Respiratory and nutritional physiology of spiny lobster juveniles in culture

by

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Statements and declarations

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Paper 4: Effect of dietary protein on energy metabolism including protein synthesis in the spiny lobster *Sagmariasus verreauxi*.

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Candidate was the primary author. Author 1, 2, 3 and 4 contributed to the conception and design of the research project, interpretation of the research data and paper revision.

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General abstract

The spiny lobster *Sagmariasus verreauxi* is the largest species in the Palinuridae family and an important commercial seafood product in the Southern Hemisphere. The recent closure of the lifecycle of *S. verreauxi* from eggs to adult in captivity has improved the pathway to sustainable aquaculture for this species. The ability to culture an aquaculture species to harvest in a sustainable manner requires a plethora of nutritional and physiological knowledge. One piece of this puzzle is understanding the processes involved in energy metabolism. In particular, understanding nutritional physiology in terms of nutritional status will help develop effective nutritional regimes to optimize growth and achieve more sustainable aquaculture. The goal of this thesis was to investigate the nutritional physiology in cultured juvenile *S. verreauxi* of different nutritional status. In detail, this thesis consists of four linked but self-standing chapters addressing the following: the contribution of protein synthesis to energy metabolism using a protein synthesis inhibitor cycloheximide before being measured directly (Chapter 2), the potential use of a non-destructive stoichiometric approach to examine metabolic energy substrate (protein/amino acid, lipid or carbohydrate) use in aquatic ectotherms (Chapter 3), the use of the stoichiometric approach to examine metabolic energy substrate use and specific dynamic action (SDA) in lobsters fed a natural feed squid (*Nototodarus sloanii*) (Chapter 4), and the effects of different dietary protein on energy metabolism including protein synthesis (Chapter 5).

Lobster metabolic rates declined with starvation and cycloheximide-sensitive protein synthesis in unfed lobsters represented a minor component of energy metabolism. In contrast, protein synthesis accounted for a major proportion of SDA in squid-fed lobsters. These results suggest the contribution of protein synthesis to energy metabolism in crustaceans varies with nutritional status, *S. verreauxi* SDA is predominantly a post-absorptive process mainly related to growth, and that high dietary digestible energy content is essential as growth-related protein

synthesis is energetically expensive. Thereafter, to better understand energy metabolism in aquatic ectotherms, a review was provided to illustrate the use of the stoichiometric approach to examine metabolic energy substrate use. This approach is based on the evaluation of respiratory quotient (RQ) and nitrogen quotient (NQ), determined by simultaneous measurements of respiratory gas (O_2 and CO_2) exchange and nitrogenous (ammonia and urea) excretion. This review is timely both because of technical advances in measuring total CO_2 in seawater and the benefits that the stoichiometric approach provides for understanding energy metabolism. This approach enables repeated evaluations of major metabolic energy substrate use in the same animal at any one time, allowing the interpretation of bioenergetic change within a daily cycle. Protein was the primary energy substrate for 2-day fasted lobsters with lipid accounting for the remainder, and lipid became the main energy substrate after 10-day starvation, indicating lipid oxidation increased with the extent of fasting. Squid-fed lobsters predominantly oxidized protein to provide energy during SDA, and lipid and carbohydrate provided significant energy at different postprandial times. This suggests suitable amounts of high-quality protein with major non-protein energy-yielding macronutrients, lipid and carbohydrate, are oxidized and therefore should be included in lobster feeds. It is important to note that the stoichiometric approach is only feasible in conditions where the RQ is within the theoretical range (0.71-1). However, RQ exceeded 1 on a number of occasions in various aquatic ectotherms, including *S. verreauxi* in my study, indicating the need for additional studies to decipher the limitations and applicability of the stoichiometric approach.

To investigate the effects of different dietary protein on energy metabolism, the measurement of SDA and metabolic energy substrate use using the stoichiometric approach was combined with the assessment of whole-body protein synthesis using a non-destructive endpoint stochastic model. Three isoenergetic feeds were formulated with varying crude protein: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively.

Similar to the SDA magnitude in squid-fed lobsters estimated by the stoichiometric approach and traditional oxycalorific coefficient approaches, there was no difference in the SDA magnitude estimated by varying approaches in lobsters fed each formulated feed. Total CO_2 and ammonia excretion, SDA magnitude and coefficient, and protein synthesis in the CP_{60} treatment were higher compared to the CP_{40} treatment. These differences demonstrate dietary protein influences post-prandial energy metabolism. The average contribution of protein oxidation was lowest in the CP_{50} treatment, suggesting the mechanisms underlying the most efficient retention of dietary protein and the optimum dietary inclusion. The combined information advances the understanding of how deficient and surplus dietary protein affects energy metabolism and provides an approach for the fine-scale evaluation to support sustainable aquaculture.

Overall, this thesis provides the first stoichiometric investigation of the metabolic energy substrate use and SDA in a spiny lobster species, by successfully measuring carbon dioxide excretion combined with simultaneous measurement of oxygen consumption and nitrogenous excretion. In addition, this thesis provides the first examination of the contribution of protein synthesis to decapod energy metabolism by using a protein synthesis inhibitor. Furthermore, the thesis determined whole-body protein synthesis using an endpoint stochastic model, and explored the relationships between protein synthesis, SDA and energy substrate oxidation. The combined measurements provide detailed nutritional and physiological information relevant to crustacean growth and have applications for the evaluation of feeds and feeding regimes. In future, it would be beneficial to investigate the use of the stoichiometric bioenergetic approach in other emerging aquaculture species and expand applications of the endpoint stochastic model when examining the effect of dietary protein quality on protein metabolism.

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Chapter 1 General introduction

This chapter provides a general overview of specific dynamic action (SDA), metabolic energy substrate use and protein synthesis in aquatic ectotherms and introduces the aim and structure of this thesis. An understanding of SDA, substrate use and protein synthesis will advance the knowledge of bioenergetics and nutritional physiology in aquaculture species, which has great potential to optimize feeds and feeding regimes to achieve sustainable aquaculture, especially in emerging species such as spiny lobster (*Sagmariasus verreauxi*) (Halver & Hardy 2003; National Research Council 2011; Carter & Mente 2014).

1.1 Specific dynamic action

Specific dynamic action (SDA) is the increased metabolism (measured as oxygen consumption or heat production) after feeding, representing energetic costs from ingestion, digestion, absorption and metabolic processing of all major energy substrates (Whiteley *et al.* 2001a; Secor 2009). A typical SDA response includes a rapid increase in oxygen consumption, followed by a gradual decline to the pre-prandial level (Jobling 1983; Carter & Brafield 1992b; Secor 2009) (Figure 1. 1). Specific dynamic action has been extensively researched over two centuries. Max Rubner was the first to introduce the term *SDA* in his book *Die Gesetze Des Energieverbrauchs Bei Der Ernährung* to describe the heat increment after feeding (Rubner 1902). However, this book was published in German and *SDA* in the book was written as *specifisch-dynamische wirkung* which, it has been suggested, may be more reliably translated as *specific dynamic effect* (Pike & Brown 1984). The earliest SDA studies were conducted before the 1870s by Antoine-Laurent Lavoisier (1774-1780), Bidder and Schmidt (1852), and Pettenkpfer and Voit (1862) (Borsook 1936; Schoffelen & Plasqui 2018). Before 1950, research on SDA was concentrated on mammals such as dogs and rats (Lusk 1912; Rapport

1924). After 1950, more SDA research has been performed in ectotherms, including reptiles (Coulson & Hernandez 1968; Coulson & Hernandez 1979; Secor & Diamond 1995), fish (Tandler & Beamish 1981; Carter & Brafield 1992b; Fitzgibbon *et al.* 2007), and invertebrates (Dall & Smith 1986; Houlihan *et al.* 1990; Whiteley *et al.* 2001a). Research on SDA in mammals is also ongoing after 1950 and demonstrates that the autonomic nervous system and exocrine hormones contribute to the SDA response (Gallivan & Ronald 1981; Diamond & LeBlanc 1988). There also have been excellent SDA reviews on both single taxa including mammals (Blaxter 1989), reptiles (Wang *et al.* 2001), fish (Jobling 1981a; Beamish & Trippel 1990) and crustaceans (Whiteley *et al.* 2001a), and multi-taxa such as amphibians and reptiles (Wang *et al.* 2001; Andrade *et al.* 2005; McCue 2006) illustrating the mechanisms of SDA, the effect of abiotic and biotic factors on SDA, and the significance of SDA in comparative and integrative physiology.

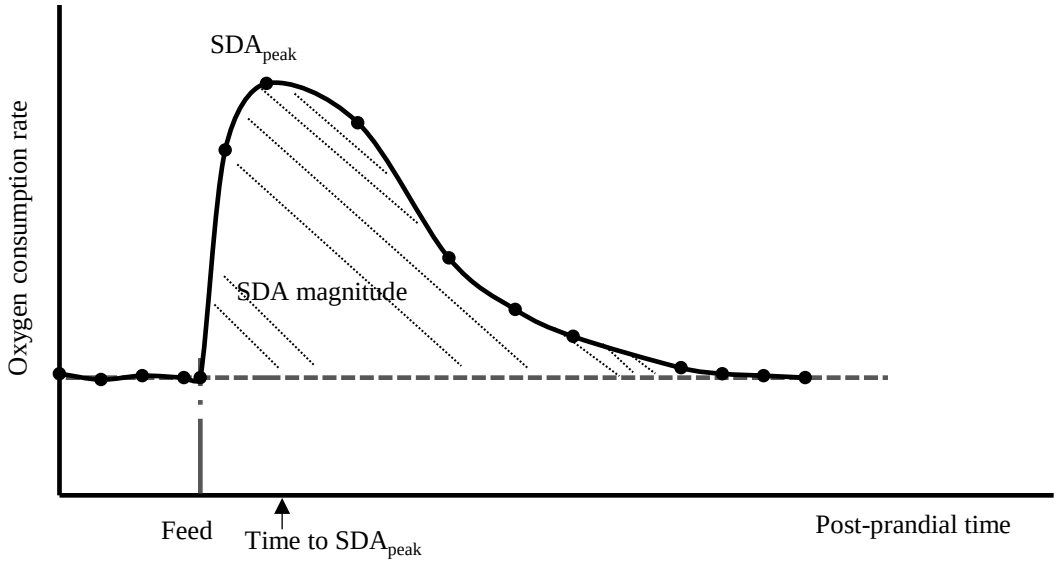

Figure 1. 1 Diagrammatic representation of post-prandial metabolism (Specific dynamic action, SDA) to time. The horizontal dash line represents the routine metabolic rate.

