High resolution analysis of metabolic cycles in the intertidal mussel *Mytilus californianus*

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ABSTRACT
Inhabitants of the marine rocky intertidal live in an environment that alternates between aquatic and terrestrial due to the rise and fall of the tide. The tide creates a cyclical availability of oxygen with animals having access to oxygenated water during episodes of submergence, while access to oxygen is restricted during aerial emergence. Here we performed liquid chromatography and gas chromatography-mass spectrometry enabled metabolomic profiling of gill samples isolated from the California ribbed mussel, *Mytilus californianus*, to investigate how metabolism is orchestrated in this variable environment. We created a simulated intertidal environment in which mussels were acclimated to alternating high and low tides of 6 hr duration, and samples were taken every 2 hr for 72 hr, to capture reproducible changes in metabolite levels over 6 high and 6 low tides. We quantified 169 named metabolites of which 24 metabolites cycled significantly with a 12 hr period that was linked to the tidal cycle. These data confirmed the presence of alternating phases of fermentation and aerobic metabolism and highlight a role for carnitine conjugated metabolites during the anaerobic phase of this cycle. Mussels at low tide accumulated 8 carnitine-conjugated metabolites, arising from the degradation of fatty acids, branched-chain amino acids, and mitochondrial β-oxidation end products. The data also implicate sphingosine as a potential signaling molecule during aerial emergence. These findings identify new levels of metabolic control whose role in intertidal adaptation remains to be elucidated.

KEYWORDS
Hypoxia, anaerobic, metabolomic, intertidal
INTRODUCTION

The biology of organisms is strongly influenced by temporal changes of environmental factors in their habitat. The marine intertidal is a particularly variable habitat because it represents the zone where the marine aquatic and terrestrial environments merge, and the inhabitants are subjected to aquatic factors during high tide and terrestrial factors during low tide. This has made intertidal inhabitants valuable study systems for investigating the mechanisms that allow life to flourish in a highly variable environment (18). Mussels, of the genus *Mytilus*, have long been a favored study organism in the intertidal because they have a worldwide distribution, and being sessile, must endure fluctuations in their environment. Therefore, they are likely to possess particularly robust mechanisms to deal with changing environmental conditions. During immersion at high tide, the seawater provides mussels access to food and dissolved oxygen, and body temperatures are similar to the prevailing sea surface temperature. In contrast, during aerial exposure the mussels cannot filter feed and body temperatures can rise or fall due to differences between air and seawater temperature or because of the heating effects of solar irradiance (21, 41). Furthermore, access to oxygen is severely restricted because emerged mussels must close their valves to prevent desiccation (10).

Studies in *Mytilus edulis* have documented a number of physiological changes that are associated with alternating periods of immersion and aerial emergence that include compensatory changes in metabolic rate (41), oxygen consumption (9), heart rate (20), valve opening/gape (33, 40). Further insights into the functional significance of these responses arose from analysis of intermediary metabolites and how their abundance changes with the ebb and flow of the tide, as well as other environmental variables (41). The results of these studies, along with similar studies of other invertebrates (27), have yielded a model that defines the core metabolic pathways associated low tide aerial exposure (41). According to this model, immersed mussels undergo aerobic metabolism and synthesize ATP using coupled citric acid cycle/electron transport pathways. Upon emergence, bivalves close their valves and oxygen concentrations in the mantle cavity drop quickly and anaerobic metabolism commences at the onset of hypoxia (2). Under these hypoxic conditions, glucose and aspartate are fermented to
produce succinate and alanine via the glucose-succinate, and aspartate-succinate pathways, respectively (24). If the duration of hypoxia extends for days then succinate is further converted to propionate which yields additional ATP as well as aiding in acid-base balance via the production of bicarbonate (24, 30). Studies into these pathways indicate that these end-products produce a greater amount of ATP per unit of glucose, and produce less metabolic protons, compared to the chief anaerobic pathway in vertebrates in which glucose is converted to lactate (27). At the same time, mussels reduce the activities of many processes such as digestion, respiration, and heart activity, and glycolysis, which allow mussels to maintain energy balance and reserve glycogen stores in anticipation of long term periods of hypoxia (27, 28, 41). Upon re-submergence the valves open within minutes, nourishing tissues with oxygen and food, and this period is characterized by increased heart rate and heat production linked to the re-synthesis of aspartate and glycogen, filter feeding, digestion, and excretion of anaerobic end-products into the aquatic medium (see review in (15)).

