model with family, time point, and temperature
347 as main effects, sample nested within date (for repeated measures), and measurement date as

348 random intercepts in the *lme4* package (Bates et al., 2014) in R Statistical Programming v4.4.1
349 (R Core Team, 2022). For all models, significance was assessed using Type III Analysis of
350 Variance tests with Satterthwaite's approximation in the *lmerTest* package (Kuznetsova et al.,
351 2015) and post hoc tests were conducted using estimated marginal means tests in the *emmeans*
352 package (Lenth, 2018).

F. Objective 5: Conduct a case study to identify relationships between metabolism and performance in *Crassostrea virginica*

(1) Selective breeding program

357 We conducted resazurin assays on selectively bred families to test relationships to
358 predicted performance at the Virginia Institute of Marine Science (VIMS). The Aquaculture
359 Genetics and Breeding Technology Center (ABC) at VIMS has been performing family-based
360 breeding of *Crassostrea virginica* since 2004. A full description of the diploid family breeding
361 program at ABC is outlined in (Allen et al., 2021). Current traits for selective breeding include
362 survival, total weight, meat yield and shell shape characteristics across low and moderate/high
363 salinity environments, with a strong genetics x environment effect for survival and growth traits
364 between low and moderate/high salinity sites. Note that moderate/high salinity test sites often
365 have annual disease pressure from *Perkinsus marinus* and *Haplosporidium nelsoni*, both of
366 which are linked to oyster mortality. Phenotypic information is collected on families at 18
367 months of age and selection of broodstock candidates for subsequent spawns and terminal
368 broodstock line creation is based on two selection indexes, one for low salinity performance and
369 one for moderate/high salinity performance. Selection locations have varied over the years;
370 however, current selection locations include those in **Table S1**. Resazurin assays in this study
371 were conducted using thermal stress tests of metabolism as an indicator of stress tolerance to
372 assess whether metabolism corresponds to general performance.

(2) Resazurin assays on selectively bred families

375 For this study, all seed were spawned in spring 2025 at the Acuff Center for Aquaculture
376 at VIMS by members of ABC. Resazurin assays were performed on *C. virginica* oysters from 50
377 families at VIMS with a total of 28 oysters per family measured (n=1,400 oysters). Family
378 spawns were performed using 1x1 crosses of broodstock candidates selected for specific
379 performance traits (described below) where each cross was kept separate through the entire
380 larval and setting period. Larvae for family crosses were reared in 60 L static, aerated tanks
381 whereas larvae for lines were reared in 200 L tanks. Larval husbandry was generally the same for
382 all cultures, including daily feeding of live microalgae cultured at the Acuff Center, water
383 changes, tank cleaning and larval health assessments every other day. When the larvae
384 approached 300 µm in size and developed an eye spot, they were added into coffee filters

385 dampened with seawater and removed from each tank. Harvests were stored at 4°C and occurred
386 every other day for 3 days. Harvests were then combined and eyed larvae were set on 400 µm
387 microcultch in the downwelling setting system within the Acuff Center. While in the setting
388 system, setters were fed daily a ration of live microalgae, and each setting tank was cleaned
389 every other day. Once the setters reached ~1mm in size, the cultch was removed through sieving,
390 and all seed were transferred to the land-based upweller system in the VIMS boat basin. One day
391 before the resazurin assays, a subset of seed from each family was removed from each silo and
392 set aside by ABC and placed into an individually labeled mesh pouch. Pouches were then held
393 briefly in three upweller silos before being transferred to the Acuff Center broodstock room.
394 Seed used for resazurin measurements ranged from 7-25 mm in size.
395   Families were randomly chosen to include those selectively bred for either moderate/high
396 salinity (n=25 families) and low salinity (n=25 families) performance. Assays were conducted in
397 24-well plates (2.5 mL) with n=4 blanks and n=20 oysters per plate. Prior to assays, we
398 conducted preliminary observations to select temperature treatments that maximized stress
399 exposure but did not cause mortality. We selected 40°C for 2.5 h followed by a recovery period
400 for another 1.5 h as our case study trial. Oysters were randomly allocated to plates for a total of
401 n=70 plates with n=22-24 plates run each day over three days. Resazurin solutions were prepared
402 as described and measurements were collected on a Varioskan Lux (Thermo Scientific,
403 Waltham, MA, USA) multi-mode plate reader at an excitation at 530 nm and emission at 590 nm
404 with a bandpass of 12 nm using SkanIt software. Chilled (4°C) resazurin was added to plates for
405 initial measurements and plates were then added to an incubator set at 40°C. Plates were added
406 to incubators at staggered intervals to ensure each plate was read every hour. Plates were
407 measured again at 1 and 2 h time points. At 2.5 h, plates were moved out of the incubator for a
408 recovery phase. Measurements were then collected at 3 and 4 h time points. Temperature was
409 recorded in one randomly selected well in 12-16 plates hourly. 145 samples (10%) reached
410 oversaturation and were removed from the analysis. Temperature profiles of the wells are
411 displayed in **Fig S1.** Temperatures were 12.3±0.3°C at initial measurements, 34.2±0.1°C at 1 h,
412 35.6±0.2°C at 2 h, 29.0±0.3°C at 3 h, and 23.2±0.2°C at the final measurement at 4 h. We
413 assessed each individual for mortality at the end of the trial.

