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Establishing typical values for hemocyte mortality in individual California mussels, *Mytilus californianus*

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ABSTRACT

Hemocytes are immune cells in the hemolymph of invertebrates that play multiple roles in response to stressors; hemocyte mortality can thus serve as an indicator of overall animal health. However, previous research has often analyzed hemolymph samples pooled from several individuals, which precludes tracking individual responses to stressors over time. The ability to track individuals is important, however, because large inter-individual variation in response to stressors can confound the interpretation of pooled samples. Here, we describe protocols for analysis of inter- and intra-individual variability in hemocyte mortality across repeated hemolymph samples of California mussels, *Mytilus californianus*, free from typical abiotic stressors. To assess individual variability in hemocyte mortality with serial sampling, we created four groups of 15 mussels each that were repeatedly sampled four times: at baseline (time zero) and three subsequent times separated by either 24, 48, 72, or 168 h. Hemocyte mortality was assessed by fluorescence-activated cell sorting (FACS) of cells stained with propidium iodide. Our study demonstrates that hemolymph can be repeatedly sampled from individual mussels without mortality; however, there is substantial inter- and intra-individual variability in hemocyte mortality through time that is partially dependent on the sampling interval. Across repeated samples, individual mussels' hemocyte mortality had, on average, a range of ~6% and a standard deviation of ~3%, which was minimized with sampling periods ≥72 h apart. Due to this intra-individual variability, obtaining ≥2 samples from a specimen will more accurately establish an individual's baseline. Pooled-sample means were similar to individual-sample means; however, pooled samples masked the individual variation in each group. Overall, these data lay the foundation for future work exploring individual mussels' temporal responses to various stressors on a cellular level.

1. Introduction

Hemocytes are immune cells found in the hemolymph of many invertebrates and are functionally comparable to macrophages in humans [1]. These dynamic cells are involved in tissue repair, transport and digestion of nutrients, neuroendocrine regulation, and immune function, and therefore are important to the organism's overall health [2–4]. Moreover, in mollusks, hemocyte mortality has been identified as a cellular index of the animal's responses to various stressors (e.g. heat, cold, salinity, and pollutants) [5–7]: in mussels, hemocyte mortality is inversely related to animals' survival rate after stress [1,3,8,9]. Hemocyte sampling also provides a non-destructive way to assess an organism's temporal responses to a given stressor; in contrast, many “omics” or biochemical approaches require between-individual comparisons because the animal must be sacrificed to obtain a sample [10]. Thus, hemocytes offer a powerful study system that allows scientists to ask mechanistic questions that do not require sacrificing the animal, such as how invertebrates respond to, and recover from, certain stressors and diseases [3]. It is therefore important to develop improved methodologies for hemocyte sampling and analysis—notably when looking at stressor effects over time.

Here, we approach these methodological issues by examining an intertidal mollusk, *Mytilus californianus*, which has long served as an important study organism in ecological physiology. This species is frequently the dominant competitor for space in rocky intertidal ecosystems along the Pacific Coast of North America, providing homes for smaller animals and food for larger predators, thus making them a key player in the intertidal ecosystem [11]. Living in the intertidal zone means that mussels spend time both emersed in air and immersed in the sea, and as such are an ideal organism in which to explore mechanistic questions about how factors relevant to climate change—like heat stress and ocean acidification—will impact intertidal ecosystems.

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Evolutionarily, mussels, like other mollusks, have a nervous system that encompasses many of the major neurotransmitters, neuropeptides, and hormones found in vertebrates [1]. This, combined with the fact that mussels live in a highly variable environment, makes them an excellent model system to study the fundamental mechanisms of neuroendocrine-immune system cross-talk and disease states in response to environmental stressors [12]. Furthermore, mussels are important from economic and human-health perspectives. Mussels comprise a part of the billion-dollar commercial aquaculture industry that provides food and jobs for millions worldwide [13,14], and are also used in many places around the world to monitor ocean pollutants for human protection [15]. Therefore, further understanding the physiological responses of mussels to environmental change, including those due to anthropogenic influences, is warranted. However, in order to better understand hemocytes' responses to typical abiotic stressors (e.g. temperature, salinity, hypoxia), we first need to develop improved methods for their study by characterizing baseline hemocyte mortality in the absence of these stressors.

While previous studies of mussels have explored some aspects of hemocyte mortality and activity, there are important limitations to this literature. Specifically, previous studies in mussels have utilized pooled hemocyte samples (e.g., from 4 to 5 individuals), an experimental approach that does not allow characterization of individual responses to stressors. It is clear that pooled hemolymph samples can track a stress response in a group of mussels over time [5,7,16]. However, due to the high inter-individual variation in stress responses on a cellular and molecular level, it is equally important to be able to track individuals' temporal responses to stressors [10]. Many studies exposing mussels to abiotic stressors, such as heat stress, have found that a percentage of animals die, while others survive, from the same stressor [17,18]. If one tracks mussel hemocyte responses to such a stressor, utilizing pooled hemolymph samples—which incorporate hemolymph from all individuals (including those that died)—might confound the results. For example, if hemocyte mortality of the pooled sample increased post-stressor, it might be concluded that all individuals' hemocyte mortality increased from the given stressor. However, in reality, it could be that only the individuals who died had a high hemocyte mortality, which would subsequently raise the pooled group mean and thus lead to potential misinterpretation. This is just one reason why being able to measure each individual's hemocyte mortality over time will augment our ability to uncover adaptive physiological mechanisms that distinguish between those that survive (vs. die) from a given stressor. Furthermore, we do not have a deep understanding of the temporal development of cellular stress responses before, during, and after stress because most current methods require animal sacrifice and thus do not allow tracking of stress responses in the same individuals over time [10,19]. It is therefore important first to establish whether hemolymph can be drawn repeatedly from a single individual over a given time period relevant to the stress response, without creating artifacts from the sampling regimen. Next, it is crucial to understand the typical intra- and inter-individual variability in hemocyte mortality that might be expected with repeated sampling in mussels free from typical abiotic stressors. These data will help create a foundation (and a baseline dataset) for comparison to future studies where individual mussels may be repeatedly sampled before and after exposure to a stressor.

Previous studies in other mollusks (the snails *Pomacea canaliculata* and *Lymnaea stagnalis*) have found that repeated hemolymph sampling does not increase hemocyte or animal mortality [20,21]. Although one of the studies [21] addressed individual variability in hemocyte mortality over time, it was specific to snails and involved a non-invasive methodology not applicable to mussels. Hemolymph samples from snails can be obtained by tickling the foot [21], whereas in mussels, sampling requires a more invasive procedure involving withdrawal of hemolymph by a needle inserted into the adductor muscle [5,7]. For such serial sampling, it is important to determine whether this more invasive sampling procedure kills the individual or causes a sustained increase in hemocyte mortality after the first sample is obtained.

Lastly, to obtain deeper insights into hemocyte function, new methods should be developed that exploit contemporary analytical technologies. For example, fluorescence activated cell sorting (FACS) has become one of the main tools for analyzing variations in the cellular composition of organisms. Although some studies have utilized FACS for some *Mytilus* species [22,23], we are unaware of any studies that have established FACS analysis for *M. californianus*. Hemocyte cell sizes reportedly vary among different *Mytilus* species [2,24], which necessitates different FACS gating methods and analyses for each species.

