Natural History and Conservation of Freshwater Mussels (Order: Unionida) of Wisconsin

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

(Freshwater and Marine Sciences)

At the

UNIVERSITY OF WISCONSIN-MADISON

2022

Date of final oral examination: 4 May 2022

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45 *"There is no question that all of the better mussel streams are capable of supporting*

46 *mussel resources many times as abundant as they do now, for they did so a score or less*

47 *of years ago." – Coker 1917-18*

48

49

50

51   Naiads were goddesses of lakes, rivers, and springs in ancient Greece. Protectors of freshwaters,

52 known for their beauty, the gifts they provided, and their long, though not everlasting lives. For anyone

53 who has ever had the opportunity to cool their face in a stream and had the good fortune of a naiad

54 appear to them, peering out from between two, small, unassuming "rocks", understands the powers

55 they possess.

56   And although their numbers are fewer today than they were yesterday, their powers to protect

57 their homes remains. For I have not met a person who has experienced the presence of a naiad and not

58 felt a sense of wonder and connection, and a deep desire to share the story of the naiads. A story that

59 has called a growing number of people to dedicate their lives to protecting our freshwaters. May the

60 story of the naiads reach enough people, that they can return to every lake, river, and spring they once

61 called home.

62 **Thesis Introduction:**

63   North America is home to approximately 300 species of freshwater mussels—the highest

64 diversity found anywhere the world. They inhabit a wide range of freshwater ecosystems from small,

65 headwater streams to large rivers, lakes, and ponds across which they provide numerous ecosystem

66 services (Vaughn and Hakenkamp 2001). Over the past 200 years, threats from a rapidly growing

67 human-dominated landscape have resulted in immeasurable losses to our freshwater mussels (Haag

68 2012; Ricciardi and Rasmussen 1999).

69   The full extent of the damage to freshwater mussel populations in North America is difficult to

70 assess. But we are far from the world described in the accounts of early naturalists where "…one could

71 not step for a mile without treading on a living mussel." (Simpson 1899) or where finding streams devoid

72 of mussels was "…somewhat of a new experience, for we had grown so used to finding shells in every

73 creek…At times it almost got monotonous" (Remington and Clench 1925).

74   The first alarms for concern of their conservation came over 100 years ago, as entire mussel

75 communities were seemingly depleted from whole rivers due to overexploitation from the pearl and

76 button industries (Kunz 1898; Coker 1919). These alarms prompted the first concerted efforts to study

77 their biology and inventory their numbers and distributions in the hopes to manage for stable

78 populations (Pritchard 2001). In the years following, threats continued to emerge—most notably the

79 widespread impoundment of North American rivers (USACOE 2020) and continued decreasing water

80 quality, the combination of which resulted in loss of habitat, fish hosts, and fragmentation of

81 populations (Vaughn and Taylor 1999; Strayer and Malcom 2012; Hamstead et al. 2019; Sousa et al.

82 2020). The total effects of which can still be seen in most rivers today (Anthony and Downing 2001; Haag

83 2012).

84   The latter half of the 20th century in the United States saw federal protections for numerous

85 mussel species enacted under the Endangered Species Act, passage of the Clean Water Act, and a

86   slowing of new dam construction (USACOE 2020). Despite these needed changes, mussel communities

87   continued to decline (Bogan 1993). The combination effects of a century of stressors may have been too

88   high a barrier for many mussels to recover from.

89   Since the 1970s a growing community of mussel conservationists have helped raise awareness

90   for these often-overlooked animals. A renewed effort to study their ecology and reproduction, identify

91   vulnerable species and important habitats, develop propagation techniques, and assess the impacts of

92   ongoing and potential future risks have greatly improved our ability to protect what remains of their

93   populations (Haag and Williams 2014; FMCS 2016; Ferreira-Rodríguez et al. 2019). But threats to their

94   persistence have not ceased. Mussels are experiencing a new wave of threats in a rapidly changing

95   climate (Inoue and Berg 2017; Baldan et al. 2021), invasive species (Ricciardi et al. 1996; Strayer and

96   Malcom 2007), and inexplicable mass-mortality events (Cummings et al. 1988; Wengström et al. 2019;

97   Vaughn 2022). Without conservation intervention, North America may lose up to half of its species in

98   the next 100 years (Ricciardi and Rasmussen 1999).

99   Effective conservation of our freshwater mussels will require a deeper understanding of their

100   ecology: in how they respond to a changing world, especially to novel and growing threats, as well as a

101   close monitoring of current populations (FMCS 2016; Ferreira-Rodríguez et al. 2019). It is to this end that

102   my dissertation is aimed. In the following three chapters I explore aspects of freshwater mussel ecology

103   in relation to environmental controls on growth, physiological response to threats from an invasive

104   species, and report on the current status and recent changes of the freshwater mussel communities of

105   Wisconsin.

106   Chapters one and two focus particularly on mussel populations in lakes as they respond to

107   natural changes in their environment. These are contributions to a very small body of research that

108   focus on lake-dwelling populations (see Strayer et al. 1981; Cyr 2008; 2020). The study of mussels in

109   lakes and lentic systems is particularly important in that these are the preferred habitats of the invasive

110   zebra mussels (*Dreissena polymorpha*) (Mellina and Rasmussen 1994; Allen and Ramcharan 2001) which

111   are spreading throughout Wisconsin as well as much of North America (Benson et al. 2021).

112   Specifically, in chapter one, I examine how growth of the freshwater mussel *Lampsilis*

113   *siliquoidea* has responded to long-term changes in an oligotrophic lake. I compare ~30 years of

114   schlerochronology records from mussel shells to long-term ecosystem-wide data. I show that annual

115   mussel growth in oligotrophic lakes can be dynamic, highly variable between individuals, and associated

116   with landscape-level environmental changes unique to lake ecosystems.

117   In chapter 2, I document physiological responses of *Lampsilis siliquoidea* during the initial stages

118   of an invasion by zebra mussels in a eutrophic lake. I use the emerging tool of metabolomics to assess

119   how metabolic processes in *L. siliquoidea* are affected by zebra mussels. I show that *L. siliquoidea* display

120   signs of starvation with increased levels of infestation by zebra mussels. I demonstrate that

121   metabolomics can be a useful tool when applied in natural environments by identifying processes

122   through which zebra mussels harm native mussel communities.

123   In chapter 3, I report on the current status of freshwater mussel communities in Wisconsin and

124   how they have changed in the past 50 years. I show that losses have occurred for the majority of species

125   and that these losses are geographically distributed across the state. I show that these losses may

126   potentially be offset by gains in other locations, but warn that that is likely an overly optimistic

127   interpretation. I describe the level of uncertainties in species assessments and identify watersheds that

128   should be of highest priority for future surveys.

129   Freshwater mussels seem almost uniquely poorly suited for survival in a human-dominated

130   world. Their conservation will require immense efforts across academia, government agencies, non-

131   governmental organizations, community engagement, and individuals[1]. I hope that this work in one way

132   or another is helpful to this cause.

133

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[1] Nearly 2,000 surveys were conducted by one person, Harold A. Mathiak who took it upon himself to conduct the first Wisconsin-statewide mussel surveys nearly 50 years ago

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210

211   **Chapter 1**

212   **Environmental controls on long-term growth of freshwater mussels in an oligotrophic lake**

213

216

217   **Abstract:**

218   Freshwater mussels are one of the most endangered groups of animals and live in some of the

219   fastest changing ecosystems in the world. Unfortunately, very little is known about how their life history

220   and ecology are influenced by their environment, especially for lake-dwelling populations. In this study,

221   we paired ~30 y of extensive environmental data to lifetime and annual growth rates of a population of

222   Fatmucket mussels (*Lampsilis siliquoidea*) living in a cool-water oligotrophic lake in northern Wisconsin,

223   USA. This population displayed one of the slowest growth rates and longest lifespans within its

224   phylogenetic tribe (Lampsilini). Growth rates were highly variable between individuals and, in contrast

225   to studies in other systems, not related to temperature, growing season, or any indicators of primary

226   production. However, growth rates were positively correlated with changes in lake level. We

227   hypothesize that mussel growth in this system is linked to landscape-level environmental conditions and

228   allochthonous resource limitation.

229   **Introduction:**

230   Freshwater mussels (Order: Unionida) are one of the most imperiled groups of organisms on

231   Earth (Ricciardi and Rasmussen 1999, Lopes-Lima et al. 2017) and inhabit some of the fastest changing

232   ecosystems worldwide (Vörösmarty et al. 2010, Carpenter et al. 2011). It is important to better

233   understand the ecology and life history of freshwater mussels because these aspects of mussel biology

234   affect how mussels will respond to future global environmental changes. Growth rate is a fundamental

235   life-history attribute that dictates a mussel's survival and reproductive success; however, the

236   environmental conditions that control growth are understudied (Haag 2012). Understanding how mussel

237   growth is controlled by divergent environmental conditions may be of significance to mussel

238   conservation in our changing world.

239   Mussels maintain long-term records of lifetime growth archived in their shells, in some cases

240   providing 30 to 300 y of annualized growth information (Schöne et al. 2005, Helama and Valovirta 2008,

241   Rypel et al. 2008). In the shells of most species, a conspicuous and often narrow dark band appears in

242   the shell record during periods of low to no growth. In temperate climates, these dark bands coincide

243   with growth cessation during winters, which can allow for exact dating of historical mussel growth

244   (Helama et al. 2006, Haag and Commens-Carson 2008, Rypel et al. 2008, Schöne 2013). Interpretation of

245   growth rings (annuli) can be used to estimate ages of individual mussels as well as describe long-term

246   variations in annual growth rates. When mussels are collected from closely monitored ecosystems, past

247   growth records can be compared to environmental records to explore how mussel growth is related to

248   their environment (i.e., sclerochronology; Schöne et al. 2004, 2005, Black et al. 2010).

249   Linking mussel growth to environmental conditions using sclerochronology has been reasonably

250   well documented in both marine and freshwater systems. In marine systems, bivalve growth has been

251   shown to correlate strongly with diverse environmental controls, such as temperature (Archambault et

252   al. 1999, Schöne et al. 2005), primary productivity (Smaal and van Stralen 1990), chlorophyll *a* (Chl *a*)

253 (Page and Hubbard 1987, Archambault et al. 1999), particulate organic C (Page and Hubbard 1987), and

254 zooplankton biomass (Wanamaker et al. 2009). In freshwaters, most research on mussels has been

255 conducted in fluvial systems (but see Kendall et al. 2010) where growth is strongly associated with

256 physical variables, such as discharge or temperature (Schöne et al. 2003, 2004, Rypel et al. 2009, Black

257 et al. 2010, Dycus et al. 2015,) and in which biological or chemical environmental variables have not

258 been detected as controls on growth.

259 Freshwater mussels are understudied in lake ecosystems, with the notable exception of a large

260 number of studies on the ecological effects and autecology of invasive species, such as Zebra (*Dreissena*

261 *polymorpha* Pallas, 1771) and Quagga (*Dreissena rostriformis bugensis* Andrusov, 1897) mussels (reviews

262 by Higgins and Vander Zanden 2010, Karatayev et al. 2015). Native lake mussels may have received less

263 research attention because of reduced species diversity and abundances compared to those found in

264 fluvial ecosystems. However, lake-dwelling mussels pose an interesting opportunity for studying growth

265 dynamics.

266 Flow is often the primary determinant of annual growth of mussels in fluvial ecosystems (Rypel

267 et al. 2008, Rypel 2009, Black et al. 2010, Dycus et al. 2015), which leads us to ask: How do drivers of

268 freshwater mussel growth change when there is little to no flow, such as in a lake? One might expect

269 that lakes are particularly challenging environments for mussels (these challenges may be the reason for

270 lower abundances and lack of species diversity found in lakes). Unique lake ecosystem characteristics

271 like the lack of flowing water and seasonal stratification, which controls important environmental

272 conditions such as temperature, dissolved oxygen, and plankton assemblages, may greatly hinder the

273 food-capturing abilities of filter-feeding mussels. The environmental characteristics of lake

274 environments lead us to ask a 2nd question: Is the growth of lake-dwelling mussels controlled by food

275 availability or an environmental condition that is unique to or more pronounced in lake ecosystems?

276 Griffiths and Cyr (2006) found that lake-dwelling Eastern Elliptio (*Elliptio complanata* Lightfoot, 1786)

277   had higher growth rates in upwind sites compared to downwind sites despite lower chlorophyll

278   concentrations and colder water temperatures at upwind sites. The unique environmental conditions

279   mussels experience in lakes and unexpected growth responses in systems in which they have been

280   studied, like the findings of Griffiths and Cyr (2006), should inspire greater attention to lake-dwelling

281   mussels in our attempts to better understand how these animals are influenced by their environment.

282   Here, we describe lifetime growth dynamics of a lake-dwelling population of Fatmucket mussels

283   (*Lampsilis siliquoidea* Barnes, 1823). Our specific goals were to: 1) develop a von Bertalanffy growth

284   curve to describe lifetime growth trajectories for this population and place the growth of *L. siliquoidea* in

285   context to that of related species; 2) construct a chronology describing long-term growth variations of

286   mussels within the lake; and 3) describe any relationships found between annual mussel growth and

287   long-term environmental conditions (abiotic or biotic) in the lake. We hypothesized that lake

288   ecosystems (especially cool, soft-water, oligotrophic environments like the one in this study) pose

289   unique environmental challenges that strongly limit mussel somatic growth. We predicted that there

290   would be variability in growth rates among years that is synchronous among individuals in the

291   population and that this variability would correlate with at least 1 environmental driver consistent with

292   the potential difficulties of living in a lake ecosystem.

293

294   **Methods:**

295   *Site description*

296   Trout Lake is located in a temperate climate in the Northern Highland Lake District of Wisconsin,

297   USA. It is a cool-water, dimictic, oligotrophic lake that freezes annually and has an area of 1608 ha, a

298   mean depth of 14.9 m, and a maximum depth of 35.7 m. It has an average summer Chl *a* concentration

299   of 2.4 µg/L and a long-term average calcium concentration of 12.6 mg/L (Magnuson et al. 2019a, b).

300   Sediment characteristics within the lake and at the sample site are primarily sand with some cobble; the

301 sample site was characterized as having very shallow sloping bathymetry, and there were no

302 macrophytes present. As one of the pioneering sites of the Long-term Ecological Research program,

303 Trout Lake has been continuously studied for physical, chemical, and biological variables since 1981.

304 *Lampsilis siliquoidea* is the most abundant unionid species found in the lake. Plain Pocketbook (*Lampsilis*

305 *cardium* Rafinesque, 1820) and Giant Floater (*Pyganodon grandis* Say, 1829) are also present but fewer

306 in number (VLB, personal observation).

307

308 *Sample collection and processing*

309   From Trout Lake, we collected individual mussels from depths of 2 to 3 m within the same

310 mussel bed (~46°01'00"N, 89°40'35"W) during the summers of 2014 and 2017. We chose this site based

311 on qualitative pilot surveys indicating that this site had noticeably higher mussel density than any other

312 known locations. We collected only live mussels of 1 species (*L. siliquoidea*), and we sacrificed them

313 immediately after collection in both years. We collected a total of ~75 mussels but used only a subset of

314 these in our analyses (see below for additional explanation). Of the mussels that we used in the

315 analyses, 7 were collected in 2014 (4 female, 3 male) and 19 in 2017 (11 female, 8 male). Sex was

316 determined by shell morphology because sexual dimorphism is readily apparent in *L. siliquoidea*. We

317 generally focused on collecting larger (and presumably older) individuals to develop the longest

318 chronologies possible. However, large size might also result from faster growth rates and lead to a

319 potential over-estimate of average growth rates of individuals in the population.

320   In the lab, we measured the length and width of each shell. We used a rock-cutting saw to cut 1

321 valve of each shell ~halfway between the major and minor axes of growth from the umbo to the shell

322 margin (the valve used varied by which valve could be best gripped by the saw's vice in a proper

323 orientation for the cut). We smoothed the mussel half-shells with 14-μm grit suspended in water until

324   polished, then adhered each of them to a transparent glass slide with epoxy. After the epoxy set, we cut

325   the shells to ~1-mm thin sections and again polished them.

326   Two independent observers interpreted shell thin sections for annuli counts (Fig. 1). Annuli were

327   identified and measured at the boundary of the nacre and prismatic layer for consistency in

328   identification of annuli between observers and between shells—annuli at this location are commonly

329   referred to as internal annuli in contrast to lines observed on the external shell. Discrepancies between

330   observers were compared until both observers agreed on the presence of each annulus. There was a

331   low threshold for excluding thin sections from analysis based upon readability; we included in the final

332   analysis only shells for which both observers self-reported having a high level of certainty in accurate

333   dating of the internal annuli to their associated years. Of the ~75 individuals initially collected from the

334   lake, 61 were cut into shell thin sections, and 35 of these were omitted because 1 or both observers

335   reported less than a high level of confidence in annuli detection. Consequently, we used 26 shell

336   sections in this study's analyses. To estimate annual growth, we measured the distance between annuli

337   to the nearest 0.01 mm by using a Leica S8AP0 microscope (Leica, Wetzlar, Germany) with the Leica

338   Application Suite software (version 3.7.0; Leica Microsystems, Buffalo Grove, Illinois).

339

340   *Chronology validation*

341   We quality checked each chronology of annual growth increments with COFECHA (Fritts 1976,

342   Holmes 1983), a software program that uses crossdating to check appropriate dating of chronologies.

343   Each chronology was crossdated in COFECHA following the methods of Rypel et al. (2008). Briefly, each

344   annual growth increment chronology was 1st detrended by using an exponential curve and then

345   smoothed with a cubic spline that retained 50% of variability over 32-y periods to remove ontogenetic

346   and low frequency patterns in the chronology prior to crossdating (Fritts 1976). In the crossdating

347   process, all the chronologies were averaged together to create a master chronology against which each

348   individual chronology was compared in a leave-1-out design as a first assessment for an appropriately

349   dated chronology (Fritts 1976). Each chronology was also cut to sequential series of 8 y in length and

350   lagged –3 to +3 y, and its correlation to the master chronology was assessed. We considered

351   chronologies to have been validated if their unlagged position had the highest correlation with the

352   master chronology. If a chronology exhibited a substantially higher correlation when lagged, both

353   reviewers reexamined the shell, and it was only adjusted to the lagged position if both reviewers agreed.

354   Otherwise, it was included in its original position. We assessed variation by sex in interannual growth

355   rates with a *t*-test assessing whether the standard deviation differed between male and female

356   chronologies. We checked assumptions for normality (graphically with histograms and QQplots) and

357   equal variance (using Levene's test) for all *t*-tests and analysis of variance (ANOVA) models (below). We

358   conducted all statistical analyses in the programming language R (version 3.6.3; R Project for Statistical

359   Computing, Vienna, Austria).

360

361   *Master chronology construction*

362   We imported validated chronologies into ARSTAN (version 44h3; Cook and Holmes 1984), a

363   software program designed for detrending individual chronologies and constructing a master

364   chronology for time series analysis. Each chronology was fit with a negative exponential model to create

365   a model of predicted growth for a mussel for any year, and deviations from this model represent above-

366   or below-expected growth for that year. We chose the "stiffer" fit of a negative exponential model

367   compared to the spline used in the chronology validation process to retain as much low-frequency and

368   climate signal as possible (Fritts 1976, Rypel et al. 2009). Each model fit was confirmed by eye, and none

369   contained any obvious bias in the residuals of the model fit.

370   We calculated standardized growth indices (SGIs) by dividing the residuals by the model

371   predictions. This approach is a standard process in sclerochronology to remove age-related growth

372   dynamics from chronologies (Fritts 1976). SGIs >1 represent above-expected growth for that year,

373   whereas SGIs <1 represent below-expected growth. A master chronology was created from the 'RESID'

374   (residual) output from ARSTAN using methods described by Cook and Holmes (1984). This is a common

375   process that first whitens out (i.e., diminishes) any autocorrelation in individual chronologies by making

376   the time series behave more like white noise, then calculates a robust biweight mean that is designed to

377   enhance common signals among individual chronologies (Kadafar 1983, Cook and Holmes 1984).