Aquafeeds with optimum digestible protein (amino acid) to energy (DP/DE) ratios and amino acid balances can efficiently spare dietary protein to maximize protein synthesis retention efficiency (SRE), growth rates and reduce energy loss via SDA (Carter & Brafield 1992b; Eliason *et al.* 2007; Hu *et al.* 2008). In contrast, imbalanced aquafeeds where the DP/DE ratio or amino acid balance is outside of the optimum range will stimulate mechanisms for regulating excess amino acids via protein synthesis or deamination and oxidation with a resultant elevation of SDA (LeGrow & Beamish 1986; Carter *et al.* 1993). The examination of SDA and metabolic energy substrate use is vital to understand the physiological basis of growth, potentially helping formulate cost-effective aquafeeds and optimize feeding regimes (Clifford & Brick 1979; Cho & Kaushik 1990; Hewitt & Irving 1990).

1.2 Metabolic energy substrate use

Traditionally, metabolic energy substrate use is determined by a compositional approach, where the animal is slaughtered after a long period to assess the change of the whole-body biochemical composition (Lauff & Wood 1996b). In comparison, the use of a stoichiometric bioenergetic approach, based on the simultaneous determination of nitrogenous (ammonia and urea) excretion and respiratory gas (O_2 and CO_2) exchange, provides a non-destructive way to examine the balance of substrate oxidation in aquatic ectotherms at any one time during aerobic metabolism (Clifford & Brick 1979; Kaushik *et al.* 1989; Ferreira *et al.* 2019). The stoichiometric bioenergetic approach allows repeated assessments of substrate oxidation in the same animals, thus providing precise measurements on substrate use under different feeding conditions (Kaushik *et al.* 1989; Lauff & Wood 1996b; De Boeck *et al.* 2001). However, stoichiometry has not been widely used in aquatic ectotherms, mainly due to technical difficulty in accurately determining total CO_2 concentrations in water (Nelson 2016; Ferreira *et al.* 2019).

1.3 Protein synthesis determination

Protein synthesis is central to animal growth (Hawkins *et al.* 1986; Carter & Mente 2014). Investigation of protein synthesis in aquaculture animals provides a sensitive way to examine the dietary protein (amino acid) efficiency to achieve long-term growth (Fraser & Rogers 2007; Carter *et al.* 2012). Two approaches have been widely used to estimate protein synthesis in aquatic ectotherms. The first is a flooding dose technique, which uses an intravenous injection of a single large dose of labelled (tracer) and unlabelled (tracee) amino acid to rapidly force equilibration of labelling between the intra- and extracellular amino acid pools (Garlick *et al.* 1980). Radioactive isotope tracers such as ^{3}H-labeled phenylalanine (^{3}H-PHE) (Houlihan *et al.* 1990; Rastrick & Whiteley 2017) and stable isotope tracers such as ^{15}N-PHE (Owen 1996) have been widely used. Recently, a stable isotope tracer, ring-D_5-PHE has proved available with the use of gas chromatography-mass spectrometry (GC-MS) (Lamarre *et al.* 2015; Lamarre *et al.* 2016). The flooding dose technique can be used to estimate protein synthesis in whole-body (Carter *et al.* 1993; Rastrick & Whiteley 2017), tissues and isolated tissues or cells (Pannevis & Houlihan 1992; Carter *et al.* 2012). Whist the flooding dose technique has many advantages, several potential drawbacks have been identified and discussed (Carter *et al.* 1994b; McCarthy *et al.* 2016). For example, this method is only available in a few hours (usually less than 4 hours) due to ^{3}H- or D_5-PHE decline in the amino acid free pool (Carter *et al.* 1994b; Rastrick & Whiteley 2017), and protein synthesis may vary during a daily cycle due to post-prandial stimulation (Carter *et al.* 1994b; McCarthy & Fuiman 2011). Moreover, a rapid injection of a large amount of tracee may itself increase protein synthesis (Liu & Barrett 2002), although this is not the case with ^{3}H-PHE (Houlihan *et al.* 1990). In addition, the injection may become impractical if the animal is too small. This issue has been solved by bathing the animal in radio-labelled amino acid (Fauconneau 1984; Houlihan *et al.* 1992). Moreover, the flooding

dose technique is invasive and terminal (Carter *et al.* 1994b; McCarthy *et al.* 2016). As a result, it is more difficult to interpret the relationship between protein accretion (measured in a long term) and protein synthesis (measured in a short term) using the flooding dose technique (Carter *et al.* 1994b; Carter & Houlihan 2001).

The second approach is an endpoint stochastic model, which is non-invasive and non-terminal as it determines protein synthesis through the measurement of nitrogenous excretion (Carter *et al.* 1994b; Duggleby & Waterlow 2005; Fraser & Rogers 2007). In this approach, animals are fed a known concentration of stable isotope (usually ^{15}N) labelled amino acid or protein uniformly distributed in a single meal (Waterlow 1981; Carter *et al.* 1994b; McCarthy *et al.* 2016). The advantages of this model have been identified and discussed (Carter *et al.* 1994b). For example, the use of stable isotopes reduces the risk to the experimenter and removes the need to dispose of radioactively labelled biological material (Fraser & Rogers 2007). Protein synthesis is measured over a relatively long period, typically 24-72 hours, compared to the flooding dose technique, promoting the accuracy for SRE estimations (Carter & Houlihan 2001; Fraser & Rogers 2007). Moreover, this model is non-invasive, allowing repeated protein synthesis measurements on the same individuals (Carter *et al.* 1998; McCarthy *et al.* 2016), critical for illustrating individual differences in growth efficiency (McCarthy *et al.* 1994; Carter & Houlihan 2001; Mente *et al.* 2001). This model has been predominantly used in fish (Carter *et al.* 1994b; Fraser *et al.* 1998; McCarthy *et al.* 2016), and not yet tested in crustaceans, partly due to their "messy" feeding behaviour (Carter & Mente 2014; McGaw & Penney 2014).

1.4 Protein synthesis and energy metabolism

Protein synthesis is energetically expensive, with a minimum cost of 11-42% of oxygen consumption in a range of endotherms and ectotherms (Houlihan *et al.* 1995b; Bowgen *et al.*

2007). The investigation of the contribution of protein synthesis to energy metabolism is crucial for the development of cost-effective aquafeeds (Lyndon *et al.* 1992; Carter & Mente 2014). Many protein synthesis inhibitors, such as cycloheximide and actinomycin-D, have been used to examine such the contribution (Brown & Cameron 1991a; Pedreira *et al.* 1996; Rastrick & Whiteley 2017). Cycloheximide is a preferred inhibitor (Houlihan 1991), as it primarily prevents cytosolic translation elongation (McKeehan & Hardesty 1969; Saini *et al.* 2009) and does not affect protein degradation (Pestka 1977) or block mitochondrial protein synthesis (Fraser & Rogers 2007). There are large variations in the contribution of cycloheximide-sensitive protein synthesis to total oxygen consumption in aquatic ectotherms (9-100%, Brown & Cameron 1991a; Houlihan *et al.* 1995c; Thor 2000) (Table 1. 1). In terms of decapod crustaceans, the contribution of cycloheximide-sensitive protein synthesis to energy metabolism has not been reported.

Table 1. 1 The contribution of cycloheximide (CHX)-sensitive protein synthesis to energy metabolism in aquatic ectotherms

Method	CHX concentration	Species	Body weight (BW)	Nutritional status	Temperature (°C)	Contribution (%)	References
Bath	2.5 g L^{-1}	*Oreochromis mossambicus*	16 mg	After 2-3-day starvation	26	9	Houlihan *et al.* (1993b)
Bath	2.5 g L^{-1}	*Oreochromis mossambicus*	16 mg	During 24-h post-feeding	26	31	Houlihan *et al.* (1993b)
Bath	1.0 g L^{-1}	*Clupea harengus*	0.13 mg dry weight	During 8-10-h post-feeding	8	79	Houlihan *et al.* (1995c)
Bath	1.0 mg L^{-1}	*Acartia tonsa*	NA	During 8-h post-feeding	14	93	Thor (2000)
Bath	1.0 mg L^{-1}	*Calanus finmarchicus*	NA	During 8-h post-feeding	14	88	Thor (2000)
Infusion	1.0 mg kg^{-1} BW	*Ictalurus punctatus*	850-1000 g	During 24-h post-infusion	22	100	Brown and Cameron (1991b)

Injection	5.0 mg g^{-1} BW	*Glyptonotus antarcticus*	42.8 g	After 24-h starvation	0	66.4	Whiteley *et al.* (1996)
Injection	5.0 mg g^{-1} BW	*Idotea rescata*	311 mg	After 24-h starvation	4	21.8	Whiteley *et al.* (1996)
Injection	8.4 mg kg^{-1} BW	*Nacella concinna*	NA	After 24-h starvation	0	56	Bowgen *et al.* (2007)
Injection	8.4 mg kg^{-1} BW	*Nacella concinna*	NA	After 24-h starvation	3	47	Bowgen *et al.* (2007)
Injection	0.05 mg g^{-1} BW	*Gammarus oceanicus*	0.08 g	After 24-h starvation	13	25	Rastrick and Whiteley (2017)
Injection	0.05 mg g^{-1} BW	*Gammarus oceanicus*	0.27 g	After 24-h starvation	5	26	Rastrick and Whiteley (2017)

NA, not available.