414   *(3) Data analysis and correlation to predicted performance*
415
416   Metabolism was calculated as described previously for each oyster. We analyzed
417 metabolism over time using a linear mixed effect model with cube-root transformed fluorescence
418 as the response and family and time point and their interaction as main effects using the *lme4*
419 package in R (Bates et al., 2014). Well, plate, and date were included as random intercepts to
420 account for repeated measures. We also tested for the effect of family phenotype (i.e., low or
421 high salinity selected) using mixed effect models with the same structure but with time point and
422 phenotype as main effects. Significance of main effects was evaluated using Type III ANOVA
423 tests with Satterthwaite's approximation method. Normality of residuals and homogeneity of

424 variance were assessed using quantile-quantile plots and Levene's tests, respectively. AUC was
425 analyzed using a linear mixed effect model with family or phenotype as the main effect with
426 plate and date as random intercepts. We used a repeatability estimation in the *rptR* package
427 (Stoffel et al., 2017) to evaluate intraclass correlation and describe within and between-group
428 sources of variation. Family, as the grouping variable, was included as a random intercept with a
429 main effect of time point and fold change in fluorescence as the response with Gaussian data.
430   Predicted performance data after progeny testing is analyzed annually using linear mixed
431 animal models using a genetic groups structure in AsReml (Gilmour et al., 2015) as in (Allen et
432 al., 2021). Growth traits are analyzed using a five-trait, multivariate model whereas survival is
433 analyzed in a separate model. "Predicted performance" values were calculated for each family as
434 estimated breeding values for survival and growth in low or moderate/high salinity environments
435 (percentage gain) generating four metrics: predicted survival in moderate/high salinity, predicted
436 survival in low salinity, predicted growth in moderate/high salinity, and predicted growth in low
437 salinity environments. We then correlated AUC of each oyster with the predicted performance
438 metrics for survival and growth using non-parametric Spearman correlation tests without data
439 transformations. We conducted correlation analyses between metabolism and predicted
440 performance metrics for high and low salinity selected families separately.

Results

A. Objective 1: Adaptation of resazurin assay to measure whole organism oyster metabolism

(1) Positive correlation between resazurin and oxygen measurements of metabolism

447   We first explored the relationship between metabolism measurements made through
448 resazurin fluorescence measurements and oxygen consumption. There was a significant positive
449 correlation between oxygen consumption and resazurin fluorescence (Pearson correlation;
450 p=0.012, r=0.321; **Fig S2**). This correlation was weakly positive with r=0.321.

(2) Clear fluorescence signals from live animals

453   We compared fluorescence signals from blank, empty shells, and live oysters to confirm
454 that metabolism of live animals is not confounded by signals originating from microbial
455 communities on oyster shells or reactions between the shell material and resazurin solution. We
456 found that signals from empty shells were higher than blanks (ANOVA; SS=14.4, F=111.4,
457 p<0.001; **Fig S3**), but that live oyster metabolism was dramatically higher than empty shells
458 (ANOVA; SS=4.9, F=96.4, p<0.001; **Fig S3**).

459   *(3) Metabolism correlates positively with oyster size*

460

461   Total metabolism over the course of the trials (calculated as AUC) positively correlated

462 with oyster size (Spearman correlation, S=2.82e6, p<0.001) with a moderately positive

463 relationship (r=0.624; **Fig S4**). Length effects on AUC were significant (LM; t=13.90, adj.

464 R^2=0.13, p<0.001) with a slope of 0.13, indicating a one unit increase in AUC with every 0.13

465 mm increase in length.

466   **B. Objective 2: Strong temperature effects on metabolism**

467

468   Metabolism was strongly influenced by temperature. We examined temperature effects

469 on metabolism using the resazurin assay in small seed (3-8 mm) measured at the University of

470 Washington as well as seed (5-15 mm) measured at the USDA Pacific Shellfish Research Unit

471 (Newport, OR). Metabolism in small seed (3-8 mm), including only those that survived the trial,

472 varied across temperatures (LMM; SS=3.05, DF=16/2260, F=15.49, p[time point x

473 temperature]<0.001). Total metabolism over the course of the incubation, measured as area

474 under the curve (AUC), changed with temperature (LMM; SS=5.28, DF=4/562.5, F=22.36,

475 p<0.001) with rates peaking at 36°C and decreasing at 38-42°C (**Fig 2A**). Q_{10} values were

476 highest from 20-36°C (Q_{10}=1.22) followed by decreased thermal sensitivity from 36-42°C

477 (Q_{10}=0.28; **Table S2**). Similarly, in larger seed (5-15 mm) metabolism differed across

478 temperatures (LMM; SS=4.91, DF=24/625.53, F=37.24, p[time point x temperature]<0.001).

479 AUC varied strongly across temperatures (LMM; SS=7.29, DF=5, F=32.85, p<0.001) with

480 metabolic activity increasing from 21-26°C to a peak at 37-42°C and declining sharply at 45°C

481 (post hoc p<0.05; **Fig 2B**). Thermal sensitivity (Q_{10}) peaked between 26-32°C (3.52) and was

482 lowest in temperatures exceeding the thermal optimum between 32-45°C (Q_{10}=0.001-1.22;

483 **Table S2**).

484   **C. Objective 3: Clear metabolic response to acute stress and metabolic depression as an**
485   **indicator of tolerance**

486

487   We observed strong metabolic responses to acute high temperature stress using the

488 resazurin assay and identified metabolic indicators of resilience. In large seed (15-35 mm) that

489 survived the entire 4 h incubation (n=92 at 42°C, n=201 at 18°C), metabolism was significantly

490 different between 18°C and 42°C (LMM; SS=1.28, DF=4/11184, F=116.62, p[time point x

491 temperature]<0.001) with oysters exposed to 42°C exhibiting metabolic depression (**Fig 3A**).

492 Metabolism was significantly lower at 42°C from 2-4 h of incubation (post hoc p<0.001). Total

493 metabolism (calculated as AUC) in oysters that lived during the 4 h incubation was lower at

494 42°C than 18°C (LMM; SS=1.54, DF=1/291, F=63.39, p<0.001; **Fig 3B**). Note that previous

