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REGULAR ARTICLE

METHOD DEVELOPMENT FOR RAPID STREAMSIDE ASSESSMENT OF FRESHWATER MUSSEL (ORDER UNIONIDA) HEMOCYTE CONCENTRATION AND MORPHOMETRICS

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ABSTRACT

Contemporary freshwater mussel population declines often lack discriminant explanations, and diagnoses are commonly post hoc, subjective, expensive, and/or lethal. To avoid further compromising imperiled populations, we need sublethal metrics and biomarkers that identify early warning signs of infection, stress responses, and deviations from physiological homeostasis or potential resilience to environmental pressures. Immune modulators can be differentiated by quantifying multiple physiological endpoints based on hemocyte form and function. Spontaneous hemocyte aggregation, cell instability, travel times from mussel collection sites, and a lack of access to costly instrumentation currently limit immune status assessments. Widespread capacity to understand immune responses to disease, toxicants, and other stressors relies on effective hemolymph handling and preservation techniques. In this study we optimized methods to measure total hemocyte concentration and stability across five antiaggregants (Alsever's Solution, K_2EDTA and sodium citrate Microtainers, Isoton II, and Leibovitz's L15) over time using a Beckman Coulter Multisizer 4e (a laboratory benchtop unit) to validate results from a Millipore Scepter (a portable, field-ready hand-held unit). K_2EDTA Microtainers held on ice for up to 6 h preserved hemolymph most effectively based on consistency in total hemocyte concentration and morphometrics over time. There was a highly significant agreement ($R^2 = 0.9156$, $P < 0.0001$) in total hemocyte concentration between the Multisizer 4e and Millipore Scepter. Portable and compact cell counters provide a less expensive, advantageous alternative to benchtop instruments for instantaneous evaluation at mussel collection sites when timely quantification would otherwise require extensive logistical planning. Our results lay the foundation for accessible streamside assessment, which can expedite the establishment of reference ranges among species and populations and identify how anticipated stressors such as infection and starvation alter the immune response. This approach also may be useful for quantifying freshwater mussel health among propagation facilities and in stable or declining ecosystems.

KEY WORDS: hemolymph, immune biomarkers, conservation, *Lampsilis radiata*

INTRODUCTION

Unionids (Class Bivalvia) are a highly imperiled, species-rich, environmentally important family of freshwater mussels facing enigmatic die-offs and widespread population declines (Graf and Cummings 2007; Haag 2012; Haag and Williams 2014; Vaughn 2018). Historically, these declines followed conspicuous impacts of habitat destruction, pollution, invasive species, and stream impoundments (Haag 2019). In contrast, contemporary mussel die-offs often lack such discriminant explanations, develop rapidly, and do not always affect other sympatric freshwater mussel species or aquatic organisms, thus prompting questions about the role of disease (Waller and Cope 2019). Though historic declines and associated factors have been documented since the 1950s, research considering

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freshwater mussel health status and infectious diseases has not progressed, nor are tools readily available or optimized to investigate immune status (Grizzle and Brunner 2009; Carella et al. 2016). Conservationists and mussel propagation biologists have expressed the need to move beyond post-hoc study of the presence of disease to include individual and population competence and resilience against infection, xenobiotics, and other exogenous stressors with added resources and approaches (Mueller et al. 2025).

Mollusks rely on a primitive but robust innate immune system consisting of a physical defense barrier (shell, epithelium, and mucus secretion) and internal cellular and humoral mechanisms (Song et al. 2010; Song 2016). Hemocytes are multifunctional hemolymphatic cells distributed in hemolymph, a fluid analogous to vertebrate blood, and moved through an open circulatory system transporting metabolites and oxygen (Auffret 1988). Hemocytes are the base of the innate immune response. They also serve a biomechanical purpose in biomineralization processes, shell formation, neuroendocrine regulation, nutrient digestion and transport, tissue repair, and general homeostatic maintenance (Zannella et al. 2017). Hemocytes work in tandem with humoral factors dissolved in hemolymph, including antimicrobial peptides, lytic enzymes, and cytokines, all of which serve in defense and signaling (Chu 1988).

Bivalve cellular and humoral defenses efficiently control infections despite lacking adaptive components (van Niekerk et al. 2016). Hemocytes engage in non-self-recognition defense against invaders through aggregation, phagocytosis, encapsulation, and cytotoxic reactions that produce reactive oxygen or nitrogen compounds and lysosomal enzymes (Canesi et al. 2002; Farcy et al. 2011). Hemocyte-bound protein-protein recognition factors trigger the production of cytokines, antimicrobial peptides, and chemokines; recruit additional hemocytes; and increase phagocytosis, autophagy, and apoptosis (Allam and Raftos 2015).