1.5 Thesis aims

The spiny lobster *Sagmariasus verreauxi* is the largest species in the Palinuridae family and an important commercial seafood product in the Southern Hemisphere (Phillips 2013). The recent closure of the lifecycle of *S. verreauxi* from eggs to adult in captivity has improved the pathway to sustainable aquaculture for this species (Fitzgibbon & Battaglene 2012a). A plethora of nutritional and physiological knowledge is imperative to achieve sustainable aquafeeds and therefore aquaculture (Halver & Hardy 2003). Compared with other decapod crustaceans, information of the effect of dietary protein on *S. verreauxi* nutritional physiology, and other spiny lobsters in general, is limited (Glencross *et al.* 2001; Ward *et al.* 2003). This thesis aimed to investigate the nutritional physiology in cultured juvenile *S. verreauxi* of different nutritional status, by exploring metabolic energy substrate use and SDA using a stoichiometric approach, examining the contribution of protein synthesis to energy metabolism using cycloheximide, and determining whole-body protein synthesis using an endpoint stochastic model. The relationships between protein synthesis, SDA and metabolic energy substrate use were also elucidated. The comprehensive information improves the understanding of the relationship of amino acid flux to energy metabolism and the nutritional bioenergetics in aquaculture animals on a fine level, which has great potential to optimize the feed and feeding regimes to support more sustainable aquaculture.

1.6 Thesis structure

The thesis is composed of six chapters, among which Chapter 2 to 5 have been published or under review in scientific journals. Therefore, some content is repeated particularly in the Introduction and Materials and methods sections.

Chapter 1

General introduction. This chapter provides a general overview of SDA, metabolic energy substrate use and protein synthesis in aquatic ectotherms. Major gaps in knowledge and the approaches required to address the gaps are provided.

Chapter 2

This chapter aims to examine the contribution of cycloheximide-sensitive protein synthesis to energy metabolism in *S. verreauxi* under different nutritional conditions.

Wang S, Fitzgibbon QP, Carter CG, Smith GG (2019) Effect of protein synthesis inhibitor cycloheximide on starvation, fasting and feeding oxygen consumption in juvenile spiny lobster *Sagmariasus verreauxi*. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **189**: 351-365. https://doi.org/10.1007/s00360-019-01221-z

Chapter 3

This chapter aims to examine the effect of abiotic and biotic factors on respiratory quotient and stoichiometrically determined metabolic energy substrate use in aquatic ectotherms, understand how the knowledge of stoichiometric bioenergetics can be used to improve feeding, nutrition and production variables for sustainable aquaculture and to interpret physiological mechanisms for survival and development of aquatic ectotherms in the wild.

Wang S, Carter CG, Fitzgibbon QP, Smith GG (2021) Respiratory quotient and the stoichiometric approach to investigating metabolic energy substrate use in aquatic ectotherms. *Reviews in Aquaculture*. Accepted. https://doi.org/10.1111/raq.12522

Chapter 4

This chapter aims to use the stoichiometric bioenergetic approach to measure metabolic energy substrate use in *S. verreauxi* of different nutritional status, examine SDA magnitude using different approaches, and re-calculate the contribution of protein synthesis to SDA.

Wang S, Carter CG, Fitzgibbon QP, Smith GG (2021) The use of stoichiometric bioenergetics to elucidate metabolic energy substrate use and specific dynamic action in cultured juvenile spiny lobsters (*Sagmariasus verreauxi*) of different nutritional status. *Aquaculture* **532**: 736021. https://doi.org/10.1016/j.aquaculture.2020.736021

Chapter 5

This chapter aims to examine the effects of dietary protein on SDA, metabolic energy substrate use, and whole-body protein synthesis in *S. verreauxi* using a stoichiometric bioenergetic approach and an endpoint stochastic model.

Wang S, Carter CG, Fitzgibbon QP, Codabaccus BM, Smith GG. Effect of dietary protein on energy metabolism including protein synthesis in the spiny lobster *Sagmariasus verreauxi*. *Scientific Reports*. Under review.

Chapter 6

General discussion and conclusions. This chapter aims to synthesize the key findings from Chapter 2 to 5, discuss how the knowledge of protein synthesis, SDA, and metabolic energy substrate use can be integrated into current research in optimizing feeds and feeding regimes in farmed animals and unveiling physiological adaptations to the environment, and provide future directions relevant to nutrition and physiology in aquatic ectotherms.

Chapter 2 Effect of protein synthesis inhibitor cycloheximide on starvation, fasting and feeding oxygen consumption in juvenile spiny lobster *Sagmariasus verreauxi*

Part of the research contained within this chapter has been published as Wang S, Fitzgibbon QP, Carter CG, Smith GG (2019) Effect of protein synthesis inhibitor cycloheximide on starvation, fasting and feeding oxygen consumption in juvenile spiny lobster *Sagmariasus verreauxi*. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **189**: 351-365. https://doi.org/10.1007/s00360-019-01221-z

2.1 Abstract

Metabolism in aquatic ectotherms evaluated by oxygen consumption rates reflects energetic costs including those associated with protein synthesis. Metabolism is influenced by nutritional status governed by feeding, nutrient intake and quality, and time without food. However, little is understood about the contribution of protein synthesis to crustacean energy metabolism. This study is the first using a protein synthesis inhibitor cycloheximide to research contribution of cycloheximide-sensitive protein synthesis to decapod crustacean metabolism. Juvenile *Sagmariasus verreauxi* were subject to five treatments: 2-day fasted lobsters sham injected with saline; 2-day fasted lobsters injected with cycloheximide; 10-day starved lobsters injected with cycloheximide; post-prandial lobsters fed squid *Nototodarus sloanii* with no further treatment; post-prandial lobsters injected with cycloheximide. Standard and routine metabolic rates in starved lobsters were reduced by 32% and 41%, respectively, compared to fasted lobsters, demonstrating metabolic downregulation with starvation. Oxygen consumption rates of fasted and starved lobsters following cycloheximide injection were reduced by 29% and 13%, respectively, demonstrating protein synthesis represents only a minor component of energy metabolism in unfed lobsters. Oxygen consumption rate of fed lobsters was reduced by

96% following cycloheximide injection, demonstrating protein synthesis in decapods contributes to a major proportion of specific dynamic action (SDA). SDA in decapods is predominantly a post-absorptive process mainly related to somatic growth. This study extends previously limited knowledge on the contribution of protein synthesis to crustacean metabolism, which is crucial to explore the relationship between nutritional status and feed quality and how this will affect growth potential in aquaculture species.

2.2 Introduction

Metabolism evaluated by the rate of oxygen consumption (MO_2) reflects energetic costs of aquatic ectotherms, and as such is a fundamental property that reflects life history strategy (Brafield 1985; Moyano *et al.* 2018). There are a variety of components to metabolic rate, including the standard metabolic rate (SMR) and routine metabolic rate (RMR) (Fry 1971; Brett & Groves 1979; Brafield 1985). The SMR is the minimum metabolic rate, measured in a post-absorptive and non-reproductive resting ectotherm at a specific temperature, which reflects the energy spent on self-maintenance (Kleiber 1975; Janča & Gvoždík 2017) and excludes any spontaneous activity (Fu *et al.* 2005c; Clark *et al.* 2013). After meeting the lowest energy requirement, an aquatic ectotherm can allocate excess energy to spontaneous activities such as movement, defined as RMR (Fitzgibbon *et al.* 2017). Many factors affect base level metabolism of aquatic ectotherms such as species (Carvalho & Phan 1997; White *et al.* 2006), body mass (Ikeda *et al.* 2001; Jensen *et al.* 2013b), temperature (Beamish 1964; Fitzgibbon *et al.* 2017) and nutritional conditions (Dall & Smith 1986; Auerswald *et al.* 2009; Simon *et al.* 2015). The nutritional status of aquatic ectotherms is of high commercial importance for aquaculture species as the nutritional status influences protein synthesis and therefore, somatic growth (Jobling 1983; Carter & Houlihan 2001; Carter & Mente 2014). Aquatic ectotherms under conditions of energy and nutrient limitation are forced to reallocate energy resources to

maintain energy and nutrient requirements (Sacristán *et al.* 2016) and as a result, RMR and performance decrease (Regnault 1981; Dall & Smith 1986; Fu *et al.* 2005b).

Following feeding, aquatic ectotherms experience a rapid post-prandial increase in metabolic rate (or heat production), followed by a gradual decrease to the pre-prandial level, in a process termed specific dynamic action (SDA) (Whiteley *et al.* 2001a; Secor 2009). Specific dynamic action in aquatic ectotherms represents energetic costs from pre-absorptive (gut peristalsis, digestive enzyme induction and secretion, protein catabolism, intestinal remodeling), absorptive (nutrient transport across membranes), and post-absorptive (protein, lipid and glycogen synthesis, nitrogenous wastes production and excretion) processes (Carter & Brafield 1992b; Jobling 1993; Grigoriou & Richardson 2008). Specific dynamic action in aquatic ectotherms can be described in terms of the peak post-prandial MO_2 (SDA_{peak}), the time when post-prandial MO_2 reaches the peak (T_{peak}), the time when the post-prandial MO_2 returns to RMR (SDA duration) and, total post-prandial rise of MO_2 above the RMR (SDA magnitude) (McCue 2006; McGaw & Curtis 2013) and SDA coefficient (C_{SDA}, %) (McCue 2006; McGaw & Curtis 2013). The SDA peak can be 2-4 times RMR for larval insects (McEvoy 1984; Bennett *et al.* 1999), 2-7 times RMR for teleost fish juvenile (Luo & Xie 2009; Wang *et al.* 2012a), and 2-4 times RMR for crustaceans (Crear & Forteath 2000; Robertson *et al.* 2001b; Radford *et al.* 2004). The SDA magnitude in some aquatic ectotherms has a positive linear relationship with growth rates (Jobling 1985; Carter & Brafield 1992b; Grigoriou & Richardson 2008), and is related to many aspects such as body weight (McGaw & Curtis 2013; Diawol *et al.* 2016), feed composition (Carter & Brafield 1992b; McGaw & Penney 2014; Palafox *et al.* 2017), feed size (Fu *et al.* 2005c; Secor 2009; McGaw & Curtis 2013), salinity (Curtis & McGaw 2010; Penney *et al.* 2016) and temperature (Robertson *et al.* 2001b; Whiteley *et al.* 2001a; McGaw & Whiteley 2012). The SDA coefficient is the proportion of ingested feed energy expended as SDA (McCue 2006; McGaw & Curtis 2013) and can be used to compare

SDA responses among species, without considering different experimental conditions such as body size, feed type, and temperature (McCue 2006; Secor 2009; McGaw & Curtis 2013).