495   stress exposure had no effect on metabolic rates (ANOVA-like test for random effects; log
496   likelihood=1905.1, likelihood ratio test=0.00, DF=1, p=1).
497   Mortality was higher at 42°C than 18°C during the 4 h resazurin measurement (GLMM;
498   X^2=115.31, DF=1, p<0.001; **Fig 3C**). During the trials, 58±3% of oysters died at 42°C while
499   only 4±1% died at 18°C. Given the degree of mortality at 42°C, we then examined differences in
500   metabolism between oysters that lived (n=92) and those that died (n=127) during the 4 h
501   exposure at 42°C.
502   First, we examined metabolism in oysters depending on time of mortality and observed
503   that oysters that died earlier in the trial (1-3 h) showed slower metabolic rates than those that
504   died at the end of the trial (4 h) (**Fig S5**). Further, oysters that remained alive showed continued
505   increases in metabolism throughout the 4 h measurements, in contrast to reduced metabolism in
506   those that died (**Fig S5**). Overall, metabolism was lower in oysters that lived compared to those
507   that died during the 4 h exposure to 42°C. Metabolism was lower in large seed (15-35 mm) that
508   lived compared to those that died during the 4 h measurements (LMM; SS=0.51, DF=4/849.95,
509   F=133.19, p[time point x mortality]<0.001; **Fig 4A**) as was AUC (LMM; SS=1.26,
510   DF=1/212.58, F=95.791, p<0.001; **Fig 4B**). Metabolism was lower in oysters that survived (post
511   hoc p<0.001; **Fig 4A**) with a 52% decrease in AUC in oysters that survived (post hoc p<0.001;
512   **Fig 4B**). Similarly, in small seed (3-8 mm), metabolic rates (LMM; SS=0.30, DF=4/404, F=5.15,
513   p[time point x mortality]<0.001) and AUC (LMM; SS=3.0, DF=82, F=14.71, p<0.001) were
514   lower in oysters that survived the 42°C exposure than those that died during the incubation (**Fig
515   4CD**).
516   We further evaluated whether metabolism at a particular time point predicted mortality at
517   the subsequent time point under acute stress. Higher metabolism was significantly associated
518   with increased odds of mortality (GLMM; estimate =4.0, X^2=67.09, DF=1, p<0.001; **Fig 5**). In
519   other words, oysters with high metabolism are at greater risk for mortality (**Fig 5**).

520   **D. Objective 4: Genetic variation in metabolism response**

521

522   We conducted resazurin measurements on five families of oyster seed (13-25 mm)
523   exposing each individual to 3 h at 18°C followed by 3 h at 40°C to examine genetic variation in
524   metabolism. Responses varied between families (LMM; SS=0.05, DF=4/934.37, F=3.45,
525   p=0.008) and metabolic rates were higher at 40°C than at 18°C (LMM; SS=0.02, DF=1/923.53,
526   F=6.09, p=0.014; **Fig 6A**), but there were no significant interactive effects between temperature
527   and family (LMM; SS=0.01, DF=4/926.87, F=0.44, p=0.782; **Fig 6B**). Within each family, there
528   was variation between individuals, but metabolism was higher in families A and B than in
529   families C and D (Fig **6AB**).

530   **E. Case study: Metabolism correlates with predicted performance in *Crassostrea***
531   ***virginica***

532

We applied the resazurin metabolic assay in a case study of *C. virginica* seed (7-24 mm) to examine whether metabolism under heat stress correlates with predicted performance in 50 selectively bred families. All oysters were exposed to 2.5 h of acute heat stress followed by a recovery period (**Fig S1**). There was significant variation in metabolism between families (LMM; SS=30.74, DF=196/4788, F=4.56, p[family x time]<0.001; **Fig 7A**). There was also a significant effect of family selected phenotype (i.e., high/moderate salinity or low salinity selected families) on metabolic rates (LMM; SS=1.29, DF=4/4980, F=8.24, p[phenotype x time]<0.001; **Fig 7B**). Similarly, total metabolism over the course of the trials (AUC) varied significantly between families (LMM; SS=136.83, DF=49/1146, F=5.02, p<0.001; **Fig 7C**) and between selected phenotypes (LMM; SS=5.60, DF=1/1239.8, F=8.77, p=0.003; **Fig 7D**). Specifically, metabolism was higher in the low salinity selected families. Repeatability analyses indicated that most variation in metabolism was due to individual response within families with little structure between families (R=0.11, p<0.001).

We then correlated mean AUC of each family with the family's predicted field performance metric. We tested for correlations between AUC and growth and survival in each phenotype group (high/moderate salinity or low salinity selected). We found that AUC in low salinity selected families was significantly positively correlated with predicted survival in a low salinity environment (Pearson correlation; t=3.16, df=22, r=0.56, p=0.004; **Fig 8**). However, there was no significant correlation within the high salinity selected families, but there was a trend for a negative relationship (Pearson correlation; t=-1.38, df=22, r=-0.28, p=0.181; **Fig 8**). Interestingly, there was no significant correlation between AUC and predicted survival in high salinity environments (Pearson correlation; p>0.05). There was no significant correlation with predicted growth for any phenotype group (Pearson correlation; p>0.05). There was no mortality observed during or after the resazurin trials.

Discussion

This study establishes resazurin metabolic assays as a reliable tool for measuring oyster metabolism, with clear utility for both scientific research and aquaculture applications. By adapting a dye widely used in a variety of cell viability, microbial, and biomedical assays (e.g., Anoopkumar-Dukie et al., 2005; Petiti et al., 2024; Zare et al., 2015) and more recently applied in fish (Reid et al., 2018; Renquist et al., 2013), we demonstrate that resazurin provides a means of quantifying whole-organism metabolism in oysters. Quantifying metabolic responses is key to understanding health, performance, and survival in challenging and energy-limiting environments (Sokolova, 2013). Our experiments show that this approach can provide a high-throughput and reliable method to capture variation in thermal performance, acute stress responses, and family-level differences in metabolism. Our application in an aquaculture case study further demonstrates that metabolism can be correlated to predicted performance in oysters, providing a foundation from which future work can identify linkages between metabolism and field performance. The adaptation of this assay for use in shellfish opens a wide