Major cell types are generally differentiated by morphology, physiological activities, biological functions, and enzymatic cytochemistry; see reviews in Anisimova (2013) and de la Ballina et al. (2022). Currently, the two accepted main hemocyte types are granulocytes and agranulocytes (hyalinocytes). Subtypes may be further differentiated by carbon: nitrogen morphometrics or organelle content and characterized as precursors to other hemocytes (Giamberini et al. 1996; Burkhard et al. 2009; Evariste et al. 2016).

Hemolymph morphological and functional biomarkers are integral to understanding organism health, disease, and ecological risks integrated over time (King et al. 2020; Kataoka and Kashiwada 2021). Perturbations to the immune system and other associated responses are likely to manifest in hemolymph, but hematological and immunological utility as biomarkers is typically limited due to their nonspecific responses to multiple stressors. However, these biomarkers are sensitive, reliable, reproducible, and closely related to other physiological systems, and they can be measured rapidly and relatively inexpensively. Hemolymph can be sampled nonlethally with no significant difference in mortality after hemolymph extraction in mussels greater than 40 mm in length after 3 yr of observation (Gustafson et al. 2005a, 2005b; Fritts 2015a, 2015b; Richling and Krause 2022). Further, some biomarkers, such as heat shock proteins or lysozymes, may be conserved across species. Thus, it is possible to identify additional universal biomarkers of health in surrogates for threatened and endangered species or to use full communities as environmental sentinels (Van der Oost et al. 2003; Anisimova 2013).

Potential cytotoxic or immunotoxic biomarkers include the hemogram (cell concentration, relative percentage of hemocyte morphotypes) and hemocyte morphology (volume, diameter). Hemograms operate under the same principles as differential cell counts in vertebrates (Levine et al. 2022). Changes in hemocyte counts are considered a general, early warning sign of stress and activation of the first-line defense against invaders (Auffret et al. 2006).

Despite the health-assessment advantages of using hemolymph, wide validation and adoption of optimal conditions to withdraw and isolate viable unionid hemocytes severely lag behind marine mollusk models. Particle-size analysis is a valuable high-throughput tool that can replace or supplement microscopy in molluscan immunology (Nguyen and Alfaro 2019). However, limited progress has been made to experimentally validate particle-size analysis of multiple immunological biomarkers in freshwater bivalves. A single protocol may not optimize accurate measurement of all immune parameters, necessitating multiple assays for a holistic description of immune status (Burkhard et al. 2009). Additionally, current methods require expensive instrumentation and extensive preparation protocols that are not feasible for streamside use.

Cell aggregation is one primary challenge of total and differential cell counts (Chakraborty et al. 2021). Aggregation and adhesion are physiological cell behaviors that occur spontaneously when hemolymph is removed (Chen and Bayne 1995). Antiaggregants are prone to interference with the matrix. Some common antiaggregant solutions used during hemolymph extraction and preparation include heparin, EDTA, Alsever's solution, Leibovitz's L15-medium, Coulter Isoton II diluent, and N-ethylmaleimide (Evariste et al. 2016; Grandiosa et al. 2018; Matozzo et al. 2018) held either at room temperature or on ice (Gagnaire et al. 2004). Accurate measurement of other immunopathological and functional parameters relies on combating the potential toxicity of preservatives, reducing holding times, and ensuring access to affordable instrumentation. Therefore, our study aimed to optimize methods for measuring total hemocyte concentration and stability across five antiaggregant solutions and to compare a handheld cell counter with the standardized automated benchtop cell counter.

METHODS

Mussel Care and Hemolymph Sampling

A total of 125 adult Eastern Lampmussels (*Lampsilis radiata*) with an average total shell length of 63.7 ± 3.04 mm were obtained from the U.S. Fish and Wildlife Service's Harrison Lake National Fish Hatchery (Charles City, Virginia) and transported in coolers containing aerated hatchery pond water in a three-hour trip to North Carolina State University's Yates Mill Aquatic Conservation Center in Raleigh, North Carolina. Mussels were acclimated for 5 d to a temperature of 22.0°C in a 530-L Living Stream System water tank (Frigid Units, Toledo, OH, USA) using pond water filtered to 25.0 µm. Water changes were conducted daily prior to feeding with a commercial feed mixture of *Nannochloropsis* sp., *Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira pseudonana*, and *Thalassiosira weissflogi* (Reed Mariculture Nanno 3600 and Shellfish Diet 1800). Mussels were isolated in pairs in 9.5-L pulse flow through tanks containing sand substrate (500–1000 µm grain size) with daily water turnover of 25.0 µm pond water; following hemolymph withdrawals, they were monitored for behavioral changes (e.g., decreased filtering, burrowing) and mortality for at least 2 wk.