Respiratory metabolic rates, including SMR, RMR and SDA, largely reflect protein synthesis in aquatic ectotherms (Brown & Cameron 1991a; Lyndon *et al.* 1992; Houlihan *et al.* 1995c). Following feeding, the rate of protein synthesis and the rate of oxygen consumption can experience a 2-3-fold increase in crustaceans (Houlihan *et al.* 1990; Robertson *et al.* 2001b) and teleosts (Lyndon *et al.* 1992; Houlihan *et al.* 1993b). Protein synthesis and growth of aquatic ectotherms are both closely correlated with feed intake (Jobling 1983; Carter & Houlihan 2001; Carter & Mente 2014). Ingested energy exceeding maintenance requirements will be converted into growth, and the efficiency whereby ingested energy surpasses maintenance requirements can be determined by SDA, with the proviso that the feed is nutritionally balanced (Kiørboe *et al.* 1987; Carter & Houlihan 2001). Evidence that SDA is positively associated with growth is increasing (Jobling 1985; Carter & Brafield 1992b; Lyndon *et al.* 1992; Grigoriou & Richardson 2008). As protein synthesis underpins growth in aquatic ectotherms (Brown & Cameron 1991b; Houlihan *et al.* 1993a; Carter & Houlihan 2001), understanding the contribution of protein synthesis to SDA and the retention efficiency of synthesized protein is central to assessing growth potential of a feed (Carter & Brafield 1992b; Carter & Houlihan 2001; Carter & Mente 2014) and to determining dietary protein (i.e., amino acid) requirements (Jobling 1985; Carter & Houlihan 2001; Carter & Mente 2014). Following starvation, protein synthesis in aquatic ectotherms may drop to a relatively stable level (Smith 1981; Loughna & Goldspink 1984; Carter & Mente 2014), in a similar pattern to RMR (Regnault 1981; Dall & Smith 1986). The downregulation of protein synthesis and RMR in aquatic ectotherms following starvation is likely due to the redistribution of energy substrates and the reduction of activity (Sacristán *et al.* 2016).

To estimate the contribution of protein synthesis to energetic costs in aquatic ectotherms, a protein synthesis inhibitor cycloheximide (CHX) has been widely used (Houlihan 1991; Houlihan *et al.* 1995c; Rastrick & Whiteley 2017). The decrease of CHX-sensitive oxygen consumption rate is regarded as the contribution of CHX-sensitive protein synthesis to energetic costs (Pannevis & Houlihan 1992; Houlihan *et al.* 1995c; Whiteley *et al.* 1996). Cycloheximide primarily prevents cytosolic translation elongation by inhibiting the translocation of peptidyl-tRNA from the aminoacyl site to the peptidyl site (McKeehan & Hardesty 1969; Saini *et al.* 2009), leading to a decrease of oxygen consumption rate in aquatic ectotherms (Houlihan *et al.* 1993b; Whiteley *et al.* 1996). However, CHX does not affect protein degradation (Pestka 1977) or block mitochondrial protein synthesis (Fraser & Rogers 2007). With the use of CHX, protein synthesis in aquatic ectotherms was estimated to account for 9-24% of RMR in fasted teleost fish (Houlihan *et al.* 1988; Carter *et al.* 1993; Houlihan *et al.* 1993b), 20-57% of RMR in fasted molluscs (Fraser *et al.* 2002; Bowgen *et al.* 2007), 22-66% of RMR in fasted amphipod and isopod crustaceans (Whiteley *et al.* 1996; Rastrick & Whiteley 2017), and 20-100% of SDA in fed teleost fish (Brown & Cameron 1991a; Lyndon *et al.* 1992; Houlihan *et al.* 1993b; Houlihan *et al.* 1995c). However, the contribution of protein synthesis to oxygen consumption in some research would likely have been underestimated, because CHX does not completely inhibit protein synthesis even at very high concentrations (Garlick *et al.* 1983; Aoyagi *et al.* 1988). Reports on the contribution of protein synthesis to SDA in crustaceans are few, using contrasting methodologies and recording contrasting results (Houlihan *et al.* 1990; Thor 2000; Whiteley *et al.* 2001a; Mente *et al.* 2003). Using the "flooding dose" method and the minimal theoretical cost of protein synthesis, Houlihan *et al.* (1990) found that protein synthesis represents 20-37% of decapod *Carcinus maenas* SDA. In contrast, using CHX, Thor (2000) found that protein synthesis represents 93% of copepod *Acartia tonsa* SDA. The contribution of protein synthesis to decapod crustacean metabolism

using CHX has not been previously reported. Considering the limited information and large discrepancies in findings, further research is required to better understand the contribution of protein synthesis to energy use in crustaceans (Houlihan *et al.* 1990; Whiteley *et al.* 1996; Thor 2000; Robertson *et al.* 2001b; Rastrick & Whiteley 2017).

Recorded discrepancies of the contribution of protein synthesis to oxygen consumption among aquatic ectotherms could be due to different species, ages and nutritional conditions (Bowgen *et al.* 2007), or failures on methodological approaches used between studies (Houlihan *et al.* 1995c; Rastrick & Whiteley 2017). Houlihan *et al.* (1995c) suggested that the measured contribution of protein synthesis to larval herring *Clupea harengus* SDA could have been an underestimation due to the limitation of the "flooding dose" method using labelled amino acids to measure protein synthesis. The incubation time of larval herring in labelled amino acids had to be expanded in order to obtain sufficient labelling, leading to starvation during label incorporation (Houlihan *et al.* 1995c). Conversely, the injection of high doses of CHX at 5.0 g kg^{-1} body weight (BW) has been considered to be in excess and may cause aberrant effects in crustaceans, resulting in an overestimation of the contribution of protein synthesis to RMR (Whiteley *et al.* 1996; Fraser & Rogers 2007; Rastrick & Whiteley 2017). Injection of CHX below 50 mg kg^{-1} BW has proven to be effective and safe to repress the MO_2 in the amphipod *Gammarus oceanicus* (Rastrick & Whiteley 2017), while the limpet *Nacella concinna* was administrated CHX at a rate of 8.4 mg kg^{-1} BW (Bowgen *et al.* 2007), and channel catfish *Ictalurus punctatus* injected at a rate of 1.0 mg kg^{-1} BW (Brown 1988; Brown & Cameron 1991a; Brown & Cameron 1991b).

In addition to high doses of CHX, the process of injection in crustaceans may cause stress, which leads to an overestimation of the contribution of protein synthesis to energetic costs (Bowgen *et al.* 2007; Rastrick & Whiteley 2017). Stress is a common physiological response in crustacean aquaculture and results from handling, tail-flipping, movement of limbs

(Houlihan *et al.* 1984; Jensen *et al.* 2013c), as well as air exposure (Whiteley & Taylor 1992; Forgan *et al.* 2014; Day *et al.* 2019). Stress may alter the physiological status of crustaceans beyond the normal range (Stoner 2012) and result in an increase in oxygen consumption rates associated with stress and increased activity (Taylor & Waldron 1997; Schock *et al.* 2010; Rastrick & Whiteley 2017). Hence, it is vital to choose an appropriate CHX concentration and to minimize or control for stress, such as the use of sham injection, to accurately determine the contribution of protein synthesis in aquatic ectotherms.

Crustacean aquaculture, almost exclusively based on decapod crustaceans, is an ever-growing global industry (FAO 2008; FAO 2018). International crustacean aquaculture production has increased dramatically from 4.7 million tons (USD 18.1 billion) to 7.9 million tons (USD 57.1 billion) from 2006 to 2016 (FAO 2008; FAO 2018). The spiny lobster *Sagmariasus verreauxi*, naturally distributed in coastal reefs of south-eastern Australia and New Zealand, is the largest Palinuridae species and has high commercial value (Leland *et al.* 2013). Recent successful cultivation of *S. verreauxi* from eggs in Australia has made this species a promising candidate for closed-cycle aquaculture (Fitzgibbon & Battaglene 2012a; Fitzgibbon & Battaglene 2012b; Fitzgibbon *et al.* 2014a). A thorough understanding of the nutritional physiology of an aquaculture species is vital to develop cost-effective commercial feeds and to realize the highest growth rate (Hasan 2000; Carter & Mente 2014; D'Abramo 2018).

The present study was designed to research the rate of oxygen consumption before and after the injection of CHX in juvenile *S. verreauxi*. The aim of this study was to determine the contribution of CHX-sensitive protein synthesis to energy metabolism in *S. verreauxi* under different nutritional conditions, including fasted, starved and fed. Fasting and starvation reflect different states of energy and nutrient limitation. The main difference between fasting and starvation is that fasting is generally short-term and safe, while starvation is prolonged fasting

and can be harmful, even fatal to aquatic ectotherms (Wang *et al.* 2006; McCue 2012; Sugumar *et al.* 2013). A key objective of this study was to determine the proportion of SDA attributed to protein synthesis in juvenile *S. verreauxi*. The present study confirms the potential contribution of protein synthesis to decapod crustacean metabolism, which is particularly important in assessing growth potential of a feed and determining dietary protein (i.e., amino acid) requirements for decapod crustaceans reared in aquaculture.