378   We characterized lifetime growth trajectories by sex as well as for the general population with the von

379   Bertalanffy equation:

380    $$L_t = L_\infty\left(1 - e^{K(t - t_0)}\right) \quad \text{(Eq. 1)},$$

381   where L_t is the length (in mm) at time t (age in years), L_∞ is the mean maximum length for the

382   population, K is a growth constant that describes how quickly an individual approaches L_∞, and t_0 is the

383   time at which length = 0. We used the R packages *FSA* (version 0.8.32; Ogle et al. 2020) and *nlstools*

384   (version 1.0.2; Baty et al. 2015) for the von Bertalanffy analyses using the default approach (2nd-degree

385   polynomial) to create starting values for t_0. For the model, we calculated L_t from a ratio of the length of

386   the thin section to the length of the major axis of the valve. The von Bertalanffy model was fit to males

387   and females separately. If t_0, L_∞, or K in the von Bertalanffy model did not differ between sexes (by

388   testing for a difference in means with a 2-tailed t-test), the model was then fit to all individuals

389   combined. We assessed differences in von Bertalanffy estimates by sex via a 1-way ANOVA testing

390   whether parameter estimates differed between a model where all estimates were allowed to vary by

391   sex and a model where the parameter of interest did not vary by sex. We assessed how the von

392   Bertalanffy parameter estimates for *L. siliquoidea* related to those previously reported for the Lampsilini

393   tribe by Haag and Rypel (2011) by using a simple rank order.

394

395

396   *Environmental data filtering and analysis*

397   To assess the relationship between mussel growth and environmental characteristics, we

398   examined a number of environmental variables, including lake-scale variables as well as broader

399   macroclimate variables. Environmental variables used in our analysis include those that have been

400   shown to influence mussel growth in other systems, such as indicators of primary and secondary

401   production, water temperature, and growing season length (Rypel et al. 2009, Wanamaker et al. 2009,

402   Black et al. 2010), as well as other variables we expected may influence mussel growth directly or

403   indirectly (Table 1). We also evaluated variables for filtering environmental data based on time periods

404   (e.g., ice-off, spring mixing), which we did as an informal way of conducting a sensitivity test (e.g., if data

405   from spring mixing were included, would that drastically change our findings?). Filtering environmental

406   data to different time windows did not change our findings (likely because the numerous measurements

407   collected during the summer stratified period outweighed the effects of the inclusion of a few spring

408   samples), so these time period variables were not included in the final analysis. The Trout Lake

409   environmental data used in our analysis have been collected and curated by the North Temperate Lakes

410   site of the Long-term Ecological Research network for ~40 y (Magnuson et al. 2019b, c, 2020a, b, c, d).

411   All limnological measurements were collected at a centrally located buoy ~500 m from our mussel

412   collection location. Data on macroclimate indices, specifically the North-Atlantic Oscillation (NAO) and

413   Pacific Decadal Oscillation (PDO), were provided by the National Centers for Environmental Information

414   managed by the National Oceanic and Atmoshperic Administration

415   (www.ncdc.noaa.gov/teleconnections). NAO_{winter} and PDO_{winter} are the mean indices for the North

416   Atlantic and the Pacific Decadal Oscillations, respectively, during the immediately preceding winter

417   months (these macroclimate indices primarily reflect winter precipitation in the study region).

418   Precipitation data were downloaded from a nearby weather station in Minocqua, Wisconsin

419   (GHCND:USC00475516).

420   We filtered environmental data to best reflect the conditions most likely experienced by the mussels

421   during their growing season. Unless otherwise specified, we summarized all environmental data from

422   the lake by the mean value from data filtered to include only data from the epilimnion during the

423   summer stratified period to highlight the environmental conditions most likely to influence mussel

424   growth (Amyot and Downing 1997, Hallmann et al. 2009). We calculated only a few variables differently:

425   lake level$_\Delta$ is the difference (in m) from the previous mean summer lake level, total precipitation is the

426   cumulative water equivalent amount of precipitation that was recorded in the water year (beginning 1

427   October of previous calendar year), SRP$_{spring}$ is the mean dissolved reactive phosphorus during the

428   immediately preceding spring mixing period (a common approach to estimating summertime

429   productivity in P-limited lakes), and degree days were calculated as the area under the curve of mean

430   epilimnetic temperature beginning immediately after ice-out and ending at the end of summer

431   stratification to capture as much of the potential growing season as possible (Amyot and Downing

432   1997). All data have a minimum temporal resolution of 2 weeks except NAO$_{winter}$ and PDO$_{winter}$, which

433   have a monthly temporal resolution, calcium, water color, specific UV absorbance at 254 nm (SUVA$_{254}$),

434   slope ratio, and linear slope 275–295, which are sampled once during summer months. We chose to

435   include multiple variables associated with dissolved organic carbon (DOC) quality (SUVA$_{254}$, slope ratio,

436   linear slope 275–295) in our best attempts to characterize potentially meaningful DOC estimates (Jane

437   et al. 2017). We summarized all continuous variables to a mean value. We calculated the Pearson

438   correlation coefficient of each environmental variable to the standardized mussel growth indices. These

439   correlation coefficients are included in Table 1 strictly for thoroughness in reporting. We also calculated

440   Pearson *r* for correlations of DOC and water color with lake level$_\Delta$ because we were interested in

441   describing how changes in lake level may have been associated with allochthonous inputs.

442   We chose an exhaustive model selection approach to identify environmental variables likely to influence

443   mussel growth (R package *MuMIn*, version 1.43.17; Bartoń 2020). Prior to model selection, we

444   standardized all independent variables ($\bar{x} = 0$, standard deviation [SD] = 0.5) to more easily compare

445   their relative importance. Exhaustive model selection is similar to a stepwise model selection except,

446   instead of comparing model fitness by eliminating or adding 1 variable at a time, all possible

447   combinations of variables are examined and compared. This approach means that exhaustive model

448   selection is robust to collinearity between predictor variables because it independently assesses all

449   variable combinations and does not drop potentially important variables as would be possible in a

450   stepwise model selection approach. We used Bayesian Information Criterion (BIC) to assess the relative

451   model fit and to discourage the selection of complex models (BIC more heavily penalizes complex

452   models than does Akaike Information Criterion). Because the top model did not substantially

453   outperform the next best fitting models (ΔBIC < 2), we chose a model averaging approach that allowed

454   us to estimate average effect size for each variable across the highest performing models. This approach

455   also allowed us to report how often a variable was included in the set of highest performing models. If a

456   variable is included in more of the highest performing models, it is more likely to have a causal

457   relationship. Only models with a BIC score within 2 units of the highest performing model's BIC score

458   were considered top performing models and included in the model averaging step. We averaged model

459   estimates, or mean effect sizes, by using a conditional average approach that calculates the average

460   effect size for each parameter across all of the top performing models (within 2 units of the lowest BIC

461   score) in which that parameter is present. Variables that were identified as potentially being important,

462   based on having been included in the top 2 performing models, showed no indication of collinearity

463   (assessed via variance inflation factors). Each potentially important variable was used in independent

464   simple linear regression models to test whether the parameterized model outperformed the null model

465   according to a least squares assessment. The use of linear regression also allowed us to report the

466   relationships of the environmental variables and mussel growth in an easier to interpret fashion. We

467   ultimately identified 2 variables of potential importance (lake level$_\Delta$ and SRP$_{spring}$), and we created 2

468   independent linear regression models, 1 containing lake level$_\Delta$ and the other containing SRP$_{spring}$, to

469   assess the independent effects of these variables on standardized mussel growth indices. We used a

470   generalized least squares approach to asses the linear model of SRP$_{spring}$ on growth to account for

471   heteroskedasticity in the model.

472

473   **Results:**

474   Individual growth chronologies ranged from 14 to 32 y (n = 26), spanning 1985 to 2016, and had

475   a mean length of 20 y. Within the Lampsilini tribe of unionid mussels, this population of *L. siliquoidea*

476   displayed one of the lowest recorded growth rates as described by K in the von Bertalanffy model

477   (population K = 0.119, 95% confidence interval: 0.11, 0.13) (Table S1, Fig. 2A, B). Our population-wide t_0

478   estimate was –0.494 y (95% confidence interval: –0.81, –0.18 y). Based on 2-tailed t-tests, there was no

479   difference in K or t_0 between males and females (K: p = 0.57, t_0: p = 0.92). Males did reach a larger L_∞

480   (73.4 mm, 95% confidence interval: 70.6, 76.2 mm) than females (66.4 mm, 95% confidence interval:

481   63.7, 69.3 mm) (p = 0.02), which is not surprising given the sexual dimorphism of *L. siliquoidea* (Fig. 2C).

482   There was a moderate level of synchrony in growth within the population (series intercorrelation: 0.390)

483   (Fig. 3). However, there was substantial variability in the SGI between the different chronologies of

484   individual mussels across all years (mean of the SD: 0.39). There was no difference in interannual

485   variability by sex (p = 0.65).

486   Exhaustive model selection identified only 1 additional model within 2 BIC units of the lowest

487   BIC score (highest performing model) (Table 2). Lake level$_\Delta$ was included in both models, whereas

488   springtime dissolved reactive phosphorus (SRP$_{spring}$) was included in 1. Lake level$_\Delta$ was suggested to be

489   positively correlated with growth, whereas SRP$_{spring}$ was suggested to be negatively related to growth

490   (Table 2). No other environmental variables were identified through this method as likely controls of

491   growth.

492   Based on a linear regression approach, lake level$_\Delta$ explained a moderate amount of the total

493 variance in growth ($R = 0.57$) and was likely positively related to growth ($p < 0.01$) (Table S2, Fig. 4A).

494 SRP$_{spring}$ explained relatively little of the total variance in growth ($R = -0.36$, $p = 0.10$) (Table S3, Fig. 4B),

495 and an ANOVA test indicated that a model containing both lake level$_\Delta$ and SRP$_{spring}$ was only marginally

496 different from a model containing only lake level$_\Delta$ ($p = 0.04$; Table S4). This finding suggests that, if

497 SRP$_{spring}$ is related to mussel growth, it is likely less important than lake level. We assessed whether we

498 could detect a relationship between lake level$_\Delta$ and indicators of allochthonous C inputs but found no

499 correlation between lake level$_\Delta$ and water color (Pearson $r = 0.08$) or lake level$_\Delta$ and DOC (Pearson $r =$

500 0.16).

501   We attempted to describe the relationship of each individual mussel chronology with lake level$_\Delta$

502 and SRP$_{spring}$ to see whether the relationship of the population growth dynamics with these

503 environmental conditions was characteristic of a general population relationship or just the result of a

504 strong relationship for only a few individuals. Standardized effect sizes (standardized estimates of the

505 strength of the relationship between the environmental variable and mussel growth) were all positive

506 for lake level$_\Delta$ and generally, but not all, negative for SRP$_{spring}$ (Fig. 5).

507

508

509 **Discussion:**

510   In this study, we were interested in describing lifetime growth dynamics of a lake-dwelling

511 population of *L. siliquoidea*. We also wanted to construct a chronology describing long-term growth

512 variations of mussels within the lake and describe any relationships found between annual mussel

513 growth and long-term environmental conditions in the lake. In this system, we were able to detect a

514 relationship between growth and lake level, but other environmental variables (e.g., temperature,

515 growing season) did not appear to be important controls on growth. However, this study used a limited

516   sample size to describe growth characteristics of *L. siliquoidea* and their relationship to environmental

517   conditions in 1 lake. *Lampsilis siliquoidea* has a wide geographic distribution and inhabits a range of

518   habitats, and the extent to which the observed growth characteristics are common in other populations,

519   or even in similar lake systems, is currently unknown. Lake size, depth, temperature, and trophic status

520   are classically understood to be important in mediating the ecology of other freshwater taxa (Magnuson

521   et al. 1979, Eadie and Keast 1984, Jeppesen et al. 2000), and future studies exploring their influence on

522   lake mussels could be instructive.

523

524   *Growth characteristics*

525   Growth rates of *L. siliquoidea* mussels in Trout Lake, Wisconsin, were among the lowest

526   recorded for the Lampsilini tribe, especially for a medium-sized species. Our focus on collecting larger

527   individuals during sampling may have resulted in a bias toward faster growing individuals, so the true

528   growth rate for this population may be even lower than what we found here. As is common with slower

529   growing mussels, the maximum observed age was high compared to other Lampsilines. This slower

530   growing, longer-lived life history, characteristic of an equilibrium strategist (Haag 2012), may be

531   common in lake populations (Haag and Rypel 2011). This slow growth rate supports our hypothesis that

532   oligotrophic soft-water lakes, such as Trout Lake, present unique environmental challenges to mussels,

533   and these challenges are likely to impose a strong limitation on somatic growth.

534

535   *Relationship of growth and environment*

536   Despite large variation among individuals, growth was strongly positively correlated with

537   changes in lake level. There were no relationships with temperature or measures of productivity as have

538   been found in marine (Page and Hubbard 1987, Smaal and van Stralen 1990, Archambault et al. 1999,

539   Schöne et al. 2005) and fluvial systems (Schöne et al. 2004, Black et al. 2010). The relationship between

540   growth and lake level suggests that mussels are responding to broad-scale ecosystem characteristics.

541   Lake level is an aggregating environmental variable indicative of regional dynamics in precipitation and

542   hydrology that link lake dynamics with the surrounding terrestrial landscape. It is unlikely that lake level

543   had a direct influence on mussel growth, but rather it may act as a proxy for changes in other

544   environmental characteristics. In fluvial systems, it has been hypothesized that a simple model for

545   mussel growth has a parabolic relationship to discharge (Strayer 2008). During low to moderate flow,

546   growth may be positively related to discharge as allochthonous resources and food capture rates

547   increase. During high flow, the energetic costs of maintaining body position and expelling ingested

548   suspended solids can outweigh the benefits of increased flow and result in a negative relationship

549   between discharge and growth. The inverse relationship of growth to discharge has been well

550   documented in systems of moderate to high levels of discharge (Black et al. 2010, Black et al. 2015,

551   Dycus et al. 2015), but studies supporting the hypothesized positive effects of increased allochthonous

552   resources are rare (but see Schöne et al. 2007). We propose that lakes represent an extreme case of a

553   low-flow system and that the positive response of mussel growth to increased lake level reflects changes

554   in allochthonous subsidies during wetter years.

555   Terrestrial subsidies likely play an important role in the littoral habitats of Trout Lake, given that

556   they are often responsible for the bulk of C in the lakes of this region (Wilkinson et al. 2013) and can

557   provide a surprisingly large proportion of C for higher trophic levels (Weidel et al. 2008, Cole et al. 2011).

558   We were, however, unable to detect changes in water color or DOC quantity or quality (proxies for

559   allochthonous inputs) associated with changes in lake level or mussel growth. This lack of connection

560   may be explained by differences between the sampling location and the location where water quality

561   metrics were measured. The mussel bed was just meters from shore and relatively close to a small inlet

562   (~300 m), where individuals are likely to be exposed to allochthonous inputs immediately after runoff

563   events. In contrast, lake water-quality variables were measured at a centrally located buoy in deep

564   water ~500 m from our sampling site.

565   Mussel growth was not related to any other metrics indicative of food availability that we were

566   able to include in our analysis. Neither Chl *a*, as a measure of phytoplankton biomass, nor cladoceran

567   density had any relationship to growth. This lack of a relationship may result from multiple reasons: 1)

568   there could be a mismatch in concentrations between littoral and pelagic habitats, as mentioned above

569   for DOC; 2) pelagic resources may not be important or are not the limiting food sources for mussels in

570   littoral habitats; 3) mussels may be integrating across or shifting between food sources, obscuring any

571   clear relationship with any one potential source; or 4) food availability does not limit mussel growth in

572   this system. Mussel diets vary by system and species, with feeding occurring across benthic and

573   suspended sources that can include diatoms, phytoplankton, zooplankton, bacteria, cyanobacteria,

574   fungi, and possibly dissolved organic matter (Newton et al. 2013, Fujibayashi et al. 2016, Weber et al.

575   2017). Mussel growth still may be limited by sources other than terrestrially derived food availability in

576   this system, but the ability to detect these potential controls would be difficult because of potential

577   shifting between food sources and the lack of data on certain sources (e.g., bacteria, fungi).

578   In addition to food, temperature is a fundamental determinant of metabolism and growth for all

579   living things and is commonly associated with mussel growth rates in other systems (Hanson et al. 1988,

580   Schöne et al. 2004, 2005, but see Cyr 2020). In this population, however, we failed to detect a

581   relationship between water temperature and growth. Closely related variables often used as proxies for

582   growing season, such as degree days and the duration of the summer stratified period, also surprisingly

583   showed no relationship to growth. The most likely explanation for growth being unrelated to

584   temperature could be that the range of summer epilimnetic temperatures in Trout Lake is small (μ =

585   19.3 ± 1°C) and may not be ecologically relevant for this population. Another possible explanation is that

586   mussels may be regulating their temperature by moving within their habitat. Mussels are not entirely

587   sedentary animals, and they move both vertically in the substrate and horizontally in response to

588   environmental cues, such as temperature (Amyot and Downing 1997, Schwalb and Pusch 2007,

589   Hernandez 2016).

590

591   *Potential drivers of variation*

592   Numerous factors likely contribute to the variability in growth among individuals. Despite the

593   population level synchrony in growth, an interseries correlation of 0.39 is relatively low compared to

594   fluvial mussel populations (Rypel et al. 2009, Black et al. 2010, Sansom et al. 2013). Within a lake, the

595   distribution of mussels can be highly patchy, suggesting that there may be spatial heterogeneity in

596   habitat quality or the environmental controls within a lake. The moderately high variance in growth

597   among individuals in this system is interesting, especially considering that these mussels were all

598   comparable in age, residing in a similar substrate, and located within meters of each other. Although the

599   open water is often fairly well mixed, benthic littoral habitats are more spatially heterogeneous

600   (Downing and Rath 1988, Stoffels et al. 2005, Cyr 2019), and even mussels in the same bed may be

601   experiencing different conditions.

602   Environmental conditions are but 1 set of factors that influence mussel growth, and

603   unmeasured biotic drivers may play a stronger role in controlling growth. Physiological constraints on

604   growth and the causes of physiological differences are often obscured and difficult to assess. We have a

605   limited understanding of how characteristics such as sex and age affect growth dynamics of an

606   individual. These effects are further complicated through differential investment in gonad development

607   or glochidia brooding (instead of somatic in growth), which may vary substantially among individuals

608   and over their lifetimes (Haag and Staton 2003, Moles and Layzer 2008). These factors are all overlaid

609   upon the genetic variation between individuals, which can also be substantial (Larson et al. 2014). Other

610   factors, such as the effects of competition, predation risk, parasites, and pathogens, may affect the

611   physiology and growth of individuals within an assemblage non-uniformly and may be important drivers

612   of variation in growth among individuals as well (Gangloff et al. 2008, Vaughn et al. 2008) but

613   unfortunately have received relatively little attention. Our study of the growth of a small sample of 1

614   species of mussels from a single bed in 1 lake provides only a limited view into the dynamics of how

615   mussel growth is related to the conditions of their environment. Additional studies of other species in

616   different systems will undoubtedly be insightful for better understanding environmental controls on

617   mussel growth.

618

619   *Conclusion*

620   The alarming collapse of freshwater mussel assemblages worldwide should inspire increased

621   effort to understand the ecology of these animals and the environmental challenges they face. Globally,

622   lakes host numerous mussel populations and may be preferred habitat for some species (Nedeau et al.

623   2009, Haag 2012). Lakes impose divergent environmental challenges for mussels in comparison to fluvial

624   environments, and we know very little about the ecology of lake-dwelling mussels, their responses to

625   changing environmental conditions, or the ecosystem services they provide. Here, we show that the

626   growth of mussels in lakes can be dynamic, can be highly variable between individuals, and may be

627   correlated to landscape-scale environmental changes unique to lake systems. Further investigation of

628   the ecology and life history of lake-dwelling mussels is important for developing a broader

629   understanding these enigmatic animals.

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812   **TABLES AND FIGURES**

813   **Table 1**

814   Environmental variables explored for potential relationships to growth of the freshwater mussel *Lampsilis siliquoidea*. Pearson *r* reports the

815   linear correlation between the master growth chronology and the environmental variable. Data for all limnological variables were collected and

816   curated by the North Temperate Lakes Long-term Ecological Research site, NAO_{winter} PDO_{winter} data were downloaded from the National Centers

817   for Environmental Information, and precipitation data were downloaded from a local weather station in Minocqua, Wisconsin, USA. Lake level$_\Delta$

818   is the difference (in m) from the previous mean summer lake level. Color refers to water color.

