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Research Article

Assessing the Potential of Adenosine and L-Theanine as Metabolic Suppressants for Improving Shipping of the New Zealand Scampi (*Metanephrops challengeri*)

Jaime R. Willis,[1] **Andrew G. Jeffs**,[1,2] **Kevin Heasman**,[3] **and Anthony J. R. Hickey**[1]

[1]*School of Biological Sciences, University of Auckland, Auckland, New Zealand*
[2]*Institute of Marine Science, University of Auckland, Auckland, New Zealand*
[3]*Cawthron Institute, Nelson, New Zealand*

Correspondence should be addressed to Jaime R. Willis; jaime.willis@auckland.ac.nz

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Live crustaceans, especially lobsters, crabs, and shrimp, fetch premium prices in many international seafood markets, especially in parts of Asia. To access these market opportunities, live crustaceans frequently need to be transported over long distances, which can involve prolonged air exposure resulting in elevated stress and increased morbidity and mortality. Interventions which deliver metabolic suppression to live crustaceans during their transport have the potential to improve outcomes from live shipping. In this study, the administration of adenosine (Ado) and L-theanine (L-th) were assessed for metabolic suppression in the New Zealand scampi, *Metanephrops challengeri*, a deep sea lobster which is highly prized as seafood, and with excellent prospects for supply into premium live seafood markets. The administration to scampi of Ado and L-th in isolation or as a mixture (Ado/L-th), caused a significant decrease in heart rate (HR) with a lasting effect for the 4 hr experimental period. However, this depression of HR did not translate into a systemic downregulation of metabolism, as measured by the key metabolites, i.e., glycogen utilization and the accumulation of lactate and ammonia. The lack of systematic metabolic downregulation would preclude the potential use of Ado and L-th for commercial application in live shipping of crustaceans.

1. Introduction

The New Zealand scampi, *Metanephrops challengeri*, is a species of deep sea lobster that is endemic to New Zealand. It is a reptant species found in offshore waters of depths of 200–600 m around much of New Zealand typically within a temperature range of 6–14°C [1, 2]. The bottom trawl fishery for scampi has an annual total catch of ~1,200 tonnes, generating around NZ$ 34 million in sales from mostly frozen whole lobsters [3]. In addition to an established fishery, there is also interest in increasing the supply of quality scampi through developing aquaculture [4], with recent successes in larval rearing to juveniles, which could provide a seed source for aquaculture production [2, 4].

Supplying live New Zealand scampi from both wild capture and aquaculture to premium seafood markets, especially in Asia, requires the transport of live lobsters over long distances quickly, using airfreight. Aerial shipment of crustaceans is typically undertaken out of the water to lower freight costs, but this is problematic for a species that is reliant on aquatic respiration. Exposure to air leads to gill collapse and a shift to the less efficient metabolic pathway to continue to supply energy in the absence of oxygen, i.e., anaerobic glycolysis [5–7]. This shift leads to a mismatch between energetic supply and demand and an accumulation of metabolic waste products; ammonia, lactate, and carbon dioxide (CO_2), which are detrimental to survival of the transported crustaceans [8–10].

Chemical anaesthetics and cold anaesthesia are two commonly used interventions for decreasing metabolic stress in crustaceans undergoing live transportation out of water, and both interventions have shown some success [11–13]. Chemical anaesthetics work primarily through depressing the central nervous system but remain understudied in crustaceans as their use on species destined for human consumption is

limited by food safety and organoleptic concerns [11, 14]. Studies have also shown that anaesthetics commonly used in finfish aquaculture and farming are largely ineffective in crustaceans, or exceedingly high concentrations are frequently required to emulate the equivalent responses [14].

Cold anaesthesia modulates metabolic rates in ectothermic species and hence its wide use in the transport of live crustaceans. However, this requires cooling systems or masses of ice, and it is frequently a time-intensive process that requires steadily lowering water temperature over several hours to a species-specific set point to avoid adverse stress from more rapid thermal shock [12]. Cold anaesthesia has less effect on cold water species such as *M. challengeri*, due to adaptation to the low temperatures (7–10°C) of their cold temperate water habitat, and further lowering and maintaining low temperatures is costly [15, 16]. Recent evidence indicates that cold anaesthesia may have limited metabolic benefits and could be detrimental to long-term survival due to a delayed thermal shock effect upon rewarming [11, 13, 17, 18]. Viable food safe alternatives to modulate metabolic rate are needed to overcome the stressors associated with transport in live crustaceans.