572 range of new opportunities to further optimize and develop standard operating procedures for
573 this approach to measure performance, monitor stress, identify resilient stocks, investigate
574 physiological responses of shellfish for scientific and aquaculture applications.
575   Our first goal was to validate the use of resazurin assays in reliably measuring
576 metabolism in oysters. Resazurin assays provide a robust measure of metabolism by acting as an
577 intermediate electron acceptor and changing from the oxidized, non-fluorescent form (blue) to
578 the fluorescent resorufin (pink) form (Rampersad, 2012). As the organism conducts metabolic
579 activity the dye is reduced by NADH in the presence of reductases and fluoresces (Candeias et
580 al., 1998; Chen et al., 2018; O'Brien et al., 2000), which is measured using a plate reader. Our
581 results confirm that the resazurin assay measures metabolic signals as biologically expected from
582 live animals (i.e., metabolism was far higher in live animals than from empty shells), that signals
583 increase with animal size, and that metabolism measured via resazurin is positively correlated
584 with oxygen consumption. These tests further highlight differences in measurement approaches
585 between resazurin fluorescence and other widely used methods such as oxygen consumption.
586 The relationship between oxygen consumption and resazurin fluorescence was positive, but
587 weak, demonstrating variability between the two measurements. This may reflect differences in
588 metabolic pathways measured (e.g., aerobic *versus* anaerobic processes), underscoring that
589 resazurin provides complementary, rather than redundant, information about metabolism
590 compared to oxygen consumption approaches. Variability in measurements between the two
591 approaches is likely driven by individual oyster day-to-day variation in responses as well as the
592 difference in mechanism of measurement of respiration (i.e., oxygen consumption) *versus*
593 resazurin (i.e., electron transport). Under temperature stress, *Crassostrea gigas* oysters exhibit
594 transitions from aerobic to anaerobic metabolism (Li et al., 2017), suggesting a need for
595 metabolic assays that capture multiple metabolic pathways and metabolic plasticity. Indeed,
596 resazurin assays may capture a wider range of metabolic responses than anaerobic metabolism
597 alone and can be adapted to capture metabolic activity in hypoxic conditions (Lavogina et al.,
598 2022).
599   One promising application of the resazurin assay is the rapid and high throughput
600 assessment of metabolic performance across environmental performance curves (e.g., thermal
601 performance curves). In our results, we observed peak metabolism at 32-36°C followed by
602 metabolic depression at higher temperatures in multiple experiments. Similar patterns have been
603 observed in previous studies in oysters. For example, observations of heart rate in *Ostrea edulis*
604 show peak cardiac activity 30-34°C followed by decreases near the lethal temperature of 34-
605 36°C (Eymann et al., 2020; Götze et al., 2025). Similarly, Tropical *Isognomon nucleus* oysters
606 exposed to increasing temperatures showed peak oxygen consumption at 39-41°C, depending on
607 thermal history, after which oxygen consumption declined (Giomi et al., 2016). Respiration rates
608 in *Crassostrea gigas* also display increased metabolic activity at 35°C compared to 20°C (Li et
609 al., 2017). Further, temperature sensitivity of metabolism, calculated as Q_{10}, ranged from <1 to
610 ~3.5 in our study, which are similar to observations in respiration of oysters in previous studies
611 (e.g., Q_{10} values of ~1.4 (Pan et al., 2021) and ~1.3-2.2 (Casas et al., 2018)). Our observation of

16

612 expected temperature-dependent metabolic activity using the resazurin assay demonstrates the
613 utility of this approach in characterizing thermal performance curves in oysters at high
614 replication. However, we did not conduct oxygen saturation measurements during trials and even
615 though plates were unsealed, allowing for oxygen diffusion into the resazurin solution, it is
616 possible that thermal stress effects were combined with oxygen stress. We recommend that trials
617 include oxygen saturation measurements to confirm oxygen saturation in solution.
618   Importantly, the resazurin assay detected metabolic signatures associated with survival
619 under acute stress conditions in our study. In acute stress tests, oysters that survived exhibited
620 stronger metabolic depression compared to those that died, indicating that metabolic
621 downregulation may serve as a protective mechanism. Metabolic depression may serve as a
622 strategy to conserve resources and allow for survival in the short term (Pörtner, 2008; Sokolova,
623 2021) but if prolonged, it becomes unsustainable with a risk for negative consequences
624 (Sokolova, 2013). In intertidal tropical oysters (*Isognomon nucleus*), metabolic depression may
625 play a role in energy conservation during periods of short-term heat stress (triggered at 37°C) at
626 low tides (Hui et al., 2020). Oysters also exhibit metabolic depression in response to
627 environmental stressors including high pCO_2/acidification conditions (Lannig et al., 2010; Le
628 Moullac et al., 2007) and starvation (García-Esquivel et al., 2002). We hypothesize that under
629 short term acute stress tests, oysters with greater capacity for metabolic depression may represent
630 more resilient stocks to acute stress events and our results suggest that short-term resazurin
631 assays could provide a rapid means of assessing stock resilience to thermal stress. Future
632 applications of acute stress tests using the resazurin assay must first consider local environmental
633 context and conduct preliminary testing to determine acute temperatures which may elicit
634 metabolic depression as a survival response, without complete mortality. It is important to note
635 that even after a behavioral determination of mortality there is still residual metabolic activity
636 detected using the resazurin assay due to ongoing metabolic activity in oyster tissues and
637 microbial communities. Therefore, any determinations of mortality made using resazurin assays
638 should be complemented and confirmed by behavioral and observational determinations of
639 survival. While responses will need to be contextualized for each system and species, resazurin
640 assays are a potential option for rapid stress-testing in hatcheries and monitoring programs. A
641 critical next step is to track and compare resazurin stress responses with field deployment of
642 oyster stocks to determine if lab-based stress tests provide realistic estimates of performance in
643 natural and aquaculture environments.
644   Genetic and family-level signatures in metabolic response highlight the potential
645 application of resazurin in selective breeding programs and to provide an index of stock
646 resilience. We observed variation in metabolism between oyster families, and in our case study
647 with *C. virginica*, metabolism under temperature stress varied depending on the selective
648 breeding phenotype. We utilized thermal stress testing as a rapid assay with resazurin to provide
649 an indicator of oyster metabolic response under stress. Although the oysters in this case study
650 were not bred specifically for thermal tolerance, the conserved energetic responses to stress
651 provide an opportunity to relate single stressor response to generalized performance.