Variable	Mean	Range	Units	Pearson *r*
Lake level$_\Delta$	0.004	−0.25, 0.21	m	0.58
Soluble reactive phosphorus (SRP_{spring})[a]	0.58	0.19, 0.87	µg/L	−0.36
Chlorophyll *a*	2.4	0.29, 5.9	µg/L	−0.02
Total cladoceran[b]	10.6	4.4, 18.5	no./L	0.13
Total phophorus	0.74	0.19, 0.95	µg/L	0.03
O_2	9	8.4, 9.6	mg/L	−0.23
pH	8.3	8.0, 8.5	–	−0.08
Ca^{2+}	12.6	11.2, 14.2	mg/L	−0.13
$NO_3 + NO_2$	3.1	0.1, 10.5	µg/L	−0.01

Total organic carbon	3	2.5, 3.4	mg/L	−0.05
Dissolved organic carbon (DOC)	3.1	2.4, 3.4	mg/L	−0.09
Specific ultraviolet absorbance (SUVA) at 254 nm	1.84	0.92, 5.15	$L\,mg^{-1}\,C\,m^{-1}$	0.2
Slope ratio	1.38	0.84, 2.1	(ratio of $S_{275-295}$ to $S_{350-400}$)	0.06
Linear slope 275–295	−0.024	−0.03, −0.014	log(slope of the abs scan over 275–295 nm)/nm	0.17
Color	17.7	2.5, 108.5	abs at 254 nm (1 m path length)	0.11
Total precip water year	107.7	3.5, 331.2	cm	0.44
North Atlantic Oscillation index (NAO_{winter})	0.1	−1.6, 1.4	–	0.26
Pacific Decadal Oscillation index (PDO_{winter})	0.27	−1.6, 2.5	–	0.34
Mean wind speed	1.25	0.3, 1.8	m/s	0.38
Duration stratification	141.3	123, 183	d	−0.03
Degree days	3336	2922, 3751	°C × d	0.001
Date last ice	113.3	79, 137	Day of year	−0.11
Date first ice	346.8	332, 369	Day of Year	−0.11
Water temperature (minimum)	13.3	9.9, 16.6	°C	0.02
Water temperature (10th percentile)	14.8	11.5, 17.3	°C	0.12

Water temperature (25th percentile)	17.3	14.5, 20.8	°C	0.1
Water temperature (mean)	19.3	17.6, 21.2	°C	0.15
Water temperature (85th percentile)	21.5	18.3, 23.6	°C	0.17
Water temperature (90th percentile)	22.6	20.1, 24.7	°C	0.26
Water temperature (maximum)	23.5	20.2, 26.3	°C	0.19

819   [a] Calculated from spring mixing period only.

820   [b] only including individuals of the taxa *Daphnia*, *Holopedium*, Bosminidae, and *Diaphanosoma*.

821 **Table 2**

822 Conditional averages of the top performing models (within 2 Bayesian information criterion units of best

823 performing model) from an exhaustive model selection. Independent variables were standardized ($\bar{x} = 0$,

824 standard deviation = 0.5) to easily compare effect size between variables. Lake level$_\Delta$ is the difference (in

825 m) from the previous summer's mean lake level. SRP$_{spring}$ is the mean dissolved reactive phosphorus

826 (μg/L) from the immediately preceding spring mixing period.

Parameter	Estimate	Standard error	z-value	Pr(>|z|)	No. of models included
Intercept	0.93	0.03	26.16	<0.001	2
Lake level$_\Delta$	0.1	0.03	2.82	0.005	2
SRP$_{spring}$	−0.05	0.03	1.39	0.16	1

827

828

829   **Figure 1**
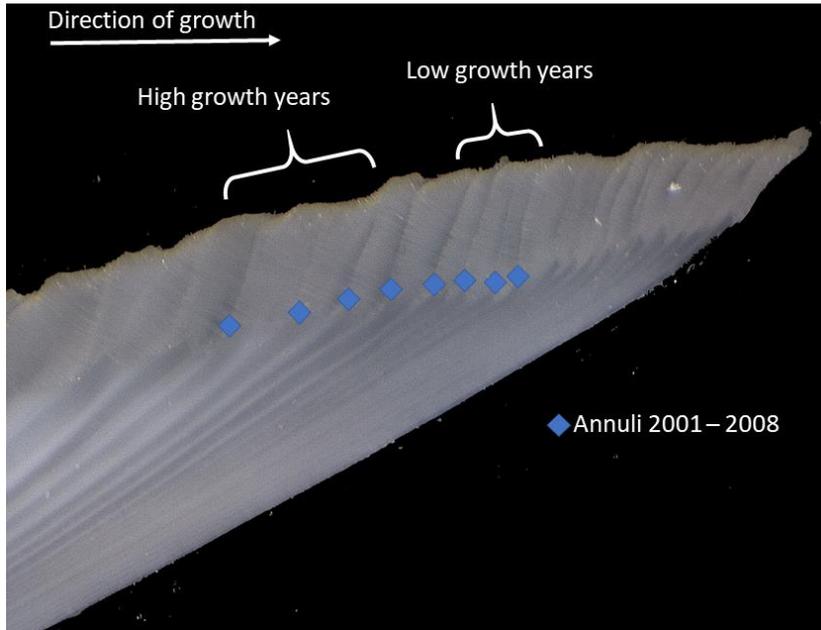
830

831   Figure 1: Close-up of shell thin-section from *Lampsilis siliquoidea*. Note conspicuous dark bands

832   indicative of winter growth cessation. Diamonds indicate reference points of annuli at the prismatic

833   layer/nacre margin from where measurements of annual growth were recorded.

834

835 **Figure 2**
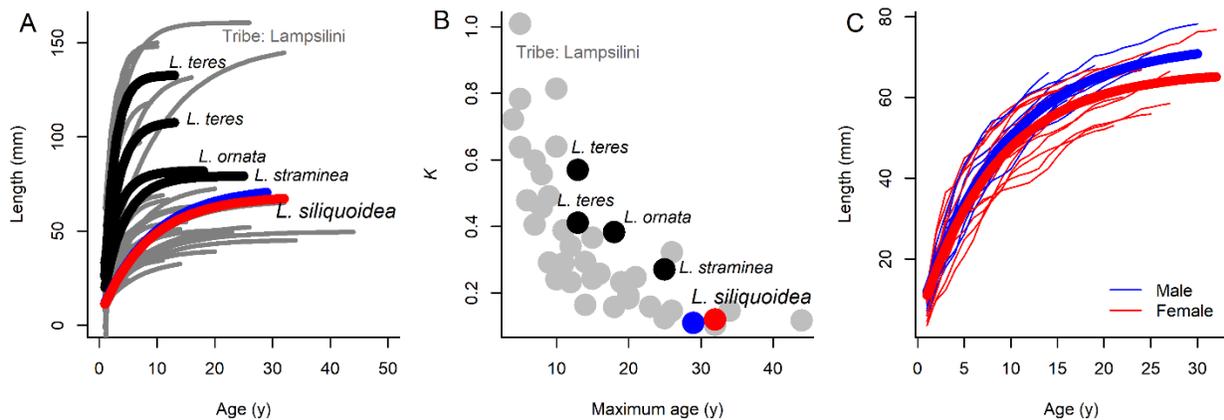
836

837 Figure 2: Growth characteristics of *Lampsilis siliquoidea* and its context within the Lampsilini tribe. A.—

838 Growth trajectories of individual mussels color-coordinated by sex. Thick lines are growth trajectories

839 for the population calculated using the von Bertalanffy equation: $L_t = L_\infty\left(1 - e^{K(t - t_0)}\right)$, where L_t is

840 the length (mm) at time *t* (age in years), L_∞ is the mean maximum length for the population, *K* is a

841 growth constant that describes how quickly an individual approaches L_∞, and t_0 is the time at which

842 length = 0. Males: *K* = 0.11, L_∞ = 73.4, t_0 = −0.59, *n* = 11; females: *K* = 0.12, L_∞ = 66.4, t_0 = −0.51, *n* = 14;

843 only L_∞ was substantially different between sexes. Males are color-coded in red, females in blue, genus

844 *Lampsilis* in black, and Lampsilini tribe in gray. B.—The von Bertalanffy growth coefficient *K* plotted

845 against maximum observed age for populations within the Lampsilini tribe (gray dots) and genus

846 *Lampsilis* (black dots) to highlight the unique growth characteristics of *L. siliquoidea* (data for panels A

847 and B include all members of the Lampsilini tribe reported in Haag and Rypel 2011; Table S1). C.—The

848 same colored growth curves from panel A with each individual chronology plotted underneath to display

849 variation among individuals.

850

851   **Figure 3**
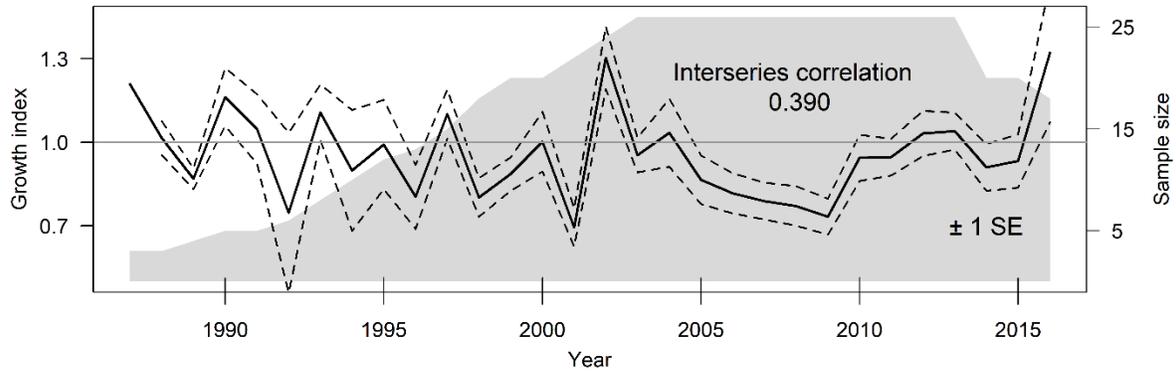
852

853   Figure 3: Master growth chronology of Trout Lake's *Lampsilis siliquoidea* population (black line) ± 1

854   standard error (SE) (dashed lines) developed from the standardized growth indices (see Methods for

855   description of detrending and standardization procedure). Values >1 indicate growth exceeded model

856   expectations for that year, whereas values <1 indicate growth was less than expected. Gray shading

857   indicates the number of chronologies contributing to that year's estimate.

858

859 **Figure 4**
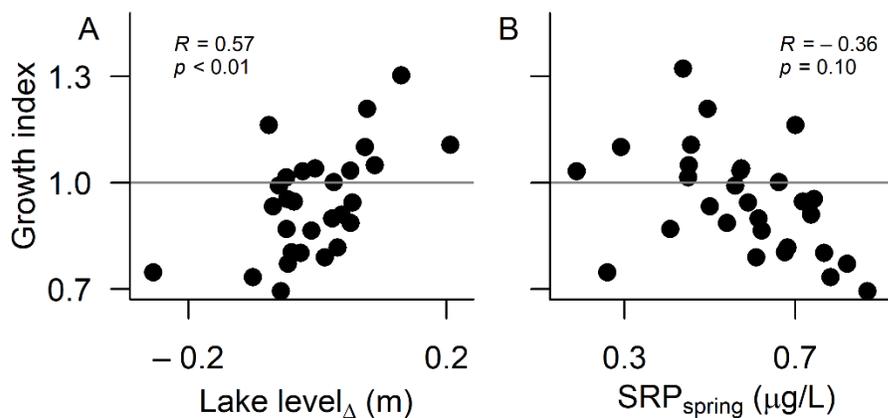
860

861 Figure 4: A.—Regressions of standardized growth indices (SGIs) on lake level$_\Delta$. B.—Soluble reactive

862 phosporus (SRP$_{spring}$). Lake level$_\Delta$ is the difference (in m) from the previous mean summer lake level;

863 SRP$_{spring}$ is the mean dissolved reactive phosphorus during the immediately preceding spring mixing

864 period. Each linear regression was run independent of other environmental variables. Each dot

865 represents average standardized growth of the population in 1 y. Growth indices >1 reflect a higher than

866 growth for that year, whereas growth indices <1 reflect growth lower than expected for that year.

867

868   **Figure 5**
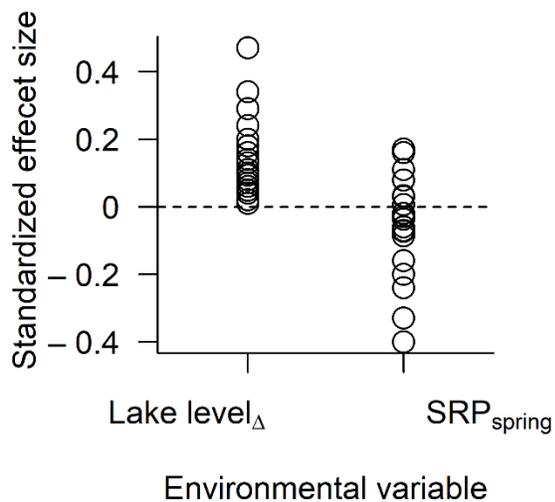
869

870   Figure 5: Estimates of effect size of lake level$_\Delta$ and soluble reactive phosphorus (SRP$_{spring}$) on individual

871   mussel (*Lampsilis siliquoidea*) chronologies. Positive values indicate that the variable likely had a positive

872   effect on that individual's growth; negative values indicate a likely negative effect on growth. Estimates

873   farther from 0 suggest that the environmental variable had a stronger effect on mussel growth.

874   Estimates were calculated independently of one another using linear models containing only the

875   environmental variable (standardized; $\bar{x} = 0$, standard deviation = 0.5) being estimated and each

876   individual chronology. Lake level$_\Delta$ is the difference (in m) from the previous mean summer lake level;

877   SRP$_{spring}$ is the mean dissolved reactive phosphorus during the immediately preceding spring mixing

878   period.

879

Chapter 2: Detecting stress in unionids in a natural ecosystem experiencing a zebra mussel invasion using metabolomics

Abstract:

Freshwater mussels (Order: Unionida) are globally endangered and face numerous threats including the now widespread zebra mussel (*Dreissena polymorpha*). Conservation efforts can benefit from a better understanding of how unionids are affected by their numerous threats. Burden from colonization is likely a strong driver of unionid decline in systems where zebra mussels are present, but other factors such as unionid species, spatial aggregation of zebra mussels, and co-habitation history have been found to have an effect as well. Understanding why some unionids are more severely impacted by zebra mussels may benefit from a better mechanistic understanding of how unionids are affected by zebra mussels. In the present study we examined physiological profiles of *Lampsilis siliquoidea* across a natural gradient of zebra mussels in a eutrophic chain of lakes using a non-targeted metabolomics approach to analyze whether physiological profiles of stress were detectable in this unionid population, and/or if other life history processes within their natural ecosystem were impacted. Twenty-four of 99 metabolites were significantly associated with zebra mussel burden, many of which displayed consistent responses to laboratory studies of unionids under stress. This suggests that the physiological profiles of stress may be similar among species and detectable in unionids in natural systems. Metabolites integral to important physiological functions such as energy production did not appear to be negatively affected by zebra mussels; however, numerous metabolites secondarily associated with energy production were consistently negatively associated with mussel shell colonization. We propose that these metabolites are being used to supplement energy production and caution that this could conceal indicators of stress if only metabolites primarily associated with energy production are examined. We suggest that metabolomics is a useful tool to better understanding how unionids respond to stressors across various systems.

Introduction:

904

905   Freshwater mussels (order Unionida) are one of the most endangered taxa globally (Williams et

906   al. 1993; Lopes-Lima et al. 2018). They experience numerous and varied threats to their persistence

907   including historical overexploitation (Anthony and Downing 2001), ongoing habitat modification,

908   decreasing water quality (Gillis 2011), invasive species (Ricciardi et al. 1996; Strayer 1999), climate

909   change (Inoue and Berg 2017; Beggel et al. 2017), and emerging pathogens (Richard et al. 2021). Long-

910   term conservation of unionids will require an understanding of how these mussels are influenced by

911   anthropogenic factors at all scales.

912   One ubiquitous threat to unionids is the expanding geographic range of zebra mussels

913   (*Dreissena polymorpha*) (Benson et al. 2021). Zebra mussels are one of the most aggressive invasive

914   species in freshwater systems globally (Karatayev et al. 2015). Their relatively fast life history integrates

915   a unique free-floating larval stage that allows rapid dispersal, high population growth rates, and high

916   local densities and biomasses (Strayer and Malcom 2006). For example, zebra mussels often colonize all

917   available hard substrates of benthic habitats (Nalepa et al. 1995; Mellina and Rasmussen 1994; Spear et

918   al. 2022), including occupied and unoccupied unionid shells (Schloesser et al. 1996). Zebra mussels also

919   trigger drastic changes to entire ecosystems and their presence negatively impacts survival and growth

920   of competing filter-feeding unionids (Ricciardi et al. 2002).

921   Understanding how zebra mussels affect native unionids at an individual scale would assist in

922   untangling why unionid communities display varied responses to zebra mussel invasions. Multiple

923   factors such as hyper-localized spatial interactions (Beason and Schwalb 2022; Baker and Hornbach

924   2008; Ricciardi et al. 1996), species-specific characteristics of the unionid (Gillis and Mackie 1994), as

925   well as community dynamics (Burlakova et al. 2014) shape the nature and severity of the impact of

926   zebra mussel on unionids. This multifaceted response in turn increases difficulty in predicting long-term

927   conservation status of unionid species in at-risk ecosystems or those currently experiencing zebra

928   mussel invasions (Strayer and Malcom 2007).

929   Unionids, and bivalves in general, are enigmatic animals and changes in body condition or

930   overall health are often not readily apparent from a cursory observation. However, measurements of

931   physiological status are often useful in identifying mechanistic drivers of fitness loss and mortality in

932   mussels and other freshwater animals. For unionids, attributes such as glycogen concentration in the

933   foot or mantle are common assessments, as they are useful and relatively robust in evaluating general

934   body condition (Naimo et al. 1998). While the role of glycogen as a primary energy storage biomolecule

935   in unionids makes it well-suited for assessing general body condition, it provides little insight into

936   identifying how and which physiological processes may be disrupted. Further, baseline glycogen

937   concentrations can differ among species, naturally fluctuate throughout the year, and are affected by

938   reproductive effort (Monroe and Newton 2001; Nagabhushanam and Lomte 1971). Unionids respond

939   differently depending on the threat they are experiencing and conditions of their ecosystem. Thus, it is

940   often difficult to identify specific threats to their well-being (Strayer et al. 2004; Downing et al. 2010).

941   Physiological assays may provide insight into stressors and how mussels respond to specific

942   anthropogenic impacts. For example, subtle changes in glycogen levels, or other metabolic products,

943   may signal less severe or early-stage changes in ecosystem conditions.

944   Non-targeted metabolomics is a bioassessment approach that estimates concentrations of an

945   array of low molecular-weight biochemicals relevant to important biological processes at the cellular

946   scale. Studies of metabolomics allow for direct insight into cellular activity and how specific biological

947   processes may be altered, including the identification of processes by which mussels are negatively

948   affected by stressors. Compared to marine bivalves, metabolomics studies in freshwater mussels have

949   been rare (see Leonard et al. 2014; Roznere et al. 2014; 2017), but those that have been completed have

950   shown the ability to identify specific metabolic pathways that have been altered in controlled

951 experimental studies of stress such as exposure to an endocrine disruptor (Leonard et al. 2014), food

952 limitation (Roznere et al. 2014), and translocation (Roznere et al. 2017).

953 In this study, we used metabolomics to expand our understanding of how lake unionids are

954 affected by zebra mussel invasion at a physiological level. We focused our assessment specifically on

955 whether metabolomic profiles of stress are readily identifiable in natural ecosystems. We were able to

956 study these by analyzing metabolomic profiles of a common freshwater mussel (*Lampsilis siliquoidea*) as

957 they responded to initial years of a zebra mussel infestation across a gradient of zebra mussel densities

958 throughout a chain of eutrophic lakes.