Technological advances in metabolite analysis have the potential to reveal new insights into metabolic adaptations to intertidal life. Whereas earlier studies were limited to measurements of a single metabolite at a time, contemporary techniques such as $^1$H NMR, liquid chromatography (LC)-mass spectrometry, and gas chromatography (GC)-mass spectrometry (LC/MS and GC/MS respectively) have the potential to quantify hundreds to thousands of metabolites in a single sample. Recent studies employing $^1$H NMR have shown that succinate and alanine accumulate in *M. edulis* tissue during hypoxia (22, 38), confirming previous studies, but have yet to yield a truly global insight into the metabolic reprogramming that occurs with each period of aerial emergence and immersion. Here we report results from an unbiased LC/MS and GC/MS metabolomic screen of a simulated tidal cycle in the California ribbed mussel, *Mytilus californianus* Conrad, the species that dominates rocky intertidal wave exposed sites from Baja California to Alaska (37). *M. californianus* is well adapted to periods of immersion and aerial emergence and appears to share all of the characteristic responses exhibited by *M. edulis* (2). By taking repeated measurements of metabolites every 2 hrs over 3 days and 6 tidal cycles we provide an unprecedented temporal overview of the relationship
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between tidal cycles of immersion and aerial emergence and changes in the metabolome.

MATERIALS AND METHODS

Animals

*Mytilus californianus* of 4-5 cm length were collected at Zuma Beach, north of Los Angeles, CA. In order to simulate intertidal conditions in the lab, the mussels were maintained in aquaria and grown as a single layer of animals on shelves at the mid-level height of the aquarium. The ebb and flow of the tide was simulated using computer-controlled water pumps that regulated the depth of water by pumping seawater into and out of the aquarium. The room was maintained at a constant temperature of 17°C to ensure that the body temperature of the mussels remained constant whether submerged or emerged in air. Food as liquid algal cultures (Shellfish Diet 1800, Reed Mariculture, Campbell, California) was continuously added to the water during each episode of high tide submergence. The seawater in the aquaria was constantly replaced such that 10% of the volume of the system was changed every day. Cardiac activity was monitored continuously in a subset of mussels non-invasively using an infra-red phototransducer that was attached to the exterior of the shell and allows heart rate to be measured (13).

A tidal regime of alternating periods of 6 hrs in and 6 hrs out of the water was established and mussels were acclimated to this regime for 4 weeks prior to the commencement of sampling. Low tides occurred from 12 am to 6 am and 12pm to 6 pm. A light/dark cycle was imposed with period of darkness occurring from 6 pm to 6 am. Animals were sampled every 2 hrs over a period of 72 hrs with the initial sample collected at 7 am on day 1 of the experiment (Fig. 1A). This sampling regime ensured that 3 samples were collected per episode of low or high tide, with the first sample collected 1 hr into the episode, the second sample taken at the middle 3 hr time-point and the third sample taken 1 hr prior to the change in tidal episode. Four individual mussels were collected at each time-point. An equal mass of gill tissue (50 mg) was
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dissected from each individual, pooled into a 2 ml cryovial, snap frozen in liquid
nitrogen, and stored at -80°C.