Adenosine and theanine may offer alternatives to provide metabolic suppression in live crustaceans whilst potentially having the advantages of rapid induction, safety for human consumption, and have negligible impact on organoleptic qualities of the seafood end product [19, 20]. Adenosine, is a "retaliatory" metabolite with deep evolutionary origins that have been implicated in prolonged survival of animals during extreme environmental conditions [21, 22]. Adenosine exerts its influence through cell surface G-protein coupled receptors with distinct adenosine binding affinities and signal transduction pathways [21]. These receptors (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) have been well-characterized in mammalian tissues and show distinct local distribution across tissue types [21]. Activation of these receptors leads to a cascade of secondary messengers that coordinate a receptor-specific response. Adenosine receptors have been broadly characterized by their effects on cyclic AMP (cAMP), whether they stimulate ($A_{2A}R$ and $A_{2B}R$) or inhibit (A_1R and A_3R) adenosine formation. Although receptor sequence similarities have diverged between vertebrates and invertebrates, adenosine has proven efficacy in crustacean tissues [23–25].

Extracellular adenosine levels increase during periods of high ATP utilization (exercise) or decreased ATP supply (hypoxia) where they have been shown to induce forms of metabolic depression in vertebrate and invertebrate species alike [26–29]. Few studies have investigated the effects of adenosine in decapod crustaceans. However, neurons were found to respond to extracellular adenosine as spontaneous and evoked responses are depressed in the Caribbean spiny lobster (*Panulirus argus*) [23] and the injection of adenosine ($0.0125\,mg\,g^{-1}$ animal biomass) decreases heart rate (HR) for up to 3 hr in immersed giant Malaysian prawns (*Macrobrachium rosenbergii*) [19].

Theanine is a natural bioactive compound derived from the tea plant (*Thea sinesis*, Theaceae) that has gained popularity as a stress-relief supplement in humans [30]. Naturally available predominately in the L-form stereoisomer, L-theanine is structurally similar to the excitatory neurotransmitter glutamate and has been shown to bind to glutamate receptors and act as an antagonist [31]. It is reported to have antitumor, antioxidant, and neuroprotective effects and has been subject to extensive research [32]. Experimental treatment with a mixture of L-theanine and the inhibitory neurotransmitter, γ-Aminobutyric acid (GABA), significantly increased sleep duration while decreasing sleep latency in mammals [31]. This synergistic effect is relevant to this current study as GABA concentrations increase during hypoxic exposure and play a role in overall metabolic depression [31, 33–35]. Application of theanine has also been shown to increase the concentration of GABA and has proven a potent inhibitor of the glutamate response at the crayfish neuromuscular junction [36].

This study investigates the role that adenosine and L-theanine may play in decreasing metabolic stress associated with air exposure in the New Zealand scampi, *M. challengeri*. Heart rate provides an accurate real-time proxy for metabolic rate, while measures of metabolic substrates and end products provide a direct measure of the outcomes of any induced shift in metabolic rate. For example, tail tissue glycogen, haemolymph glucose, and lactate provide direct measures of energy utilization during air exposure and the subsequent shift to anaerobic metabolism [7, 37]. Measurement of haemolymph ammonia and urea provides insight into protein catabolism [38, 39].

2. Materials and Methods

2.1. Experimental Scampi and Housing. Female scampi (average weight 90 g) from wild capture (caught within 100 km of 42.924395°S, 177.260249°E) were transferred, acclimated, and maintained in a bespoke recirculating aquaculture system at the Cawthron Aquaculture Park (Glenduan, Nelson, New Zealand), with seawater temperatures maintained at 11°C [2]. Experimental animals were acclimated in the aquaria for a period of 4 months after capture. Exclusively female scampi were used in this study as they had been held in captivity at Cawthron Aquaculture Park as part of an observational study into the reproductive biology of scampi. The animal welfare regime in New Zealand promotes the multiple use of experimental animals in research if it is feasible without compromising the experimental rigor. Scampi were housed under dim blue light (10 lumens) at the local daylight photoperiod as a diurnal cue for the animals. However, this low level of lighting made it difficult to conduct fine-scale experimental procedure. As such, dim red light (915 nm; 5 W LED) was used to facilitate the experimental procedures and previous studies indicate that lobster species are insensitive to red light [40]. Experiments occurred within an insulated and temperature-controlled shipping container where the temperature was maintained at 15°C. Scampi were starved for 24 hr prior to experimentation to avoid effects of specific dynamic action. Scampi were weighed prior to experimentation to determine drug dosage calculations. The sample size for HR analysis was 10 individuals per treatment (40 total animals), this number was reduced for subsequent metabolite analyses

due to a freezer failure. The sample sizes for each assay are stated within the respective figure caption. The research was compliant with approvals under the New Zealand Animal Welfare Act of 1999 and approved by the Animal Ethics Committee of the Nelson Marlborough Institute of Technology (AEC2014-CAW-02).