959

960 **Methods:**

961 *Sampling location*

962 The Yahara Lake system is a well-studied chain of eutrophic lakes (Mendota, Monona, Waubesa,

963 and Kegonsa) located in southern WI, USA. Zebra mussels were first discovered in 2015 in Lake Mendota

964 and quickly increased to high densities by 2018 (>30,000 m^{-2}) (Spear et al. 2022). During this period,

965 downstream lakes were steadily colonized and a natural gradient of zebra mussel densities developed

966 throughout the lake chain. We used this natural gradient to examine physiological responses to varying

967 degrees of zebra mussel infestation. Unionids higher in the lake chain had significantly higher total

968 numbers of zebra mussels attached to their shells on average (ANOVA p-value < 0.01): Mendota (mean =

969 46, SD = 16.8), Monona (mean = 27, SD = 25.9), and Kegonsa (mean = 0.5, SD = 1.1). This pattern

970 correlated with zebra mussel loads on unionids as defined by total grams dry mass of zebra

971 mussels/grams dry mass of unionid soft tissue being substantially higher in the earlier invaded lakes

972 higher in lake chain: Mendota (mean zebra mussel load = 4.3, SD 1.8), Monona (mean = 1.9, SD = 1.8),

973 and Kegonsa (mean <0.01, SD = 0.01).

974

975   *Sample collection*

976   During summer 2017, Lakes Mendota, Monona, and Kegonsa were surveyed for native mussels.

977   Near shore sites were selected for SCUBA and snorkel surveys based on suitable habitat for native

978   mussels. A total of 33 unionids were sampled across the lake chain for glycogen analysis: Mendota (n =

979   15), Monona (10), Kegonsa (8) and 32 individuals were sampled for metabolomics analysis: Mendota (9),

980   Monona (15), Kegonsa (8) (Figure 1). The breeding season of *Lampsilis siliquoidea* is mid-summer and no

981   females were observed to be gravid during collection. Mussels that were collected for glycogen analysis

982   were immediately placed on ice to preserve the tissue for later analysis (following Dunn and Ellis, 2005).

983   In the lab, individuals were sexed via external shell morphology, dried in a drying oven to a constant

984   mass, weighed, then approximately 10mg of foot tissue was collected and run for glycogen analysis

985   (following Naimo et al. 1998).

986   For mussels that were sampled for metabolomics analysis, in the field, immediately after

987   collection, approximately 200uL of hemolymph was drawn from the anterior adductor muscle using a

988   27–29-gauge syringe, stored in a 2mL cryotube, and flash frozen in liquid nitrogen. Unionids were then

989   scraped of all zebra mussels if present and returned to the benthos. All zebra mussels greater than

990   length 5mm were counted and measured along the major axis of the shell. Cumulative dry weight of all

991   zebra mussels was estimated using a standard conversion of length to dry weight for zebra mussels

992   (Coughlan et al. 2021). Zebra mussel loads were calculated as total estimated zebra mussel dry mass

993   divided by the total dry mass of the unionid soft tissue. Hemolymph samples were stored at -80˚C until

994   they were shipped on dry ice to West Coast Metabolomics Center at UC-Davis for non-targeted analysis

995   of primary metabolites. Metabolites were identified using gas chromatography time-of-flight mass

996   spectroscopy (GC-TOF MS and their peak heights were normalized to the sum peak height of annotated

997   compounds (mTIC-normalization) by the West Coast Metabolomics Center. Each metabolite was

998   independently normalized to zero and standardized to one standard deviation.

999   Metabolites were identified as being significantly affected by the presence of zebra mussels via

1000   a t-test on the relative metabolite concentrations between the groupings of unburdened and burdened

1001   mussels (p-value threshold of 0.05). The cutoff between unburdened and burdened was selected to

1002   favor natural breaks in the data as well as balance the sample size of each factor level. Model fits

1003   displayed in the scatter plots on all figures of relative metabolite concentration and zebra mussel load

1004   are back-transformations of linear regressions fit to ln(relative metabolite concentration + α) ~ ln(zebra

1005   mussel load +1) where α is the absolute value of the minimum metabolite relative concentration + 0.5.

1006   They are included only in plots of significantly affected metabolites and are intended only to provide a

1007   general description of the relationship between the metabolite and zebra mussel load. Non-metric

1008   multi-dimensional ordination on metabolomic data was calculated using Canberra distance to assess

1009   dissimilarity using the vegan package in R (Oksanen et al. 2020; R Core Team 2020).

1010   **Results:**

1011   *Glycogen*

1012   Glycogen concentrations were lowest for mussels found in Mendota; mean glycogen in foot

1013   tissue was 70.6 mg/g (SD 21). Mussels from Lake Monona had a mean glycogen concentration of 78.1

1014   mg/g (SD 25.8), while Kegonsa residents had a mean concentration of 113mg/g (SD 8.5). Glycogen

1015   concentrations of unionids were significantly negatively related to zebra mussel load (Figure 1). Unionids

1016   burdened by zebra mussels had a mean glycogen concentration in foot tissue at 77.0 mg/g (SD 21.0)

1017   where unionids unburdened by zebra mussels had mean foot glycogen concentrations of 107.0 mg/g (SD

1018   10.7). Glycogen levels were not related to sex (LMM with sex as a random intercept, p = 0.99).

1019

1020   *Non-metric multi-dimensional ordination (NMDS):*

1021   Of the 99 metabolites that were identified by West Coast Metabolomics, 24 showed a significant

1022   change in response to zebra mussel loads (Table 1). There was substantial overlap of metabolite profiles

1023   among the three lakes. Unionids had a consistent response among individuals strongly associated with

1024   zebra mussel load and not by sex or mass of unionid (Figure 2). Metabolite concentrations that were

1025   affected by zebra mussel load included molecules important in energy production, the citric acid cycle,

1026   and the urea cycle.

1027   *Carbohydrate metabolism*

1028   Energy sources for carbohydrate metabolism showed the opposite pattern from glycogen

1029   concentrations. Unionids burdened with zebra mussels displayed positive relationships with relative

1030   metabolite concentrations of glucose ($p < 0.01$), galactose ($p < 0.01$), and fructose ($p = 0.01$) (Figure 3).

1031

1032   *Citric Acid Cycle*

1033   Primary metabolites centrally involved in the citric acid cycle were able to maintain consistent

1034   concentrations across all levels of zebra mussel loads. There was no detectable relationship between

1035   relative metabolite concentration and zebra mussel load in three of four of the metabolites central to

1036   the citric acid cycle: fumarate ($p = 0.78$), succinate ($p = 0.06$), or citrate (p 0.72), while only malate

1037   displayed a significant positive relationship ($p = 0.02$) (Figure 4 left column). However, unionids

1038   burdened with zebra mussels experienced consistent and marked decreases in many metabolites that

1039   can act as substrates to support the citric acid cycle; secondary metabolite concentrations negatively

1040   related to zebra mussel loads included asparagine ($p = 0.03$), lysine ($p < 0.01$), methionine ($p = 0.02$),

1041   serine ($p < 0.01$), tryptophan ($p = 0.02$), threonine ($p = 0.03$), and valine ($p = 0.01$) (Figure 4 middle and

1042   right columns).

1043

1044   *Urea Cycle*

1045   Metabolites central to the urea cycle did not display consistent patterns in relation to zebra

1046   mussel loads. Zebra mussel load was not correlated with metabolites urea ($p = 0.84$) nor citrulline ($p =$

1047   0.05); however, zebra mussel load was negatively correlated with ornithine ($p < 0.01$) (Figure 5).

1048

1049   **Discussion:**

1050   Freshwater mussel metabolomic profiles displayed several consistent patterns associated with

1051   increasing zebra mussel load. This was consistent regardless of sex or mass of mussel (Figure 3). The

1052   most affected metabolites were amino acids known to be secondarily involved in energy production via

1053   the citric acid cycle (asparagine, lysine, methionine, serine, threonine, tryptophan, valine) and the urea

1054   cycle (ornithine), suggesting that processes necessary for energy production, protein metabolism and

1055   possibly excretion have been affected. Although the exact set of metabolites identified in this study

1056   were different, the specific metabolite expressions and direction of their changes are largely consistent

1057   with laboratory studies of physiological profiles of unionids experiencing stress from starvation (Roznere

1058   et al. 2014) such as reduced levels of free amino acids associated with the citric acid cycle and the urea

1059   cycle.

1060   Glycogen is the primary energy storage molecule for mollusks, and its depletion in response to

1061   stressful situations is well-documented (de Zwaan and Wijsman 1976; Hummel et al. 1989), including in

1062   other studies of unionids affected by zebra mussel invasions (Haag et al. 1993; Beason and Schwalb

1063   2022; Baker and Hornbach 2000). In this study, the negative relationship between foot glycogen

1064   concentration and zebra mussel load suggests unionids with moderate and high zebra mussel loads are

1065   likely tapping more into their stored glycogen in order to sustain basal metabolic function. This process

1066   cannot be maintained indefinitely and depleted glycogen levels may decrease reproductive capacity

1067   and/or increase mortality risk during periods of lower food availability.

1068   Circulating metabolites important for energy production such as glucose, galactose and fructose,

1069   seem to not only be maintained but are slightly elevated in unionids burdened with zebra mussels. This

1070   is likely due in part to the mobilization of stored glycogen as well as an increase in gluconeogenesis (the

1071   process of synthesizing glucose from non-carbohydrate substrates such as amino acids). Elevated

1072   glucose levels during stress is not uncommon (McCue 2010; Roznere et al. 2014) and is likely an

1073   overcompensation for a lack of sufficient carbohydrate intake from food sources to balance energy

1074   demands (McCue 2010). However, this strategy of overcompensation cannot be maintained long-term

1075   as stores of glycogen become depleted along with the substrates used for gluconeogenesis. These

1076   patterns of increased glycolysis and diminishing gluconeogenic substrates closely mirrors that of

1077   laboratory studies of stress from starvation in *Amblema plicata* (Roznere et al. 2017), suggesting that

1078   metabolic patterns of stress are likely similar among unionids and may be easily identifiable through

1079   metabolomic analyses.

1080   The citric acid cycle relies on circulating glucose as a primary source of energy. In this study,

1081   there were no significant changes in three of the four primary intermediates directly involved in the

1082   cycle, with only malic acid indicating an increase in response to zebra mussel load (Figure 4 left column);

1083   however, numerous amino acids capable of supplementing the citric acid cycle were consistently

1084   depleted in unionids with moderate and high zebra mussel loads (Figure 4 middle and right columns). It

1085   is likely that these amino acids are being depleted in order to supplement the citric acid cycle in an

1086   attempt to meet energy demands. In addition, another likely consequence of amino acids depletion is its

1087   effect on protein metabolism, specifically diminished opportunities for protein synthesis. The increased

1088   demand for energy sources without a concomitant upregulation of citric acid cycle intermediates may

1089   indicate an increased reliance on anaerobic respiration in mussels undergoing higher zebra mussel loads

1090   as energy demands are not being met through aerobic respiration alone. Venter et al. (2018) have

1091   reported that decreases in asparagine with high zebra mussel load are consistent with stress from

1092   hypoxia in abalone and may serve as additional support for increased reliance on anaerobic metabolism.

1093   In the present study, only one of three identified metabolites in the urea cycle showed

1094   significant changes in response to zebra mussel load (Figure 5). These minor changes in metabolites

1095   involved in the urea cycle suggest that if mussels have increased their rate of protein catabolism, it may

1096   only be to a minor extent. However, it is important to note that the urea cycle is not the most common

1097   pathway for removal of metabolic nitrogen in freshwater mussels, as a majority of nitrogen is excreted

1098   as ammonia prior to the urea cycle. Thus, if protein catabolism is increased in unionids experiencing

1099   higher levels of zebra mussel load, its effects on the urea cycle may be diminished as well as our ability

1100   to detect it.

1101   Metabolomic analyses provide a powerful tool to identify changes in multiple metabolic

1102   pathways important to the physiology of an organism. Therefore, because patterns across individuals

1103   appear to be consistent, physiological impacts from zebra mussel infestation likely scale to population

1104   and perhaps even ecosystem level impacts. It is important to remember, however, that metabolites

1105   rarely have a single purpose and their interactions in multiple cellular processes make interpreting

1106   changes in the concentration of individual metabolites challenging. For example, changes in important

1107   physiological functions such as energy production through the citric acid cycle can be obscured by other

1108   processes such as gluconeogenesis. In addition, the variability of any one metabolite between species,

1109   time of year, and/or reproductive stage can make assessments based on one or only a few metabolites

1110   limited in their usefulness. A holistic approach that focuses on pathways as a unit rather than individual

1111   metabolites should be employed as it would provide greater confidence in identifying which pathways

1112   have been affected and how they have changed.

1113   Metabolomics is broadly applicable to studies of mussel conservation and ecology. Metabolomic

1114   profiles associated with specific stressors such as hypoxia and temperature stress have been previously

1115   described in marine bivalves (Ellis et al. 2014; Dunphy et al. 2015; Tuffnail et al. 2009), as well as in

1116   mussels responding to harmful chemicals such as endocrine disruptors in freshwater systems (Leonard

1117   et al. 2014). Within the focus of this study, the mechanisms through which zebra mussels negatively

1118   affect unionids are not well-defined, but likely include reduced filter-feeding ability and respiration, and

1119   hampering of burrowing and movement (reviewed by Strayer, 1999) and multiple undefined effects

1120   associated with physiological stress may be occurring in conjunction. Identifying metabolomic patterns

1121   associated with specific types of stress is an important next step, as it has broad reaching applications

1122   and may be particularly helpful when trying to identify specific threats to mussels in their environments

1123   (Tuffnail et al. 2009). In addition, very little is known about zebra mussel impacts on unionid

1124   reproduction, and with the increasing number of systems in which cohabitation is occurring, this is an

1125   important area of study.

1126   In this study, we were able to identify signs of stress in unionids in response to zebra mussel

1127   fouling in their natural environment, but caution that physiological signs of stress may not always be

1128   readily apparent even for key physiological processes. Our study also highlights a potential for how

1129   metabolomics can be a useful tool in wild mussel health assessments, likely over a range of questions

1130   and applications. Finally, since freshwater mussels are also quite long-lived (Haag and Rypel 2011),

1131   approaches, like metabolomics, that allow sub-lethal characterization of fitness of these animals, will be

1132   critical to their future conservation management. The threats that freshwater mussels face are

1133   numerous and varied; the toolbox we will need to best understand, monitor, and manage mussel health

1134   will likely require an equally diverse approach.

1135

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1253

1254

1255 **Figures and Tables**

1256 **Table 1**

1257 List of metabolites identified as affected by zebra mussel burden. Each metabolite was independently

1258 standardized to a mean of zero (1 SD). Values for unburdened (n =17) and burdened (n = 15) columns

1259 correspond to mean metabolite value across all individuals. P-values were calculated using a t-test

1260 between unburdened and burdened groups for each metabolite.

			Zebra mussel load			
	KEGG ID	metabolite	Unburdened	Burdened	Response	p-value
Carbohydrate metabolism	C00152	asparagine	0.359	-0.407	-	0.028
	C02336	fructose	-0.446	0.506	+	0.005
	C00984	galactose	-0.524	0.594	+	0.001
	C00800	gluconic acid	-0.403	0.457	+	0.013
	C00221	glucose	-0.531	0.602	+	0.001
	C00047	lysine	0.658	-0.745	-	0
	C00711	malic acid	-0.373	0.423	+	0.022
	C00073	methionine	0.403	-0.457	-	0.013
	C00079	phenylalanine	0.354	-0.402	-	0.03
	C00065	serine	0.55	-0.623	-	0
	C00183	threonine	0.366	-0.414	-	0.025
	C00078	tryptophan	0.392	-0.444	-	0.016
	C00183	valine	0.406	-0.461	-	0.012
Urea cycle	C00077	ornithine	0.516	-0.585	-	0.001
	C00064	glutamine	0.398	-0.451	-	0.014
	C02989	methionine sulfoxide	0.396	-0.449	-	0.014
		methylhexose	-0.362	0.41	+	0.027
	C00138	putrescine	0.358	-0.406	-	0.028
	C00795	tagatose	-0.355	0.403	+	0.03
	C01620	threonic acid	0.346	-0.392	-	0.035
	C00106	uracil	-0.37	0.419	+	0.023
		5-methoxytryptamine	0.387	-0.439	-	0.017
		5-hydroxynorvaline	0.337	-0.382	-	0.04
	C02721	2-aminobutyric acid	0.4	-0.454	-	0.013

1261

1262 **Figure 1**
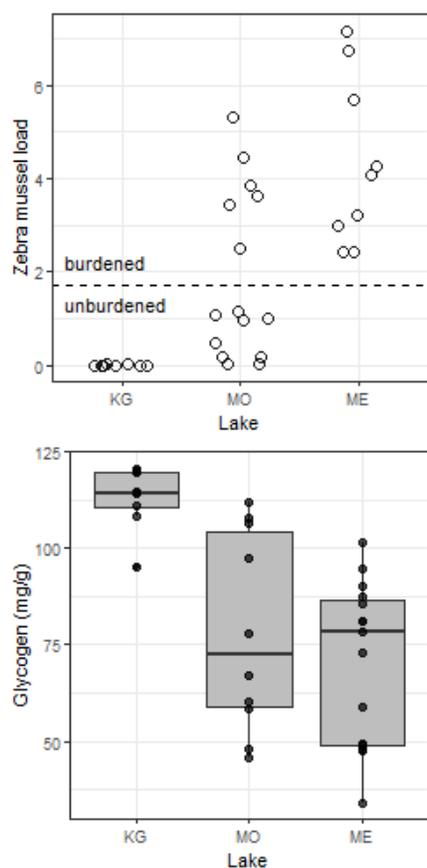
1263

1264

1265 Figure 1: Distribution of zebra mussel loads on unionids used for metabolomics analysis among the

1266 sampled lakes (top). Zebra mussel loads were calculated as the total grams dry mass of zebra

1267 mussels/grams dry mass of unionid soft tissue. Unburdened (n = 17) / burdened (n=15) cutoff was

1268 chosen to favor natural breaks in the data as well as balance the sample size of each factor level. Points

1269 are offset horizontally for visualization purposes only. Unionid foot glycogen concentrations across lakes

1270 in relation to zebra mussel loads (bottom). Glycogen concentrations are reported in mg of glycogen/g

1271 dry weight foot tissue. Lake IDs stand for Lakes Kegonsa (KG), Monona (MO), and Mendota ME).