Metabolite analysis
Gill samples were shipped on dry ice to Metabolon (Durham, NC) for metabolite
analysis. Samples were prepared for LC and GC separation and MS analysis at
Metabolon. The tissue samples were ground (Glen Mills Genogrinder 2000) in methanol
for 2 mins which served to dissociate small molecules bound to protein and precipitate
proteins. The sample was centrifuged and the resulting supernatant was split into equal
volumes for analysis on the LC+, LC-, and GC platforms, and vacuum-dried. The LC/MS
portion of the platform incorporated a Waters Acquity UPLC system and a Thermo-
Finnigan LTQ mass spectrometer, including an electrospray ionization (ESI) source and
linear ion-trap (LIT) mass analyzer. Aliquots of the vacuum-dried sample were
reconstituted, one each in acidic or basic LC-compatible solvents containing 8 or more
injection standards at fixed concentrations (to both ensure injection and
chromatographic consistency). Extracts were loaded onto columns (Waters UPLC BEH
C18-2.1 x 100 mm, 1.7 μm) and gradient-eluted with water and 95% methanol
containing 0.1% formic acid (acidic extracts) or 6.5 mM ammonium bicarbonate (basic
extracts). Samples for GC/MS analysis were dried under vacuum desiccation for a
minimum of 18 hours prior to being derivatized under nitrogen using bistrimethyl-silyl-
trifluoroacetamide (BSTFA). The GC column was 5% phenyl dimethyl silicone and the
temperature ramp was from 60° to 340° C in a 17 minute period. All samples were then
analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass
spectrometer using electron impact ionization. The instrument was tuned and calibrated
for mass resolution and mass accuracy daily. For monitoring of data quality and
process variation, multiple replicates of a pool of human plasma were injected
throughout the run, interspersed among the experimental samples in order to serve as
technical replicates for calculation of precision. Signatures for each metabolite were
identified by matching to a database of 1,205 authentic compound standards (16).
Quantitative comparisons of each compound in each sample were based on integrated
peak ion counts of the quantification ion peak and were adjusted for minor day-to-day
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instrument gain drift by Metabolon as described (26). Null values were imputed with the minimum value detected for that compound among all samples based on the assumption that the values were below the level of detection.

Metabolites that exhibited a rhythmic pattern of abundance were identified using JTK_CYCLE (23) and p-values were corrected for a false discovery rate of <0.05 using a standard method (36). As an additional measure of significance a Welch’s t-test was performed to test the null-hypothesis that metabolite levels were unchanged between the 18 samples collected during low tide and the 18 samples collected during high tide.

RESULTS

Metabolomic profiling identified a total of 169 named compounds in gill samples isolated from mussels during a simulated tidal regime. To identify metabolites whose abundance was linked to the tidal cycle we used the JTK_CYCLE algorithm to identify 24 metabolites which exhibited statistically significant rhythmic changes in abundance (Table 1). The JTK_CYCLE algorithm was developed to identify rhythmic patterns in large scale time-series, and is a non-parametric test that detects orderings of values that correlate with predefined period lengths (23). The metabolites that were assigned the most significant p-values by JTK_CYCLE were those that exhibited cyclical levels of abundance of large amplitude. Overall, the relative abundance of 17 metabolites peaked in the samples collected during low tide, while abundance of 7 metabolites peaked in high tide collected samples. Interestingly, the abundance of all 24 metabolites peaked every 12 hrs, indicating that their rhythmic pattern was associated with changes in the tidal cycle, and we found no evidence for metabolite abundance patterns that adhered to a circadian cycle. All but 2 of these metabolites were deemed statistically significant in a Welch’s t-test that compared all the samples collected during low tide to those collected during high tide (Table 1). Simultaneous monitoring of cardiac activity confirmed previous studies (19) and showed that aerial emergence caused an abrupt cessation of heart rate which persisted for the duration of low tide, and that cardiac activity resumed quickly upon submergence at high tide (Fig. 1B).
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The most significantly oscillating metabolite in this dataset was succinate, the end-product of anaerobiosis in mussels (12), which exhibited a strong rhythmic pattern of abundance, with levels increasing upon aerial emergence and then declining rapidly during the first hour of immersion (Fig. 2A). Similarly, levels of malate, which is the intermediate product formed during the reduction of oxaloacetate to succinate, oscillated and increased during low tide (Fig. 2A). Consistent with glucose serving as the primary substrate for anaerobiosis (12) our data revealed that glucose abundance generally declined during low tide (Fig. 2B). The other anaerobic pathway in mussels is the reduction of aspartate resulting in the formation of succinate as well as alanine via glutamate and the abundance of these three metabolites was cyclical, with alanine and aspartate exhibiting broadly anti-correlated abundance profiles (Fig. 2C). As evidence of a resumption of aerobic metabolism at high tide, we observed a sharp rise in citrate levels after 1 hr of immersion (Fig. 2D) suggesting that the accumulated malate and succinate were metabolized through an active TCA cycle. Propionate which is produced by a further reduction of succinate was not detected in any of these samples consistent with reports that propionate production is initiated only after longer periods of hypoxia (24).