2.2. Experimental Design. The effect of adenosine, L-theanine, and an adenosine/L-theanine mixture on overall metabolism was assessed in *M. challengeri* by following HR, quantifying tail muscle and haemolymph glucose/glycogen, lactate, ammonia, and urea content. Ten scampi were each randomly assigned to one of four treatment groups; vehicle (control for a drug vehicle effect), adenosine (Ado), L-theanine (L-th), or adenosine/L-theanine (Ado/L-th). Treatment involved injection of each corresponding test substance at a volume-matched dosage, adjusted for the weight of individuals, to expose individuals to the same concentrations. Scampi in the vehicle treatment were dosed with the vehicle (H_2O) (0.5 μL/10 g), adenosine was dosed at 2.5 mM (0.25 μL/10 g), L-theanine was dosed at 5 mM (0.5 μL/10 g), and the adenosine/L–theanine mixture was dosed at 2.5/5 mM (0.75 μL/10 g), respectively. Dosages were informed by previous studies [19, 23, 31]. Scampi were injected through the coxa membrane at the base of the third walking leg using a cooled needle (20 g × 1.5") and syringe. A fifth group of 10 scampi was culled immediately from the tanks and served as a baseline with no intervention, hereafter referred to as untreated controls. Following injection, individuals were placed within 6 L experimental tanks that contained a layer of damp paper towels to maintain humidity. Scampi HRs were monitored for 4 hr, at which point individuals we removed and euthanised in a salted ice bath for 10 min. Following this, haemolymph (~100 μL) was extracted through the pericardial cavity using a precooled hypodermic needle, and then snap frozen on dry ice and stored at −80°C for later metabolite analysis. Muscle tissue samples were excised following euthanasia and frozen at −80°C for later metabolite analysis. For analysis, tail muscle samples were homogenized in 1 × PBS (phosphate buffered saline) using a Tissuelyser II (Qiagen) and centrifuged at 500 *g* for 10 min at 4°C. Supernatant was collected; with 20 μL used to quantify total protein (Pierce™ BCA protein assay kit; Thermo Fisher, United States), while the remaining supernatant was deproteinated with 0.6 M ice-cooled perchloric acid (PCA). This was spun at 10,000 *g* for 10 min in a 4°C centrifuge to separate the deproteinated debris, and the resultant supernatant was taken for later metabolite analyses. Haemolymph was deproteinated with 0.6 M PCA and spun at 10,000 *g* for 10 min in a 4°C centrifuge, and the resultant supernatant was used for metabolite analysis. All metabolite assays samples were analyzed in 96-well plates, and absorbances were measured using the SpectraMax 340PC-384 plate reader and SoftMax pro software v1.23. Metabolite data are adjusted for total protein of the individuals and are presented as g mL^{-1}. All chemicals and consumables were sourced from Thermo Fisher (United States) and Becton Dickinson and Company (BD, United States) unless stated otherwise.

2.3. Heart Rate Assessment. Heart rate was monitored non-invasively with bespoke infrared (IR) photoplethysmography sensors [19, 41]. Briefly, the sensors consisted of an IR-LED coupled to an IR-specific photodiode within a miniature 3D-printed housing (5.5 × 2.0 × 2.0 cm). A 5V USB connector powered the sensor, and the analog photodiode voltage signal was analyzed with a two-channel A/D recorder (PowerLab15T Model ML4818, AD Instruments, New Zealand). Scampi were removed from housing tanks and sensors were attached dorsally above the heart on the cephalothorax with miniature cable ties. After sensor attachment scampi were placed within 6 L holding tanks containing aerated seawater held at 11°C and left undisturbed for 1 hr before experimentation to allow recovery from handling. Baseline heart rate measurement was undertaken at the end of this recovery period and prior to experimental intervention, i.e., $T = 0$. Ten scampi were randomly assigned to one of the four treatment groups (as outlined above) at the start of the experimental period, removed from their holding tank, and injected with the corresponding treatment through the coxa membrane at the base of the fifth pereopod. Following injection, scampi were placed in 6 L experimental tanks without seawater that contained damp paper towels to maintain humidity, and heart rate was monitored for 4 hr. Baseline HR was variable across groups prior to treatment. To account for these differences and accurately present the effects of treatment, we have presented the data as fractional HR relative to baseline [19].

2.4. Metabolite Assays

2.4.1. Glycogen/Glucose. Muscle tissue glycogen was converted to glucose enzymatically with amyloglucosidase (AMG). The resulting glucose was measured *via* an enzymatic assay kit (K-GLUHK-110A/K, Megazyme, Ireland) that quantified NADH (reduced nicotinamide adenine dinucleotide) absorbance (340 nm). Free glucose was measured separately and subtracted from the total glucose to determine glycogen (presented as mM g^{-1} L^{-1} glucose). Haemolymph glucose was measured using the same assay kit and is presented as mM g^{-1} L^{-1} glucose.