1272

1273   **Figure 2**
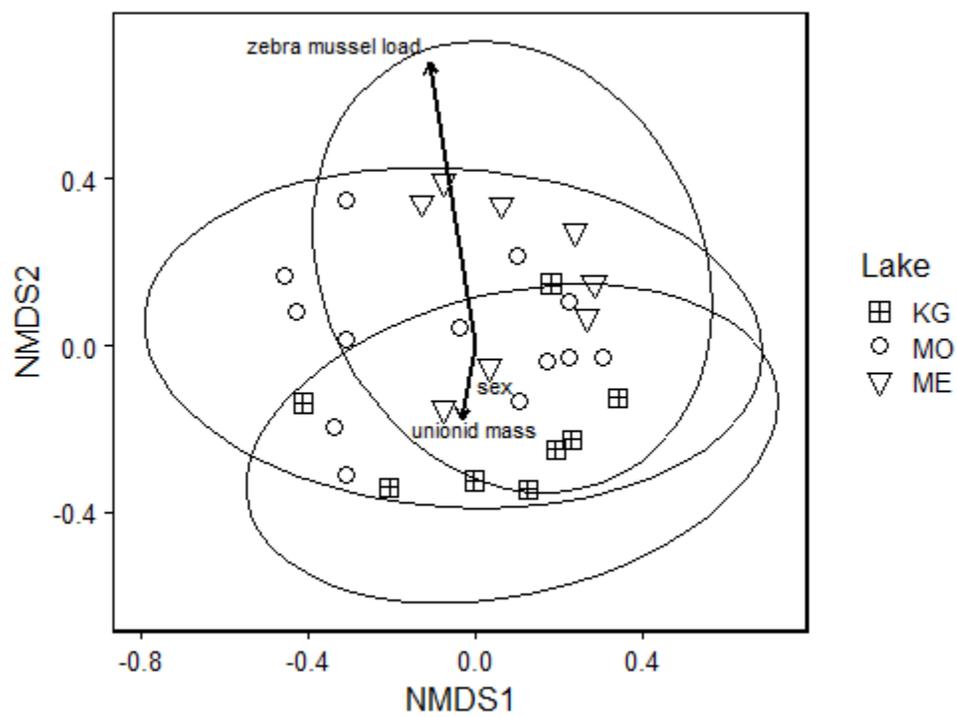
1274

1275   Figure 2: Non-metric multi-dimensional scaling of metabolomic profiles of unionids. All 99 identified

1276   metabolites were included in NMDS. Dissimilarity was calculated using Canberra distance. Ellipses were

1277   calculated with the default level of 0.95. The direction of the factors zebra mussel load, unionid mass,

1278   and (unionid) sex and their distance from the origin are proportional to the direction and strength of

1279   association of that factor with the ordination axes. KG Kegonsa; MO Monona; ME Mendota

1280 **Figure 3**
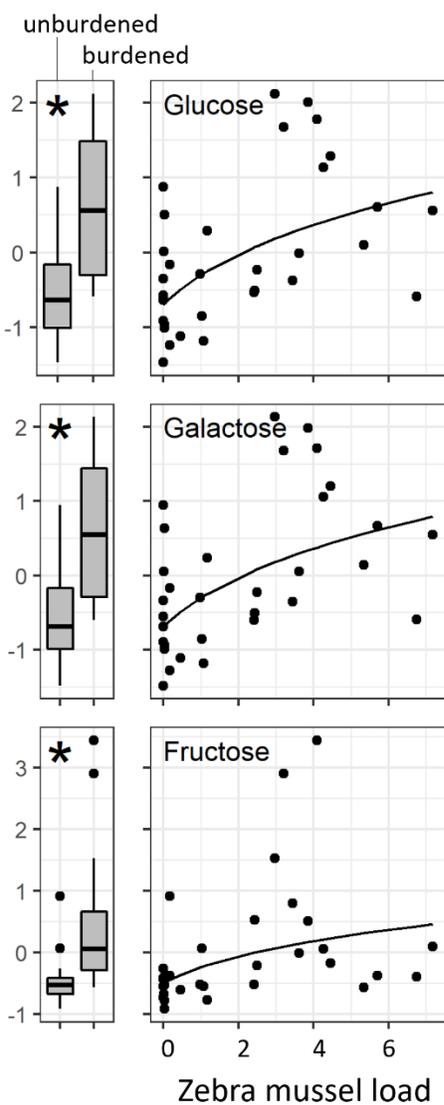
1281

1282 Figure 2: Metabolites important in carbohydrate metabolism/energy production and their relationship

1283 to zebra mussel load. Boxplots show metabolite relative concentrations grouped by

1284 burdened/unburdened status. *denote significant differences between groups. Scatterplots display the

1285 same data across the range of zebra mussel loads. Modeled lines are back-transformations of linear

1286 regressions fit to ln(metabolite + α) ~ ln(zebra mussel load) and are only intended to generally describe

1287 the relationship.

1288   **Figure 4**
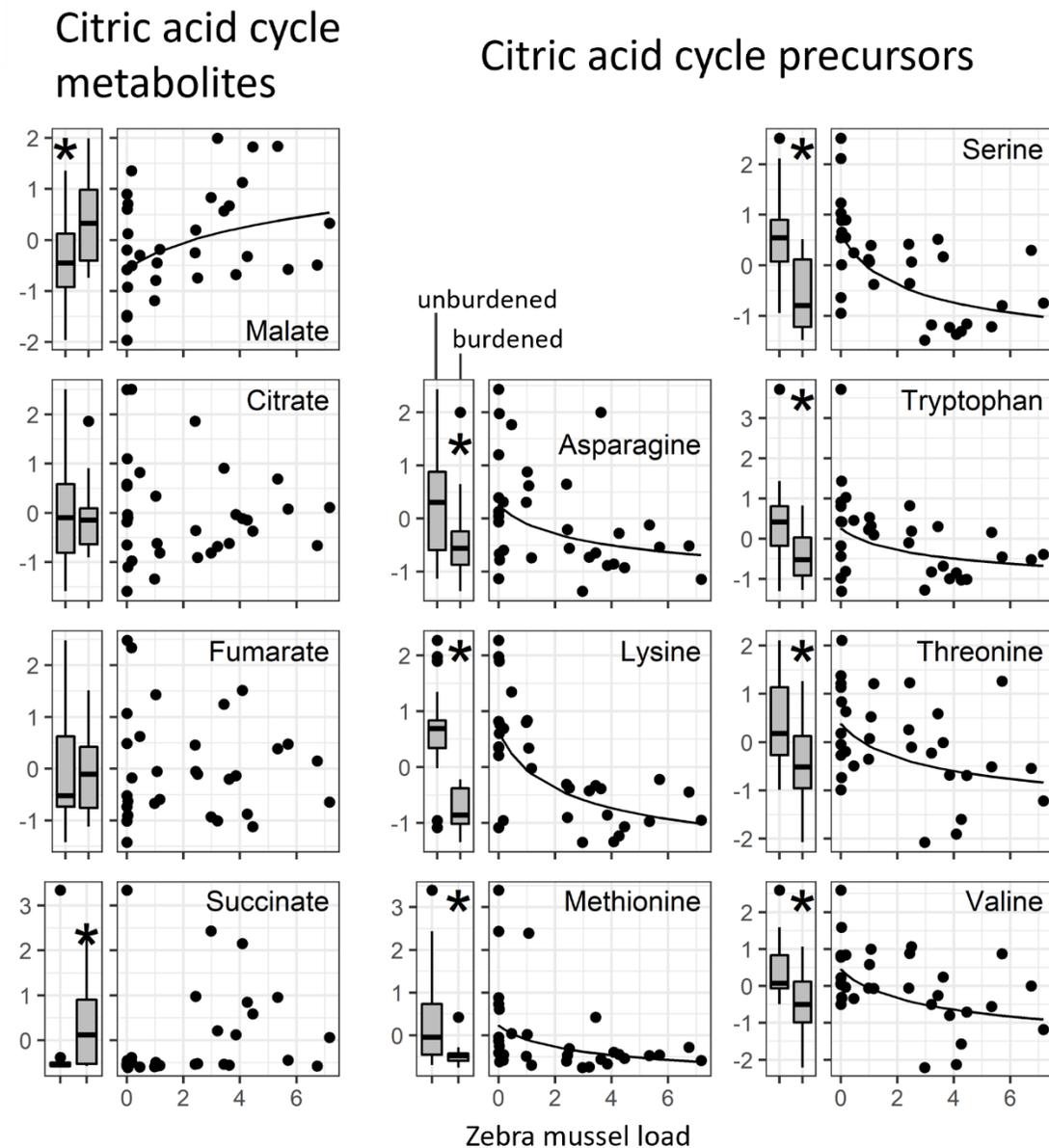
Zebra mussel load

1289

1290   Figure 4: Relative concentrations of metabolites associated with citric acid cycle and their relationship to

1291   zebra mussel load. Left column comprised of metabolites central to the citric acid cycle, middle and right

1292   columns comprised of metabolites that can supplement the citric acid cycle. Boxplots show metabolite

1293   relative concentrations grouped by burdened/unburdened status. *denote significant differences

1294   between groups. Scatterplots display the same data across the range of zebra mussel loads. Modeled

1295   lines are back-transformations of linear regressions fit to ln(metabolite + α) ~ ln(zebra mussel load) and

1296   are only intended to generally describe the relationship.

1297

1298

1299   **Figure 5**
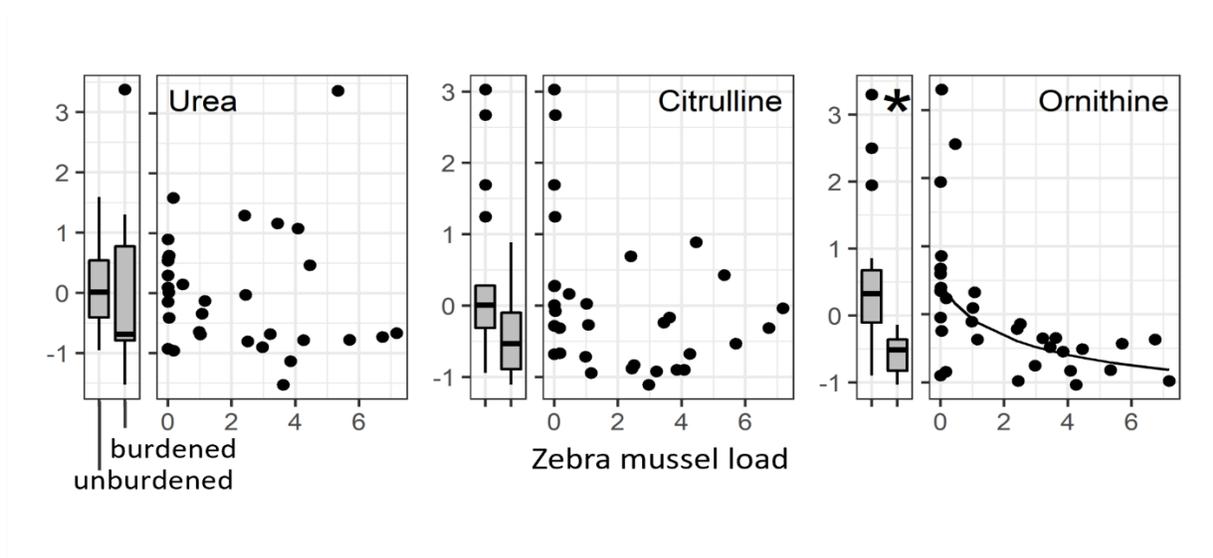
1300

1301   Figure 5: Relative concentrations of metabolites central to urea cycle across zebra mussel load levels.

1302   *denote metabolites that are significantly negatively correlated with zebra mussel load (p < 0.05).

1303   Modeled lines are back-transformations of linear regressions fit to ln(metabolite + α) ~ ln(zebra mussel

1304   load) and are only intended to generally describe the relationship.

1305

1306 **Supplementary Table 1:**

1307 Full list of identified metabolites and their association with zebra mussel burden (n = 99). Values

1308 correspond to mean peak height for that metabolite within unburdened/burdened group. All

1309 metabolites were independently standardized to a mean of zero (1 SD). P-values were calculated using a

1310 t-test between unburdened and burdened groups for each metabolite.

KEGG ID	Metabolite	Unburdened	Burdened	p-value
C00147	Adenine	-0.239	0.271	0.153
C00212	Adenosine	0.075	-0.085	0.658
C00041	Alanine	0.146	-0.166	0.388
C01904	Arabitol	0.066	-0.075	0.698
C00152	Asparagine	0.359	-0.407	0.028
C00049	Aspartic acid	0.184	-0.208	0.276
C00099	Beta alanine	0.002	-0.002	0.991
C01571	Capric acid	-0.074	0.084	0.664
C00090	Catechol	-0.057	0.065	0.736
C01971	Cellobiose	0.192	-0.218	0.253
C00187	Cholesterol	0.017	-0.019	0.921
C00158	Citric acid	0.06	-0.068	0.722
C00327	Citrulline	0.322	-0.365	0.051
C01420	Cystine	0.223	-0.253	0.184
	Deoxypentitol	-0.299	0.339	0.071
C06593	Epsilon caprolactam	0.003	-0.004	0.984
C02336	Fructose	-0.446	0.506	0.005
C00122	Fumaric acid	0.048	-0.055	0.777
C01235	Galactinol	-0.044	0.049	0.798
C00880	Galactonic acid	-0.223	0.253	0.184
C00984	Galactose	-0.524	0.594	0.001
C00800	Gluconic acid	-0.403	0.457	0.013
C00221	Glucose	-0.531	0.602	0.001
C00103	Glucose 1 phosphate	-0.32	0.363	0.052
C01172	Glucose 6 phosphate	0.098	-0.111	0.562
C00025	Glutamic acid	0.294	-0.333	0.077
C00064	Glutamine	0.398	-0.451	0.014
C00489	Glutaric acid	-0.186	0.211	0.269
C00258	Glyceric acid	-0.113	0.128	0.505
C00116	Glycerol	0.053	-0.06	0.754
C05401	Glycerol 3 galactoside	0.064	-0.072	0.707
C03189	Glycerol alpha phosphate	0.092	-0.105	0.587

C00037	Glycine	0.123	-0.139	0.468
C00387	Guanosine	0.232	-0.263	0.166
C00530	Hydroquinone	-0.167	0.19	0.322
C00294	Inosine	0.268	-0.304	0.107
C00407	Isoleucine	0.262	-0.296	0.117
	Isopentadecanoic acid	-0.183	0.208	0.277
C00639	Isothreonic acid	-0.314	0.356	0.057
C07064	Lactulose	-0.146	0.165	0.388
C00123	Leucine	0.148	-0.167	0.382
C00047	Lysine	0.658	-0.745	0
C00532	Lyxitol	0.025	-0.028	0.883
C00711	Malic acid	-0.373	0.423	0.022
C00208	Maltose	0.31	-0.351	0.061
C01835	Maltotriose	0.173	-0.196	0.306
C00392	Mannitol	-0.255	0.289	0.126
C08243	Melezitose	-0.16	0.181	0.344
C00073	Methionine	0.403	-0.457	0.013
C02989	Methionine sulfoxide	0.396	-0.449	0.014
	Methylhexose	-0.362	0.41	0.027
C00137	Myo inositol	0.227	-0.257	0.176
C00645	N acetylmannosamine	-0.159	0.181	0.345
C00253	Nicotinic acid	-0.015	0.017	0.931
C00756	Octanol	-0.208	0.235	0.216
C00077	Ornithine	0.516	-0.585	0.001
C01879	Oxoproline	0.243	-0.276	0.146
C00079	Phenylalanine	0.354	-0.402	0.03
	Pipecolinic acid	0.286	-0.324	0.085
C00148	Proline	0.114	-0.129	0.501
C02067	Pseudo uridine	-0.003	0.003	0.988
C00138	Putrescine	0.358	-0.406	0.028
C01108	Pyrogallol	-0.276	0.313	0.097
C00296	Quinic acid	-0.147	0.166	0.385
C00492	Raffinose	-0.304	0.345	0.066
C01685	Ribonic acid	-0.246	0.279	0.141
C00121	Ribose	-0.287	0.325	0.084
C00805	Salicylic acid	-0.015	0.017	0.929
C00065	Serine	0.55	-0.623	0
C00493	Shikimic acid	-0.045	0.051	0.793
C00042	Succinic acid	-0.306	0.347	0.064
C00089	Sucrose	0.032	-0.037	0.85
C00795	Tagatose	-0.355	0.403	0.03
C16884	Threitol	-0.161	0.183	0.34
C01620	Threonic acid	0.346	-0.392	0.035
C00188	Threonine	0.366	-0.414	0.025
C00214	Thymidine	0.07	-0.079	0.681

C01083	Trehalose	0.175	-0.199	0.299
C00078	Tryptophan	0.392	-0.444	0.016
C00082	Tyrosine	-0.136	0.154	0.423
C00106	Uracil	-0.37	0.419	0.023
C00086	Urea	0.035	-0.04	0.835
C00183	Valine	0.406	-0.461	0.012
C00181	Xylose	-0.061	0.069	0.721
C05437	Zymosterol	0.024	-0.028	0.886
C02814	1, 2, 4-benzenetriol	-0.26	0.294	0.119
C07326	1,5-anhydroglucitol	-0.108	0.122	0.525
C01885	1-monopalmitin	-0.036	0.04	0.834
D01947	1-monostearin	0.243	-0.275	0.147
C02721	2-aminobutyric acid	0.4	-0.454	0.013
	2-deoxytetronic acid	-0.009	0.011	0.956
	2-hydroxyvaleric acid	0.183	-0.208	0.277
C00322	2-ketoadipic acid	0.101	-0.114	0.552
	2 methylglyceric acid	-0.148	0.168	0.382
C01197	3 4 dihydroxycinnamic acid	0.144	-0.163	0.394
C05145	3 aminoisobutyric acid	-0.069	0.079	0.684
	5 hydroxynorvaline NIST	0.337	-0.382	0.04
	5 methoxytryptamine	0.387	-0.439	0.017
C08352	6 deoxyglucose	0.225	-0.255	0.179

1311

1312   **Chapter 3**

1313   **Changes in freshwater mussels (Order: Unionida) of Wisconsin in the past 50 years**

1314

1315   **Abstract:**

1316   Wisconsin, U.S.A., located primarily in the Upper Mississippi River drainage, is home to 50

1317   species of freshwater mussels. Over the past 200 years, they have experienced numerous threats from a

1318   rapidly growing human-dominated landscape resulting in nearly half of them now being endangered,

1319   threatened or whose conservation status is of special concern to the state. Systematic surveys have

1320   been conducted across the state semi-regularly since the 1970s; however, recent synthesis of these

1321   surveys and analysis of how species status may have changed has yet to be done. In this study, we used

1322   a paired-survey design to assess state-wide changes that have occurred to mussel populations over the

1323   past 50 years. We found evidence that population changes were variable among species as well as

1324   geographically distributed across the state. Our analysis suggests that species losses within the past half-

1325   century may be substantial, but we show that challenges associated with species detection, especially of

1326   uncommon species, makes accurate population assessment difficult.

1327 **Introduction:**

1328   Freshwater mussels (Order: Unionida) are one of the most endangered group of organisms

1329 worldwide (Lopes-Lima et al., 2018; Williams et al., 1993). They have faced numerous threats from

1330 decreased water quality (Ellis, 1936; Gillis, 2011), overexploitation (Anthony & Downing, 2001; Kunz,

1331 1898), loss of habitat, loss of fish hosts, fragmentation of populations from impoundments (Modesto et

1332 al., 2018; Ortmann, 1918; Sousa et al., 2020; Vaughn & Taylor, 1999), and competition and direct

1333 negative effects from invasive species (Ricciardi et al., 1996; Strayer, 1999). Thorough monitoring of

1334 their populations is challenging, but needed for effective conservation management. The numerous

1335 ecosystem services mussels provide should underscore the importance of their preservation (Vaughn &

1336 Hakenkamp, 2001).

1337   There are approximately 300 species of freshwater mussels native to North America. Wisconsin,

1338 situated within the Upper Mississippi River drainage, is home to 50 of these species. Four of Wisconsin's

1339 species are federally-listed as endangered, 11 are state-listed as endangered with an additional 13

1340 whose conservation status is threatened or considered to be of special concern (WI-DNR 2021). As is

1341 common in North America, Wisconsin's species are almost entirely of the Unionidae family, with one

1342 member of the Margaritiferidae family (*Cumberlandia monodonta*).

1343   Concerns regarding conservation of freshwater mussels in the region date back to the turn of

1344 the 20th century (Kunz, 1898; Coker, 1919). In the past 200 years, Wisconsin's rivers have experienced

1345 substantial increases in sediment loads as its forests were almost completely clear cut and its prairies

1346 were converted to cultivated land following settlement by Euro-Americans (Fitzpatrick & Knox, 2000;

1347 Knox, 2006). Nearly 4000 dams of varying sizes have been constructed, sundering Wisconsin's rivers,

1348 modifying local habitats, and limiting host fish movement. Overexploitation from the pearl and button

1349 industries from the late 1800s to the 1950s was widespread throughout the region and dramatic

1350 depletions of mussels beds were commonplace (Coker, 1919). The effects of the combined stressors of

1351   decreased water quality, depleted numbers, and limited population connectivity are still apparent nearly

1352   a century later (Anthony & Downing, 2001).

1353   It was not until the 1970's that any state-wide assessment of Wisconsin's native mussel

1354   community occurred, when one retired scientist took it upon himself to inventory mussels from over

1355   600 sites across the entire state (Mathiak, 1979). Since then, there has been greater effort to monitor

1356   Wisconsin's mussel communities: approximately 6,500 additional surveys have been conducted

1357   throughout the state, with nearly 800 standardized surveys having been conducted since 2000 primarily

1358   by Wisconsin's Department of Natural Resources.