A recurring pattern in these metabolomic data was the increase in carnitine-conjugated metabolites during low tide, with a total of 8 carnitine-conjugated metabolites exhibiting a rhythmic abundance profile. For example, we identified that the carnitine derivatives of the end products of β-oxidation of fatty acids with even or odd numbers of carbons, acetylcarnitine and propionylcarnitine, respectively, oscillated and peaked during low tide (Fig. 3A). We detected corresponding changes in free carnitine whose level trended towards decreasing at low tide (Fig. 3B). Long-chain fatty acids have to be conjugated to carnitine for transfer into the mitochondrial matrix for β-oxidation, and samples taken during low tide had significantly higher levels of both stearoylcarnitine and butyrylcarnitine (Fig. 4A). Similarly, animals at low tide had significantly higher levels of 4 carnitine-conjugated intermediates of branched-chain amino acid (BCKA) catabolism, isobutyrylcarnitine, 2-methylbutyroylcarnitine, isovalerylcarnitine and
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hydroxyisovaleroyl carnitine (Figs 4B & 4C). The primary step in their catabolism is the formation of branched-chain keto acids which are then oxidized in the mitochondria to form acetyl-CoA and propionyl-CoA through a pathway that requires the formation of CoA intermediates, but instead these intermediates accumulated as carnitine-conjugates during low-tide. Closer inspection of the time-course of the accumulation of these carnitine-conjugates indicates that while acetylcarnitine and propionylcarnitine tended to continue to accumulate for the duration of the low tide episode, the abundance of the other carnitine-conjugates often peaked at the mid 3 hr time-point of low tide. Considered together, these data indicate that intermediate metabolites that are destined for metabolism in the mitochondria accumulate during low tide, and that rather than accumulating as CoA derivatives, are conjugated to carnitine. Levels of the CoA derivatives of these metabolites were always below the detection threshold of our metabolomic screen suggesting that CoA conjugates are rapidly metabolized and do not accumulate in this tissue. Consistent with this pattern, the abundance profile of pantothenate, a precursor for CoA synthesis, exhibited a low amplitude cyclical abundance pattern (Fig. 3C) with levels on average being elevated during low tide (Table 1), suggesting that the demand for CoA is decreased during periods of aerial emergence.

These data revealed other metabolites whose abundance was correlated with the tidal cycle but whose function in intertidal physiology remains cryptic. For example, levels of sphingosine, a signaling lipid and the backbone molecule of sphingolipids, cycled during the tidal regime and its abundance declined during low tide (Fig. 5A). Our metabolomic data revealed that two metabolites in pathways regulating sulfur metabolism exhibited cycles of abundance, with levels of S-adenosylhomo-cysteine (SAH) declining during low tide, whereas levels of cysteine-glutathione disulfide, an oxidative stress protectant (3), were elevated at low tide (Fig. 5C). Also peaking during low tide was gamma-glutamylalanine (Fig. 5C), which is formed by the transfer of the γ-glutamyl moiety of glutathione to extracellular alanine by γ-glutamyl transpeptidase. Another metabolite whose abundance peaked during low tide was aminoadipate which is formed during the catabolism of lysine (Fig. 5D).
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DISCUSSION
The ebb and flow of ocean tides are one of the most predictable forces on Earth and in this study we present the most comprehensive screen to date of metabolite abundance in bivalves with respect to this tidal cycle. Our findings confirm previous studies that have examined metabolism in mussels during aerial emergence, and in addition implicate additional levels of metabolic control whose role in intertidal adaptation remains to be elucidated. Previous work in bivalves has reported tissue differences in the levels of both substrates and accumulated products, as well as their relative proportions during hypoxia (25), and indicate that this variation probably is linked to differences in the prevailing composition of the energy stores in the tissues (11). This suggests that strategies of metabolic reorganization will vary according to tissue and the metabolic status of the animal. Despite these sources of variation, our data recapitulated much of what is already understood regarding the pathways and metabolites that participate in the switch to anaerobic metabolism that occurs during low tide aerial emergence (Fig. 6A). Metabolites associated with glucose and aspartate fermentation displayed particularly robust oscillations in this dataset. Malate and succinate as intermediate and end-products of these pathways accumulated quickly following emergence, increasing ~8-fold during the first hour in air, suggesting that this anaerobic pathway was active within a short period of emergence. Upon immersion, succinate levels fell rapidly and had returned to base-line levels within the first hour. Studies in other mollusks have indicated that much of the accumulated succinate is simply released into the external environment, rather than being metabolized (17), but our observation of a large increase in citrate during the first hour of immersion suggests that a proportion of the succinate is recovered and metabolized by the TCA cycle. Unfortunately, our data provides only relative abundance levels which do not allow us to estimate the proportion of the accumulated succinate that is metabolized to citrate. The reduction of aspartate is believed to be the first anaerobic pathway that is activated in response to hypoxia and our metabolomic data confirms that the aspartate-succinate
pathway was also active in emerged *M. californianus*, and revealed that the relative abundance of aspartate and alanine is in anti-phase to one another.