Experimental Design and Sampling

Ten experiments were conducted to assess (1) the effect of multiple antiaggregants and holding temperature on total hemocyte concentration (THC), volume, size, and stability over time and (2) the reliability of a handheld automated cell counter (Scepter™ 3.0 Automated Cell Counter, Millipore, Miami, FL, USA; hereafter "Scepter") compared to a benchtop particle counter/size analyzer (Multisizer™ 4e Coulter Counter, Beckman Coulter, Brea, CA, USA; hereafter "Multisizer"). Hemolymph (0.5–1.0 mL) was withdrawn from the anterior adductor muscle with a pre-chilled 27.0-gauge, 12.7-mm needle attached to a 1.0-mL sterile Leur Slip syringe and immediately dispensed into a 6.0-mL Fisherbrand™ borosilicate glass culture tube with prechilled antiaggregant. Hemolymph withdrawal practices included removing the needle and vacutainer lids before dispensing out of the syringe to maintain cell integrity. To assess the effects of antiaggregant and holding time, hemolymph was withdrawn from six randomly selected *L. radiata* and held in antiaggregants on ice and from three randomly selected *L. radiata* and held in antiaggregants at room temperature (untreated hemolymph, K_2EDTA Vacutainers, and Isoton II diluent) for the duration of the experiment and assessed after 30 min and 1, 2, and 3 h postremoval. Treatments included the following:

Untreated hemolymph, osmolarity 300 mOsm/kg

2.0 mL K_2EDTA Vacutainer, pH 7.2, osmolarity 300 mOsm/kg

3.0 mL 3.2% sodium citrate Vacutainer, pH 7.1, osmolarity 300 mOsm/kg

Isoton II diluent, pH 7.2, osmolarity 300 mOsm/kg (1:1 dilution v:v)

Alsever's solution, pH 6.9, osmolarity 300 mOsm/kg (1:1 dilution v:v)

Leibovitz's L15, pH 7.2, osmolarity 300 mOsm/kg (1:1 dilution v:v)

To validate the instrumentation, hemolymph was withdrawn from 72 randomly selected *L. radiata*, and paired samples were immediately analyzed using both the Scepter and the Multisizer.

Hemocyte Size Frequency Distribution

We determined total hemocyte concentration, size frequency distribution, and hemocyte volume by dispensing a 20-µL aliquot of hemolymph-antiaggregant (*1:1dilution v:v*) solution into a sterile 1.5-mL microcentrifuge tube (Basix™, Fisher Scientific, Waltham, MA) and aspirating into the Scepter equipped with a 60-µm sensor. A separate 100-µL aliquot of the hemolymph solution was added to 5.0 mL of Isoton II diluent in individual Accuvette™ ST sampling vials (Beckman Coulter) for Multisizer analysis. The Multisizer was equipped with a 100-µm aperture set to an aperture current of 800 µA, and a gain setting of two; cell concentration was determined by volumetric analysis. Duplicate 100-µL aliquots were passed into the Multisizer and cells between 5.0 µm and 30.0 µm were counted.

Quality Assurance

A thorough quality assurance protocol was followed through analysis of blanks, duplicates, and standard reference materials. Before each experiment, the Scepter was calibrated with 11-µm PHCC3 beads (Millipore), and the Multisizer was calibrated using certified NIST CC size standard L20 20-µm latex beads (Lot BR0072.2312; Beckman Coulter). Experiments began by blanking the instruments with each antiaggregant in Isoton II. The aperture was increased from 50 µm to 100 µm after preliminary analysis to decrease the rate of aperture blockage, monitored for further blockages, and flushed after every 10 samples. Samples were visually inspected for debris before transferring to Isoton II in accuvettes and mixed well by gently rolling the accuvette and removing air bubbles. Duplicate measurements were taken on each sample with an average coefficient of variance of 3.94% ± 3.69% (range 0.00–16.9%; $n = 108$). Samples were removed and repeated if an individual produced significantly apparent discrepancies. Particles less than 5 um were gated out to avoid cellular debris and improve consistency between instruments based on the manufacturer's specifications.