2.3 Materials and methods

2.3.1 Collection and maintenance of juvenile lobsters

Juvenile *Sagmariasus verreauxi* were hatchery reared from eggs as described by Fitzgibbon and Battaglene (2012b). Lobsters were maintained in a 4000 L fiberglass tank at 21 ± 0.2 °C, salinity 35 ± 0.1 ppt, dissolved oxygen 100 ± 10% saturation, pH 8.1 ± 0.1 at the Institute for Marine and Antarctic Studies (IMAS), Hobart, Australia. To avoid interference from natural circadian rhythms, lobsters were acclimated to a constant dim light for 4 weeks before experimentation. During the acclimation period, juvenile lobsters were fed fresh blue mussels (*Mytilus galloprovincialis*) and frozen squid (*Nototodarus sloanii*) twice weekly *ad libitum*. Lobsters were observed daily for moulting and were individually marked with a waterproof label adhered to the carapace on the first day after moulting. All experiments were conducted on lobsters that were 10 d post-moult. A pleopod was removed from each lobster and their moult stage was confirmed with microscopy (Olympus SZ-ST, Olympus, Tokyo) (Turnbull 1989).

2.3.2 Experimental lobsters

Thirty intermoult *S. verreauxi* [mean ± standard error (SE) BW 350 ± 72 g, range = 229-460 g] were randomly assigned into five groups:

Fasted followed by lobster saline injection (FS Group, control group). Six lobsters (342 ± 36 g, range = 229-430 g) were fasted for 2 d prior to experimentation and injected with saline (0.462 mol L^{-1} NaCl, 0.016 mol L^{-1} KCl, 0.026 mol L^{-1} $CaCl_2$, 0.008 mol L^{-1} $MgCl_2$, 0.011 mol L^{-1} glucose, and 0.01 mol L^{-1} HEPES, pH 7.4) (Chang *et al.* 1999) in a volume equivalent to that of injected CHX in the other groups through the blood sinus of the 5th walking leg using a 1.0 mL syringe and 27-G needle (Terumo Co., Ltd., Japan) (Houlihan *et al.* 1990; Whiteley *et al.* 1996; Jiménez-Morales *et al.* 2018).

Fasted followed by CHX injection (FC Group). Cycloheximide stock solution was prepared at 2.0 mg mL^{-1} in saline to achieve an active lobster concentration of 2.0 mg kg^{-1} BW. Six lobsters (314 ± 22 g, range = 234-401 g) were fasted for 2 d and injected with CHX at 2.0 mg kg^{-1} BW.

Starved followed by CHX injection (SC Group). Six lobsters (404 ± 30 g, range = 260-450 g) were starved for 10 d and injected with CHX at 2.0 mg kg^{-1} BW.

Fed with no further treatment (FED Group). Six lobsters (340 ± 24 g, range = 275-408 g) were fed frozen squid at 3% ration level.

Fed followed by CHX injection (FEDC Group). Six lobsters (354 ± 38 g, range = 238-460 g) were fed frozen squid at 3% ration level and injected with CHX at 2.0 mg kg^{-1} BW within 10 min after the lobster consumed all the squid.

The injection of saline or CHX was delivered over 10 seconds to ensure the solution was released thoroughly into the blood sinus. In addition, seepage of the injection solution was avoided by waiting for 3 s before removing the needle to allow for some circulation (Whiteley *et al.* 1996). The concentration of CHX used in the present study (2.0 mg kg^{-1} BW) was determined by previous studies on other aquatic ectotherms (Brown & Cameron 1991b; Bowgen *et al.* 2007) and a 48-h pilot experiment on *S. verreauxi*. Previous studies demonstrated that the injection of CHX at 1.0-8.4 mg kg^{-1} BW is effective to inhibit protein synthesis (Brown & Cameron 1991b; Bowgen *et al.* 2007). Note that the BW of limpet *N. concinna* in Bowgen

et al. (2007) was determined by the total weight minus the shell weight, hence, the concentration of CHX in Bowgen *et al.* (2007) (8.4 mg kg^{-1} BW) was less than 8.4 mg kg^{-1} total weight. The pilot experiment examined two concentrations of CHX (2.0 and 5.0 mg kg^{-1} BW) with two lobsters each. Lobsters injected with CHX at 2.0 mg kg^{-1} BW survived and the rate of oxygen consumption (MO_2) dropped rapidly during the experimental procedure, however, lobsters injected with CHX at 5.0 mg kg^{-1} BW died within 15 h after injection. Bowgen *et al.* (2007) suggested it is not necessary to inhibit all cytosolic protein synthesis, as long as protein synthesis and oxygen consumption are measurably decreased. Therefore, the concentration of 2.0 mg CHX kg^{-1} BW was used in the present study. Lobsters that did not consume the entire squid of the calculated ration within 45 min were excluded from analysis to ensure equal feed intake amongst individuals. Body weight (g) of lobsters was measured at the start of the experiment. Lobsters, except for the SC Group, were fed fresh mussels and frozen squid *ad libitum* for 10 d then left fasted for 48 h to ensure all lobsters were at the same post-prandial status prior to any measurements. Lobsters in the SC Group were starved for 10 d.

2.3.3 Oxygen consumption

Rates of oxygen consumption were measured using an intermittent-flow respirometer system (Jensen *et al.* 2013b). Two 3.55 L gas-tight acrylic respiration chambers (internal diameter = 9.6 cm, length = 48 cm, thickness = 1 cm) were used simultaneously, the lobsters and the treatment groups were chosen randomly for each experiment. The respirometers were immersed in a 300 L ambient tank kept at 21 °C to maintain thermal equilibrium. The seawater was kept air saturated with a constant flow of air. Each lobster was placed into a respirometer in the late afternoon and following 16 h of acclimation (started from 16:00), during which MO_2 was recorded for the last 8 h to establish the baseline metabolism (SMR and RMR). Standard

and routine metabolic rates in each lobster were calculated as the mean of the lowest 10% of MO_2 readings and the mean of the MO_2 readings, respectively (Jensen *et al.* 2013c; Fitzgibbon *et al.* 2014b). In the following morning at 08:00 after baseline measurements, lobsters in the FS treatment were taken out of the chamber carefully and injected with saline, then replaced into chambers for 24 h during which new SMR (recorded as SMR_i) and RMR (recorded as RMR_i) were calculated using the same method for SMR and RMR measurement. The increased metabolic rates due to handling and saline injection in the FS treatment was subtracted from the recorded metabolic rates of each lobster in FC and SC treatments to correct for the influence of handling and injection stress. The corrected values were used to determine the SMR_i and RMR_i in FC and SC treatments. For the fed (FED and FEDC) treatments, the respiration chamber was opened while emerged and the lobster fed (08:00) in the chamber to investigate the SDA response for 36.5 h. Preliminary data suggested that SDA duration in *S. verreauxi* at the same size and same ration was less than 32 h. The open and close feed presentation procedures were undertaken within 3 min.

The experiment was conducted under constant dim light. Artificial shelters made of oyster mesh were placed inside of chambers to provide lobsters with a shelter and substrate to hold. The respiratory system was surrounded by black plastic sheeting to decrease lobster visual disturbance during any experimental activities. The oxygen content in the 3.55 L respirometry chamber was recorded with a luminescent dissolved oxygen probe (HQ40d, Hach Company, USA) housed in a separate 6 mL chamber which received seawater from the respirometry chamber via a recirculating pump (Meacon Systems, TAS, Australia) at a rate of 12 mL min^{-1}. The oxygen probe having been calibrated to 100% in water-saturated air before measurements (Johnson *et al.* 2017; Klaus *et al.* 2017) logged dissolved oxygen recorded every 30 s. Two submersible aquarium pumps (Quiet one 1200, Aquasonic, Wauchope, NSW, Australia) were connected to each respirometer. One pump recirculated seawater inside the

respirometer at a rate of 1.0 exchange min^{-1} to ensure proper mixing inside the chamber. The other was a flushing pump, connected to a digital timer (DRT-1, Sentinel, China), intermittently exchanged water inside the respirometer at a rate of 1.0 exchange per min with seawater from the 300 L ambient tank for 10 min each 20 min, thus creating a 10 min closed (measuring period) and a 10 min flush (re-oxygenation period) cycle, allowing one MO_2 value per 20 min. Oxygen tensions never fell below 70% saturation at any time (Jensen *et al.* 2013b). Background MO_2 was measured in blank chambers (without a lobster) for 2 h after each measurement. Oxygen consumption rates of juvenile lobsters were thereafter determined by subtraction of background MO_2 and were calculated from the rate of decline in oxygen in the respirometer (mg O_2 L^{-1} h^{-1}).

2.3.4 Data analysis

For 2-day fasted and 10-day starved lobsters, SMR, RMR, SMR_i and RMR_i were examined in each lobster and expressed as mg O_2 g^{-1} h^{-1}. To calculate the magnitude of the decrease of MO_2 in FC and SC treatments, the decrease of MO_2 was determined by the difference of RMR before CHX injection and stress corrected metabolic rates after CHX injection. For fed lobsters, eight variables were individually identified: (1) SMR; (2) RMR; (3) SDA_{peak} (mg O_2 g^{-1} h^{-1}); (4) T_{peak} (h); (5) SDA duration (h), determined as two or three consecutive post-prandial MO_2 falling within 1 RMR ± 1 SE (Fitzgibbon *et al.* 2007); (6) SDA magnitude (mg O_2), calculated by the total increase in the rate of oxygen consumption above the RMR (McGaw & Curtis 2013); (7) E_{SDA}, where SDA magnitude was converted to energy (J) using an empirical oxycalorific coefficient (Q_{ox}) of 13.84 J mg^{-1} O_2 (Brafield & Llewellyn 1982); (8) C_{SDA}, calculated by dividing E_{SDA} by the energy in the ingested feed (J) (McCue 2006; McGaw & Curtis 2013). The contribution of CHX-sensitive protein synthesis to respiratory metabolism in all treatments

was determined by the decrease of CHX-sensitive oxygen consumption rates (Pannevis & Houlihan 1992; Houlihan *et al.* 1995c; Whiteley *et al.* 1996).