In summary, the purposes of this study are to: (1) establish FACS as a method to analyze hemocytes in *M. californianus*, (2) evaluate whether hemolymph can be repeatedly drawn from an individual mussel without adverse effects, and if so, whether the time interval between repeated samples affects hemocyte mortality or organism survival, and (3) describe the typical intra- and inter-individual variability in hemocyte mortality across repeated samples in mussels not exposed to typical abiotic stressors.

2. Methods

2.1. Experimental animals

Specimens of adult *M. californianus* (n = 59; see Table 1 for morphometric data), were collected during springtime (March–April 2019) from a mussel bed of a moderately wave-exposed shore at Hopkins Marine Station in Pacific Grove, California, USA (36.6216°N, 121.9042°W); intertidal height of sampled mussels ranged from 0.95 to 1.22 m above mean lower low water (GTS-211D Total Station, Topcon, Livermore, CA, USA). Between sampling timepoints, mussels were submerged in a flow-through aquarium with animals from the same Group (see below for definition of "Group"), and fed a commercial shellfish diet (Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA) three to four times per week [25]. Mussels were not subjected to any form of abiotic stress (e.g. temperature, pH, or salinity) during the experiment: water temperature was 13.91 ± 0.69 °C, and ranged from 12.52 °C to 15.59 °C during the three months the study was conducted. To determine baseline hemocyte mortality across repeated samples, mussels' hemolymph was sampled at varying time intervals that were set in advance (see below). However, to avoid any effects of constant

Table 1
Group morphometric data (Mean ± SD).

Sampling Interval (hours apart)	Sample Size (n)	Body Mass (g)	Shell Length (mm)	Shell Width (mm)	Shell Height (mm)
24 (1 day)	15	22.6 ± 3.4	56.6 ± 3.6	23.4 ± 1.6	26.9 ± 1.3*
48 (2 days)	15	25.1 ± 6.3*	59.0 ± 5.7*	24.5 ± 2.5*	27.9 ± 2.4*
72 (3 days)	14	19.7 ± 3.1	54.0 ± 3.0	21.9 ± 1.9	24.9 ± 1.6
168 (1 week)	15	25.8 ± 4.8*	59.6 ± 5.2*	24.0 ± 1.7*	28.0 ± 1.8*
Overall	**59**	**23.3 ± 5.1**	**57.4 ± 5.0**	**23.5 ± 2.1**	**26.9 ± 2.2**

Note: *Indicates a significant difference from the 72 h Group ($p < 0.017$); there were no other significant pairwise differences between Groups (all $p > 0.017$). Despite the differences between Groups' morphometric data, the overall percentage of dead hemocytes at baseline was not different among Groups (i.e., Sample 1; all $p > 0.05$).

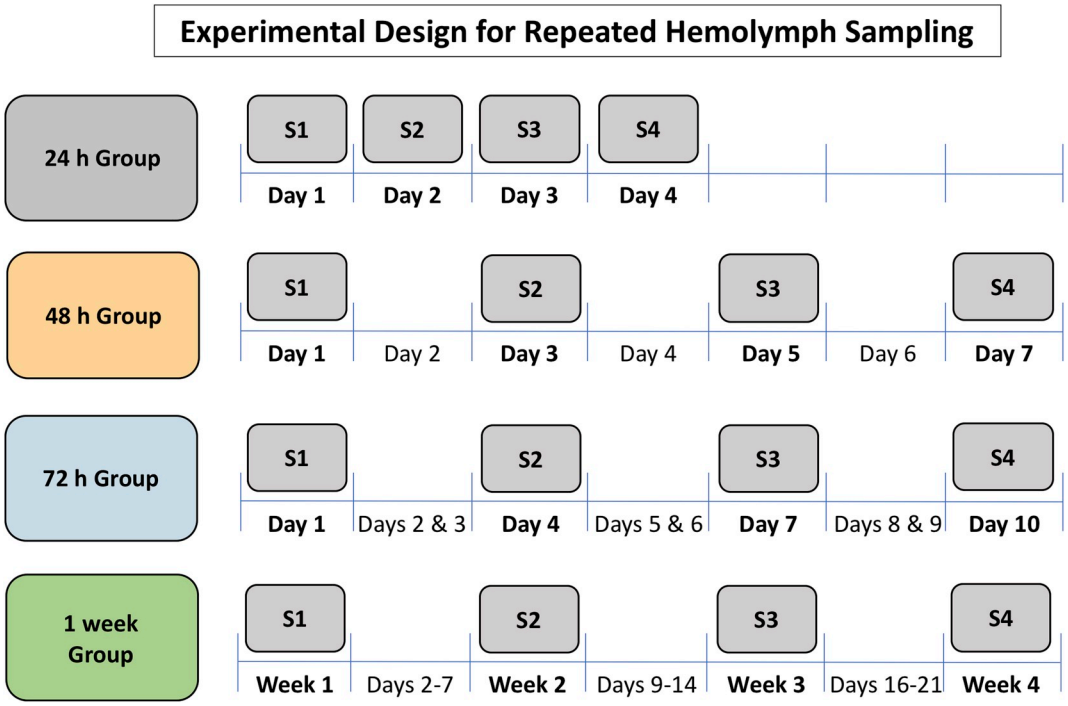

Fig. 1. Diagrammatic representation of the experimental design for each Group's (n = 14–15 individuals) repeated hemolymph sampling schedule. "S" in the gray boxes stands for "Sample" (i.e., Sample 1, 2, 3, or 4). All individuals from each Group were sampled four times, however, the time between the repeated samples varied, as denoted in the diagram by the timing indicated in each Group's row. On each of the sampling days, each individual had its hemolymph drawn, and hemocyte mortality was analyzed for each individual (i.e., n = 14–15 samples per Group). Out of those 14–15 individual hemolymph samples, two pooled samples were made by combining hemolymph from each of four randomly selected individuals; these pooled group samples were also analyzed on the same day as the individual samples to allow for comparisons between the sample means of the pooled vs. individual groups. Mussels were kept in a flow-through aquarium between the repeated sampling days and fed three to four times per week (see Methods for details).

submersion on biochemical changes [26], each mussel's first hemolymph sample was drawn within one week of field collection. Mussels were starved 24 h before each hemolymph sample was drawn, except in the 24 h Group where this was not possible. In the 24 h Group, mussels were fed within 1–2 h after their hemolymph was drawn, that is ~21–23 h before the next day's sample was drawn. After each Group's experiments were completed, morphometric measurements of shell height (the longest distance from dorsal to ventral surfaces), shell width (the widest part of the mussel across both closed valves), and shell length (the longest distance from the anterior to posterior) were made using digital calipers [27], and body mass was measured via electronic scale (accurate to the nearest 0.0001 g; see Table 1).

2.2. Experimental design

See Fig. 1 for a schematic of the experimental design. To assess whether individuals' hemolymph could be repeatedly sampled (without killing the animal), and to establish baseline hemocyte mortality values in unperturbed mussels, hemolymph was sampled from the same individuals at time zero and three subsequent timepoints, with varying intervals between subsequent samples: 24, 48, 72 or 168 h. Each of these four different sampling intervals defined a separate experimental Group (n = 14–15 mussels per Group).