Statistical Analysis

We exported data from the Beckman Coulter Particle Characterization software (version 4.03) and conducted statistical analyses in GraphPad Prism 10.3.1 (GraphPad Softward, Boston, MA). Prior to analysis, we checked data for normality using the Shapiro–Wilk test. Mixed-effects models and Tukey's post-hoc tests were used to compare differences in mean THC, cell volume, and cell size standard deviation over

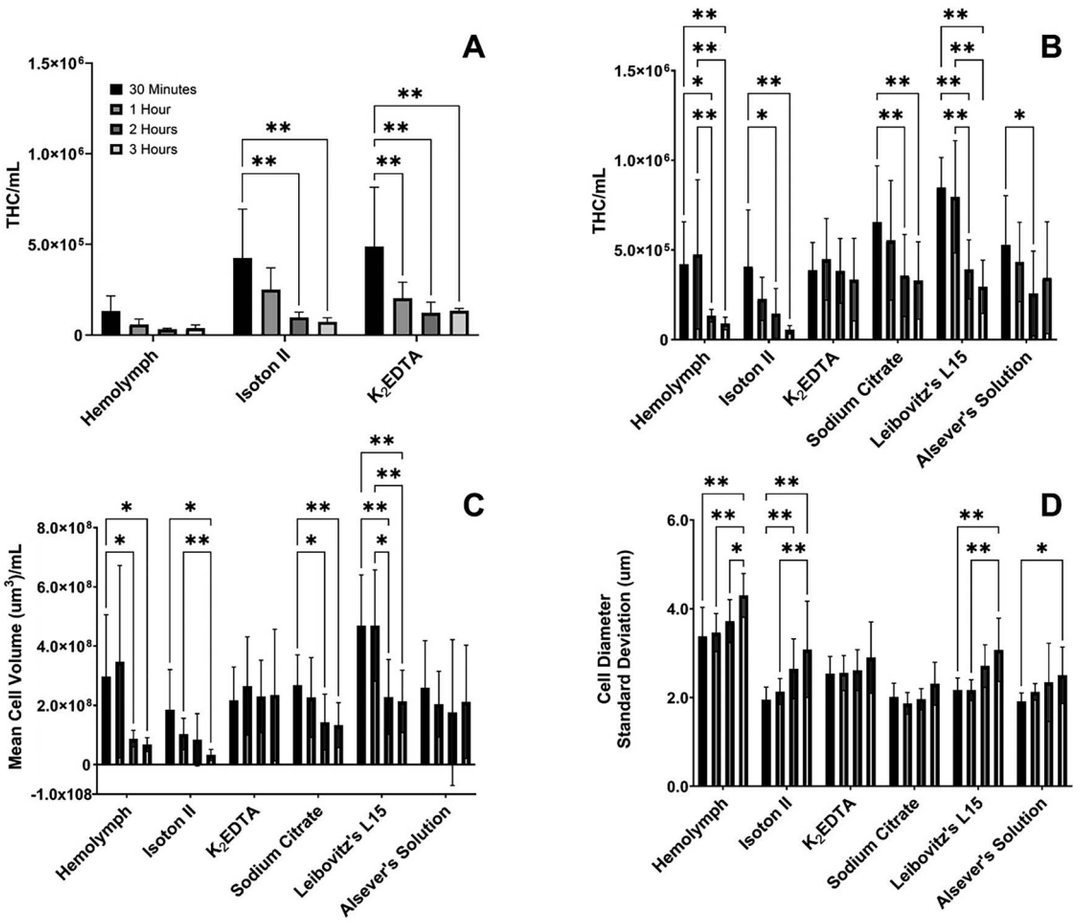

Figure 1. Changes in total hemocyte concentration (THC) in (A) treatments held at room temperature and (B) treatments held on ice. (C) changes in mean cell volume and (D) standard deviation of cell diameter. Error bars represent standard deviation. Asterisks over bars represent statistical significance at $*P < 0.05$ and $**P < 0.01$.

time. Kruskal–Wallis tests were used when data were not normally distributed (only in the case of K_2EDTA held after 6 h). We used linear regression to compare THC between the Multisizer and Scepter. Statistical significance was indicated at $\alpha = 0.05$; P values of ≤ 0.05 and ≤ 0.01 were both presented to indicate relative strength of relations.