All figures were plotted using SigmaPlot (Version 12.5, Systat Software, San Jose, USA). All statistical analysis was performed using SPSS Statistics Software (Version 24, IBM Corporation, New York, USA). Before statistical analyses, normality tests were carried out via Kolmogorov-Smirnov test, followed by the verification of homogeneity of variances via Bartlett's test. Data that were homogeneous were compared using t-tests and one-way analysis of variance (ANOVA), data that were not homogeneous were compared using the Kruskal-Wallis test. For both tests, a probability of $P < 0.05$ was considered significant in all analyses. Paired t-tests were used to examine if there were differences in the baseline metabolism before and after saline or CHX injection, and if there were differences between the RMR and the corrected metabolic rates under the same treatment. All data were expressed as mean ± SE.

2.4 Results

2.4.1 Oxygen consumption in unfed lobsters

2.4.1.1 Oxygen consumption in the FS treatment

Oxygen consumption rates of 2-day fasted and saline sham injected (FS) lobsters exhibited an immediate rise after saline injection, followed by a slight drop at the first 5 h. Oxygen consumption rates then raised over the next 2 h, followed by a slow decrease to a level not significantly different ($P > 0.05$) from the RMR (Figure 2. 1). The base levels were significantly increased after saline injection ($P < 0.05$) in the FS treatment (Table 2. 1).

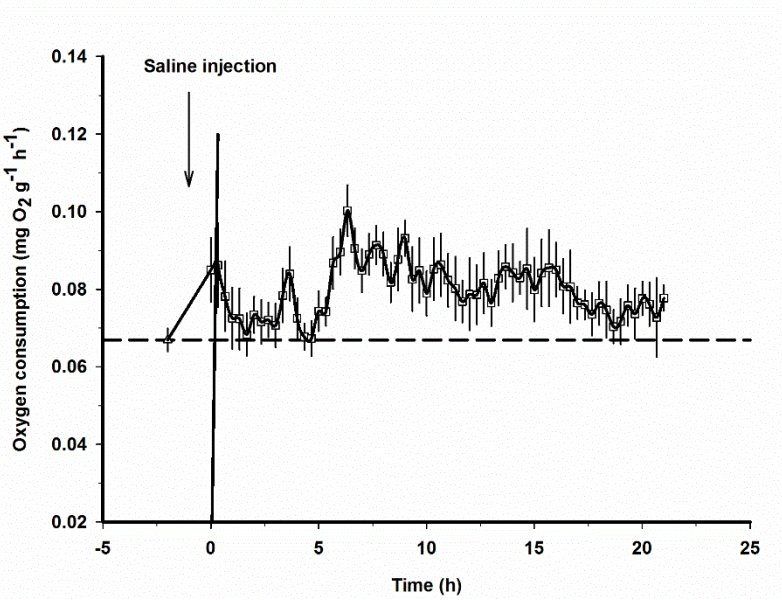

Figure 2. 1 Oxygen consumption of 2-day fasted and saline sham injected (FS) *Sagmariasus verreauxi*. The triangle and the dashed horizontal line indicate the pre-treatment routine metabolic rate (RMR); the vertical solid line indicates when the first post-treatment oxygen consumption rate was recorded. All data represent mean ± standard error (SE) of 6 individuals.

Table 2. 1 Comparison of metabolic rates in juvenile *Sagmariasus verreauxi* among treatments before and after injection

Treatments	SMR (mg O_2 g^{-1} h^{-1})	SMR_i (mg O_2 g^{-1} h^{-1})	Significance (t-tests)
FS	0.053 ± 0.004^{ab}	$0.063 \pm 0.004^{a*}$	0.043
FC	0.057 ± 0.007^{a}	0.054 ± 0.003^{b}	0.629
SC	0.039 ± 0.003^{b}	0.041 ± 0.002^{c}	0.637
	RMR (mg O_2 g^{-1} h^{-1})	RMR_i (mg O_2 g^{-1} h^{-1})	
FS	0.067 ± 0.003^{a}	$0.077 \pm 0.004^{a*}$	0.039
FC	0.080 ± 0.006^{a}	0.072 ± 0.003^{a}	0.264
SC	0.047 ± 0.005^{b}	0.053 ± 0.003^{b}	0.410
	FC	SC	

Decrease of MO_2 (%)	28.51 ± 2.82	13.12 ± 4.04**	< 0.001

[a] FS, 2-d fasted and saline sham injected; FC, 2-d fasted and cycloheximide injected; SC, 10-d starved and cycloheximide injected. SMR, the standard metabolic rate; SMR_i, new SMR calculated after injection; RMR, the routine metabolic rate; RMR_i, new RMR after injection; MO_2, oxygen consumption rate. All data represent mean ± standard error (SE) of 6 individuals. The superscript (*) indicates significant differences in each treatment ($P < 0.05$), the superscript (**) in the last column indicates significant differences between FC and SC treatments ($P < 0.05$). *P* values of paired t-tests are shown in the last row. Different superscripts (a, b) in each column indicates significant differences among treatments ($P < 0.05$)

2.4.1.2 Oxygen consumption in the FC treatment

Corrected oxygen consumption rates of 2-day fasted and CHX injected (FC) lobsters decreased significantly ($P < 0.05$) compared to the RMR at the first 3.7 h post-injection (Figure 2. 2). Although MO_2 increased from 2.0 to 3.0 h, the MO_2 at 3.0 h was still significantly lower ($P < 0.05$) than the RMR. The MO_2 increased significantly ($P < 0.05$) from 3.7 to 4.7 h post-injection, then decreased significantly ($P < 0.05$) from 4.7 to 6.3 h. The lowest MO_2 occurred 6.3 h post-injection and was 51% of the RMR ($P < 0.05$), after which MO_2 increased gradually and reached a level not significantly different ($P > 0.05$) from the RMR at 16.7 h post-injection (Figure 2. 2). The differences were not significant ($P > 0.05$) between the SMR and SMR_i, or between the RMR and RMR_i in the FC treatment (Table 2. 1). The contribution of CHX-sensitive protein synthesis to the RMR in the FC treatment was determined by the decrease of CHX-sensitive oxygen consumption rates (Table 2. 1).

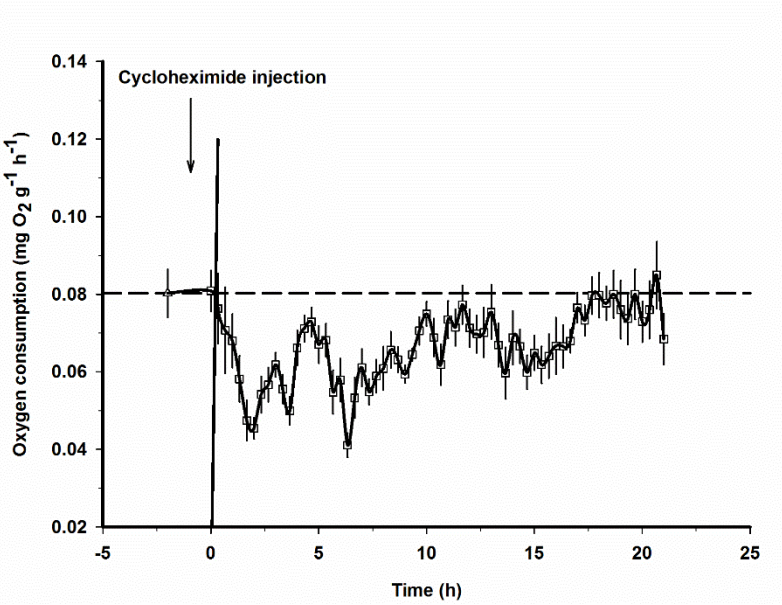

Figure 2. 2 Oxygen consumption of 2-day fasted and cycloheximide injected (FC) *Sagmariasus verreauxi*. The triangle and the dashed horizontal line indicate the pre-treatment routine metabolic rate (RMR); the vertical solid line indicates when the first post-treatment oxygen consumption rate was recorded. All data represent mean ± standard error (SE) of 6 individuals.

2.4.1.3 Oxygen consumption in the SC treatment

Corrected oxygen consumption rates of 10-day starved and CHX injected (SC) lobsters increased insignificantly ($P > 0.05$) at the first 0.67 h post-injection, then decreased significantly ($P < 0.05$) until 2.0 h (Figure 2. 3). The MO_2 fluctuated from 2.0 to 6.3 h, and the lowest MO_2 occurred 6.3 h, which was 51% of the RMR ($P < 0.05$). Thereafter, MO_2 increased and was not significantly different compared to the RMR at 7.0 h post-injection ($P > 0.05$) (Figure 2. 3). The differences were not significant ($P > 0.05$) between the SMR and SMR_i, or between the RMR and RMR_i in the SC treatment (Table 2. 1). The SMR and RMR in 10-day starved lobsters were significantly ($P < 0.05$) lowered by 32% and 41%, respectively, compared to that of fasted lobsters (Table 2. 1). The contribution of CHX-sensitive protein synthesis to the RMR in the SC treatment was determined by the decrease of CHX-sensitive oxygen

consumption rates, and the value was significantly lower ($P < 0.05$) compared with the FC treatment (Table 2. 1).

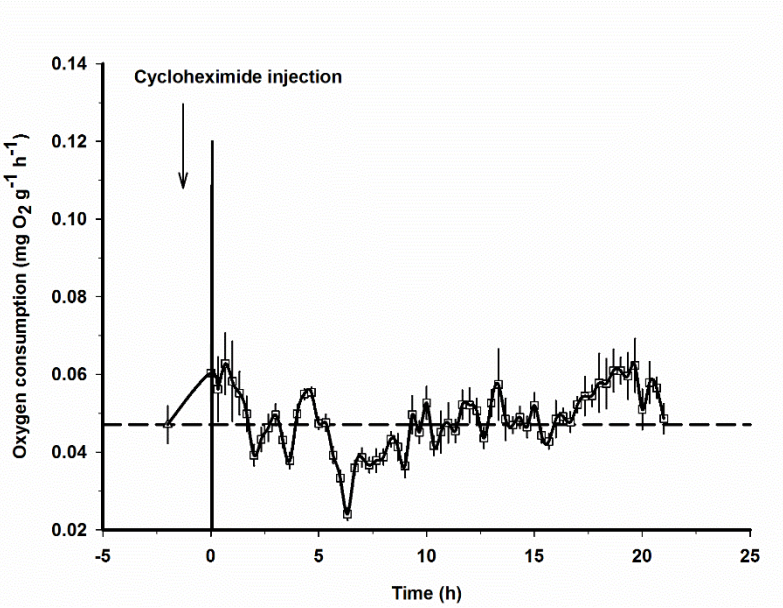

Figure 2. 3 Oxygen consumption of 10-day starved and cycloheximide injected (SC) *Sagmariasus verreauxi*. The triangle and the dashed horizontal line indicate the pre-treatment routine metabolic rate (RMR); the vertical solid line indicates when the first post-treatment oxygen consumption was recorded. All data represent mean ± standard error (SE) of 6 individuals.