On each sampling day ~1 ml of hemolymph was drawn from the adductor mussel [5–7] of each individual in a Group using a 20 gauge needle attached to a 3 ml syringe, from which 200 μl were used to analyze hemocyte mortality. On rare occasions, we were only able to draw the minimum amount of hemolymph required for FACS analyses (200 μl); however, this never occurred twice in the same individual. As we are unaware of any previous work exploring differences between pooled-vs. individual-group samples, we also assessed whether pooled samples had a similar hemocyte mortality compared to the individual samples. There is a possibility that a pooled sample of four to five individuals' hemolymph might not represent the mean of those individual samples due to a biological interaction between the four samples when combined, e.g., hemocytes from a healthier sample (i.e., low hemocyte mortality) could phagocytose unhealthy or dead hemocytes from another individual's sample. In this case, the pooled sample mean would not match the mean of the individuals that comprised the pooled sample. To determine whether creating pooled samples biologically alters an individual sample, we created two pooled samples (n = 4 individuals per pooled sample) from each Group on each sample day. The 200 μl pooled sample comprised 50 μl hemolymph from each of four individuals (picked at random from the 14–15 individuals in that Group). All individuals in a specific Group were sampled and analyzed on the same day to ensure sampling consistency within each respective group and sampling period.

2.3. FACS setup

A FACS machine (On-Chip Sort HS, On-Chip Biotechnologies Co., Tokyo, Japan) uses optical properties to characterize individual particles as they pass through the instrument's detector. Each particle from a sample is illuminated by a laser, and the resulting fluorescence and light scattering are measured, allowing one to quantify characteristics such as cell viability, size and granularity [28]. Forward scattering (FSC) is reflective of cell size, with the largest cells having the highest FSC values. Side scattering (SSC) is reflective of cell granularity, with the most granular cells having the highest SSC value (see below for the specific FSC and SSC thresholds used in this study). As particles (including both cells and debris) pass through the machine, FSC and SSC values are measured for each particle, and those values can then be plotted on a FSC vs. SSC graph e.g., where, for example, a large, granular cell would appear in the top right corner of the graph (Fig. 2).

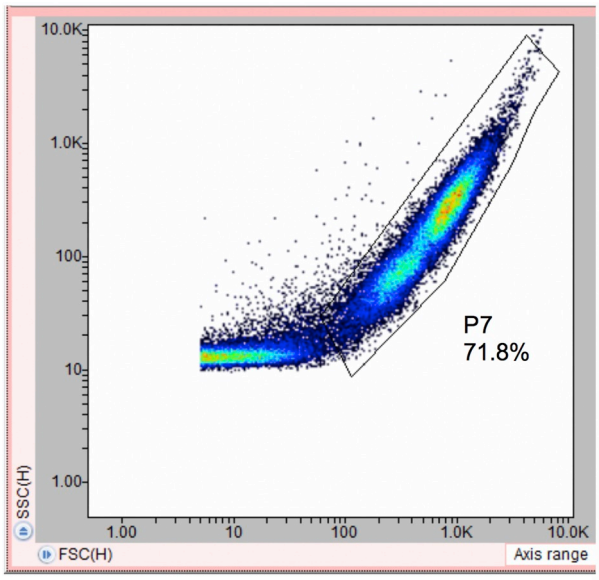

Fig. 2. Density plots of a single sample, where FSC vs. SSC data (log-log transformed) are the x and y axes, respectively. Orange/red indicates the highest density of events (each point is one event), green a moderate number of events, and blue the lowest density of events. The black P7 gate around the group of cells on the right-hand side of the plot represents the total hemocyte population. The dots (events) outside of that gate are debris (bottom left side of plot). Only this gate (P7) was used to analyze hemocyte mortality, and ~20,000 events were obtained within this gate for each hemolymph sample. These gates were initially determined through sorting and microscopy, and then applied to all samples (see Methods for more details). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

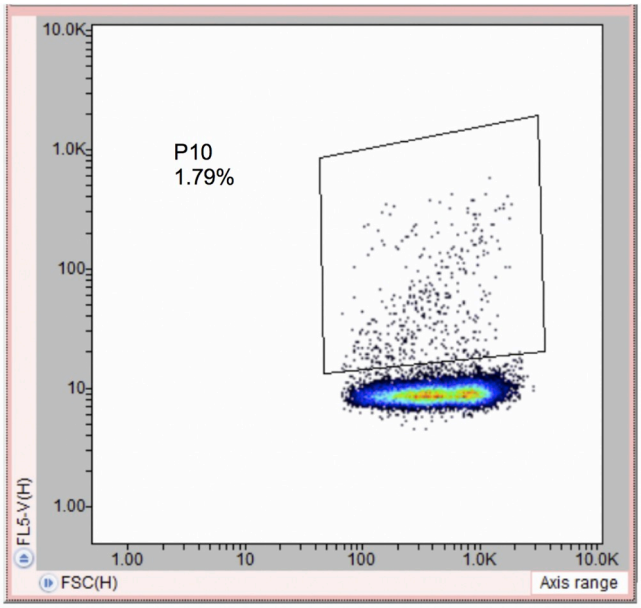

Fig. 3. Sample density plot of one individual's hemolymph sample stained with PI to determine the percentage of dead hemocytes. FSC on the x-axis vs. FL5 channel (to detect PI fluorescence) on the y-axis (log-log transformed). The gate (P10) shows the group of dead hemocytes that have high PI fluorescence relative to the high density of cells with minimal fluorescence in the FL5 channel; in this case 1.79% of the total hemocyte population were dead. For each individual hemolymph sample, these analyses were completed for the overall hemocyte population, and for the separate hyalinocyte and granulocyte populations.

Cells and debris occupy different areas (gates) on the graph. Once the cells are separated from the debris, the cells can then be analyzed separately according to their fluorescence (see Fig. 3), and this fluorescence can be used to indicate the cell's viability (see below).

Since FACS had not previously been used in studies of *M. californianus*, the first step was to establish gating methods for the hemocyte population. First, different gates were created and sorted (via FACS), and the cells comprising that gate were then imaged under a microscope to determine where the overall hemocyte population (vs. debris) was located on the FSC vs. SSC graph. Cells were imaged on a confocal microscope (Zeiss LSM 700 with 20x objective, Lens NA 0.8, Carl Zeiss Microscopy, LLC, NY, USA) with Hoechst 33342 (1:1000 ratio; a stain for double-stranded DNA; H3570, Life Technologies, Carlsbad, CA) to differentiate between debris and cells. Through repeatedly sorting and visualizing different gated populations on multiple pooled samples, we found the gate for the entire cell population to be at a FSC threshold of ~60, and a SSC threshold of ~20 (arbitrary, dimensionless units specific to this brand of FACS machine; see Fig. 2).

Next, we determined the gating properties for the two main types of hemocytes: hyalinocytes and granulocytes. Although there is still much debate as to whether these cell types come from the same cell lineage, it is agreed that the two types have different diameters (i.e. sizes), and also differ in their nucleus-to-cytoplasm ratios [22,24,29]. Based on one of the few papers exploring hemocyte properties in *M. californianus*, we expected that the diameter of the hyalinocytes would range from ~4 to 9 μm, while the granulocytes' diameter would likely range from 10 to 12 μm [2]. Additionally, previous literature has indicated that hyalinocytes have a larger nucleus-to-cytoplasm ratio than granulocytes [20,22,24,29]. See Results section for findings.

Hemocyte mortality was assessed using propidium iodide (PI): cellular entry of this dye indicates a loss of membrane integrity and thereby cell death. Briefly, 5 μl of a 1 mg·ml^{-1} solution of PI (P4864, Sigma-Aldrich, Inc., MO, USA) were added to each 200 μl hemolymph sample. PI-stained samples were then incubated in the dark for 30 min at 4 °C [5]. Each sample was analyzed within approximately 1 h of the sample being drawn to avoid any cell clumping [21]. To analyze each sample, ~100 μl of hemolymph were placed into the sample reservoir on a microfluidic chip (2-D Chip-w150, On-chip Biotechnologies, Ltd., Tokyo, Japan), and filtered sea water was placed into all sheath reservoirs to provide the cells with a fluid of the same osmolality, thereby avoiding cell death due to sheath fluid composition. To avoid any between-sample contamination, filtered seawater was used to flush the sample reservoir between samples.