One of the most striking aspects of these data was the rapid increase of carnitine-conjugated metabolites during low tide aerial emergence. Carnitine is a low molecular-weight compound obtained from the diet but which can also be synthesized from the essential amino acids lysine and methionine. Carnitine has an obligate role in the mitochondrial oxidation of long-chain fatty acids because long-chain fatty acid acyl groups must be transferred from CoA derivatives to carnitine in order to enter the mitochondria because the mitochondrial inner membrane is impermeable to polar molecules such as CoA. Carnitine serves as a carrier for this transport in a process called the carnitine shuttle and the conjugation reaction is catalyzed by Carnitine acyltransferases. Consistent with this role, gill samples collected during low tide had significantly higher levels of stearoylcarnitine and butyrylcarnitine indicating that these fatty acid transfer molecules are accumulating rather than entering the mitochondria and being catabolized. Therefore, we interpret the increase in long-chain fatty acyl carnitine conjugates as evidence that fatty acid catabolism is halted during low tide, leading to the accumulation of these intermediate metabolites (Fig. 6B). The other sources of carnitine-conjugated compounds were acyl-chain intermediary metabolites of BCKA catabolism, as well as acetylcarnitine and propionylcarnitine (Fig. 6C). In contrast to the established role that carnitine plays in fatty acid catabolism, carnitine does not play an obligate role in BCKA metabolism, nor in the metabolism of acetyl-CoA or propionyl-CoA, suggesting that carnitine-conjugation may serve a specific role in regulating these pathways during low tide. While the role that carnitines play in branched-chain amino-acid catabolism is poorly understood studies in mammals indicate that their carnitine-conjugates can be found in most mammalian tissues (4).

These data raise questions regarding the role that carnitine plays in the control of metabolism during low tide. Conjugation and removal of carnitine is regulated by Carnitine acyltransferase 1 and Carnitine acyltransferase 2, located on the outer and inner layers of the inner mitochondrial membrane respectively. In addition to carnitine's
role in fatty acid transport, it has been proposed that the formation of carnitine conjugates may represent a safety mechanism to prevent acyl-CoA accumulation in the cytoplasm and mitochondria (5). Carnitine binds acyl residues and helps in their elimination by decreasing the number of acyl residues conjugated with CoA and increasing the ratio between free and acylated CoA. In turn, changes in this ratio alter the activity of many mitochondrial enzymes involved in the citric acid cycle, gluconeogenesis, and fatty acid oxidation (35). Evidence of the detrimental effects of elevated levels of acyl-CoA arises from research into genetic disorders affecting acyl-CoA metabolism, which results in elevated levels of acyl-CoA in the mitochondria and to serious health problems and early death (34). Investigations into these diseases report that the mitochondria of affected individuals compensate for acyl-CoA imbalances by converting acyl-CoA to acyl-carnitines and that the capacity of the patient to correct this imbalance is limited by available carnitine and that dietary carnitine supplements help to relieve these metabolic diseases (31). We hypothesize that the transfer of carnitine to acyl-CoA molecules during low tide may represent a mechanism to offset the debilitating effects that elevated levels of acyl-CoA may present. In the context of fatty acid metabolism, this means that acyl-CoA molecules remain conjugated to carnitine and are not metabolized further, and in the context of BCKAs, that intermediate acyl-CoA products are transferred to carnitine. Similarly, we interpreted the increases in acetylcarnitine and propionylcarnitine as a reflection of a buildup of acetyl-CoA and propionyl-CoA during low tide which resulted in their transfer to carnitine. However, growing evidence indicates that both acetylcarnitine and propionylcarnitine play a role in the cellular stress response to oxidative damage (6), and that increased levels of these metabolites have therapeutic effects and reduce ischemia-reperfusion injury (7). Furthermore, the ratio of acetyl-CoA to CoA has important effects on overall mitochondrial metabolism by modulating the activity of Pyruvate dehydrogenase with low ratios enhancing the metabolism of carbohydrates (8). Therefore the functional significance of the increases in these metabolites during low tide remains cryptic.