652 Interestingly, metabolism was also correlated with predicted survival in low-salinity
653 environments, but only for the families that were selectively bred for high performance in this
654 environment. This finding may reflect shared physiological mechanisms between osmoregulation
655 and thermal stress, as both require substantial energetic investment in maintaining cellular
656 homeostasis (Sokolova, 2021, 2013) and the application of a thermal stress test assay of
657 metabolism may provide an indicator of stress response that relates to capacity for generalized
658 performance. In contrast, families bred for high-salinity environments are generally selected in
659 environments with stronger disease and pathogenic pressure (Bushek et al., 2012; Haskin and
660 Ford, 1982), where resilience may be shaped more by immune response (Frank-Lawale et al.,
661 2014) than metabolic or osmoregulatory functions. These differences highlight that selective
662 pressures targeting different stressors may favor distinct physiological pathways, and that
663 metabolic assays may be useful for detecting matches and mismatches between selectively bred
664 stocks and their potential environments. Further, we found that a small number of highest and
665 lowest performing families drove the differences in metabolism between high and low salinity
666 selected families, emphasizing the utility of the assay to detect high and low performers. These
667 results also demonstrate the need to sample a high sample size and sample across multiple
668 families to account for biological variability and allow for sufficient detection of high and low
669 performers. With further validation against observed field performance, resazurin assays could
670 provide hatchery managers with an accessible method to screen large numbers of individuals or
671 families for resilience traits.

672   Overall, our work demonstrates that resazurin metabolic assays provide a valid and
673 versatile approach for measuring metabolism in oysters and opens a wide range of opportunities
674 to investigate oyster physiological responses to a variety of environmental conditions. Resazurin
675 assays further offer a bridge between basic research and applied aquaculture, enabling scientists
676 to probe physiological mechanisms of stress tolerance while providing growers and managers
677 with an additional tool in aquaculture settings. Potential applications include screening for high-
678 performing or stress-resilient stocks, monitoring health and stress in hatcheries, and
679 characterizing family-level variation to inform breeding. It is critical that additional development
680 of resazurin assays includes thorough testing and consideration of local environmental context to
681 develop resilience or performance indices that are relevant for the stocks being tested. With
682 further refinement and validation in field settings, the resazurin assay could become an important
683 addition to the toolkit for oyster aquaculture and conservation under climate change.

Acknowledgements

Data Availability

All data and scripts are publicly available on GitHub for assay development (https://github.com/RobertsLab/resazurin-assay-development/releases/tag/v3.1) and the case study (https://github.com/RobertsLab/vims-resazurin/releases/tag/v1.0).

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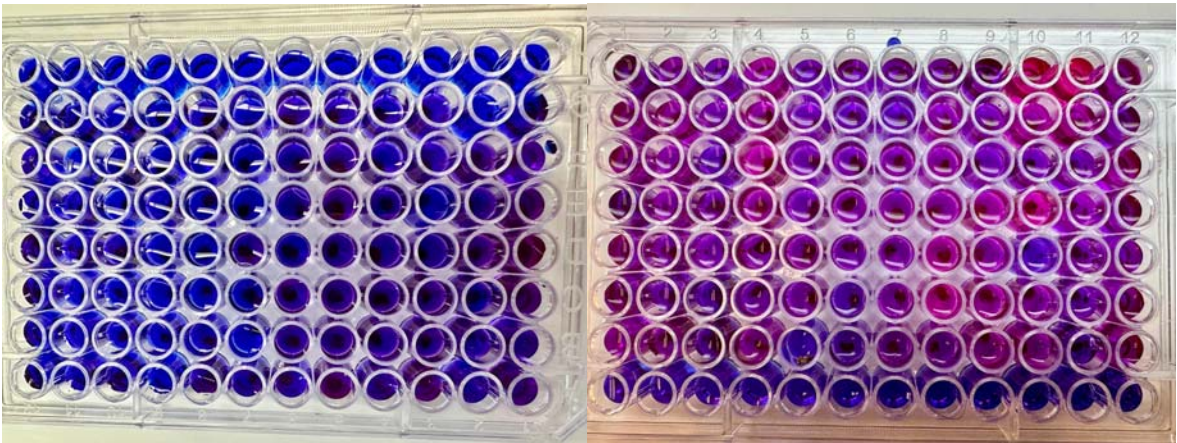
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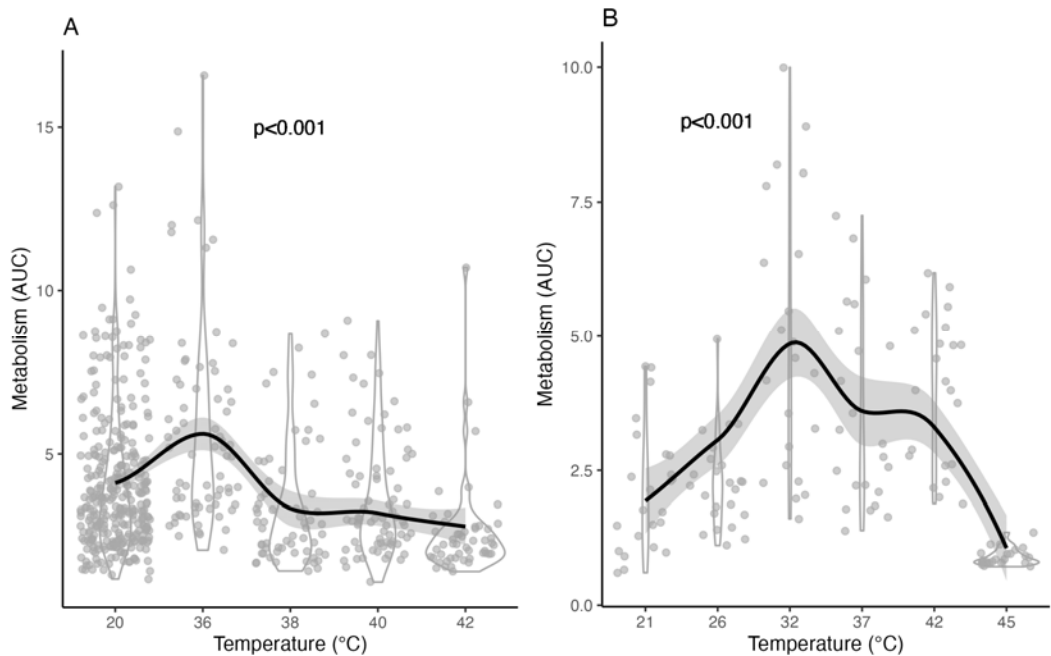
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Figures

846
847 **Fig 1.** Visual examples of resazurin assays conducted on oysters. Note that brighter pink colors
848 indicate higher metabolism as resazurin is reduced to resorufin. The left photograph shows an
849 example of oysters in 96-well plates early in an incubation (low fluorescence) and the right
850 photograph shows an example of oysters in 96-well plates late in an incubation (high
851 fluorescence). Note the bottom most row includes blank wells.