2.4.2 Oxygen consumption in fed lobsters

Oxygen consumption rates of fed (FED) lobsters exhibited an immediate rise after the commencement of feeding, followed by a progressive drop from the peak which occurred 0.89 h post-feeding, to a level not significantly different ($P > 0.05$) from the RMR at 30.26 h post-feeding (Figure 2. 4 and Table 2. 2). Corrected oxygen consumption rates of fed and CHX injected (FEDC) lobsters increased immediately after the commencement of feeding and injection, followed by a quick decrease (Figure 2. 5 and Table 2. 2). The SDA peak and the

time to peak in the FEDC treatment were not significantly different ($P > 0.05$) compared to the FED treatment; however, the SDA duration, SDA magnitude, and SDA coefficient were all significantly lower ($P < 0.05$) in the FEDC treatment compared to the FED treatment (Table 2. 2). The contribution of CHX-sensitive protein synthesis to SDA in the FED treatment was 96.40%, determined by the decrease of CHX-sensitive oxygen consumption rates (Table 2. 2).

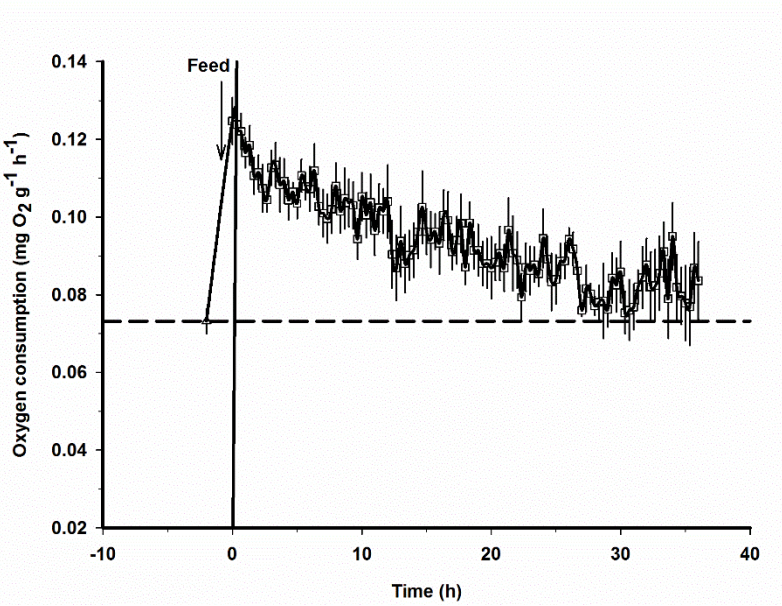

Figure 2. 4 Oxygen consumption of fed (FED) *Sagmariasus verreauxi*. Lobsters were fed squid at 3% body weight. The triangle and the dashed horizontal line indicate the routine metabolic rate (RMR); the vertical solid line indicates when the first post-prandial oxygen consumption rate was recorded. All data represent mean ± standard error (SE) of 6 individuals.

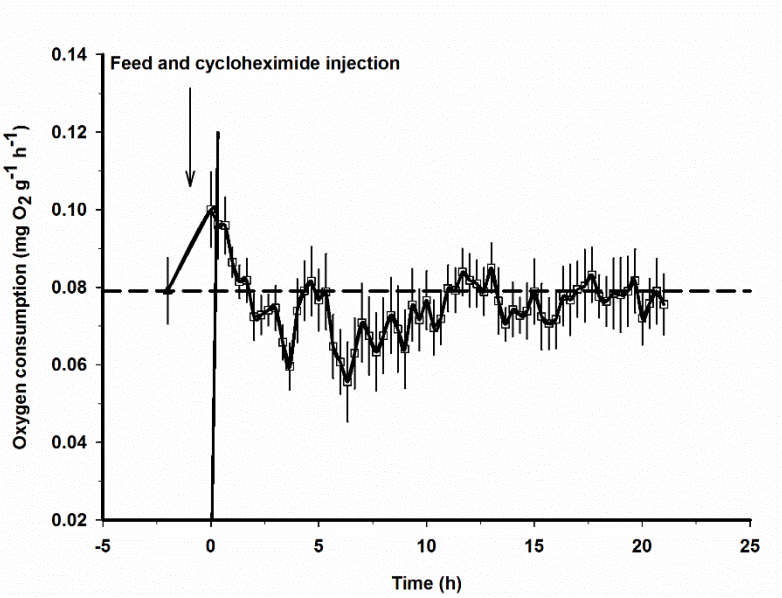

Figure 2. 5 Oxygen consumption of fed and cycloheximide injected (FEDC) *Sagmariasus verreauxi*. Lobsters were fed squid at 3% body weight. The triangle and the dashed horizontal line indicate the routine metabolic rate (RMR); the vertical solid line indicates when the first post-treatment oxygen consumption rate was recorded. All data represent mean ± standard error (SE) of 6 individuals.

Table 2. 2 Specific dynamic action (SDA) parameters in fed juvenile *Sagmariasus verreauxi*

Parameters	FED	FEDC	Significance (Paired t-tests)
SMR (mg O_2 g^{-1} h^{-1})	0.059 ± 0.003	0.061 ± 0.01	0.765
RMR (mg O_2 g^{-1} h^{-1})	0.073 ± 0.003	0.079 ± 0.01	0.527
SDA_{peak} (mg O_2 g^{-1} h^{-1})	0.13 ± 0.01	0.11 ± 0.01	0.052
T_{peak} (h)	0.89 ± 0.60	0.61 ± 0.26	0.679
Duration (h)	30.26 ± 1.33	2.01 ± 0.72*	< 0.001
SDA magnitude (mg O_2 g^{-1})	0.78 ± 0.11	0.028 ± 0.01*	< 0.001
E_{SDA} (J g^{-1})	10.84 ± 1.56	0.39 ± 0.11*	< 0.001
C_{SDA} (%)	9.39 ± 1.35	0.34 ± 0.10*	< 0.001

[a] FED, fed lobsters with no further treatment; FEDC, fed lobsters injected with cycloheximide; E_{SDA}, SDA magnitude converted to energy; C_{SDA}, SDA coefficient. All data represent mean ± standard error (SE) of 6 individuals. The superscript (*) indicates significant differences between the FED and FEDC treatments ($P < 0.05$), and the *P* values of paired t-tests are shown in the last row

2.5 Discussion

The present study has enlarged the current knowledge of crustacean nutritional physiology by providing previously restricted information on the contribution of protein synthesis to crustacean metabolism. Understanding the contribution of protein synthesis to energy metabolism is a key to investigate the relationship between nutritional status and feed quality (Jobling 1985; Carter & Houlihan 2001; Carter & Mente 2014) and how this will affect growth potential (Carter & Brafield 1992b; Carter & Houlihan 2001; Carter & Mente 2014) in crustacean aquaculture. That protein synthesis accounted for 96.40% of *S. verreauxi* SDA demonstrated that protein synthesis in decapod crustaceans can represent one of the highest proportions of SDA in any aquatic ectotherms (Brown & Cameron 1991b; Houlihan *et al.* 1995c; Thor 2000). In addition, the high proportion of SDA attributed to protein synthesis demonstrated that decapod crustacean SDA is largely a post-absorptive process (Jobling & Davies 1980; Brown & Cameron 1991a; Lurman *et al.* 2013), indicating that SDA can be used to assess the growth potential of an aquafeed (Carter & Brafield 1992b; McGaw & Penney 2014; Palafox *et al.* 2017).

2.5.1 Contribution of protein synthesis to oxygen consumption

In the present study, the contribution of CHX-sensitive protein synthesis to the RMR of 2-day fasted *S. verreauxi* was 28.51%, within the range of 9-42% contribution of protein synthesis to

RMR in many aquatic ectotherms (Houlihan *et al.* 1990; Houlihan *et al.* 1993b; Carter & Houlihan 2001; Fraser *et al.* 2002), and consistent with previous studies in crustaceans where the contribution of protein synthesis to the RMR was 28% in amphipod *G. oceanicus* (Rastrick & Whiteley 2017) and 21.8% in isopod *Glyptonotus antarcticus* (Whiteley *et al.* 1996). The contribution of CHX-sensitive protein synthesis to the RMR of 10-day starved *S. verreauxi* was 13.12%, comparable to previous research where protein synthesis was estimated to contribute to 7.5% of the RMR of 5-day starved isopod *Saduria entomon* (Robertson *et al.* 2001b) and 8% of the RMR of 10-day starved teleost *Gadus morhua* (Lyndon *et al.* 1992). The contribution of protein synthesis to the RMR of 2-day fasted *S. verreauxi* was significantly higher than that of 10-day starved *S. verreauxi*, indicating that juvenile *S. verreauxi* under starvation spend more energy for substrates catabolism, rather than anabolism such as protein synthesis, to maintain major life activity (Sacristán *et al.* 2016). The relatively low contribution of protein synthesis to RMR in unfed *S. verreauxi* confirms that fasted aquatic ectotherms spend less energy on protein synthesis (Brown & Cameron 1991a; Brown & Cameron 1991b).