Finally, our metabolomic screen revealed that the CoA precursor, pantothenate, increased during low tide, suggesting that de novo synthesis of CoA is depressed. The production of CoA from pantothenate requires a large amount of energy (4 ATP) and
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therefore the transfer of carnitines to intermediate metabolites may represent an energetically favorable strategy to salvage CoA during low tide. Further investigations will be required to test these hypotheses starting with comparisons of the relative levels of total carnitine and carnitine acyltransferase activity in mussels growing under tidal versus subtidal conditions. Increases in intertidal mussels would suggest that an elevated capacity for carnitine-conjugation may serve an adaptive purpose for intertidal mussels.

While most compounds that exhibited cyclical changes in abundance tended to accumulate during emergence, levels of sphingosine declined ~2-fold. Sphingosine comprises an 18 carbon unsaturated hydrocarbon chain with an amino alcohol terminus and is component of sphingolipids, such as ceramide and sphingomyelins. Sphingosine is also involved in cell signaling and can be phosphorylated by Sphingosine kinase (SK1) to produce sphingosine 1-phosphate, and both sphingosine and sphingosine 1-phosphate are important lipid signaling molecules and have implicated as important regulators of many cellular processes, particularly cell survival, proliferation, and death (29). Of particular relevance to this study is that SK1 contains a HIF-1α element binding site that enhances the conversion of sphingosine to sphingosine-1-phosphate during hypoxia (1), with evidence that sphingosine 1-phosphate functions to reduce hypoxia-reoxygenation injury (39). Sphingosine levels declined during aerial emergence when the mussel tissues were hypoxic, and while levels of sphingosine 1-phosphate were below detectable limits in these samples, we speculate that HIF-1α regulated increases in SK1 could be responsible for the decline of sphingosine during emergence. This could mean that sphingosine abundance may represent a biomarker for the activation of hypoxia-signaling pathways during low tide hypoxic episodes and may prompt further investigations into the role that sphingolipid secondary messengers play in intertidal adaptation.

There are limitations of global metabolomic approaches such as we employed here because detection of many metabolites requires specific extraction and detection protocols (14). This approach is unable to detect changes in flux through a particular
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pathway and instead the data must be interpreted by detecting changes in one or more intermediary metabolites or more often by changes in the levels of starting substrates or end-products. Furthermore, this global profiling approach provides data on relative metabolite levels and it does not report the absolute concentrations of compounds in the tissue. The lack of absolute quantitative data restricts the conclusions that can be drawn from the data because information regarding the proportion of a metabolite pool that is subject to changes is absent, nor can changes in metabolite concentration be interpreted within the context of enzyme Km’s (32). Ultimately, absolute quantification of metabolite levels as well as metabolite flux measurements will be required to fully elucidate metabolic reorganization in this model. Regardless of these shortcomings, we demonstrate that mass-spectrometry based metabolomics can provide new insights into the metabolic reprogramming that occurs in bivalves during the switch from aerobic to anaerobic metabolism, despite the fact that this topic has been intensively studied over the last 3 decades.