Once we identified the cut-off between debris vs. hemocytes, the entire cell population was gated (see Fig. 2, gate P7), and for each hemolymph sample, ~20,000 events were recorded at an event rate of ~300–650 events·s^{-1}. Hemocyte mortality was expressed as the percentage of hemocytes showing PI fluorescence (~650 nm, gain of 30, FL5 channel) via FACS. The percentage of dead cells was analyzed both for the total hemocyte population and for the separate populations of hyalinocytes and granulocytes. These hemocyte mortality analyses were completed by drawing a gate around cells with values (on the FL5 channel) higher than the baseline fluorescence, indicating high PI fluorescence and therefore cell death (see Fig. 3 for an example). All data were analyzed by the same individual to ensure consistency in gating techniques across samples (On-Chip Sort Software).

Lastly, to determine whether repeated sampling caused animal mortality, mussel survival was monitored for two weeks after each individual's last sample was drawn.

2.4. Statistical analyses

R 3.5.2 [30] was used for all statistical analyses and models. Two-way, within (i.e. sample) – between (i.e., Group) ANOVAs were used to assess whether there was a main effect of Group or sample, and/or whether an interaction effect (Group × sample) existed for total

hemocyte mortality. Additionally, one-way repeated measures ANOVAs were used to assess 1) each individual Group's differences in hemocyte mortality across the four-sample time series, and 2) any overall changes (all Groups combined) in hyalinocyte or granulocyte percentages, and mortality of these two populations across the four samples. The individual Groups' vs. pooled groups' data were not statistically compared due to unequal sample sizes (i.e., pooled samples (n = 2) vs. individual samples (n = 14–15) for each sample timepoint within one Group). However, a one-way repeated measures ANOVA was used to evaluate overall changes in hemocyte mortality of the pooled-group samples across the four timepoints. A paired *t*-test was used to determine whether the pooled group means were similar to the theoretical pooled group means (i.e., calculated as the mean of the four individuals' hemocyte mortality comprising each pooled sample). Lastly, Groups' baseline morphometric data were compared using a one-way (between) ANOVA. For any statistically significant *F* scores ($p < 0.05$), pairwise differences were analyzed using independent *t*-tests with a Bonferroni correction.

To understand individual variation across timepoints, and what could be expected as a typical (baseline) in hemocyte mortality for control mussels, the standard deviation of each individual's four samples was calculated separately, before obtaining the mean ± SD of the entire Group. Moreover, each individual's range in hemocyte mortality was calculated by taking each individual's maximum minus their minimum hemocyte mortality across the four samples, and then the overall group mean ± SD was calculated. The differences between repeated samples for each individual were calculated by averaging the absolute difference between subsequent samples (e.g., | *Sample 4 – Sample 3* |) for each individual, and then calculating the group mean ± SD. The coefficient of variation (CV) was calculated for each individual as well, by calculating the SD of each individual's four samples and dividing it by the mean of their four samples. This value was already a percentage (% dead hemocytes), and so was not multiplied by 100.

3. Results

Morphometric data for each Group of mussels are presented in Table 1. There were minor differences between Groups. However, as there were no differences in baseline hemocyte mortality among Groups ($p > 0.05$), it appears that these morphometric disparities did not affect the study's results. Moreover, none of the morphometric variables were related (i.e., correlated) to baseline hemocyte mortality (all $p > 0.05$). Thus, despite the fact that the 72 h Group comprised slightly smaller mussels, this did not alter the hemocyte mortality at baseline. Lastly, the number of days that mussels were held in the flow-through aquarium before their first hemolymph sample (ranging from 0 to 4 days), did not affect their baseline hemocyte mortality (all $p > 0.05$).

To identify the hyalinocyte and granulocyte populations, we began sorting and imaging different subpopulations (i.e. gates) of the overall hemocyte population (P7 in Fig. 2). Through cell sorting and microscopy, as well as the presence of a low-density gap separating two groups of high-density areas in the density plot, we were able to identify the hyalinocytes and granulocytes (Fig. 4). Furthermore, previous research [2,3,24] indicated that the granulocytes would be the cell population in the top right corner of the density plots (large cells that are very granular; gate P9), and the hyalinocytes would be the cells in the bottom middle of the chart (smaller cells that are not very granular; gate P8; Fig. 4). For confirmation, these gated populations (stained with Hoechst DNA stain) were sorted and imaged on a confocal microscope, and cell diameters, along with nucleus-to-cytoplasm ratios, were evaluated [37] to confirm that the morphological properties we found were similar to that of previous research (see Fig. 5). Nucleus-to-cytoplasm ratios were measured by dividing the longest nuclear diameter by the longest cell axis (excluding lamellipodia) [24]. Similar to that of previous literature [22,24,29], we found a larger nucleus-to-cytoplasm ratio in hyalinocytes vs. granulocytes (means ± SD (range) = 0.58 ± 0.13 (0.42–0.88) vs. 0.27 ± 0.07 (0.19–0.42), respectively; n = 12 cells from each population), indicating that our FACS gating methods were correct for these two populations. From these two gates, we were able to get an approximate percentage of hyalinocytes and granulocytes relative to the total cell population (see Table 2 for details), and also to determine whether these two cell populations responded similarly to repeated sampling in terms of cell mortality.

There was no sample-by-Group interaction effect ($p > 0.05$), so data from all four Groups were combined to assess whether there were changes in hyalinocyte or granulocyte population percentages and/or cell mortality among these two populations with repeated sampling (see Table 2). Overall (all samples and Groups combined), mussels had more hyalinocytes than granulocytes (by ~7%; $p < 0.05$). There were minimal differences (< 2%) across repeated samples in hyalinocyte and granulocyte mortality, indicating that these populations are fairly stable with repeated sampling and that cell mortality (in mussels free from environmental stressors) is relatively similar in both cell populations. In terms of the overall composition of hyalinocytes vs. granulocytes among groups (i.e., main effect of Group), only the 24 and 48 h Groups were different from each other ($p < 0.05$). The 24 h Group had a higher percentage of hyalinocytes than the 48 h Group, and vice versa for the percentage of granulocytes. Yet this difference in cell type composition did not lead to any differences in the percentage of dead hyalinocytes vs. granulocytes among Groups (all $p > 0.05$).

Next, we assessed whether there was a main effect of Time within each Group (i.e. repeated sampling effect). Hemocyte mortality was not different across samples in either the 48 or 72 h Groups (all $p > 0.05$). In the 24 h Group, hemocyte mortality was significantly higher for Sample 2 vs. Sample 4 (~7.6 vs. 2.6%, respectively; $p = 0.006$); and in the 168 h Group, hemocyte mortality was significantly higher in week 1 vs. week 4 (~5.2 vs. 2.5%, respectively; $p = 0.002$; see Fig. 6). However, these sample means differed by ~3–5%, which is within the normal individual variation across the four samples (see below). When comparing hemocyte mortality among Groups for a given sample timepoint (e.g., sample 3), we found that there were no differences among Groups for samples 1 or 3; however, there were a few small differences (all < 5%) among Groups for samples 2 and 4 (see Fig. 6; $p < 0.008$). There was also a significant main effect of Group ($p < 0.05$), and pairwise comparisons with a Bonferroni correction (i.e., $p < 0.008$) indicated that the 72 h Group had a significantly lower hemocyte mortality overall vs. both the 24 and 48 h Groups ($p < 0.008$); however, the difference between Group means was < 2%, and may not be biologically significant (see Discussion for rationale). No other significant pairwise differences existed among Groups for overall hemocyte mortality across repeated samples (all $p > 0.008$).