RESULTS

Hemogram

Baseline THC (after 30 min of incubation) in all treatments held on ice averaged 5.37×10^5 cells/mL (95% CI, 4.69×10^5 to 6.05×10^5; $n = 70$). THC changed over time in all treatments except K_2EDTA Microtainers when held on ice (Fig. 1A, B and Table 1). The greatest differences were observed between untreated hemolymph and hemolymph treated with Isoton II. Hemocyte viability immediately decreased when untreated and incubated at room temperature. THC appeared roughly stable in Alsever's solution, but the highest biological variation was observed in those samples, and additional individuals had to be sampled for cells to be counted. THC remained stable for up to six hours in K_2EDTA incubated on ice (Fig. 2). Hemocyte size frequency produced a typical bimodal distribution with fewer cells between 6 μm and 10 μm and more between 12 μm and 18 μm, characteristic of hyalinocytes and granulocytes (Fig. 3). Cell volume decreased in most treatments except K_2EDTA and Alsever's solution (Table 2). However, Alsever's solution again produced the most variation between individuals (Fig. 1C), while variation in cell diameter increased in untreated hemolymph and hemolymph treated with all antiaggregants except K_2EDTA and sodium citrate (Fig. 1D and Table 3).

Instrument Validation

There was significant agreement in total hemocyte concentration between the Multisizer and Scepter ($R^2 = 0.9156$ [$F_{(1,70)} = 759.6$, $P < 0.0001$]; $n = 72$; Fig. 4). The Scepter slightly underestimated total particle concentration compared to the Multisizer; the 60-μm Scepter sensor is designed to count particles between 6 μm and 36 μm and cells between 8

Table 1. Percent change of total hemocyte counts in each antiaggregant and holding temperature compared to baseline concentrations. Boldface type represents statistical significance at $P < 0.05$.

Treatment		After 1 h	After 2 h	After 3 h
Held on ice	Compared to 30 min at room temperature			
Hemolymph	**−68.5%**	12.9%	**−68.3%**	**−78.6%**
Isoton II	4.73%	−44.2%	**−64.2%**	**−86.6%**
K_2EDTA	**26.2%**	15.8%	−0.95%	−13.5%
Sodium citrate	—	−15.6%	**−45.5%**	**−49.8%**
Leibovitz L15	—	−6.06%	**−53.8%**	**−65.2%**
Alsever's solution	—	−18.2%	**−51.4%**	−34.8%
Held at room temperature				
Hemolymph		−55.9%	−75.9%	−70.5%
Isoton II		−41.0%	**−77.0%**	**−82.8%**
K_2EDTA		**−58.3%**	**−74.9%**	**−72.5%**

and 25 μm, whereas the Multisizer 100-μm sensor has a much greater range depending on user settings. Additionally, inherent biological variability of the hemolymph matrix generated minor differences between technical replicates measured on the Multisizer ($R^2 = 0.9698$ [$F_{(1,106)} = 3401$], $P < 0.0001$; CV = 3.94% ± 3.69%; range 0.00–16.9%; 95% CI = 3.23–4.64%; $n = 108$).

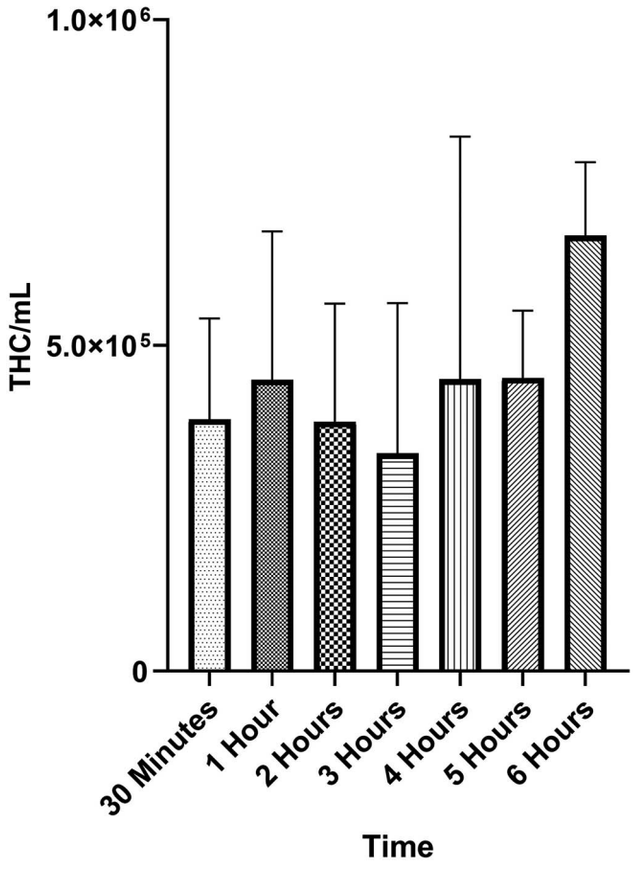

Figure 2. Changes in total hemocyte concentration (THC) in K_2EDTA held on ice for 6 h. No significant differences were recorded at $P < 0.05$ by Kruskal–Wallis test $H(6) = 11.10$, $P = 0.0852$.