2.4.2. Lactate. Tissue and haemolymph lactate were prepared and analyzed according to a modified method derived from Noll et al.'s [42] study. Briefly, lactate is converted to pyruvate with lactate dehydrogenase (LDH) and quantified by the NADH produced in this reaction. Hydrazine and NAD+ are added to the reaction to sustain the pyruvate to ensure the reaction continues and provide sufficient substrate for the production of NADH. Absorbance was measured at 340 nm and tissue and haemolymph lactate are presented in μmol g^{-1} and mmol L^{-1}, respectively.

2.4.3. Ammonia and Urea. Haemolymph ammonia and urea were quantified using a modified berthelot/indophenol blue method outlined in Pozhoth and Jeffs's [38] and Sumida et al.'s [43] studies. The indophenol blue method is a colorimetric assay that consists of reacting ammonia with hypochlorite to form monochloramine, this is then reacted with phenol to produce a blue indophenol compound that is then quantified at 625 nm. Total ammonia is proportional to the

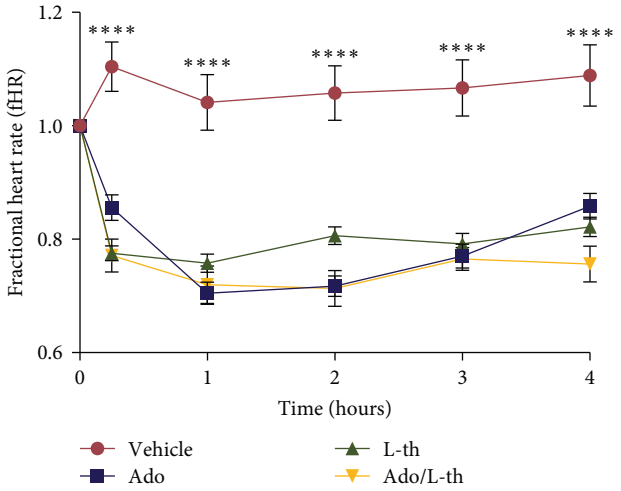

FIGURE 1: Fractional heart rate (fHR) of *M. challengeri* following administration of vehicle, adenosine (Ado), L-theanine (L-th), and the Ado/L-th mixture. fHR is presented as beats per minute (BPM) and the sample size is $n = 10$ per treatment. Significant differences of $p \leq 0.0001$ among treatments at set times are denoted (****).

amount of indophenol blue. Total urea is measured through the same method but has an additional preliminary step with the addition of the enzyme urease which converts urea to ammonia. Total ammonia and urea are quantified through a standard curve of ammonia and urea samples. Both ammonia and urea are presented here as μmol L^{-1}.

2.4.4. Calculation and Statistics. Repeated measures two-way ANOVA was performed to analyze the effect of treatment on HR relative to time (0, 0.25, 1, 2, 3, and 4 hr). One-way ANOVAs were performed to detect changes in tissue and haemolymph metabolites; glycogen/glucose, lactate, ammonia, and urea across treatment and where significant, pairwise Tukey-post hoc tests were used to isolate differences among means. Unless stated, all values are presented as mean ± SEM (standard error of the mean). All statistical tests were conducted in Prism (v9.2).

3. Results

3.1. Heart Rate. Heart rate decreased from baseline in scampi treated with adenosine, L-theanine, and Ado/L-th (16%, 21.4%, and 21.8%, respectively) at 0.25 hr while in contrast the HR increased in scampi treated with vehicle from baseline (10.4%; Figure 1(a)). The decrease in HR following treatment with Ado, L-th, and Ado/L-th was sustained across the 4 hr experimental period (~27%, 20%, and 28.6% on average, respectively; Figure 1(a); 0.25–4 hr; $p \leq 0.0001$).

3.2. Metabolite Assays

3.2.1. Glycogen. Mean tissue muscle glycogen, measured as glucose, was significantly lower in all treated groups compared to untreated control values (~45% lower on average; Figure 2(a); untreated vs. vehicle, $p < 0.0001$; untreated vs. Ado, $p = 0.0018$; untreated vs. L-th, $p = 0.0023$; and untreated vs. Ado/L-th, $p = 0.0028$). There was no significant difference in mean tissue muscle glycogen between treatment groups (Figure 2(a)). Mean haemolymph glucose was significantly higher in all treated groups compared to untreated control values (Figure 2(b); $p \leq 0.0001$). Haemolymph glucose was significantly higher in the Ado/L-th treated group compared to the Ado-treated group (Figure 2(b); $p = 0.0090$).