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853
854 **Fig 2.** (A) Metabolism (area under the curve; AUC) across temperatures (°C) for small seed (3-8
855 mm) analyzed at the University of Washington. Plot displays only seed that survived the trial.
856 (B) AUC across temperatures for seed (5-15 mm) analyzed at the Pacific Shellfish Research
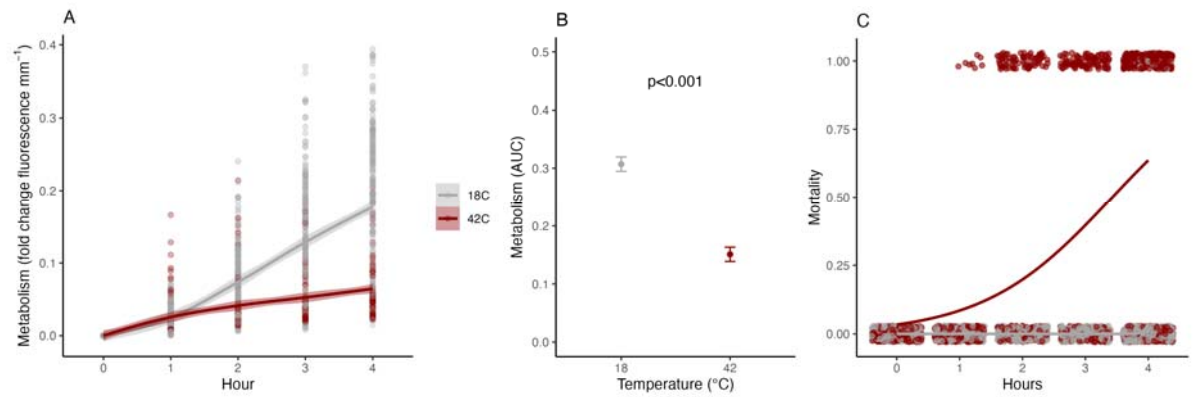857 Unit. In all plots, loess line displays response across temperatures.