Due to the low sample sizes for the pooled groups at each sample timepoint (n = 2), we combined hemocyte mortality data for all pooled-group samples at each sample timepoint and ran a one-way ANOVA to assess differences in pooled-group hemocyte mortality over time. We found no significant difference in the pooled groups' hemocyte mortality across the four samples (overall mean ± SD: 4.6 ± 2.6%; $p > 0.05$). Our data are similar to previous research in snails [20,21], indicating that despite the invasive nature of our sampling method, pooled samples are robust to repeated sampling, showing minimal variation across repeated samples ranging from 24 h to 1 week (i.e., 168 h) apart. When combining the individual Groups' samples at each timepoint (i.e., independent of sampling intervals), there was no significant difference between the individual- vs. pooled-group samples ($p = 0.75$). Furthermore, the pooled-group means were representative of the individual-group means, as there was < 0.5% difference in hemocyte mortality between the individual- and pooled-group means for any given sample timepoint (all Groups combined; see Fig. 7). Lastly,

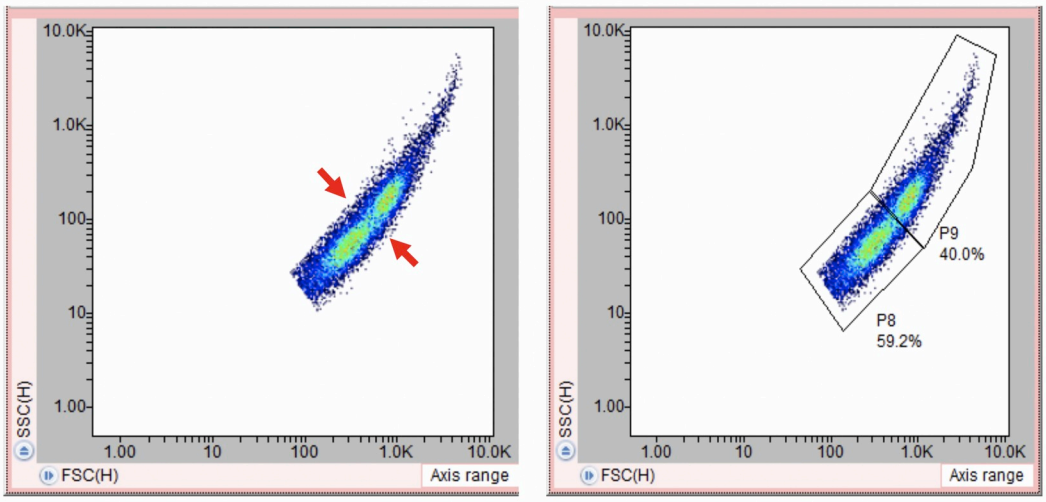

Fig. 4. Density plots of a single sample, where FSC vs. SSC data (log-log transformed) are the x and y axes, respectively. These data represent the total hemocyte population (i.e., P7 gate from Fig. 2). Orange/red indicates the highest density of events, green a moderate number of events, and blue the lowest density of events. The left-hand panel is the density plot without any gating. The slight separation of the two green densities (indicated by the red arrows) denotes the separation between two cell populations, which are gated for sorting in the right-hand panel. The P8 gate represents the hyalinocytes, while the P9 gate represents the granulocytes. The percentages below these gates represent the percentages of hyalinocytes and granulocytes relative to the total number of events in the P7 gate. These percentages are relative estimates since the total number of cells (absolute) were not counted for any samples. Note that the granulocyte and hyalinocyte percentages do not total to exactly 100%, as a small percentage of cells fall outside of these two gates due to the nature of FACS analyses (see Methods for details). The numerical values assigned to the gates (e.g. P8) are arbitrarily assigned by the software and do not bear any specific units, but simply differentiate gates from one another. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

we determined whether the actual pooled sample means were similar to the theoretical pooled means (of the four individual samples that comprised the pooled sample). Hemocyte mortality was not statistically different between the actual vs. theoretical pooled means ($p > 0.05$), the means were strongly correlated ($r = 0.84$), and the mean absolute difference was ~1%. Although three out of the 32 pooled-sample means differed from the theoretical means by > 2%, these data indicate that in most cases, pooling several individual samples together does not biologically alter the individual samples. Thus, several individual samples can be averaged to reliably estimate a pooled group mean.

There was considerable inter- and intra-individual variability in hemocyte mortality with repeated sampling (see Fig. 8). The 24 and 48 h Groups had slightly higher individual variability compared to the 72 and 168 h Groups (see Table 3). On average, an individual's percentage of dead hemocytes across the four samples varied by ~3% (i.e., SD) and ranged (i.e., maximum minus minimum) by ~6%. The maximum and minimum percentages of dead hemocytes across all individual samples were 22.9 and 0.8%, respectively. We also looked at the percentage of outliers in each group, in this case defined as data points above the boxplot's whiskers in Fig. 8 (i.e., points above $Q3 + 1.5 \times$ *interquartile range*; [31]). The 48 and 168 h Groups had the most outliers (48 h Group: n = 5 outliers or ~8% of the total samples; 168 h Group: n = 4 outliers or ~7% of total samples), whereas the 24 h and 72 h Groups had 3 and 1 outliers, respectively, across the four samples (5% and 2% of the total samples, respectively). None of the Groups' outliers were attributable to the same individual or the same sampling timepoint. In other words, if an individual had one sample that showed a high percentage of dead hemocytes (and was an outlier), their subsequent sample had a typical percentage of dead hemocytes (i.e. was within the box-and-whisker plot). We could not identify a