DISCUSSION

We optimized methods to preserve freshwater mussel hemolymph for rapid, automated quantification of multiple parameters reflective of instantaneous immune status. THC recorded by the Scepter was significantly concordant with the Multisizer measurements. Regardless of the antiaggregant used, we concurrently demonstrated that hemolymph must be held on ice as hemocytes lost integrity immediately after withdrawal at room temperature. Antiaggregants that chelate calcium ions, such as sodium citrate and EDTA, performed better than other antiaggregants we investigated, particularly when potassium ions were added to further decrease free calcium, as demonstrated in in vitro studies (Grandiosa et al. 2018). The critical role of calcium in regulating hemocyte aggregation offers a potential strategy for managing hemocyte behavior for biomarker identification, quantification, and monitoring, as well as for preserving hemocytes for hemogram characterization.

The invertebrate hemogram has informed diagnoses of stress, disease, contaminant exposure, and starvation, but determination conventionally relies on microscopy (Hinzmann et al. 2017; Steinagel et al. 2018). Cell identification and quantification, while useful, can be less practical without extensive training or significant dedicated time resources. In contrast, flow cytometry quantifies morphological, chemical, and functional endpoints (Donaghy and Volety 2011; Nguyen and Alfaro 2019; Barjhoux et al. 2020). Yet flow cytometry requires costly instrumentation and extensive preparation protocols unsuitable for field use. Coulter counters balance some of these limitations and are commonly available in mussel propagation facilities as instruments to monitor food availability and filtration rates (Carter et al. 1985; Mueller et al. 2025).

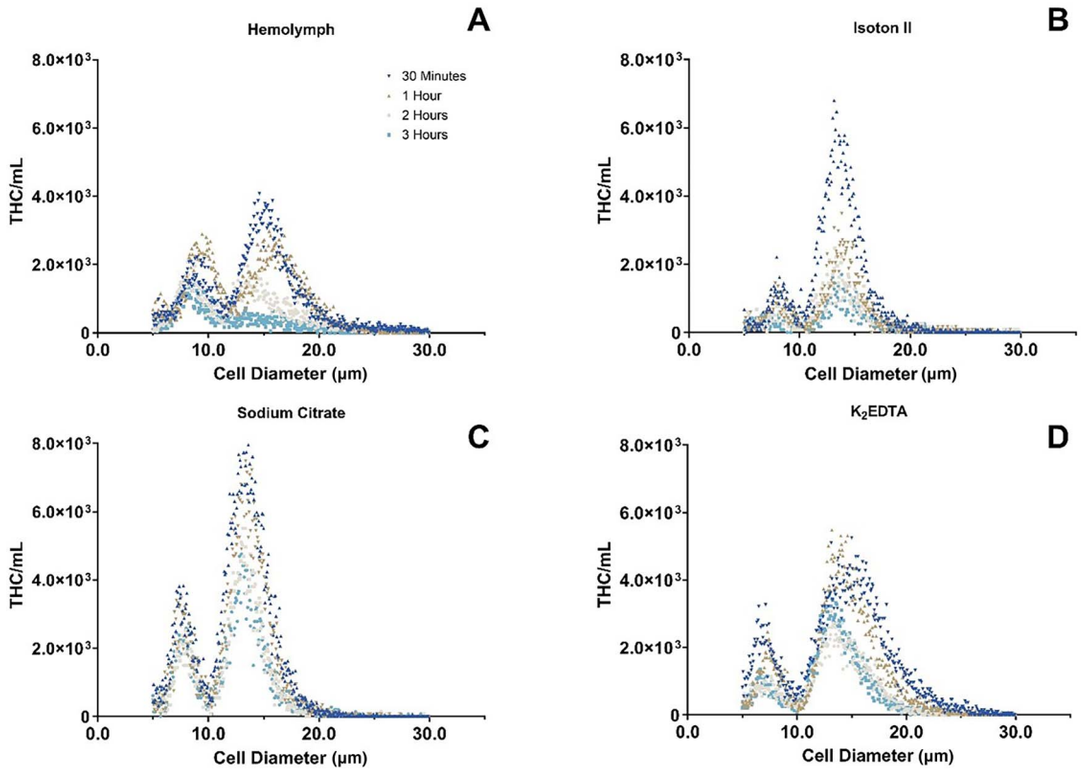

Figure 3. Total hemocyte concentration (THC) distributions averaged among six individuals reported on the Multisizer 4e.