3.2.2. Lactate. Mean tissue lactate was significantly higher (~50% on average) in vehicle, L-th, and Ado/L-th treated groups compared to untreated control values (Figure 3(a); untreated vs. vehicle, $p = 0.0171$; untreated vs. L-th, $p = 0.0015$; untreated vs. Ado/L-th, $p = 0.0143$). Mean tissue lactate in groups treated with Ado was not significantly different compared to untreated control values (Figure 3(a); untreated vs. Ado, $p = 0.5383$). Mean tissue lactate was not significantly different among treatment groups; however, mean haemolymph lactate was significantly higher by ~60% in treated groups compared untreated control values (Figure 3(b); untreated vs. vehicle, $p \leq 0.0001$; untreated vs. Ado, $p = 0.0024$; untreated vs. L-th, $p \leq 0.0001$; and untreated vs Ado/L-th, $p = 0.0038$). Haemolymph lactate was significantly lower in the Ado treated (~22% lower, Figure 3(b); $p = 0.0134$) and Ado/L-th treated groups (~25% lower, Figure 3(b); $p = 0.0087$) compared to the vehicle-treated group.

3.2.3. Ammonia and Urea. Mean haemolymph ammonia was significantly higher in all treated groups compared to untreated control values (Figure 4(a); $p \leq 0.001$). Mean haemolymph ammonia was significantly higher in the Ado (Figure 4(a); vehicle vs. Ado, $p < 0.0001$; L-th vs. Ado, $p < 0.0001$) and Ado/L-th (Figure 4(b); vehicle vs. Ado/L-th, $p = 0.0002$; and L-th vs. Ado/L-th, $p = 0.0012$) treated groups compared to both vehicle and L-th treated groups. Mean haemolymph urea was significantly higher in vehicle (70% higher, Figure 4(b); $p \leq 0.0084$), Ado (110% higher, Figure 4(b); $p < 0.0001$) and Ado/L-th (105% higher, Figure 4(b); $p < 0.0001$) treated groups compared to untreated control values. Mean urea was significantly higher in both adenosine (25% and 30%) and Ado/L-th (20% and 25%) treated groups compared to vehicle and L-th treated groups, respectively (Figure 4(b); Ado vs. vehicle, $p = 0.0064$; Ado/L-th vs. vehicle, $p = 0.0381$; Ado vs. L-th, $p = 0.0007$; and Ado/L-th vs. L-th, $p = 0.0046$).

4. Discussion

The transport of live crustaceans, such as lobsters and marine-emersed crabs, out of water results in hypoxia and metabolic stress. The collapse of the gills and impaired gas exchange leads to a switch to anaerobic metabolism, which inefficiently consumes glucose to produce ATP *via* glycolysis, and leaving lactate as an end-product and producing only two ATP per glucose molecule versus ~36–38 ATP produced *via* aerobic metabolism. The supply of ATP from anaerobic metabolism is typically deployed to meet normoxic energy demands and rapidly leads to dysfunction in hypoxia-sensitive species. Conversely, hypoxia/anoxia tolerant species show resistance to this energetic dysfunction and

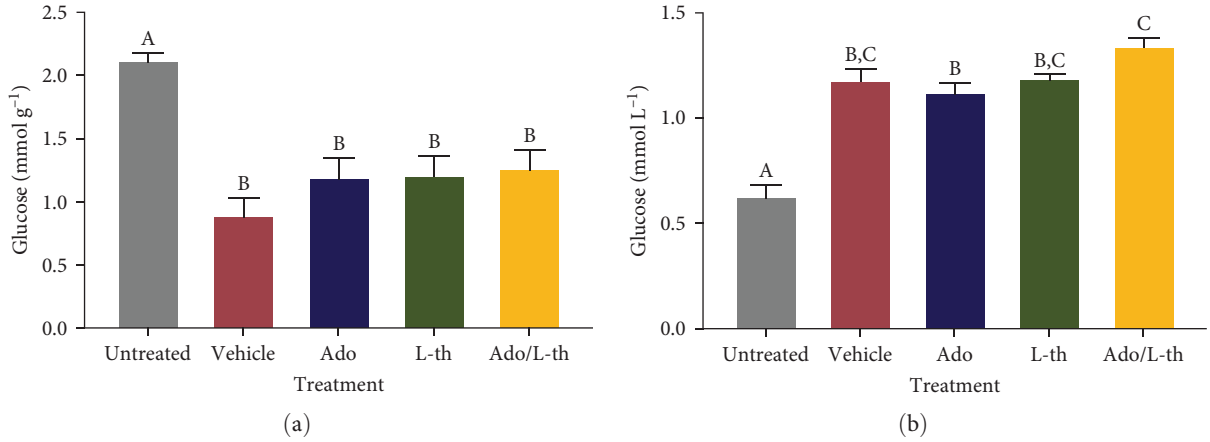

FIGURE 2: (a) Scampi tail muscle tissue glycogen concentration, presented as glucose (mmol g^{-1}), and (b) haemolymph glucose concentration (mmol L^{-1}) in untreated controls or after administration of vehicle, adenosine (Ado), L-theanine (L-th), or Ado/L-th mixture. Sample sizes for tissue glycogen (a) are $n = 10$, $n = 6$, $n = 6$, $n = 6$, and $n = 7$; respectively, for the treatments. The sample size for haemolymph glucose (b) is $n = 10$ for all groups. Significant differences ($p \leq 0.05$) among groups are marked by different superscript letters.