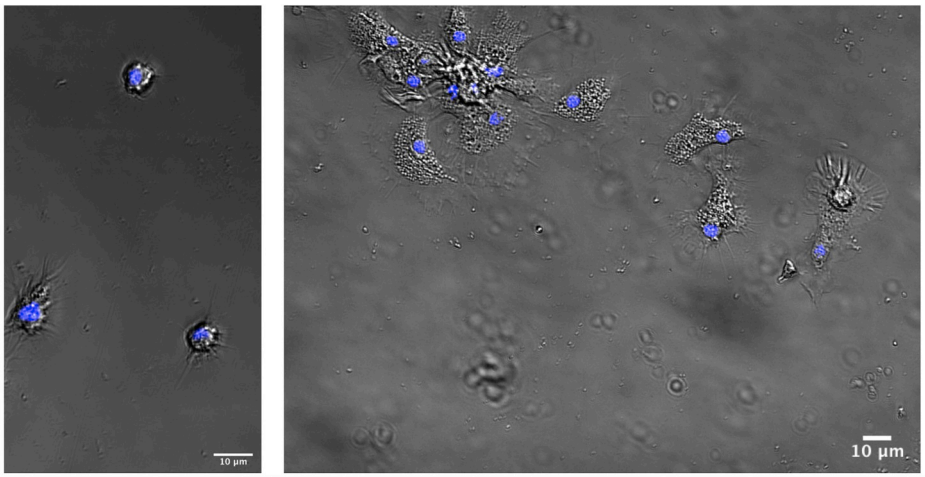

Fig. 5. Confocal microscopy images of three hyalinocytes (left) and multiple granulocytes (right), where blue stains the nucleus of the cells (Hoechst stain; imaged on Zeiss LSM 700 with 20x air objective, lens NA 0.8). These images represent live cells from pooled hemolymph samples (n = 5 individuals). A scale bar of 10 *µm* is in the bottom-right corner of each image. The hyalinocytes in general are < 10 *µm* in diameter and, relative to granulocytes, are rounder and have a larger nucleus relative to the cytoplasm [22,24,29]. Granulocytes are often > 10 *µm* in diameter and, relative to hyalinocytes, are more granular and have a larger cytoplasm relative to the nucleus. Briefly, 500 *µl* of hemolymph were treated with 2 *µl* Alsever's solution to prevent clumping of cells [7], and stained with 0.5 *µl* Hoechst and 10 *µl* of PI. Live cells were identified as previously described (see Methods), and then sorted via FACS into the two gates of hyalinocytes and granulocytes as indicated in Fig. 4. After sorting, cells were centrifuged at 400 relative centrifugal force for 5 min with slow-braking, the supernatant was removed, and the remaining cell pellet was resuspended in filtered seawater and 12 *µl* were placed into wells (µ-slide 18 Well Flat, ibidi GmbH, Munich, Germany) and allowed to settle for 30 min before imaging. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Overall hemocyte parameters for each of the four sample timepoints, with all Groups combined.

Sample Timepoint	Dead Hemocytes	Median Dead Hemocytes	Range Dead Hemocytes	Absolute n, % Outliers	Total Relative Hyalinocytes	Dead Hyalinocytes	Total Relative Granulocytes	Dead Granulocytes
1	5.1 ± 4.0	3.9 ± 2.6	1.2, 22.9	3, 5%	53.3 ± 14.9	4.9 ± 4.1	44.7 ± 14.8	6.4 ± 6.6
2	5.1 ± 3.8	4.2 ± 2.8	1.1, 20.5	4, 7%	52.4 ± 13.1	4.8 ± 3.4	45.3 ± 13.0	6.6 ± 5.4
3	4.0 ± 3.5	3.0 ± 1.8	0.8, 11.7	5, 9%	52.7 ± 16.3	3.6 ± 3.3	45.0 ± 16.3	4.7 ± 4.3
4	3.7 ± 3.4	2.6 ± 1.7	1.0, 11.0	5, 9%	51.7 ± 15.5	3.7 ± 3.2†	46.0 ± 15.5	4.5 ± 3.5†
Overall	**4.6 ± 2.6**	**3.4 ± 2.3**	**0.8, 22.9‡**	**17, 7.4%‡**	**52.5 ± 14.9***	**4.3 ± 3.5***	**45.2 ± 14.8**	**5.5 ± 5.1**

Note. All values are means ± SD, unless otherwise noted. Data comprise all four Groups (i.e., 24, 48, 72, and 168 h Groups) at each sample timepoint (n = 55–59 for all columns, except for Sample 1 of Dead Hyalinocytes and Granulocytes: n = 51). All data presented are percentages except for the "Absolute n." *Indicates a significant difference between the hyalinocytes vs. granulocytes overall ($p < 0.05$). †Indicates a significant difference between Sample 1 vs. Sample 4 ($p < 0.008$). The "Absolute n" represents the absolute number of samples that were outliers at that sample timepoint, and the "% Outliers" represents that absolute number as a percentage (see Results section for definition of outliers). ‡Overall values represent the range, absolute n, and % outliers from all samples (n = 236). Note that the "Median Dead Hemocytes" values are median ± median absolute deviations (i.e., MAD). The granulocyte and hyalinocyte percentages do not total to exactly 100%, as some small percentage of cells fall outside these two gates due to the nature of FACS analyses (see Methods for details).

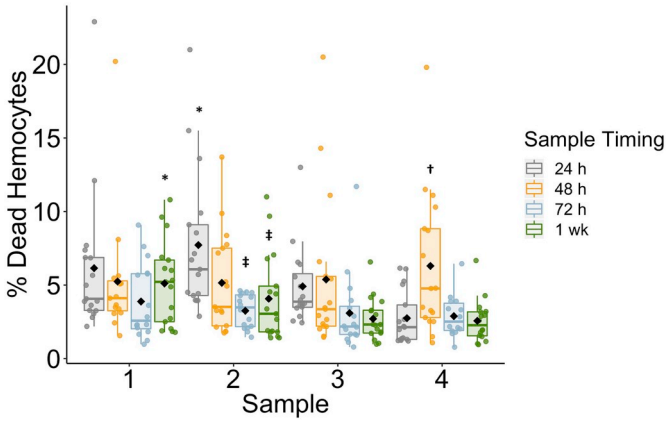

Fig. 6. Group hemocyte mortality across samples. Boxplots outline the 25th and 75th percentiles, and the midline indicates the median percentage of dead hemocytes (assessed by PI using FACS) in the four Groups (n = 14–15 each) that were each sampled repeatedly at four timepoints. Groups were repeatedly sampled four times, with different time intervals between samples: 24, 48, 72, or 168 h. Black diamonds represent the Group mean. *Indicates a significant difference between that sample timepoint vs. Sample 4, for that specific Group ($p < 0.008$). ‡Indicates a significant difference from the 24 h Group for that specific sample timepoint (all $p < 0.05$). †Indicates a significant difference from all other Groups for that specific sample timepoint (all $p < 0.05$).

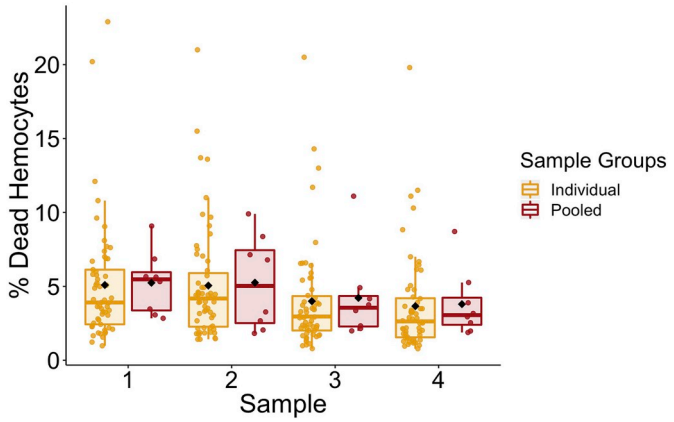

Fig. 7. Hemocyte mortality in pooled vs. individual samples. Boxplots outline the 25th and 75th percentiles, and the midline indicates the median percentage of dead hemocytes (all four Groups combined) for the individual (yellow) vs. pooled (red) group samples. Black diamonds indicate the group mean. No statistical analyses were used to compare pooled-vs. individual-group means for a given sample timepoint due to unequal sample sizes. However, there was < 0.5% difference in mean hemocyte mortality between the individual- vs. pooled-group means for any given sample timepoint (see Results). Overall, the grand means of the pooled-vs. individual-groups were not significantly different from each other ($p = 0.75$). The percentage of outliers (defined as data points above the box and whisker plots) ranged from 5 to 9% of the total population. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

reason for these random outliers, as they occurred on different days, and so there did not appear to be any effect of sampling time or day, nor machine error on a specific day. As expected, the median value (as opposed to the mean) of each Group's hemocyte mortality was much less influenced by outliers, and therefore was more replicable across sampling timepoints among Groups (the median hemocyte mortality across Groups ranged from 2.5 to 4%), and the between-sample variation across each Group was minimized (varying by ~1.5–3.5% across each Group's four samples; see Table 2). Thus, the median may be a better descriptive statistic to use when trying to detect a biologically meaningful change in hemocyte mortality when repeatedly sampling the same group of individuals over time.