Antiaggregant Effectiveness

Hemocyte stability varied widely across treatments. We found that THC, volume, and cell diameter variation changed significantly in hemolymph alone with no treatment. Aggregation can be attributed to a combination of factors as hemocytes adapt to new conditions outside the circulatory system.

Spontaneous hemocyte aggregation occurs as a part of the innate immune response activated in part by calcium serving as an intracellular signaling molecule in transduction pathways involved in cell adhesion (Cheng et al. 2006). Specifically, calcium ions are essential for the activation of adhesion molecules, such as cadherins and integrins, that mediate cell–cell and cell–substrate interactions (Shozawa and Suto 1990; Alberts et al. 2002). This cascade of events begins with the activation of phospholipase C, leading to the production of inositol trisphosphate, which triggers the release of calcium ions from intracellular stores into the cytoplasm (Putney and

Table 2. Percent change of hemocyte volume in each antiaggregant and holding temperature compared to baseline concentrations. Boldface type represents statistical significance at $P < 0.05$.

Treatment		After 1 h	After 2 h	After 3 h
Held on ice	Compared to 30 min at room temperature			
Hemolymph	**−61.7%**	16.5%	**−70.7%**	**−77.5%**
Isoton II	17.9%	−44.0%	−54.7%	**−82.4%**
K_2EDTA	**70.6%**	22.1%	5.88%	8.0%
Sodium citrate	—	−15.5%	−46.7%	**−50.4%**
Leibovitz L15	—	0.04%	**−51.5%**	**−54.6%**
Alsever's solution	—	−21.5%	−32.3%	−18.3%
Held at room temperature				
Hemolymph		−66.9%	**−78.9%**	**−78.5%**
Isoton II		−27.8%	−69.4%	**−80.1%**
K_2EDTA		−38.3%	**−64.4%**	**−61.2%**

Table 3. Percent change of hemocyte diameter standard deviation in each antiaggregant and holding temperature compared to baseline concentrations. Boldface type represents statistical significance at $P < 0.05$.

Treatment		After 1 h	After 2 h	After 3 h
Held on ice	Compared to 30 min at room temperature			
Hemolymph	5.0%	2.5%	10.1%	**27.2%**
Isoton II	−2.5%	9.5%	**35.7%**	**58.1%**
K_2EDTA	21.8%	0.7%	3.19%	14.3%
Sodium citrate	—	−7.3%	−2.7%	14.7%
Leibovitz L15	—	−0.18%	24.9%	**41.8%**
Alsever's solution	—	11.4%	22.6%	30.9%
Held at room temperature				
Hemolymph		−2.8%	13.1%	4.6%
Isoton II		27.0%	**70.0%**	**81.5%**
K_2EDTA		37.7%	**46.1%**	34.1%

Tomita 2012). Elevated calcium levels then activate protein kinases and other signaling molecules, enhancing the affinity of adhesion molecules and further promoting hemocyte aggregation (Ramos Martínez et al. 2012; Meier et al. 2024).

Hemocyte aggregation mediates phagocytosis, one of the primary functions of hemocytes to defend against foreign pathogens and eliminate cellular debris across multiple taxa (Liu et al. 2020; de la Ballina et al. 2022). Hemocytes also aggregate to facilitate wound healing and tissue repair by releasing signaling molecules such as cytokines to promote healing (Chakraborty et al. 2021). Once removed from circulation, hemocytes are activated from a resting state without inhibitory signals (Chen and Bayne 1995). Similarly, while demonstrated to be nonlethal, handling and hemolymph withdrawal may stimulate a rapid release of cytokines and chemokines (Gustafson et al. 2005a). Environmental effects, such as changes in temperature or oxygen levels, can also promote an emergency response (Ericson et al. 2024).

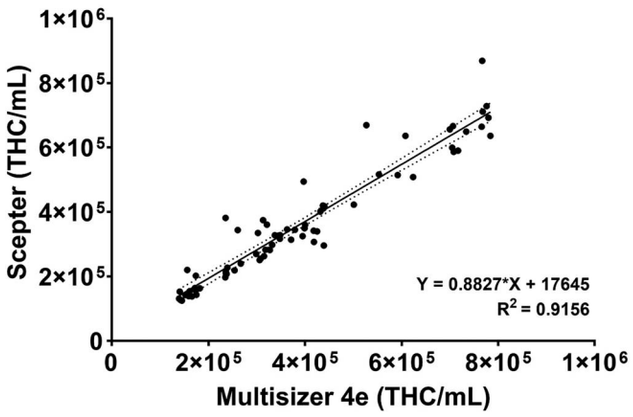

Figure 4. Relationship between total hemocyte concentration (THC) simultaneously measured on both the Beckman Coulter Multisizer 4e and Millipore Scepter ($P < 0.001$).