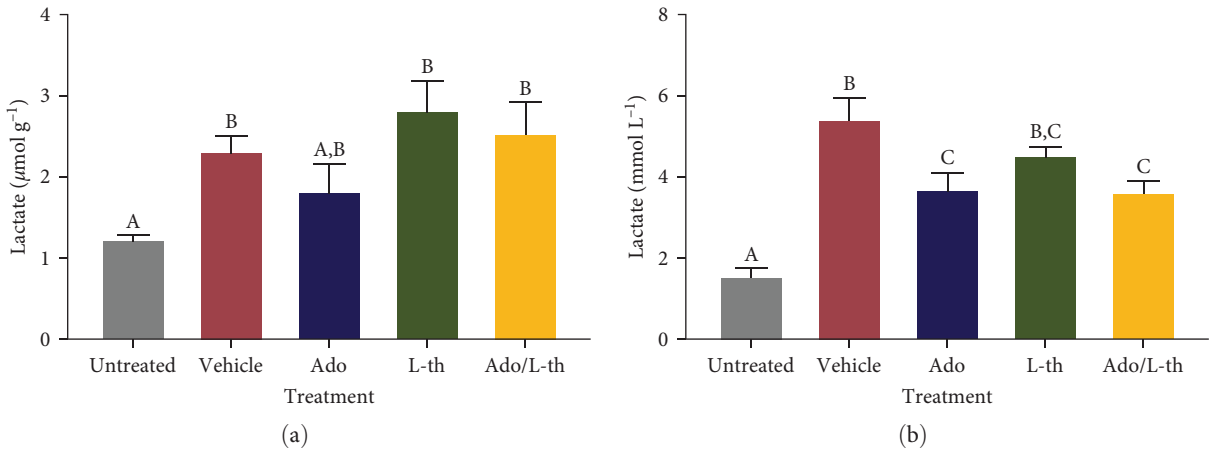

FIGURE 3: Mean lactate concentration in (a) scampi tail muscle (μmol g^{-1}) and (b) haemolymph (mmol L^{-1}) in untreated controls or after administration of vehicle, adenosine (Ado), L-theanine (L-th), or Ado/L-th mixture. Sample sizes for tail muscle lactate (a) are $n = 7$, $n = 9$, $n = 5$, $n = 6$, and $n = 5$; respectively, for the treatments. Sample size for haemolymph lactate (b) is $n = 10$ for all groups. Significant differences ($p \leq 0.05$) among groups are marked by different superscript letters.

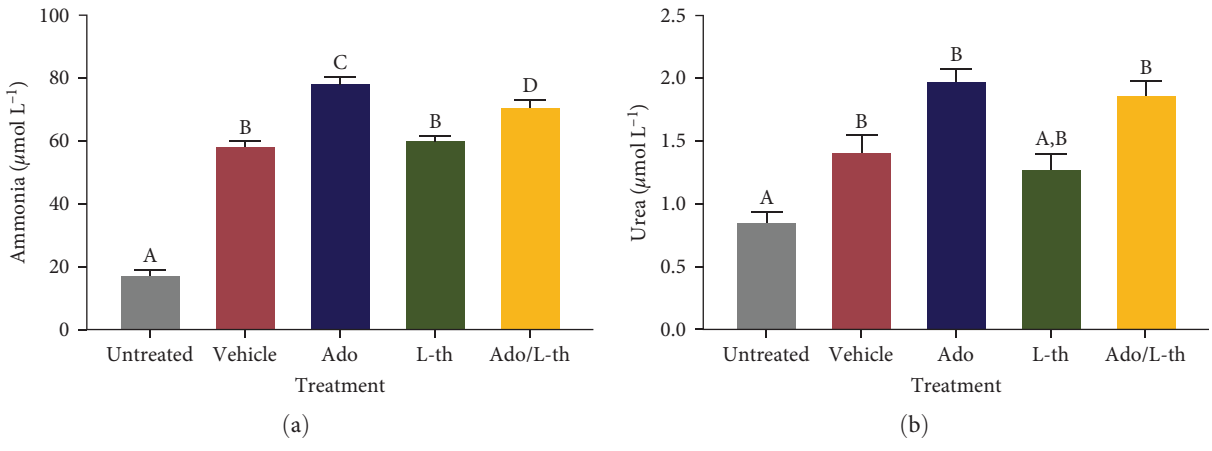

FIGURE 4: Mean scampi haemolymph concentrations of (a) ammonia (μmol L^{-1}) and (b) urea (μmol L^{-1}) in untreated controls or after administration of vehicle, adenosine (Ado), L-theanine (L-th), or Ado/L-th mixture. Sample sizes are $n = 6$ for all groups. Significant differences ($p \leq 0.05$) among groups are marked by different superscript letters.

downregulate energetic processes to align with changes in the status of metabolic energy production. Decreasing the expenditure of energetically expensive tissues (heart and nervous tissue) is a common strategy employed by hypoxia/anoxia tolerant species, which frequently experience low O_2 [44]. Consequently, chemical intervention to downregulate metabolic energy demands in animals may provide greater tolerance during periods of hypoxia/ anoxia, such as for the transport of live crustaceans in air.