1359   Prior to the first surveys of Wisconsin's waters, losses due to intense harvesting for the button

1360   industry, decreased water quality, and loss of habitat from dams likely had a strong influence on

1361   Wisconsin's mussels (Anthony & Downing, 2001; Coker, 1919), but while many threats to mussels have

1362   remained, new threats have also emerged. Mussels still face a fragmented landscape, diminished

1363   population sizes, in addition to an increasing number of invasive species—most notably, the zebra

1364   mussel (*Dreissena polymorpha*), which has spread to nearly 300 lakes and rivers throughout the state

1365   within the 30 years since its arrival (WI-DNR, 2022). Effective conservation of these species requires

1366   accurate up-to-date information as well as analysis of long-term dynamics of the communities. These

1367   assessments require substantial effort over many years as many species are long-lived (thus seeing

1368   changes in populations requires comparably long sampling records), are highly spatially heterogeneous,

1369   and often exist in environments difficult to sample. A state-wide assessment of how Wisconsin's mussel

1370   communities have changed since comprehensive surveys began 50 years ago has yet to be done prior to

1371   this study.

1372   In this study, we assessed whether changes have occurred in the freshwater mussel

1373   communities of Wisconsin over the past 50 years. We compared presence/absence at sites located

1374   across the state at which comparable historical (pre-2000) and recent surveys (post-2000) had been

1375 conducted. We assessed whether population changes were associated with species commonness as well

1376 as whether changes in communities were different across major watersheds of Wisconsin.

1377

1378 **Methods:**

1379 *Study Area*

1380   Wisconsin, USA is a water-rich region with 32,000 miles of perennially flowing water and over

1381 10,000 lakes. Most of the state is located in the Upper Mississippi River drainage, with the rest draining

1382 to the Great Lakes. In general, the northern half of the state is characterized by mixed secondary forests

1383 with substantial lake and wetland features and little, but growing exurban development. The southern

1384 half of Wisconsin is an agriculturally dominated landscape with suburban and urban development

1385 (Carpenter et al., 2007).

1386

1387 *Data Overview*

1388   All data were provided by the Department of Natural Resources of Wisconsin (WI-DNR). This

1389 includes surveys conducted by the WI-DNR, public and private researchers, contracted organizations, as

1390 well as specimens collected by community members. From these surveys, 50 species and over 40,000

1391 individuals have been recorded in Wisconsin's inland waters. Some of Wisconsin's first surveys date back

1392 to the late 19th century, with significant contributions in the 1970s (Mathiak, 1979), 1980s, 1990s, and

1393 late 2010s.

1394   We were interested in assessing whether changes in the mussel communities were detectable

1395 within the past 50 years across Wisconsin. In order to do this, we separated the data into surveys

1396 conducted prior to the year 2000 (hereafter "historical surveys"), for which 6503 records exist, and

1397 surveys conducted between 2000-2020 (hereafter "recent surveys"), for which 777 records exist (Figure

1398 1). The year 2000 was chosen as a cutoff because there was a natural break in the data for state-wide

1399   number of surveys and it also provided sufficient sample size in number of recent surveys in comparable

1400   locations to historical surveys. We identified all historical surveys that were within 500 meters of recent

1401   surveys and had specified that they took place in the same river, then joined these historic records to

1402   recent records. We omitted surveys that were conducted using methodologies that were not

1403   appropriately comparable (filtering process is described below) resulting in 149 sites (hereafter "paired"

1404   sites) in which recent surveys were comparable to historical records.

1405

1406   *Data Management*

1407   *Filtering methodologies*

1408   Prior to identifying suitable surveys for our paired survey analyses, we omitted surveys that

1409   were described as shoreline surveys, surveys that targeted single species, and those that used a kicknet;

1410   we also did not consider observations of specimens reported through WI-DNR's Citizen-based

1411   Monitoring program to be surveys because they are rarely standardized and often include one or only a

1412   few specimens per submission. Unidentified mussels, shell collections not containing live mussels, and

1413   specimens only identified as "juvenile unionid" were omitted from all analyses. All data were

1414   transformed to presence/absence to best standardize across different sampling techniques. It is

1415   important to note that surveys in both historical and recent time periods were not randomly selected

1416   across Wisconsin's waters and that multiple survey methodologies were used (supplemental figure 1).

1417   Apart from the survey methodologies described above, we did not omit any other survey types

1418   within the historical data; however, we omitted surveys that were quadrat-based from recent methods

1419   as this methodology is not designed to be sensitive to detecting uncommon species and was associated

1420   with a high rate of species suspected to be missing from sites (supplemental table 1). We did this to

1421   minimize biases in estimates of losses while maintaining as large of a sample size as possible. As we

1422   were more interested in assessing whether noticeable losses had occurred, we prioritized only including

1423 survey types that were sensitive to detecting uncommon species in recent surveys. We acknowledge

1424 that this approach likely inflates estimates of newly found species at sites and caution the reader to

1425 keep this bias in mind when comparing estimates of missing and newly found species.

1426 *Assessment of species status*

1427 Species assessments were based on changes in their presence/absence at each paired-survey

1428 location. Species were considered "retained" if they were recorded both historically and recently at a

1429 site; species that were present historically and not resampled between 2000-2020 were classified as

1430 "missing", while species that were not recorded historically but were recorded between 2000-2020

1431 were considered "newly found".

1432 We examined whether regional patterns were associated with changes in species

1433 presence/absence. To do this, we calculated changes among species within Wisconsin's major

1434 watersheds (hydrologic unit code level 6). We estimated changes in species commonness within

1435 watersheds by calculating the difference in the number of sites in which a species was recently present

1436 and the number of sites in which it was historically present divided by the total number of paired sites

1437 within that drainage basin. We only calculated these changes for the ten watersheds with 5 or more

1438 paired surveys. For plotting purposes only, in figure 5, species that were only recently found in a

1439 watershed were assigned a value of one for change in proportion. To summarize general patterns of loss

1440 at the watershed scale we counted the total number of species whose change in proportion within that

1441 watershed was less than zero; similarly, we counted the total number of species whose change in

1442 proportion within the watershed was greater than zero to characterize the general patterns of apparent

1443 gains across watersheds. We chose to report gains and losses independently as compared to their

1444 difference to remove the effect of differences in methodologies between historical and recent surveys in

1445 different locations as well as to be able to differentiate watersheds with only a few species changing in

1446 commonness from watersheds with many increasing and many decreasing in commonness.

1447   All data analyses were done using R statistical software (R Core Team, 2020). Non-metric

1448   multidimensional scaling of mussel assemblages was calculated using individual survey

1449   presence/absence data using all historical and recent surveys (excluding kicknet, shoreline, and

1450   community member collected observations). The scaling was calculated using Chao dissimilarity

1451   distances within the vegan package (Hsieh et al., 2016; Oksanen et al., 2020; R Core Team, 2020).

1452

1453   **Results:**

1454   Wisconsin is home to 50 species of freshwater mussels, 24 of which are state-listed as

1455   endangered (n = 11), threatened (n = 8), or whose status is of special concern (n = 5). Species-rich

1456   communities are distributed across much of the state. However, endangered, threatened, and species of

1457   special concern are disproportionately found in western regions of the state that are more closely

1458   connected to the Upper Mississippi River (Figure 2).

1459   Forty-three species were present among the 149 paired survey sites. Of the seven that were not

1460   present among the paired survey sites, three were species of special concern (*Elliptio complanata*,

1461   *Megalonaias nervosa*, *Utterbackiana suborbiculata*), and one is endangered (*Arcidens confragosus*).

1462   Twenty-one species (10 of listed status) were found in more sites recently compared to historical

1463   surveys while 13 (seven listed) species were found in fewer sites recently (Figure 3). There was no

1464   relationship between the direction of change in the number of sites in which a species was present and

1465   species commonness as assessed by logistic regression of direction of change in commonness by rank of

1466   historical number of sites at which a species was present (p = 0.12; supplemental figure 2). The rate at

1467   which species were resampled at sites in which they were historically present was variable between

1468   species and was positively associated with species commonness as assessed by linear regression of rank

1469   resampling rate by rank commonness across all recent surveys (p < 0.01; Figure 4). Nine species were

1470   resampled at a rate of 0.75 or above, 17 species were resampled at a rate between 0.50-0.75, seven

1471   species were resampled at a rate between 0.25-0.50, and seven species were resampled at a rate below

1472   0.25 with five of these species having not been retained in any recent surveys within the paired sites.

1473   The survey methodology used for recent surveys had a significant effect on whether a species was

1474   retained, newly found, or missing at that site as assessed by a Chi-squared test of recent methodology

1475   and retained/newly found/missing status across paired sites (p < 0.01, Table 1).

1476   Species richness within paired sites was variable among watersheds, ranging from 14 in central

1477   Wisconsin and Milwaukee watersheds to 38 in the St. Croix watershed (calculated only for watersheds

1478   with 5 or more paired surveys). Species-rich watersheds were more likely to contain uncommon species

1479   as assessed by linear regression of the rank of species commonness within paired recent surveys by

1480   watershed species richness (p <0.01; supplementary figure 3; calculated using only watersheds with 5 or

1481   more paired surveys). Across the ten watersheds with five or more paired survey sites, there were 168

1482   instances of species being retained at any site within a watershed in which they were historically

1483   present. Across the same ten watersheds, there were 20 instances in which species were not observed

1484   at any paired survey sites within a watershed in which they were historically present and 34 instances of

1485   species being newly found in recent surveys within watersheds in which they weren't documented at

1486   paired survey sites historically (Figure 5).

1487   The number of species found in more sites within a watershed recently ranged from 3 (central

1488   Wisconsin River watershed) to 24 (St. Croix watershed) with a median of 8 and the number of species

1489   found in fewer sites within a watershed recently as compared to historically ranged from 6 (Wolf River

1490   watershed) to 12 (lower Chippewa River watershed) with a median of 8 (Figure 6). The proportion of

1491   species found in more sites recently within a watershed ranged from 0.21 (central Wisconsin watershed)

1492   to 0.71 (Wolf River watershed with a median of 0.40 and the proportion of species found in fewer sites

1493   within a watershed ranged from 0.21 (Wolf River watershed) to 0.57 (central Wisconsin and Milwaukee

1494   River watersheds) with a median of 0.46. Watersheds that had a lower total species richness had a

1495   higher proportion of species decreasing in commonness ($p < 0.01$) as assessed by linear regression of the

1496   proportion species less common among recent surveys by watershed species richness (supplemental

1497   figure 4).

1498

1499   **Discussion:**

1500   Wisconsin is home to a diverse community of freshwater mussels. Over the past 50 years,

1501   thousands of surveys have been conducted to document the diversity present across the state as well as

1502   provide opportunities to assess community changes that may be occurring. Recent surveys suggest that

1503   many species have likely experienced substantial losses within the past 50 years and that these losses

1504   are unevenly distributed among species as well as across Wisconsin's major watersheds.

1505   The number of sites in which a species was missing or newly found was often substantial relative

1506   to the number of sites at which it was retained. Also, for most species, the number of sites at which the

1507   species was newly found was higher than the number of sites in which it was missing (Figure 3). An

1508   optimistic interpretation of this could be that species ranges may be increasing and highly dynamic,

1509   where losses at one site are offset by gains elsewhere and to some extent, this may be true. However, it

1510   is likely that many instances of species being only recently found at sites were missed in historical

1511   surveys and do not represent true gains at a site as it is much less likely that mussels are highly spatially

1512   dynamic in Wisconsin's rivers given their sedentary lifestyle and their dependence on the movement of

1513   their host fish to access new watersheds—an opportunity that Wisconsin's nearly 4000 dams likely limit.

1514   However, the magnitude of newly found species indicate that many of these communities are more

1515   species rich than historical surveys suggested. Although, instances of newly found species were

1516   disproportionately common species, evidence that uncommon species are not more common than

1517   previously thought.

1518   Due to the nature of the paired survey data, a more informative analysis may be to examine

1519   whether species were resampled at sites in which they were known to be historically present. The

1520   proportion at which a species was resampled in recent surveys was variable among species and

1521   positively associated with species commonness (Figure 4). It is often the case that mussel beds are

1522   dominated by only a few common species and many species make up only a fraction of the total number

1523   of individuals (Haag, 2012). It is unlikely that all species present at a site will be found in any one survey

1524   and uncommon species are inherently less likely to be detected. The positive relationship between

1525   commonness of species and the proportion of sites in which a species was resampled is very likely

1526   affected by this regardless of whether population changes are occurring at the site level. However, even

1527   if no losses were occurring, we would expect that species that are similarly common would be

1528   resampled at a similar rate to one another and that does not appear to be the case. We see a wide

1529   range in the proportion of sites in which a species was resampled for both common and uncommon

1530   species suggesting that low resampling rates are not solely a product of detection sensitivity in recent

1531   surveys, but are evidence of diminished population numbers at the site scale. Notably, species that are

1532   often dominant species at a site such as *Pygandon grandis* and *Strophitus undulatus* being found in less

1533   than half of the sites in which it was historically recorded is eye-catching and worrisome.

1534   There is an added level of difficulty when comparing changes in populations when different

1535   methodologies are conducted at different times across different sites. Methodology can have a strong

1536   effect on likelihood of detecting species, esepcially uncommon species (Vaughn et al., 1997). As

1537   expected, in this study, detection was associated with survey methodology. For example, timed surveys,

1538   a common qualitative survey technique, designed to detect species uncommon at a site, reported both

1539   the highest proportion of newly found species per survey, as well as the highest proportion of newly

1540   found species relative to missing species (Table 1). In contrast, methodologies that are quantitative such

1541   as random point sampling, or semi-quantitative such as collections of groups of 20 or 40 individuals had

1542   lower proportions of newly found species per survey and newly found species relative to missing

1543   species. A strength of assessing resampling rate (as compared to total change in number sites in which a

1544   species is present) is it diminishes the effect of different pairings of survey methodologies across sites by

1545   only assessing changes in species that were detected in historical surveys regardless of the detection

1546   sensitivity of the historical survey. The proportion of species missing that is a product of survey

1547   detection compared to being truly lost from a site can only be relatively assessed and is beyond the

1548   scope of this study. However, it is important to note that even at sites where a timed survey was

1549   conducted, an alarmingly low resampling rate of 65% of species were found again that were known to

1550   be historically present. Determining whether these high numbers of missing species are truly lost at a

1551   given site should be a priority as it is either indicative of the need to conduct multiple qualitative surveys

1552   at a site to fully capture the community composition or that these losses may be better described as

1553   catastrophic.

1554   The major watersheds of Wisconsin were characterized by different mussel communities as well

1555   different rates of community change. Uncommon species were disproportionately present in species-

1556   rich watershed such as the Saint Croix and Lower Chippewa as well as the Wolf River as compared to

1557   species-poor watersheds, however, uncommon/rare species were distributed across most of Wisconsin.

1558   Changes in species populations were often variable across major watersheds in Wisconsin, with species-

1559   poor watersheds having a higher proportion of species that are less common across recent surveys than

1560   species-rich watersheds. To some extent, these differences may be a product of different methodologies

1561   in recent surveys being used in different proportions across major watersheds. But it may also indicate

1562   that communities may be changing at broad regional patterns, and species seem to be doing worse in

1563   watersheds that historically may pose more challenging of habitats for species (i.e., species-poor

1564   watersheds). Thus, underscoring the need of state-wide monitoring efforts that include species-poor

1565   watersheds to accurately assess changes in vulnerable populations or risk underestimating losses at the

1566   state scale.

1567   Assessing changes in mussel populations is difficult, especially when survey methods vary

1568   between time periods. The magnitude and widespread nature of potential losses found in this study are

1569   alarming, but not unexpected as the patterns of losses found in this analysis are consistent with findings

1570   of changes in mussel communities from other studies in the region. A study of Illinois mussel

1571   communities found that species richness in rivers across the state was significantly lower than what

1572   would be expected were the rivers under natural conditions with up to nearly half of the species missing

1573   from river segments with the most impaired mussel communities (Cao et al., 2017). A study of mussel

1574   communities across a land-use gradient in Minnesota found that mussel abundances were significantly

1575   negatively associated with agriculture land use (a common land use especially in southern Wisconsin)

1576   (Hornbach et al., 2019). Even in the federally protected St. Croix river on the border between Minnesota

1577   and Wisconsin, mussel communities have reported losses in species richness and abundance over a

1578   recent 20-year period (Hornbach et al., 2018).

1579   Mussel communities are likely changing across most of Wisconsin, and the scale of work needed

1580   to monitor even only communities that contain vulnerable species is immense. Across all recent survey

1581   sites, nearly half (332) contain species that are endangered, threatened, or of special concern, with 89 of

1582   the sites containing endangered species. Although these vulnerable species are disproportionately

1583   found in western regions of the state, populations of vulnerable species are found throughout the entire

1584   state. As this study has shown that there are likely regional patterns to mussel loss, monitoring mussels

1585   throughout their range is necessary for accurate assessment of species status. Developing a monitoring

1586   program capable of detecting population changes—even if focused on only the most vulnerable

1587   species—would be a challenge, especially at the state-wide scale. Current methodologies that

1588   incorporate both qualitative and quantitative approaches are an effective strategy for detecting changes

1589   to species as well as communities. In addition, the Citizen-Based Monitoring program developed and

1590   managed by the Wisconsin Department of Natural Resources has demonstrated its value in identifying

1591   important sites for future monitoring efforts through 169 observations (as of 2021) of endangered,

1592   threatened, or species of special concern around the state.

1593   Unfortunately, despite the great effort by state biologists and contributing community

1594   members, much remains uncertain about the current status and trajectories of freshwater mussels in

1595   Wisconsin. There are six species that are currently listed as endangered, threatened or of special

1596   concern who were not found at any of the paired survey sites in this analysis (*Elliptio complanata*,

1597   *Megalonaias nervosa*, *Utterbackiana suborbiculata*, *Arcidens confragosus*, *Elliptio crassidens*, and

1598   *Reginaia ebunus*, many of these are only found in large rivers) and this analysis cannot provide any

1599   assessment of their population trajectories. There are numerous species whose current conservation

1600   status may need to be reassessed as the number of sites from which they were missing are substantial

1601   relative to the total number of sites in which they have recently been found. For eight species in the

1602   most severe of cases, the number of sites from which they were missing equates to nearly half or more

1603   of the total number of known sites in which they have recently been found (*Cyclonaias nodulata*,

1604   *Obovaria olivaria*, *Plethobasus cyphus*, *Potamilus ohiensis*, *Quadrula fragosa*, *Quadrula quadrula*,

1605   *Simpsonaias ambigua*, *Truncilla donaciformis*). The task of continued monitoring of currently known

1606   sites of vulnerable populations, re-surveying sites in which vulnerable populations were historically

1607   present that have not been visited in at least 20 years, managing community-based observations, as well

1608   as developing up-to-date status assessments of species is no small task and it requires more attention

1609   and support than is currently be awarded to Wisconsin's freshwater mussel communities.

1610   Freshwater mussels in Wisconsin have faced an intense and dynamic landscape of threats in the

1611   past 200 years and major losses very likely occurred prior to any systematic surveys. This analysis

1612   suggests that mussel communities of Wisconsin have continued to experience substantial losses over

1613   the past 50 years. These losses are likely variable among species as well as across regions of the state.

1614   Increased efforts to document the current distributions and status of populations, especially rare and

1615   vulnerable species, would greatly support effective conservation of Wisconsin's freshwater mussels.

1616

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1683

1684   **Table 1**

1685   Table of species survey status (e.g., "missing", "retained", "newly found") by survey methodology of

1686   recent survey in paired surveys (also including "area quadrat" although not included in paired analysis).