We suggest that the increased statistical rigor provided by profiling changes in metabolite levels at high temporal resolution over 6 tidal cycles has led to the detection of metabolites that cycle robustly through periods of tidal immersion and emergence. Note that in nature, the duration and timing of bouts of emergence changes each day because the tidal cycle advances by 25 min every tide, and the heights of each high tide are typically different. In contrast, in this study we employed a simulated tidal cycle, consisting of equal periods of submergence and emergence that were repeated at the same times each day. This experimental design was chosen because it allowed patterns of metabolite abundance to be accurately correlated with episodes of immersion or emergence and further studies will be required to confirm that these patterns persist under more variable conditions in the field. These data emphasize the overwhelming effect that tidal cycles of emergence and immersion have on the metabolism of intertidal mussels. While caution should be exercised in extrapolating these findings directly to other species, a review of anaerobic metabolism in other species of bivalves reveals that they deploy similar fermentation pathways during periods of reduced oxygen availability (see review in (17)). Therefore, it will be
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interesting to investigate the extent to which carnitine-conjugation is a ubiquitous strategy deployed in organisms that regularly experience cycles of aerobic and anaerobic metabolism.

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REFERENCES
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FIGURE LEGENDS

Fig. 1. Cardiac activity is correlated with the tidal cycle in a simulated intertidal environment. A: Representation of the environmental conditions used in the simulated tidal environment. Animals were sampled every 2 hr starting at 7 am. Animals were emerged during low tides which occurred from 12 am – 6 am and from 12 pm – 6 pm, while sunrise and sunset occurred at 6am and 6 pm. B: Heart rate of two representative mussels illustrates the rapid cessation of cardiac activity upon aerial emergence.

Fig. 2. Products of anaerobiosis exhibit a cyclical abundance profile and accumulate during low tide. x-Axes, time as represented by the tidal cycle; y-axis, relative metabolite abundance plotted on a log_{10} scale. A: Succinate and malate. B: Glucose. C: Aspartate and alanine. D: Citrate.

Fig. 3. Substrates for the TCA cycle accumulate as carnitine conjugates during low tide. A: Propionylcarnitine and acetylcarntine. B: Free carnitine. C: Pentothenate

Fig. 4. Fatty acids and BCKA degradation products accumulate as carnitine conjugates during low tide. A: Fatty acid degradation products stearoylcarnitine and butyrylcarnitine. B: Valine and isoleucine degradation products ssobutyrylcarnitine and 2-methylbutyroyl-carnitine. C: Leucine degradation products isovalerylcarnitine and hydroxyisovaleroyl-carnitine.

Fig. 5. Diverse metabolites show rhythmic abundance profiles and accumulate during low tide. A: Sphingosine. B: S-adenosylhomo-cysteine. C: Gamma-glutamylalanine and cysteine-glutathione disulfide. D: Aminoadipate.

Fig. 6. Simplified metabolic schema showing the key pathways that are altered during low tide. A: Consensus anaerobic pathways. B: Catabolism of fatty acids. C: Catabolism of branched-chain amino acids. Metabolites in red and green text indicate compounds that were elevated or reduced during low tide respectively.
## Table 1. Metabolites that oscillate during the tidal cycle