This current study showed that treatment of scampi with Ado (2.5 mM), L-th (5 mM), and the Ado/L-th (2.5/5 mM) mixture resulted in a decrease in HR compared with vehicle-treated scampi (Figure 1(a)). Decreased HR is a common strategy used by hypoxia-tolerant species that reduces overall cardiac work and as such is energetically favorable during periods of low ATP supply [45]. Adenosine has been shown to reduce HR in some organisms. For example, in the anoxia-tolerant red-eared slider turtle (*Trachemys scripta*), the administration of Ado caused a transient bradycardia (lowered HR) and increased blood flow [46]. Similarly, administration of 2.5 mM Ado in the immersed freshwater prawn (*M. rosenbergii*) resulted in a decrease in HR while tissue glycogen stores were maintained compared to controls [19]. In contrast, steady-state infusion of 2–3 μmol L^{-1} adenosine into the coelomic cavity increased the ventilation and HR of the American lobster (*Homarus americanus*) [47, 48]. Collectively, these diverse responses point to a variable effect of Ado across species and dosages.

In crustaceans, it has been observed that Ado does not act directly on the myocardium but exerts its effects through the nervous system, and more specifically the cardiac ganglion (CG) [48]. The heart is thought to be exclusively under neurogenic control in malacostracan crustaceans and that a number of modulators impact heart function *via* their interaction with the CG motor pattern generation [49, 50]. Administration of Ado in the brain of the Caribbean spiny lobster (*Panulirus argus*) modulates the spontaneous and evoked responses of the neurons, leading to a decrease in overall activity [23]. This, as well as the results of our study, point to several sites of action of Ado that should be further elucidated in future studies. In the current study, the initial elevation in HR in the vehicle-treated scampi was unexpected, as an initial bradycardia (lowered HR) is common in crustaceans that are exposed to emersed conditions. The observed elevation in HR may be a consequence of the initial handling stress [13] or the injection of water, a hypotonic solution, as the vehicle control (Figure 1(a)). As this elevation in HR was sustained across the 4 hr experimental period, it may be the result of handling stress and the choice of vehicle in combination with one another. The use of a hypotonic solution as the vehicle in this study may have resulted in osmotic stress in all treatment groups which may have limited the impact of test substances on HR (Figure 1(a)) [51]. Future studies should incorporate a more appropriate vehicle that limits osmotic stress. Treatment of scampi with L-theanine showed a sustained decrease in HR across the 4 hr experimental period (Figure 1(a)). This is in contrast to the response seen in humans which shows a short-term decrease in HR following L-th administration (2 mg mL^{-1}) which returns to baseline after ~25 min [52]. Theanine's structural similarity to glutamate leads to a decrease in excitatory neurotransmission through its antagonism of glutamate receptors and inhibition of glutamine import for glutamate production [30, 52, 53]. Theanine has been shown to antagonize the action of glutamate at the crayfish neuromuscular junction [54]. During periods of hypoxia, GABA accumulates while glutamate is depleted [35]. This is a consequence of enzyme interactions with oxygen as GABA production and breakdown, as well as glutamate resynthesis is altered by oxygen availability, shifting to favor GABA production, and glutamate degradation [35]. This rise of GABA and fall of glutamate in the brain of hypoxia/anoxia tolerant species is a key feature of the decrease in systemic energy use and extended survival. Glutamate and GABA influence HR in decapod crustaceans, through interactions with the CG [50]. Like their role as neurotransmitters, in crustaceans' glutamate is excitatory activating cardio-accelerator neurons in the CG and increasing HR while GABA is inhibitory, activating cardio-inhibitor neurons that decrease HR [50]. The effect of L-theanine to decrease HR in scampi may be modulated through the glutamate and GABA-sensitive neurons innervating the CG, but this remains to be confirmed.