1687   Proportion species resampled calculated as 1 – (n species missing/n species historically present),

1688   proportion species newly found calculated as n species newly found/n species historically present. *Area

1689   quadrat surveys was not included in count totals or calculation of means

1690

Methodology of recent survey at paired sites	number surveys in paired data	number species newly found	number species missing	number species retained	proportion species retained	proportion species newly found
Area quadrat*	39	65	223	220	0.497	0.147
Groups of 20	13	40	29	111	0.793	0.286
Groups of 40	14	22	43	22	0.338	0.338
Inventory	8	22	25	30	0.545	0.400
Other	1	2	4	9	0.692	0.154
Random point	11	19	33	29	0.468	0.306
Timed	40	178	111	203	0.646	0.567
Transect	1	-	7	20	0.741	-
Undocumented	61	203	161	279	0.634	0.461
Total	149	486	413	703		
Mean					0.607	0.359

1691

1692

1693   **Figure 1**
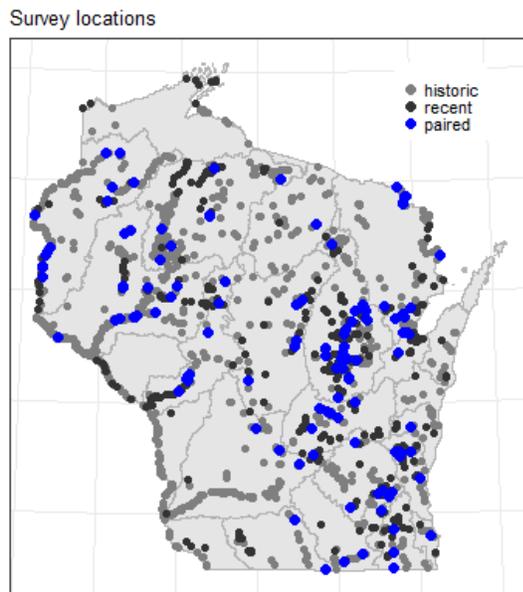
Survey locations

1694

1695   Figure 1: Point-survey locations for all WI-DNR records of historical (pre-2000, grey points n = 6503),

1696   recent (2000-2020, black points n = 777) surveys. Blue points denote locations of paired-surveys (n =

1697   149).

1698

1699   **Figure 2**
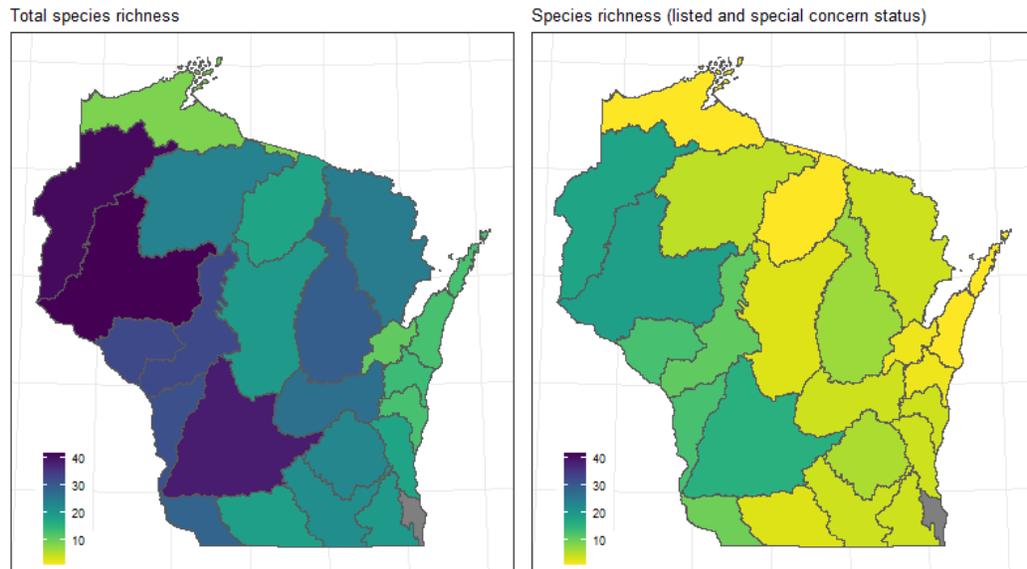
1700

1701   Figure 2: Total species richness by major watersheds (left) and species richness of state-listed and

1702   species of special concern (right). Species richness was calculated across all historical and recent surveys.

1703   Color scales are consistent between panels.

1704
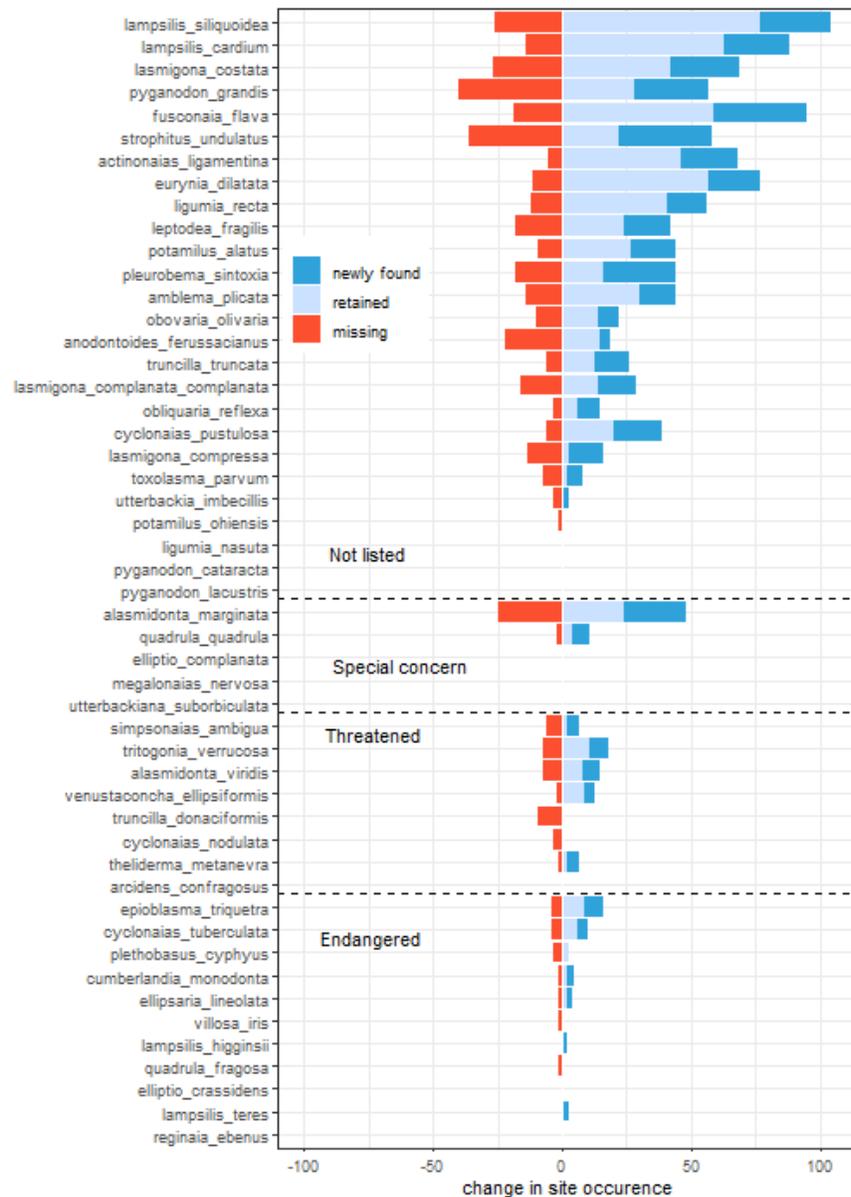
Figure 3

1706
1707
1708 Figure 3: Changes in number of sites in which a species was found across all paired sites (total number

1709 of paired sites = 149) grouped by state conservation status. Species that were present historically at a

1710 site and not resampled were considered missing, species that were present in both time periods were

1711 considered retained, while species that were only present in recent surveys were considered newly

1712 found. Species are ordered within each group by number of sites in which they were present historically.

1713   **Figure 4**
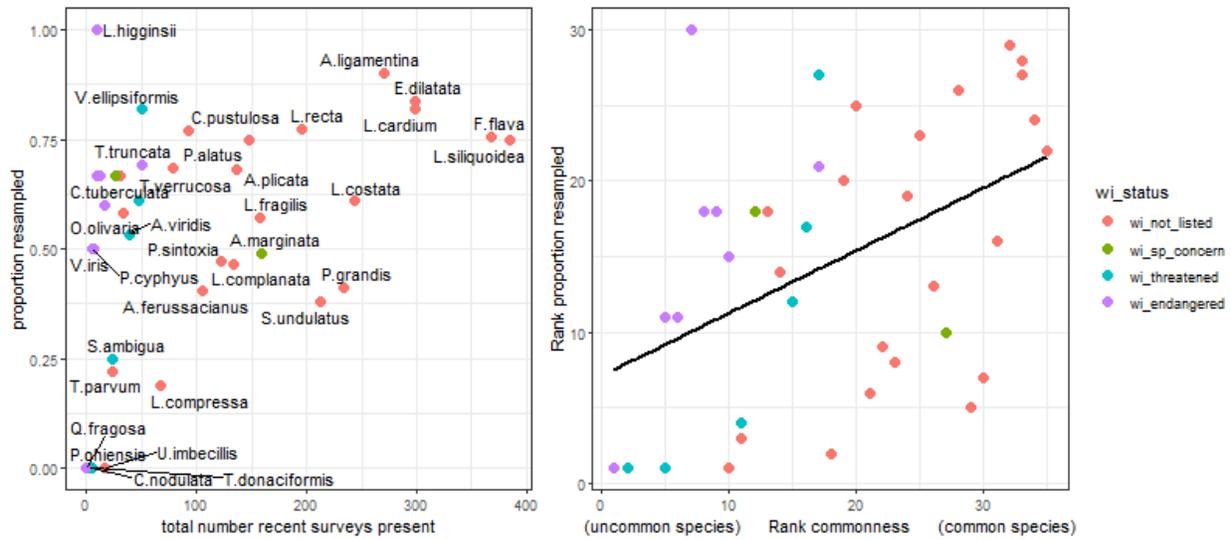
1714

1715   Figure 4: Rate of resampling for species by species commonness (left) and non-parametric regression of

1716   relationship (right). Proportion resampled calculated as 1- (n instances missing where historically

1717   present/historical abundance), total number of recent surveys is calculated from all recent surveys (not

1718   just paired sites). Linear regression of ranked proportion resampled by rank commonness suggests

1719   positive relationship ($p < 0.01$).

1720 **Figure 5**

1721
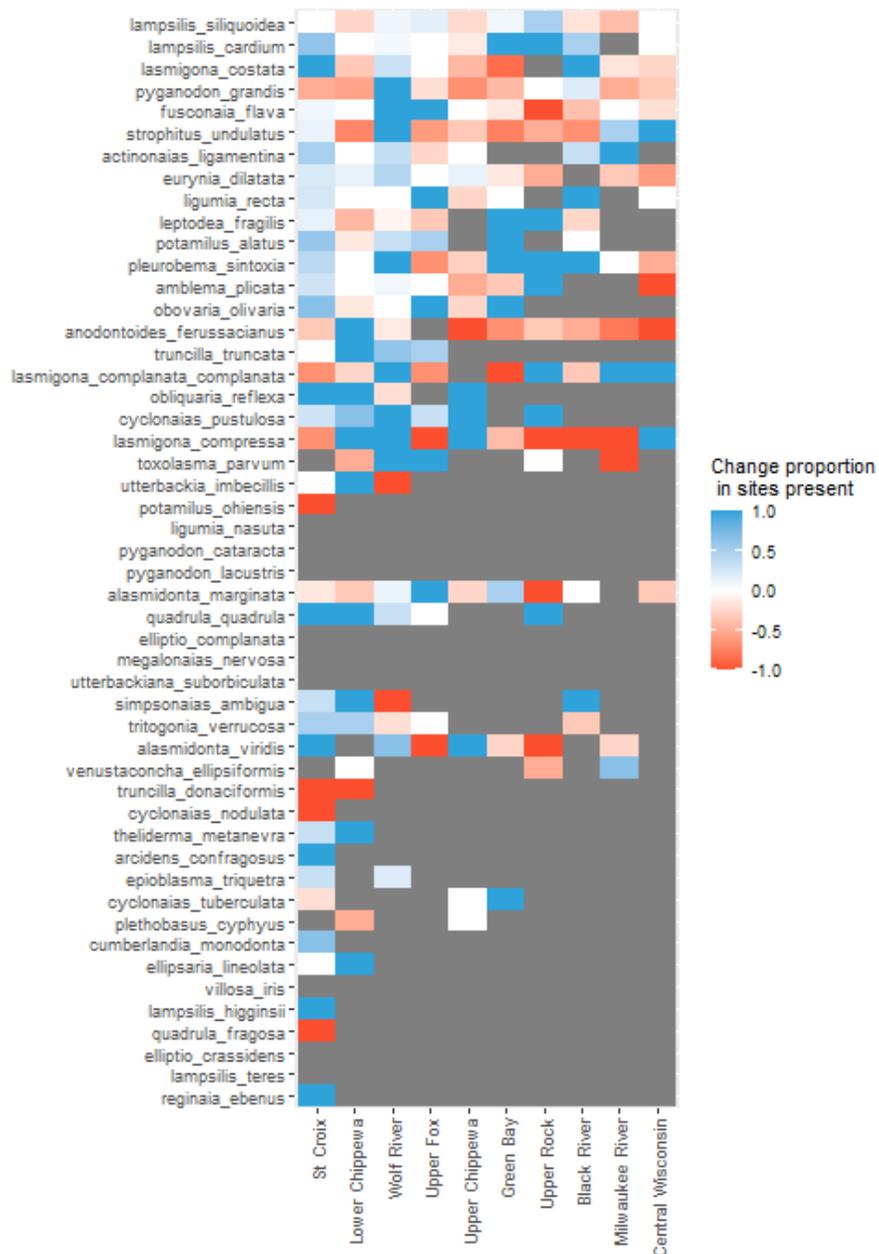
1722
1723
1724 Figure 5: Heatmap of change in species commonness by watershed. Change in proportion was

1725 calculated as follows: (n sites recently present – n sites historically present) / n sites historically present,

1726 so that 0 (white) represents a species that present in the same number of sites in both time periods, -1

1727 represents a species that was historically present in at least one survey, but not found in any recent

1728   surveys, and 1 represents a species that was found in twice as many sites recently compared to

1729   historically. Species that were only recently found in a watershed were assigned a 1 and species whose

1730   change in proportion was greater than 1 (n = 8) were assigned a 1 so as not to skew the color ramp.

1731   Species are in the same order as in figure 2, watersheds are arranged from highest total species richness

1732   at left to lowest.

1733

1734   **Figure 6**
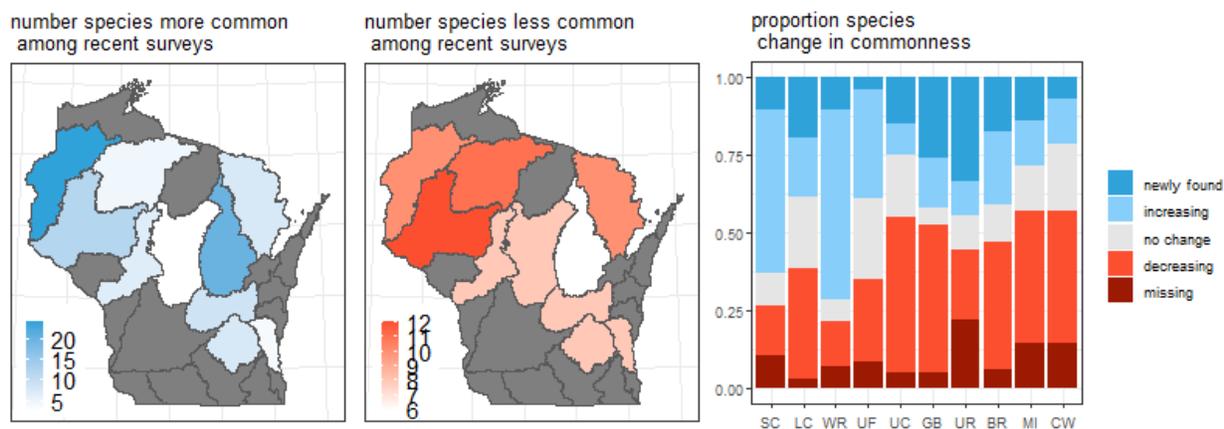
1735

1736

1737   Figure 6: Changes in species commonness at paired survey locations across watersheds. Note that color

1738   scales have different ranges between left and middle panels). Species that were found in at least one

1739   historical survey within a watershed and not in any recent surveys were considered "missing", species

1740   that were found in fewer surveys recently than historically were considered "decreasing", species who

1741   were found in the same number of sites recently as historically were considered "no change", species

1742   who were present in both time periods, but present in more sites recently than historically were

1743   considered "increasing", while species who were only found recently were considered "newly found".

1744   Watersheds are arranged from highest total species richness to lowest. Number of paired surveys by

1745   watershed are as follows: Saint Croix: 11, Lower Chippewa: 17, Wolf River: 38, Upper Fox: 8, Upper

1746   Chippewa: 9, Green Bay: 17, Upper Rock: 6, Black River: 10, Milwaukee River: 8, Central Wisconsin: 6.

1747

1748   **Supplementary Table 1**

| Species | Wisconsin status | Total observations in recent surveys | Paired surveys (n=149) | | | |
			n sites retained	n sites newly found	n sites missing	proportion resampled
Lampsilis siliquoidea	not listed	385	77	27	26	0.748
Lampsilis cardium	not listed	299	63	25	14	0.818
lasmigona costata	not listed	243	42	27	27	0.609
Pyganodon grandis	not listed	234	28	29	40	0.412
Fusconaia flava	not listed	368	59	36	19	0.756
Strophitus undulatus	not listed	212	22	36	36	0.379
Actinonaias ligamentina	not listed	271	46	22	5	0.902
Eurynia dilatata	not listed	299	57	20	11	0.838
Ligumia recta	not listed	195	41	15	12	0.774
Leptodea fragilis	not listed	158	24	18	18	0.571
Potamilus alatus	not listed	148	27	17	9	0.750
Pleurobema sintoxia	not listed	123	16	28	18	0.471
Amblema plicata	not listed	137	30	14	14	0.682
Obovaria olivaria	not listed	33	14	8	10	0.583
Anodontoides ferussacianus	not listed	105	15	4	22	0.405
Truncilla truncata	not listed	78	13	13	6	0.684
Lasmigona complanata complanata	not listed	134	14	15	16	0.467
Obliquaria reflexa	not listed	30	6	9	3	0.667
Cyclonaias pustulosa	not listed	92	20	19	6	0.769
Lasmigona compressa	not listed	67	3	13	13	0.188
Toxolasma parvum	not listed	24	2	6	7	0.222
Utterbackia imbecillis	not listed	16	0	3	3	0.000
Potamilus ohiensis	not listed	2	0	0	1	0.000
Ligumia nasuta	not listed	0	0	0	0	NA
Pyganodon cataracta	not listed	0	0	0	0	NA
Pyganodon lacustris	not listed	3	0	0	0	NA
Alasmidonta marginata	special concern	159	24	24	25	0.490
Quadrula quadrula	special concern	27	4	7	2	0.667
Elliptio complanata	special concern	6	0	0	0	NA

96

Megalonaias nervosa	special concern	0	0	0	0	NA
Utterbackiana suborbiculata	special concern	0	0	0	0	NA
Simpsonaias ambigua	threatened	24	2	5	6	0.250
Tritogonia verrucosa	threatened	47	11	7	7	0.611
Alasmidonta viridis	threatened	39	8	7	7	0.533
Venustaconcha ellipsiformis	threatened	51	9	4	2	0.818
Truncilla donaciformis	threatened	5	0	1	9	0.000
Cyclonaias nodulata	threatened	2	0	0	3	0.000
Theliderma metanevra	threatened	12	2	5	1	0.667
Arcidens confragosus	threatened	3	0	1	0	NA
Epioblasma triquetra	endangered	51	9	7	4	0.692
Cyclonaias tuberculata	endangered	16	6	4	4	0.600
Plethobasus cyphyus	endangered	6	3	0	3	0.500
Cumberlandia monodonta	endangered	12	2	3	1	0.667
Ellipsaria lineolata	endangered	10	2	2	1	0.667
Villosa iris	endangered	5	1	0	1	0.500
Lampsilis higginsii	endangered	9	1	1	0	1.000
Quadrula fragosa	endangered	0	0	0	1	0.000
Elliptio crassidens	endangered	0	0	0	0	NA
Lampsilis teres	endangered	4	0	3	0	NA
Reginaia ebenus	endangered	2	0	1	0	NA

1749

1750 **Supplementary Figure 1**
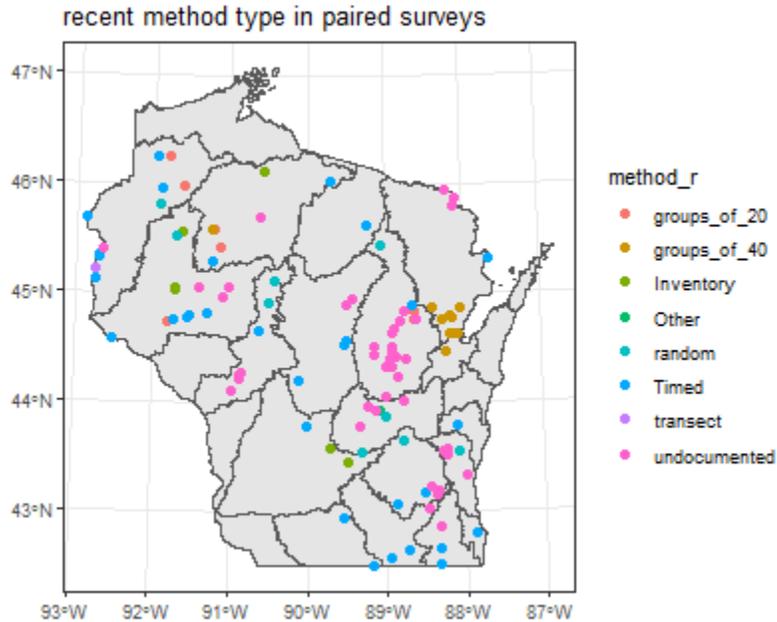
1751

1752 Supplementary Figure 1: Recent survey methodology in paired surveys. Not all categories are entirely

1753 internally consistent (e.g., methodology for "random" surveys often depends on river size, "Timed" may

1754 not be a similar duration, and "undocumented" likely contains multiple survey techniques). General

1755 characteristics of the most common types of surveys are as follows. "Groups of 20/40": group collection

1756 of a predefined number of individuals, collection stops when no new species were collected in a new

1757 group. "Inventory" surveys focused on richness, "Random" is a predefined grid projected over river

1758 within which quadrats were randomly sampled, total number of samples differed with stream size.