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861 **Fig 3.** (A) Metabolism (fold change in fluorescence mm^{-1} over time) in oysters that survived the
862 4 h incubation at 18°C (gray) or 42°C (red). (B) Metabolism (area under the curve, AUC) at
863 18°C and 42°C. (C) Mortality during the 4 h incubation period. Lines indicate binomial mixed
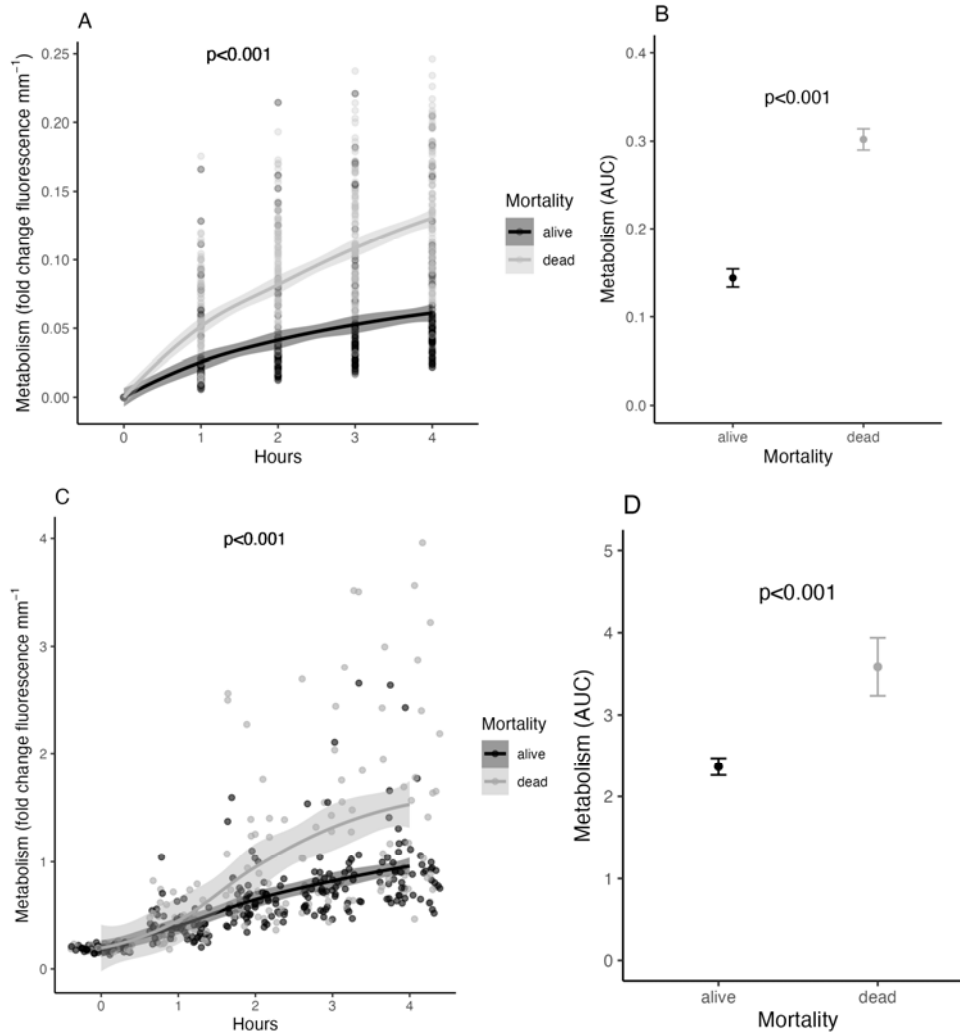864 effect model predications with a value of 1 indicating mortality and a value of 0 indicating a live
865 oyster.
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Fig 4. (A) Metabolism (fold change in fluorescence mm^{-1} over time) in oysters (15-35 mm) that
survived (black) or died (gray) by the end of the trial at 42°C. (B) Total metabolism (area under
the curve, AUC) in oysters that survived (black) or died (gray) by the end of the trial at 42°C. (C)
Metabolism (fold change in fluorescence mm^{-1} over time) in oyster seed (3-8 mm) that survived
(black) or died (gray) by the end of the trial at 42°C. (D) Total metabolism (area under the curve,
AUC) in oyster seed (3-8 mm) that survived (black) or died (gray) by the end of the trial at 42°C.
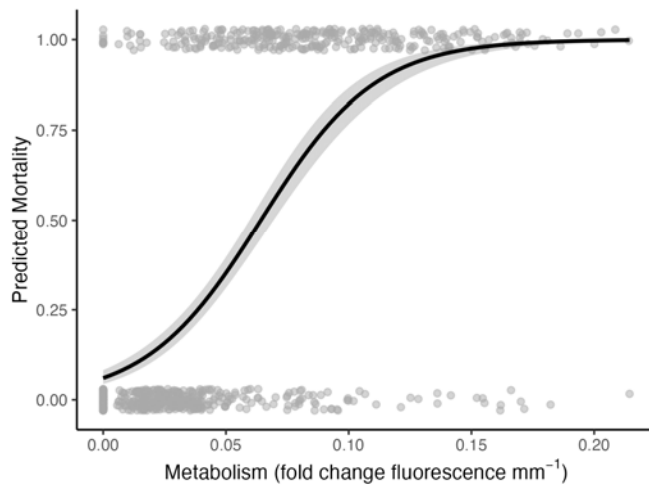
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Fig 5. Predicted mortality associated with metabolism (fold change in fluorescence mm^{-1}) measured using the resazurin assay at 42°C. Line indicates model prediction of the relationship between metabolism and mortality. Data points indicate observations in which 0 indicates a live oyster and 1 indicates a dead oyster by the end of the trial.
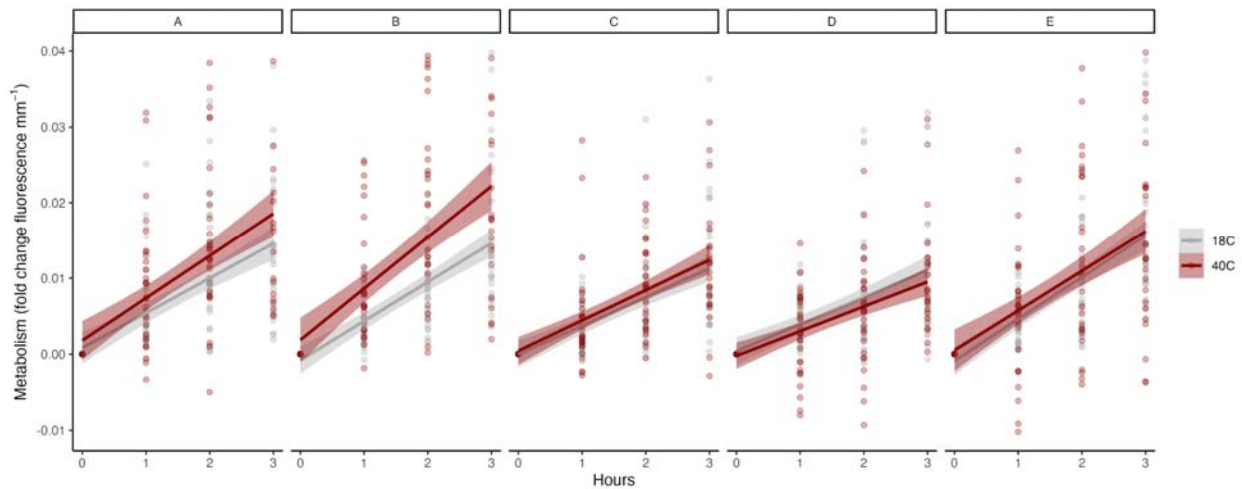