Colder temperatures slow aggregation, cell metabolism, and cell death. They also reduce metabolic rates and stabilize cell counts for longer-term holding (Chen and Bayne 1995; Lapointe et al. 2012). Hemocytes require consistent osmotic balance and nutrient supply for physiological equilibrium, or they may experience rapid apoptosis or aggregation (Hinzmann 2016). Like hemolymph, Isoton II, a salt solution, maintains osmolarity, but in our study it did not actively maintain cell integrity or prevent aggregation. Alsever's solution and Leibovitz's L15 are routinely used to preserve red blood cells in vertebrates by maintaining a balanced ionic environment and providing nutrients, but they are less effective in invertebrates (Tang et al. 2022). Calcium buffering effectively decreases cell adhesion, which has important implications for maintaining more regulated, automated cell differentiation (Eisner et al. 2023).

Hemogram Biomarkers

The Multisizer provided simultaneous quantification of multiple immunological biomarkers that can expand interpretations beyond simple cell counting. Baseline THCs were lower than what might be expected from previous research, but because mussels were collected during winter, they may have had depressed hemocyte production and lowered metabolic rates (Blaise et al. 2002; Salimi et al. 2009; Gillis 2012; Yao and Somero 2012; Evariste et al. 2016; Lubawy and Słocińska 2020).

Future studies are needed to quantify the hemogram across different seasons. For example, an average of 5.86×10^6 cells/mL ($n = 4$), an order of magnitude higher than this study, was found while determining that hemolymph withdrawal can be a nonlethal technique in *Elliptio complanata* (Gustafson et al. 2005a). Observed changes in cell concentration from baseline could be the result of either aggregation or cell lysis. Expected bimodal cell size distribution reflects the two main cell populations, hyalinocytes and granulocytes, but

intermediate cell types also have been described in bivalves—qualities that are not easily discernible by the Multisizer compared to a flow cytometer (Burkhard et al. 2009; Steinagel et al. 2018).

Changes to cell volume can reflect allometric scaling, the biological process where cell volume decreases as diameter increases, indicative of cell-shape changes and effects of osmotic pressure. The decrease in mean cell volume across multiple antiaggregants suggests either changes to the overall differential cell populations, cell lysis, atrophy, or compensatory mechanisms as cells reduce their volume to optimize metabolic efficiency (Fig. 1C). Similarly, increased heterogeneity of cell diameter indicates loss of homeostasis as cell types respond differentially to stress (Fig. 1D). Resiliency to stress among cell types varies, disrupting the expected distribution.

Instrument Validation

Freshwater mussel conservation relies heavily on in-stream, on-site surveys to monitor population level stability, collect healthy broodstock for propagation, and augment populations with propagated individuals. Many sites are relatively remote with long travel times to laboratory or facility resources. Surveys can benefit from additional tools to assess individual and population health, especially in the event of active die-offs or declines. The Scepter has been used in other invertebrate investigations to determine THC, hemocyte diameter, and hemocyte volume in the crab *Carcinus aestuarii* following exposure to bisphenols, and to observe increased hemocyte counts in *Mytilus galloprovincialis* exposed to a mixture of glyphosate and aminomethylphosphonic acid and *Crassostrea gigas* exposed to higher temperatures and *Vibrio* infection (Wendling and Wegner 2015; Matozzo et al. 2018; Fabrello et al. 2024).

Total hemocyte concentration compared between the Scepter and Multisizer was within the expected variation as measured by duplicate samples processed by the Multisizer. Dilution during sample preparation produces inherent sample heterogeneity. Further investigations should facilitate generating reference ranges across species and environmental conditions to understand if biotic and abiotic influences transcend observed instrumental variation and provide relevant diagnostic or prognostic tools. Additionally, more refined differentiation between hemocyte subpopulations' responses to immune stressors would further validate the use of instruments such as the Scepter.

Ecological Relevance and Implications for Conservation

Our study supports the use of hemolymph to assess the health status of freshwater mussels, and it offers a practical tool and solution for population conservation efforts. Rapid generation and deployment of hemogram reference ranges can expand the potential for diagnosing and predicting declines in health, support mussel-care techniques to maximize health and resilience, and connect environmental stressors to physiological conditions.

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