1759 "Timed": Predefined search time intervals, focusing on richness and rare species. "Transects":

1760 Predefined length of line where all mussels within ~1m from line are counted.

1761   **Supplementary Figure 2**
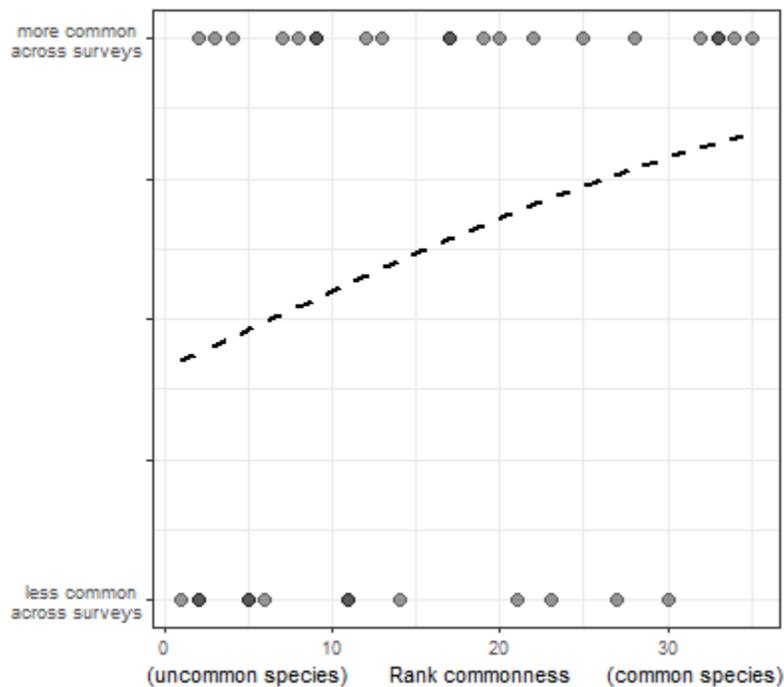
1762

1763   Supplementary figure 2: Logistic regression of change in commonness (less/more) as compared to

1764   historical commonness by rank abundance. Species were considered "net more common across surveys"

1765   if they were found in more surveys recently than historically, or "net less common across surveys" if

1766   they were found in fewer surveys recently than historically. Regression coefficient not statistically

1767   significant ($p = 0.12$).

1768

1769   **Supplementary Figure 3**
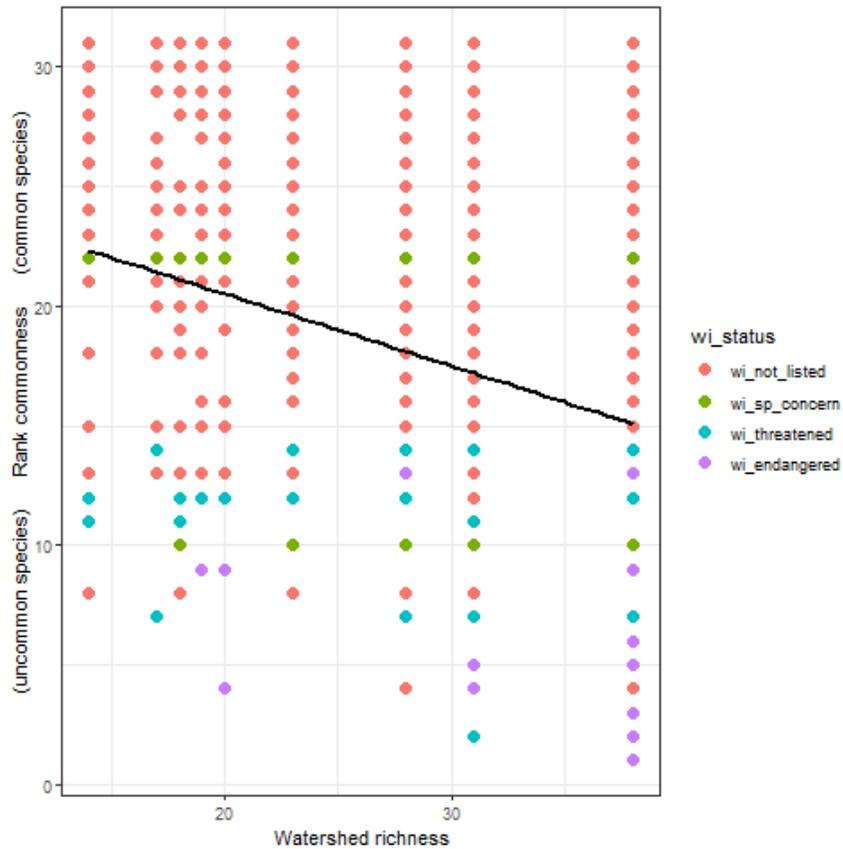
1770

1771   Supplementary figure 5: species ranked commonness by watershed richness. Rank commonness was

1772   calculated as number of sites present using paired recent surveys, watershed richness was calculated as

1773   number of species identified as ever being present among historical and recent surveys in watershed (p

1774   < 0.01).

1775

1776 **Supplementary Figure 4**
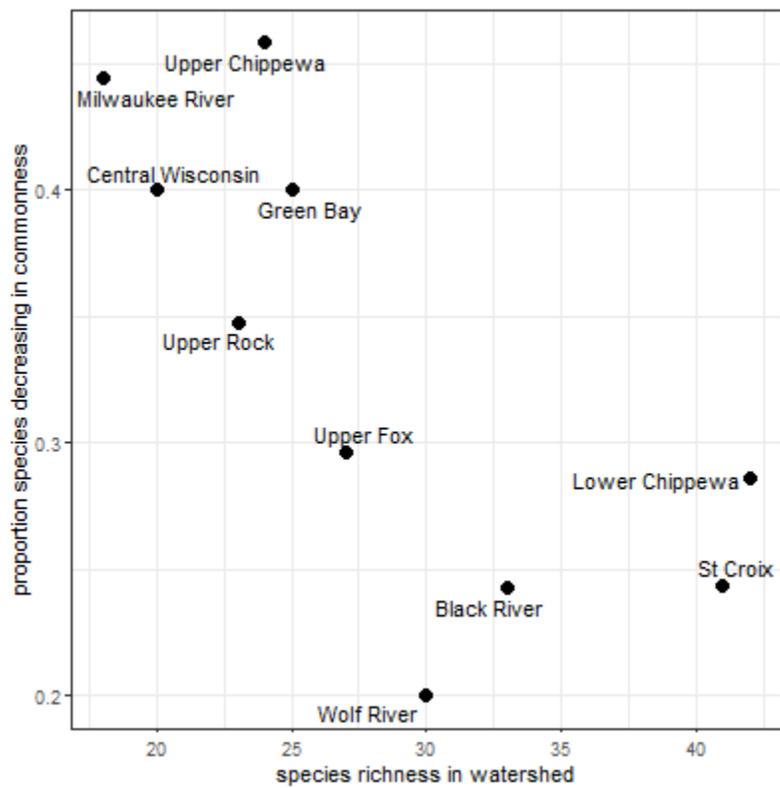
1777

1778 Supplementary figure 4: Proportion of species that are less common recently compared to historically

1779 within each watershed by total species richness in watershed. Proportion of species decreasing in

1780 commonness calculated as n species in fewer surveys recently than historically/n species richness in

1781 watershed within paired survey data. Number of paired surveys by watershed are as follows: Saint Croix:

1782 11, Lower Chippewa: 17, Wolf River: 38, Upper Fox: 8, Upper Chippewa: 9, Green Bay: 17, Upper Rock:

1783 6, Black River: 10, Milwaukee River: 8, Central Wisconsin: 6. The x-axis represents the total number of

1784 species present in all recent surveys within watershed (not just within paired sites). Linear regression

1785 suggests a negative relationship between these two variables (p=0.02).

1786

1787

1788   **Supplementary Figure 5**
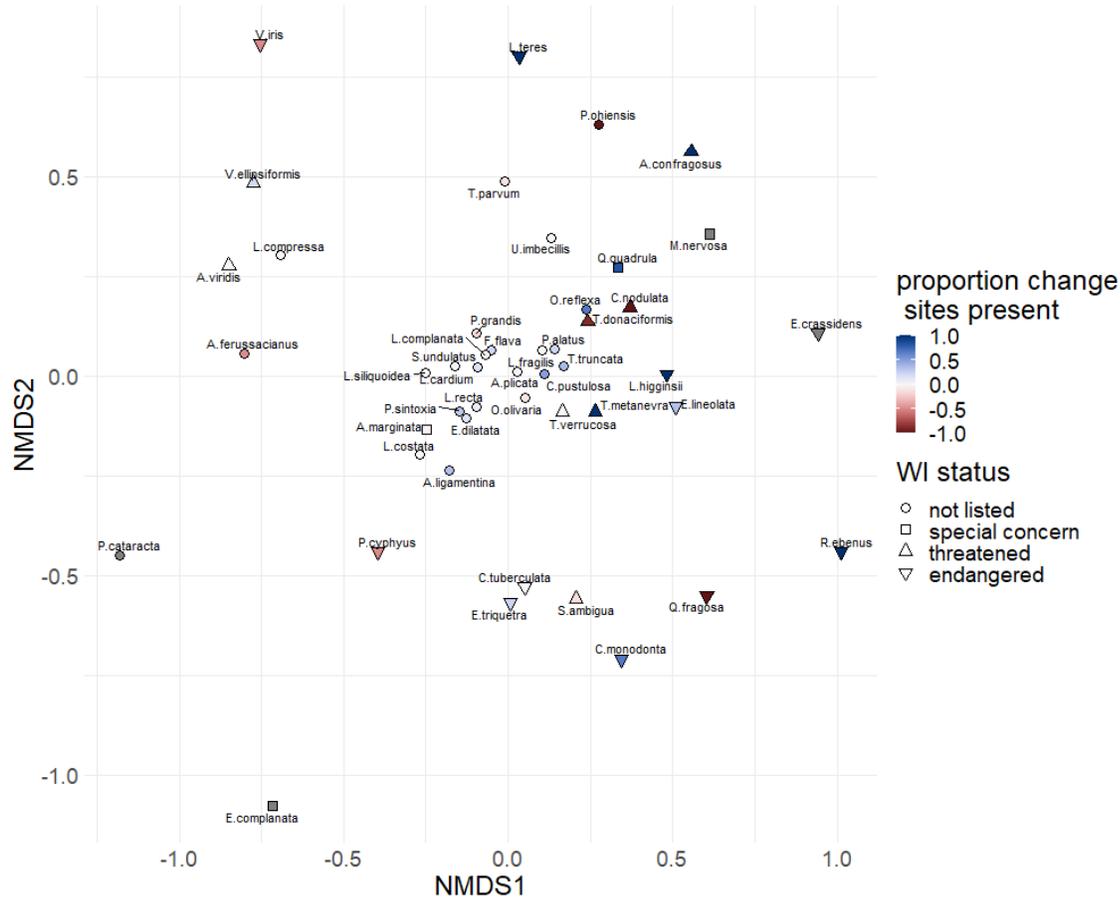
1789

1790   Supplementary figure 5: Non-metric ordination of mussel species assemblages. Ordination was

1791   calculated using Chao distances of all surveys historical and recent (n surveys = 6503, stress = 0.122).

1792   The proportion change in sites present (color) was calculated using all paired site data (n=149) as was

1793   calculated as follows: (n sites recently present – n sites historically present) / n sites historically present,

1794   so that 0 (white) represents a species that present in the same number of sites in both time periods, -1

1795   represents a species that was historically present in at least one survey, but not found in any recent

1796   surveys, and 1 represents a species that was found in twice as many sites recently compared to

1797   historically. *Arcidens confragosus*, *Lampsilis teres,* and *Reginaia ebunus* were found in 1, 1 and 3 recent

1798   surveys respectively and not found in any historical sites, they were all assigned a 1 for color coding

1799   purposes.

1800 **Chapter 4: Thesis contributions to the study of freshwater mussels**

1801   Freshwater mussels are one of the most endangered groups of animals, as the conservation

1802 status of at least 40% of mussel species are classified as imperiled globally (Lopes-Lima et al., 2018).

1803 They have faced, and continue to face, numerous threats associated with the increasing impacts of

1804 humans on freshwater ecosystems and their future is uncertain. Currently, our ability to effectively

1805 protect vulnerable freshwater mussel species is limited in part by a lack of understanding of important

1806 aspects of their life history and ecology.

1807   Recently, researchers and members from the Freshwater Mollusk Conservation Society (FMSC)

1808 created strategy plans that identified priority issues regarding the conservation of freshwater mussels

1809 considered to be of greatest immediate need (Ferreira-Rodríguez et al., 2019; FMCS, 2016). The goals of

1810 these strategy plans were to help guide research and management into conservation efforts of

1811 freshwater mollusks in North America. These strategy plans highlighted the need for research at

1812 multiple scales, from a better understanding of physiological characteristics important to population

1813 viability, to up-to-date broad-scale population assessments. The work in this thesis contributes towards

1814 the goals put forth within the strategy plans, with an emphasis on how freshwater mussels respond to

1815 changes in their environment. The studies within this thesis span a similar scale from physiological to

1816 state-wide scales. In chapter one, I showed how mussel growth can be related to broad-scale natural

1817 changes in their environment. In chapter two, I showed how native mussel communities respond

1818 physiologically to harmful invasive mussel species during the early stages of their invasion. In chapter

1819 three, I documented how native mussel communities have changed across Wisconsin over the past 50

1820 years. In the following paragraphs I highlight the specific contributions of each chapter and how they

1821 align with the goals set out in the strategy plans.

1822   In chapter one, I showed that mussel growth in lake populations can be dynamic, variable

1823 between individuals, and is associated with landscape-level environmental conditions. One of the main

1824   issues defined in the strategy plans is the need to better understand the ecology of mollusks at the

1825   individual, population, and community level and in describing the issue, specifies the need to describe

1826   life history characteristics at appropriate scales. In this chapter, I described growth characteristics of a

1827   lake-dwelling population of *Lampsilis siliquoidea*, how their life-history was unique in comparison to

1828   closely related fluvial populations, and showed that controls on their growth were associated with

1829   landscape-level environmental changes unique to lake ecosystems. These contributions demonstrate

1830   that lake-dwelling populations can be unique regarding important life-history characteristics as well as

1831   are the environmental controls influential to their growth. Globally, lakes are important habitats for

1832   numerous mussel species (Haag, 2012), yet populations residing in lakes are disproportionately

1833   understudied, likely because lake communities often do not reach the same abundances and species

1834   richness as fluvial communities resulting in less interest in their study. This chapter contributes to the

1835   very small body of work describing important life-history characteristics of mussels in lakes and how

1836   their growth is related to unique characteristics of their ecosystem. Broadly, this chapter provides an

1837   example of how unique the life-history and ecology of mussels in lakes can be, which hopefully will

1838   inspire interest in these unique and understudied communities.

1839   In chapter two, I examined physiological responses of a lake-dwelling populations of the native

1840   freshwater mussel *Lampsilis siliquoidea* as they responded to the stress of an invasive zebra mussel

1841   infestation. Health assessments and the development of new non-lethal methodologies for assessing

1842   sublethal effects from environmental stressors on freshwater mussels has been a recent focus of FMCS

1843   having organized a specific workshop for the cause in 2018, as well as highlighting the need to describe

1844   the risks and magnitudes of past, ongoing, and newly emerging stressors on mollusks as key issues

1845   within the strategy plans. In this chapter, I used non-targeted metabolomics—a relatively new and

1846   nonlethal approach in mussel health assessment—to assess sublethal effects of the stress associated

1847   with being burdened with zebra mussel attachment—a widespread and growing threat to native

1848   freshwater mussels especially in the Midwest. I demonstrated that physiological profiles of stress were

1849   identifiable in populations under natural environmental conditions and that these profiles were

1850   consistent with studies of stress under laboratory conditions. I also demonstrated that physiological

1851   signs of stress are not necessarily apparent in metabolites central to important physiological processes,

1852   but can be obscured by supplemental processes that can compensate for the immediate effects on

1853   primary metabolites. This chapter, while focused on the stress associated with the burden of zebra

1854   mussels, demonstrates the usefulness of this approach in being able to detect populations experiencing

1855   stress in natural environments and should encourage other researchers that are interested in assessing

1856   stress associated with other potential drivers. This chapter also contributes to our understanding of

1857   lake-dwelling native mussel populations, populations that are particularly vulnerable to negative effects

1858   of invasive zebra mussels that prefer lake ecosystems.

1859   In chapter three, in a collaboration with biologists from the Wisconsin Department of Natural

1860   Resources, we conducted the first state-wide assessment of how Wisconsin's mussel communities have

1861   changed since state-wide surveys were first carried out in the 1970's. The Department of Natural

1862   Resources has collected and managed thousands of records of survey data from across the state, but

1863   had yet to be able to analyze the data and synthesize how mussel communities have changed across the

1864   state of Wisconsin. The strategy plans emphasized that population assessments need to occur

1865   frequently, as effective conservation efforts are dependent on up-to-date records of the current

1866   distributions of populations as well as how these populations have changed over time. In this chapter, I

1867   showed that the extent of losses has been variable among species, but substantial losses have likely

1868   occurred for many of Wisconsin's native mussel populations within the last 50 years and I also provided

1869   evidence that the patterns of loss have not been uniform across the state. This chapter provides a large-

1870   scale assessment of conservation status of Wisconsin's mussel communities, this is useful information

1871   for state biologists by identifying species that may be in need of updated population assessments and

1872   may merit consideration for updating their conservation status as well as identify regions where

1873   vulnerable populations may be particularly at risk and require close attention for future monitoring

1874   efforts. In addition, it encourages increased investment in conservation efforts through documenting

1875   the scale of monitoring required to effectively manage for their successful conservation.

1876   The broad range of study across the chapters of this thesis has given me the opportunity to train

1877   myself broadly in areas of research important for the conservation of freshwater mussels. The future of

1878   native freshwater mussel communities is uncertain as their numbers continue to decline and human

1879   impacts on their ecosystems intensifies. To improve their outlook, effective conservation planning will

1880   require a diverse set of skills and a range of perspectives. I have demonstrated within this thesis that I

1881   have contributed broadly to efforts important for freshwater mussel conservation and have developed a

1882   diverse set of professional skills that will be a foundation for meaningful future contributions towards

1883   their conservation.

1884

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