No mussels died following the repeated sampling procedure when monitored for two weeks after each individual's last sample was drawn. Repeated hemolymph sampling with ≥24 h between samples is, thus, not lethal.

4. Discussion

The purposes of this study were, firstly, to develop FACS methods to analyze hemocyte characteristics in *M. californianus*. Next, we sought to evaluate whether hemolymph could be repeatedly drawn from an individual mussel, and if so, whether the time intervals between samples affected hemocyte mortality or organism survival. Lastly, through employing these procedures, we sought to establish a baseline dataset describing the intra- and inter-individual variability in hemocyte mortality across repeated sampling in mussels free from typical environmental stressors. To address these questions, we created four Groups of 14–15 mussels, and then repeatedly sampled their hemolymph three times after the initial (time zero) sample was taken; each Group's subsequent samples were spaced either 24, 48, 72, or 168 h apart. We evaluated each individual's hemocyte mortality (using FACS), and then compared these individual-group samples to pooled-group samples that we created (of four individuals each). Our study produced several key findings.

First, we were able to successfully establish FACS gating and analysis methods to assess hemocyte mortality in *M. californianus*, thus allowing future studies of this species to use FACS for examining mechanistic questions about hemocyte responses to stress over time. Forward and side scatter thresholds were established to separate the hemocyte cell population from debris, and the separate hyalinocyte and granulocyte populations were then identified through gating and

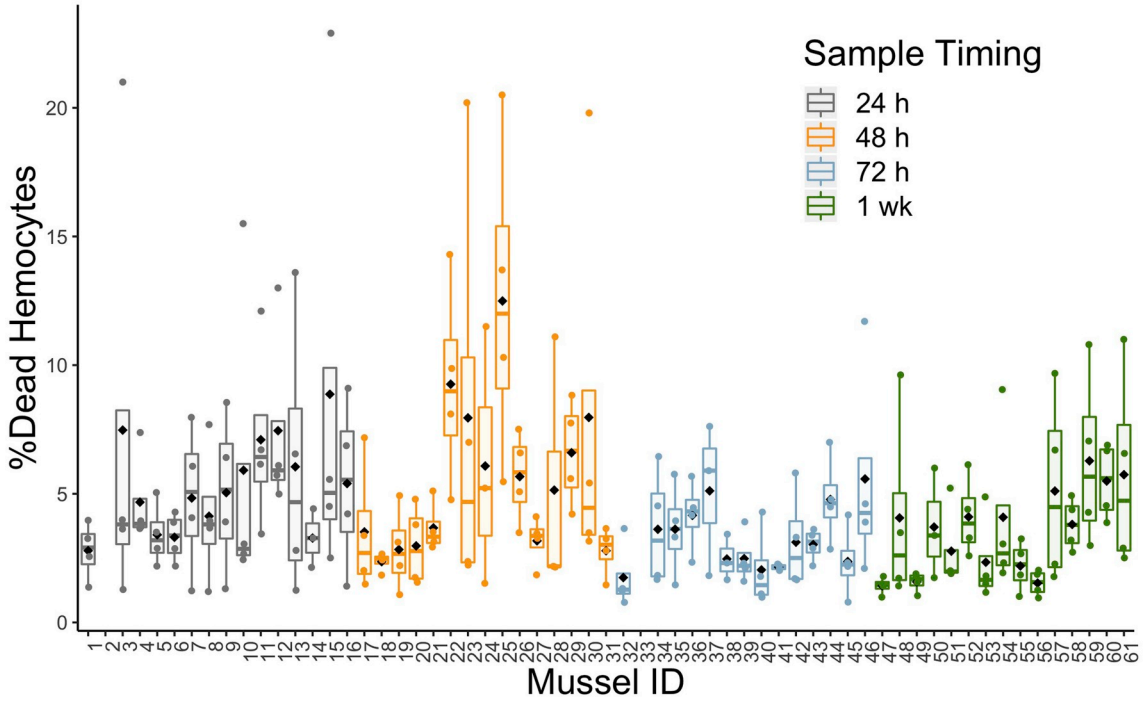

Fig. 8. Individual variability in hemocyte mortality across samples. Each boxplot outlines the 25th and 75th percentiles, and the midline indicates the individual's median percentage of dead hemocytes across the four (repeated) samples. Black diamonds indicate the individual's mean hemocyte mortality across samples. Different Groups with different sample timing intervals are represented in the colors of gray (24 h apart), orange (48 h apart), blue (72 h apart), and green (168 h apart). There is large inter- and intra-individual variability. However, even if an individual had one sample with a high percentage of dead hemocytes (e.g., Mussel ID 3), the next day its dead cell count would come back to typical values. Moreover, there was no sampling (day) effect, which means that the intra-individual variability in hemocyte mortality is likely a part of natural biological cycles in hemocyte mortality. Note that Mussel IDs 2 and 33 were excluded from this study due to the inability to obtain enough FACS events in one or more samples (these individuals are not included in the total sample size). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Mean ± SD Individual variability & reliability of hemocyte mortality across samples.

Sample Group	Individual Sample SD	Individual Sample Range	Individual Sample Differences	Individual Sample CV
24 h	3.7 ± 2.7	8.2 ± 5.8	4.3 ± 3.4	0.77 ± 0.72
48 h	3.3 ± 2.7	7.1 ± 5.6	3.5 ± 2.6	0.56 ± 0.30
72 h	1.7 ± 1.0	3.7 ± 2.2	2.3 ± 1.6	0.49 ± 0.21
168 h	2.0 ± 1.3	4.3 ± 2.8	2.0 ± 1.4	0.52 ± 0.23
Overall	**2.7 ± 2.2**	**5.9 ± 4.7**	**3.0 ± 2.5**	**0.59 ± 0.43**

Note: All data presented in the table are percentages (of hemocyte mortality, except for the CV). No statistical analyses were completed for these data, as they are simply meant to describe the overall variability and reliability of repeated sampling within the same individuals across four repeated hemolymph samples. The "Individual Sample SD" is calculated by obtaining the standard deviation of each individual's four samples, and then calculating the mean ± SD of that Group overall (n = 14–15 per group). The "Individual Sample Range" is calculated by obtaining each individual's maximum minus minimum hemocyte mortality across the four samples, and then calculating the overall Group mean ± SD. The "Individual Sample Differences" was calculated by obtaining each individual's average of the absolute differences between each of the subsequent samples (e.g., |*Sample* 3 − *Sample* 4|), and then calculating the Group mean ± SD. The "Individual Sample CV" represents that Group's mean ± SD for each individual's coefficient of variation across the four samples (i.e., an individual's standard deviation of all samples divided by the mean of all samples); these values are unitless, as the mean and SD are both already percentages.

microscopy. Based on our images, we confirmed that gated populations of the two cell types matched descriptions from previous research: hyalinocytes are smaller, rounder, less granular cells with a larger nuclear-to-cytoplasm ratio compared to granulocytes [2,24]. Using FACS, we were also able to analyze the relative percentage of hyalinocytes and granulocytes in any given sample. We found that in our population of 59 adult mussels, there was, on average, a larger percentage of hyalinocytes vs. granulocytes (by ~7%); interestingly, both cell types had a similar percentage of dead cells that did not vary with repeated sampling. In some mollusks (oysters [22]) there are more hyalinocytes than granulocytes, but in a congener of *M. californianus, M. galloprovincialis,* granulocytes are present in higher densities than hyalinocytes [24]. These differences may be due to local environmental stressors inducing hemocyte changes, differences in study methodologies, between-species differences, or even potentially due to the use of different aged individuals across studies [32–34]. It is clear that more research is required to further elucidate hemocyte cell population differences among *Mytilus* species and across various types of mollusks. Regardless, our ability to isolate specific hemocyte populations, such as hyalinocytes vs. granulocytes, opens the door to future down-stream analyses of these cell types via FACS in *M. californianus*. For example, one could explore whether these cell populations respond differently to various stressors based on changes in their transcriptional or proteomic profiles.