<table>
<thead>
<tr>
<th>Compound</th>
<th>HMDB #</th>
<th>Pathway</th>
<th>Platform</th>
<th>Low tide/High tide</th>
<th>Welch’s t-test p-value</th>
<th>JTK_CYCLE p-value</th>
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<td>LC/MS pos</td>
<td>2.88</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2-methylbutyroylcarnitine</td>
<td>HMDB00378</td>
<td>Amino-acid metabolism</td>
<td>LC/MS pos</td>
<td>1.85</td>
<td>&lt;0.001</td>
<td>0.0001</td>
</tr>
<tr>
<td>isovalerylcarnitine</td>
<td>HMDB00688</td>
<td>Amino-acid metabolism</td>
<td>LC/MS pos</td>
<td>2.02</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>hydroxyisovaleryl carnitine</td>
<td>NA</td>
<td>Amino-acid metabolism</td>
<td>LC/MS pos</td>
<td>1.24</td>
<td>0.0532</td>
<td>0.0474</td>
</tr>
<tr>
<td>S-adenosylhomocysteine</td>
<td>HMDB00939</td>
<td>Cysteine metabolism</td>
<td>LC/MS neg</td>
<td>0.80</td>
<td>&lt;0.001</td>
<td>0.0020</td>
</tr>
<tr>
<td>gamma-glutamylalanine</td>
<td>NA</td>
<td>Peptide metabolism</td>
<td>LC/MS pos</td>
<td>1.63</td>
<td>0.0142</td>
<td>0.0004</td>
</tr>
<tr>
<td>gamma-glutamylcysteine</td>
<td>HMDB11171</td>
<td>Peptide metabolism</td>
<td>LC/MS pos</td>
<td>0.84</td>
<td>0.0563</td>
<td>0.0269</td>
</tr>
<tr>
<td>cysteine-glutathione disulfide</td>
<td>HMDB00656</td>
<td>Glutathione metabolism</td>
<td>LC/MS pos</td>
<td>1.46</td>
<td>0.1047</td>
<td>0.0351</td>
</tr>
<tr>
<td>glucose</td>
<td>HMDB00122</td>
<td>Glycolysis/gluconeogenesis</td>
<td>GC/MS</td>
<td>0.55</td>
<td>0.0027</td>
<td>0.0214</td>
</tr>
<tr>
<td>2-aminoacidopate</td>
<td>HMDB00510</td>
<td>Lysine metabolism</td>
<td>LC/MS pos</td>
<td>1.10</td>
<td>0.3957</td>
<td>0.0003</td>
</tr>
<tr>
<td>pantothenate</td>
<td>HMDB00210</td>
<td>CoA metabolism</td>
<td>LC/MS pos</td>
<td>1.15</td>
<td>0.0059</td>
<td>0.0400</td>
</tr>
<tr>
<td>phenylacacetate</td>
<td>HMDB00209</td>
<td>Phenylalanine metabolism</td>
<td>LC/MS neg</td>
<td>1.49</td>
<td>0.0050</td>
<td>0.0435</td>
</tr>
<tr>
<td>sphingosine</td>
<td>HMDB00252</td>
<td>Sphingolipid metabolism</td>
<td>LC/MS pos</td>
<td>0.66</td>
<td>0.0077</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

HDMB refers to the Human Metabolome Database entry for each identified metabolite. Platform indicates the platform on which the compound was detected, and pos/neg signifies whether the extract was eluted under acidic or basic conditions. Low tide/high tide reports the average relative abundance of the compound in samples collected during low versus high tide.
Figure 1

A

Temperature (°C)  
Light/Dark
High
Tidal cycle
Low
Time-points
6am 6pm 6am 6pm 6am 6pm 6am

B

Heart rate (bpm)
6am 6pm 6am 6pm 6am 6pm 6am
Figure 3

A

Relative abundance

Propionylcarnitine

Acetylcarnitine

B

Relative abundance

Carnitine

C

Relative abundance

Pentothenate

Tidal cycle
Figure 4

(A) Relative abundance of Stearoylcarnitine and Butyrylcarnitine over the tidal cycle.

(B) Relative abundance of Isobutyrylcarnitine and 2-methylbutyroyl-carnitine over the tidal cycle.

(C) Relative abundance of Isovalerylcarnitine and Hydroxyisovaleroyl-carnitine over the tidal cycle.
Figure 5

**A**
- Relative abundance
- Sphingosine

**B**
- Relative abundance
- S-adenosylhomocysteine (SAH)

**C**
- Relative abundance
- Gamma-glutamylalanine
- Cysteine-glutathione disulfide

**D**
- Relative abundance
- Aminoadipate
Figure 6

A

glucose
phosphoenolpyruvate
aspartate
oxaloacetate
malate
fumarate
succinate
propionate

B

fatty acid
fatty acyl-CoA
stearylcarnitine/butrylcarnitine
mitochondrial β-oxidation
propionyl-CoA
propionylcarnitine
propionylcarnitine
acetyl-CoA
acetyl carnitine
acetyl carnitine

C

valine isoleucine leucine
transamination
oxidative decarboxylation
isobutyrylcarnitine
2-methylbutyrylcarnitine
isovalerylcarnitine
3-methylcrotonyl-CoA

mitochondrial β-oxidation
propionylcarnitine
acetyl-CoA
acetyl carnitine
acetyl carnitine
TCA cycle