While Ado and L-th, in isolation or administered as a mixture, caused a significant decrease in HR this did not translate into a systemic downregulation of metabolism, as measured by key metabolites. Tissue glycogen utilization increased in all treatment groups compared to the untreated controls (Figure 2(a)). Air exposure increases production of the crustacean hyperglycaemic hormone which leads to increased utilization of glycogen stores and a corresponding increase in haemolymph glucose which fuels energetic demands [7]. In hypoxia-tolerant species, greater glycogen reserves are common. This allows sufficient energy production via anaerobic glycolysis to meet energy demands during hypoxic exposure. Glucose utilization in the crucian carp is mediated by the activation of AMP-activated protein kinase (AMPK) which functions as a key regulator of intracellular energy metabolism [44, 55]. AMPK sequences are highly conserved across species and are present in crustaceans where activity has been shown to increase during hypoxic exposure (*Macrobrachium nipponense*; [56]. Adenosine activates AMPK extracellularly and upon uptake into the cells where it is converted to AMP [56, 57]. Our results are consistent with those observed in *M. rosenbergii*, showing increased glycogen utilization resulting from hypoxic exposure and treatment with Ado [3]. In the current study, haemolymph lactate shows an increase in all treated groups compared to the untreated controls (Figure 3(b)). While this was the case, haemolymph lactate was lower in Ado and Ado/L-th treated scampi compared to vehicle-treated scampi (Figure 3(b)). This is in line with the increased utilization of glycogen and the wider literature which finds elevated lactate levels following increased rates of anaerobic metabolism in crustaceans [7]. The lack of concomitant rise in haemolymph lactate that was hypothesized to follow increased glycogen utilization (Figure 2(b)) during emersion may point to some aerobic metabolism being maintained during emersion

possibly through some residual oxygen exchange across the gills [58].

Ammonia is the main nitrogenous waste of decapod crustaceans and is primarily excreted across the gills. Under air exposed conditions, the collapse of gill filaments leads to impaired ammonia excretion, which results in an endogenous accumulation of ammonia [6, 39]. Ammonium ions affect membrane potentials and are particularly harmful to nervous tissues where they cause rapid depolarization. This depolarization leads to an increased release of excitatory neurotransmitters, such as glutamate, and the consumption of ATP to restore membrane potentials [39]. Excretion of ammonia across the gill surface is not the only method of elimination or deactivation of this toxic metabolite, as when concentrations of ammonia increase it can be detoxified by conversion to urea [39, 59]. This study showed that haemolymph ammonia and urea were higher in all treated groups compared to untreated controls (Figures 4(a) and 4(b)). Treatment with Ado and Ado/L-th led to elevated levels of both haemolymph ammonia and urea compared to vehicle and L-th treated groups (Figures 4(a) and 4(b)). These results are also consistent with the presence of purine metabolism, which can increase ammonia as a result of adenosine being metabolized to inosine via adenosine deaminase (ADA), producing ammonia as a by-product [39].

To function in a commercial setting, the application of any intervention to improve the outcomes from the live transport of crustaceans must be cost-effective, minimally invasive, and easily applicable. While theanine is abundantly available as a supplement and is produced in tea leaves, adenosine is much more expensive and as such limits its applicability unless it is highly effective at a low dose. A previous study on the giant freshwater prawn found that application of adenosine into the surrounding media was ineffectual as a means of delivery [19]. The mode of action of adenosine appears to be highly localized, requiring direct injection to deliver any effect [21]. As such in this study, adenosine and L-theanine were injected through the coxa membrane at the base of the third walking leg, this limits its applicability on a commercial scale as the individual handling and injection of crustaceans is technically difficult and time consuming. The duration of the effect is an important consideration as the transport of commercial crustaceans can take up to 20 hr or more, especially for exporting to large international live seafood market hubs [7].

This study showed that Ado and L-th in isolation and in combination maintained a depressed HR across the 4 hr experimental period, while this is useful, further investigations are needed to find the extent of this effect. Dosage plays an important role in the overall duration and while the dosages (2.5 and 5 mM for Ado and L-th, respectively) showed efficacy, increasing these may improve the overall effect or influence its duration. Again, this warrants further investigation as high concentrations of adenosine can result in variable receptor activation that may negate the suppressant effects [21].

5. Conclusion

In conclusion, while separate or combined administration of adenosine and L-theanine significantly modulated HR in scampi, its usefulness in the transport of live crustaceans appears to be limited by a lack of impact on overall metabolism as overall glycogen utilization was elevated and haemolymph lactate, ammonia and urea remained high. The cost of Ado and the need for injection of these compounds limits their applicability in a commercial setting. Further analysis on the effectiveness of these compounds for crustacean transport is needed under modified simulated transport conditions, that more closely reflect industrial or commercial situations, to determine if they can significantly decrease morbidity and mortality.

Data Availability

The datasets supporting this manuscript are available upon request.

Disclosure

This article was made available as a preprint at https://papers.ssrn.com/sol3/papers.cfm?abstract_id=4455460.

Conflicts of Interest

The authors declare no competing interests or conflicts regarding funding.

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