Fig 6. (A) Metabolism (fold change in fluorescence mm^{-1}) for each oyster family (families A-E) exposed to 18°C for 3 h (gray) followed by 40°C (red) for another 3 h. Lines indicate mean response in each temperature treatment.
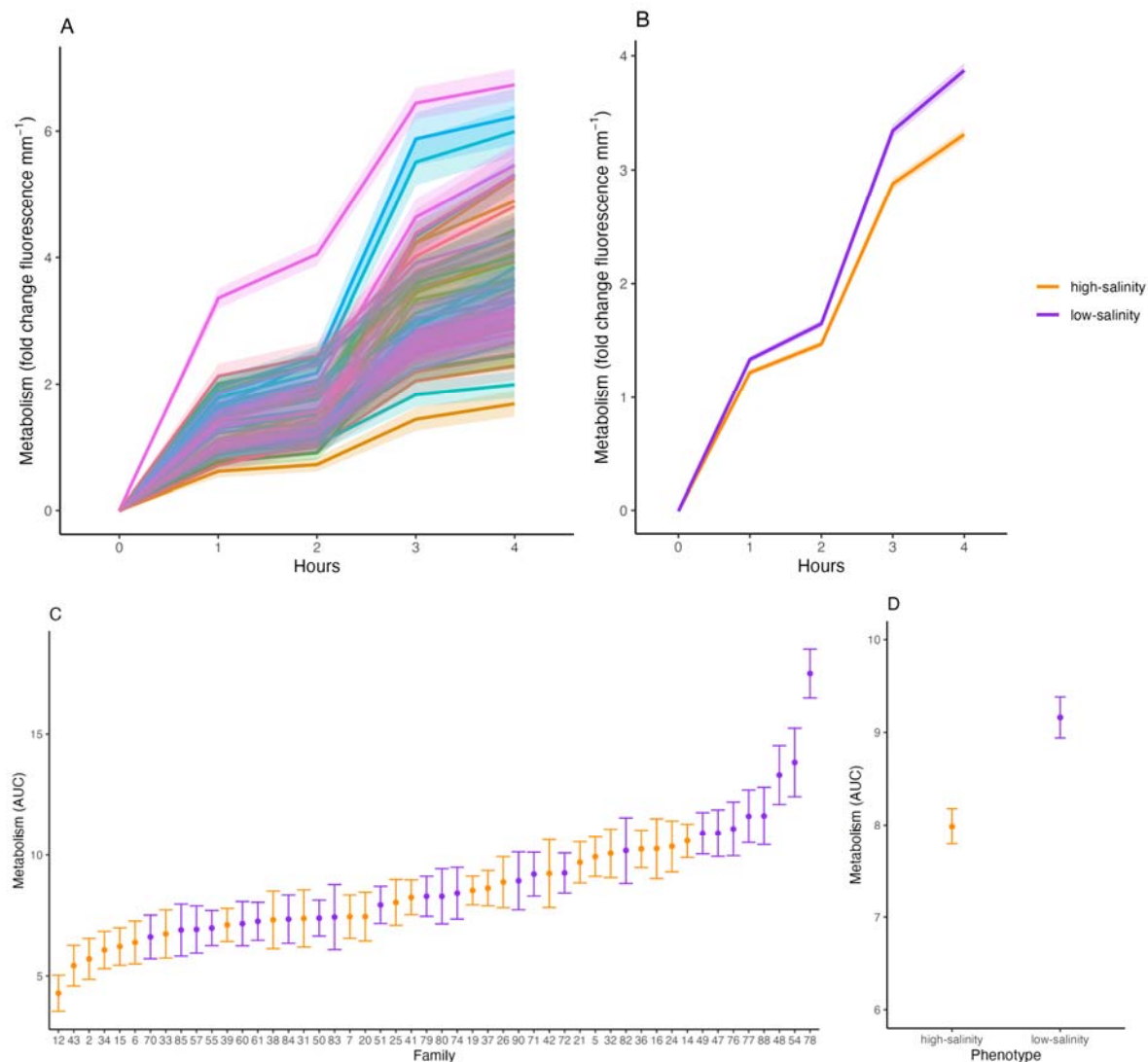
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913 **Fig 7.** (A) Metabolism (fold change in fluorescence mm^{-1}) of each family (represented by color)
914 during resazurin measurements. (B) Metabolism of each selected phenotype (i.e., selected for
915 high/moderate salinity or low salinity performance). A-B, lines indicate the modeled response
916 with error bars indicating 95% confidence intervals. (C) Total metabolism (area under the curve;
917 AUC) for each family and (D) AUC summarized by selected phenotype. In B-D, families
918 selected for high/moderate salinity environments are in orange and those selected for low salinity
919 environments are in purple.

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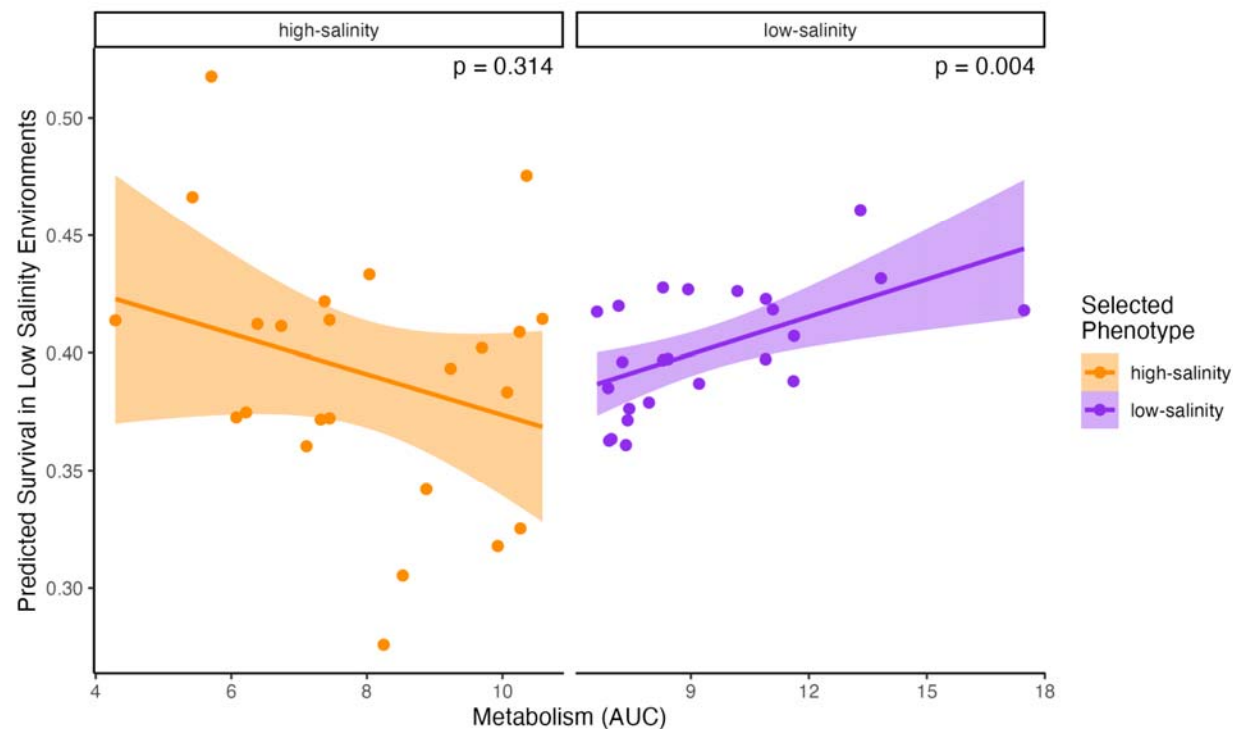922
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Fig 8. Correlation between total metabolic activity (AUC) and predicted survival (% gain) in low
salinity environments. Correlations conducted separately for families selected for performance in
low salinity (purple) and high/moderate salinity (orange) environments.

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