Our mussels' average baseline hemocyte mortality for the pooled- and individual-group samples (grand means ± SD = ~4.6 ± 2.6% and 4.5 ± 3.7%, respectively; n = 32 pooled samples and n = 230 individual samples) were similar to that of pooled hemolymph samples in previous research [5–7]. However, unique to our study, we evaluated individual samples, which allowed us to establish a dataset of baseline hemocyte mortality for individuals that were repeatedly sampled over time and not exposed to any abiotic stressors. We found that repeated hemolymph sampling of the same individual, with as little as 24 h between samples, is possible, and did not cause any discernible adverse

effects in terms of animal mortality. In addition, there were a similar number of outliers (indicating substantial above average hemocyte mortality) across samples (all Groups combined; see Table 2), whereby 5–9% of the individuals were outliers on any given sample day. The 72 h Group had the least number of outliers (~2% of individuals), and the other three Groups had a similar number of outliers overall (ranging from 3 to 5 individuals). However, none of these outliers were from the same individual or occurred on the same day, and so we suspect that this is a part of normal biological variation. Since the mean is heavily influenced by outliers, we suggest that researchers use the median of individual-group samples when comparing hemocyte mortality from repeated samples over time, as this statistic is more robust to outliers.

There was large intra-individual variability with repeated sampling. For example, there were some individuals whose hemocyte mortality was as high as 23% for one sample but had hemocyte mortalities < 5% in the other samples. This finding is similar to that of a previous study which evaluated snails' hemocyte mortality across 10 repeated samples [21]. That study also showed large inter- and intra-individual variability with repeated sampling and reported that unstressed snails had an average hemocyte mortality of ~15%, and a few individuals even experienced ranges of > 20% in hemocyte mortality across repeated samples. It is unclear what causes some individuals to experience this large, but acute, increase in hemocyte mortality one day, and then 24 h later, have more typical values of hemocyte mortality. However, our data highlight the point that a snapshot-in-time of an individual's hemocyte mortality may be misleading. Regardless of the sampling interval, it appears necessary and important to employ repeated sampling to fully understand how an individual responds to, and recovers from, a stressor over time. That being said, we recommend obtaining at least two baseline samples from each individual vs. a single baseline sample. Moreover, it is important to understand how the time interval between samples may affect study results. We found that intra-individual variability was highest in the 24 and 48 h groups vs. the 72 and 168 h groups (see Table 3). Therefore, it may be advisable to use a larger sampling window (i.e., ≥72 h apart between the two repeated samples) to minimize intra-individual variability across repeated samples and thereby reduce the risk of false positives, i.e., attributing a sample with high hemocyte mortality to the effect of the stressor, when it is actually due to normal biological variation. However, this longer time interval between samples may mean that one would miss important elements of the hemocyte "stress response" [35,36]. As such, researchers should be aware of the pros and cons of repeated sampling at different time intervals, and can use our baseline dataset as a guide for the intra-individual variability in hemocyte mortality associated with repeated sampling .

Many studies have used pooled samples to minimize variation [5–7,16], but we are unaware of any studies of hemocyte mortality that have actually described the variation among individual- vs. pooled-group samples. In our study, we did not find any differences between the pooled- and individual-group sample means, with the means differing by ~1% at each sample timepoint (see Fig. 7). However, we caution researchers against using pooled samples comprised of as few as four individuals (the number of individuals typically used in previous studies) because with such a small sample size the pooled group mean can be heavily influenced by outliers: just one individual outlier in the pooled sample can elevate the mean and lead to the potentially false conclusion that the stressor or experimental intervention had an effect on all of the individuals. For example, the two pooled-group means on Day 3 in the 48 h Group were substantially different: ~4 and 13% hemocyte mortality. This was because two out of the four individuals that comprised the 13% sample were outliers, which resulted in the highest pooled group mean for the 48 h Group across all four Days. Overall, tracking pooled-group samples across time precludes the ability to distinguish individual differences in hemocyte dynamics that may be attributable to unique physiological responses to a given stressor.

Given the observed substantial intra- and inter-individual variability in this study, we caution against concluding that a change in hemocyte mortality that is statistically significant, is necessarily biologically significant. For example, all of the statistical differences we found between- or within-Groups had mean differences of < 5%. Although this might seem like a large mean difference between Groups, this < 5% difference falls within the average range (~6%) in hemocyte mortality across an individual's repeated samples. Therefore, although these group differences are statistically significant, they may not be biologically significant. In addition, the fact that these mussels were not exposed to any stressors (other than the hemolymph withdrawal), would indicate that, in this case, statistical significance does not imply biological significance. These findings clearly lead to a host of future research questions that aim to better understand how hemocytes function and respond to stressors, as well as more basic research questions that include trying to better understand hemocyte turnover and hematopoiesis. In the meantime, we propose that our dataset can be used not only to determine the sample sizes needed (i.e., power calculations) to detect statistical differences in hemocyte mortality between- and within-groups when employing repeated hemolymph sampling, but also as a foundation for understanding normal biological fluctuations in hemocyte mortality of mussels free from typical abiotic stressors.

In conclusion, our study demonstrated that 1) FACS can be used for hemocyte analyses in *M. californianus*, where hyalinocyte and granulocyte populations can also be studied, 2) hemolymph can be repeatedly sampled in single individuals, with repeated samples occurring ≥24 h apart, 3) there is substantial intra-individual variability in hemocyte mortality associated with repeated sampling, which is minimized with longer time intervals between repeated samples (i.e., ≥72 h apart), and 4) due to this intra-individual variability, a single sample may not be representative of that individual's typical hemocyte mortality; therefore repeated sampling (≥2 samples) can better establish an individual's baseline. We encourage scientists to consider when it is important to track group changes versus individual changes over time and suggest that studying individual stress responses will help elucidate cellular mechanisms linked to different phenotypes. If time and resources allow, we recommend that researchers begin to analyze individual samples (within each treatment group), while simultaneously exploring group responses (by taking the overall group median). These recommendations also apply to researchers using hemocytometers to obtain hemocyte mortality, as this same intra-individual variability would surface with repeated sampling. Lastly, our dataset is important in describing the typical intra- and inter-individual variability associated with repeated hemolymph sampling at varying time intervals in mussels free from typical environmental stressors. As such, we believe our dataset can help scientists to determine what might be considered "biological significance" (vs. statistical significance) in hemocyte mortality when evaluating individual responses across differing treatments or experiments.

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Data Availability

Dataset available for download at doi: https://doi.org/10.17632/nsx59f4c29.2.

CRediT authorship contribution statement

Nicole E. Moyen: Conceptualization, Methodology, Visualization, Formal analysis, Investigation, Project administration, Writing - original draft, Data curation, Supervision, Funding acquisition, Resources. **Paul A. Bump:** Conceptualization, Methodology, Investigation, Writing

- review & editing, Supervision, Resources. **George N. Somero:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Mark W. Denny:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing or financial interests.

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