Fed juvenile *S. verreauxi* injected with CHX presented a significantly smaller SDA response compared to fed *S. verreauxi* without CHX injection, in a similar pattern to previous recorded on channel catfish *I. punctatus* (Brown & Cameron 1991a; Brown & Cameron 1991b), demonstrating that protein synthesis was inhibited (Brown & Cameron 1991a; Brown & Cameron 1991b; Bowgen *et al.* 2007; Rastrick & Whiteley 2017). That the lobsters in FED and FEDC treatments consumed all the squid indicated that the inhibitory effect of CHX could not be a consequence of suppression of feed ingestion or regurgitation (Thor 2000). Instead, the inhibitory effect of CHX is more likely to be a consequence of inhibition of absorption and assimilation of amino acids (Brown & Cameron 1991a). The contribution of CHX-sensitive protein synthesis to SDA in teleost fish has been widely researched, ranging from 20 to almost 100% (Houlihan *et al.* 1988; Brown & Cameron 1991a; Brown & Cameron 1991b; Lyndon *et*

al. 1992; Houlihan *et al.* 1993b; Houlihan *et al.* 1995c). However, the contribution of protein synthesis to SDA in crustaceans has only been measured twice, ranging from 20 to 93% (Houlihan *et al.* 1990; Thor 2000). In the present study, the contribution of CHX-sensitive cytosolic protein synthesis was estimated to account for 96.40% of *S. verreauxi* SDA, demonstrating that decapod crustacean SDA is predominantly a post-absorptive process (Jobling & Davies 1980; Lurman *et al.* 2013). However, the contribution of protein synthesis to oxygen consumption in the present study would have likely been underestimated, because CHX does not completely inhibit protein synthesis even at very high concentrations (Garlick *et al.* 1983; Aoyagi *et al.* 1988). The high correlation between SDA and protein synthesis in the present study suggested that SDA has some potential to predict both long-term growth and feed potential (Fu *et al.* 2005a; Radford *et al.* 2008; Carter *et al.* 2012). However, using SDA to understand feed potential to promote growth will require consideration that protein synthesis also appears to be used to regulate excess amino acid intake (Carter & Houlihan 2001; Carter & Mente 2014). Hence, further research should be performed in decapod crustaceans to examine protein accretion to better understand the inter-relationships between feeds, SDA and long-term growth.

The 96.40% contribution of protein synthesis to *S. verreauxi* SDA also confirmed that protein synthesis in decapod crustaceans can represent one of the largest proportions of SDA in any aquatic ectotherms recorded to date (Brown & Cameron 1991b; Houlihan *et al.* 1995c; Thor 2000). Protein synthesis is triggered by the presence of amino acids and is a transitory response (Dobson 2003). However, previous studies demonstrated that protein synthesis in aquatic ectotherms following a single feeding can remain significantly higher than that in unfed aquatic ectotherms for more than 24 h, particularly in muscle (Houlihan *et al.* 1990; Lyndon *et al.* 1992; Carter *et al.* 2012), indicating that the assimilation of amino acids in aquatic ectotherms may continue over relatively long periods of time. Protein requirements for decapod

crustaceans reared in aquaculture are generally high (Mu *et al.* 1998; Carter & Mente 2014), which in turn lead to an increase of protein synthesis and in part reflect temporary synthesis of protein not retained as growth (Carter & Bransden 2001; Perera *et al.* 2005; Carter & Mente 2014). In the present study where squid with a high protein content was the only feed source, the high contribution of protein synthesis to *S. verreauxi* SDA indicated that the squid was well digested and that amino acids as the main digestive products were highly absorbed (Lawrence & Lee 1997; Ward *et al.* 2003). This indication was in agreement with previous studies on channel catfish *I. punctatus* where essential amino acids as the feed were infused into the body through a catheter, demonstrating nearly 100% of SDA was used for *I. punctatus* protein synthesis (Brown & Cameron 1991a; Brown & Cameron 1991b). As a result, minimal energy would be left for other purposes such as gluconeogenesis and lipogenesis (Pannevis & Houlihan 1992), suggesting that feeds where most of the energy is consumed by protein synthesis are suitable for juvenile *S. verreauxi*. To some extent, this has been confirmed by high values for the predicted optimum dietary protein requirement in lobster feeds (Glencross *et al.* 2001; Ward *et al.* 2003).

2.5.2 Oxygen consumption under different feeding conditions

The 32% decrease of SMR in 10-day starved *S. verreauxi* in the present study was comparable to other starved aquatic ectotherms (Beamish 1964; Dall & Smith 1986; Auerswald *et al.* 2009). Aquatic ectotherms under starvation are forced to reallocate energy substrates to maintain major life activity (Sacristán *et al.* 2016), resulting in the decrease of protein synthesis (Smith 1981; Loughna & Goldspink 1984; Houlihan *et al.* 1990) and RMR (Regnault 1981; Dall & Smith 1986; Fu *et al.* 2005b). Hence, the decrease of SMR in 10-day starved *S. verreauxi* indicated a decrease of protein synthesis. Simon *et al.* (2015) reported a 52% decrease of SMR in 14-d starved *S. verreauxi*, representing a larger drop of SMR compared to the present study,

probably due to a longer starvation period. Based on two formulas, $SMR_{starved} = 0.90 * day^{-0.60}$ and $SMR_{fed} = 0.31 + 1.21 * e^{-0.70day}$ from Simon *et al.* (2015), the SMR of 10-day starved *S. verreauxi* was estimated to decline by 29%, similar to that recorded in the present study (32%). The 41% decline of the RMR of 10-day starved *S. verreauxi* in the present study resembled previous research on other decapods where the RMR declined 40-50% after 10-day starvation (Regnault 1981; Dall & Smith 1986; Auerswald *et al.* 2009), and was also comparable to research on starved teleost fish where the RMR of white sucker *Catostomus commersoni* reared at 20 °C declined by 43% (Beamish 1964).

The SDA magnitude in fed lobsters was 10.84 J g^{-1}, aligned with other decapods at 3% ration level for instance the spiny lobster *J. edwardsii* (7.64 J g^{-1}) (Crear & Forteath 2000), crab *Pugettia producta* (10.23 J g^{-1}) and *Cancer gracilis* (11.08 J g^{-1}) (McGaw & Curtis 2013). The peak of SDA response in this study (2.2 times the SMR) was comparable to previous research on spiny lobster *P. cygnus* (2.19 times the SMR) (Crear & Forteath 2001) and *P. homarus* (2.02 times the SMR) (Kemp *et al.* 2009b), but was higher compared with *J. edwardsii* (1.6-1.72 times the SMR) (Crear & Forteath 2000; Radford *et al.* 2004). The differences of SDA_{peak} were most likely due to different temperatures regimes: *S. verreauxi* (present study), *P. cygnus* (Crear & Forteath 2001) and *P. homarus* (Kemp *et al.* 2009b) were reared at 21 °C, while *J. edwardsii* was reared at 13 °C (Crear & Forteath 2000; Radford *et al.* 2004). The application of SDA coefficient allows interspecific comparison of SDA, independent of various experimental conditions such as body size, feed type, and temperature (McCue 2006; McGaw & Curtis 2013). As with other decapod crustaceans (McGaw & Curtis 2013), fed *S. verreauxi* in the present study showed a 9% SDA coefficient, suggesting that the ingested energy expended on SDA is comparable among decapods (McGaw & Curtis 2013).

2.5.3 Reversibility of protein synthesis inhibition caused by cycloheximide

In the present study, the MO_2 in 2-day fasted and 10-day starved treatments following CHX injection presented a similar pattern whereby the MO_2 dropped with fluctuations at the first 6.3 h. The MO_2 in all CHX injection treatments decreased significantly at the first 2 h, then increased significantly from 3.7 to 4.7 h post-injection, thereafter decreased significantly from 4.7 to 6.3 h. The significant decrease at the first 2 h post-injection was in agreement with previous research on *I. punctatus* (Brown & Cameron 1991a; Brown & Cameron 1991b). The lowest MO_2 occurred 6.3 h, after which the MO_2 increased and returned gradually to the pre-treatment level. The change of MO_2 following CHX injection indicated that the suppression of protein synthesis caused by CHX injection might be reversible in unfed *S. verreauxi*, however, the inhibitory effect of CHX might be irreversible in fed *S. verreauxi*, because the post-prandial MO_2 only experienced a short-term increase. The reversibility of protein synthesis inhibition has been illustrated in ciliate *Tetrahymena thermophila* bathed in CHX at 0.5 mg L^{-1}, where there was a significant depression of protein synthesis at the first hour, then protein synthesis gradually recovered (Hallberg *et al.* 1985). Similarly, oxygen consumption rates in unfed channel catfish *I. punctatus* showed a decrease at the first hour after CHX infusion, then returned to the control level within 3 h (Brown & Cameron 1991a; Brown & Cameron 1991b). Cycloheximide as a nuclear signaling agonist prolongs the usually transient induction of 'immediate early response' (IE) genes transcripts from several minutes to several hours (Fort *et al.* 1987; Edwards & Mahadevan 1992), leading to delayed transcriptional shutoff, extended half-life and stabilization of mRNA, and repressed protein synthesis (Fort *et al.* 1987; Rahmsdorf *et al.* 1987; Edwards & Mahadevan 1992). Therefore, the decrease of MO_2 in unfed *S. verreauxi* at the first 6.3 h following CHX injection could be due to the prolongation of the IE genes transcripts whereby protein synthesis is inhibited (Fort *et al.* 1987; Edwards & Mahadevan 1992). It has been confirmed that CHX prevents cytoplasmic protein synthesis but

does not repress mitochondrial protein synthesis (Fraser & Rogers 2007; Saini *et al.* 2009). Moreover, it remains unknown whether the reversibility of protein synthesis inhibition is due to CHX detoxification or exclusion (Roberts & Orias 1974). Currently, it is widely accepted that the reversibility of protein synthesis inhibition is related to an adaptation mechanism whereby the CHX-resistant protein might be synthesized on mitochondrial ribosomes instead of cytoplasmic ribosomes (Frankel 1970; Roberts & Orias 1974; Hallberg *et al.* 1985). Hence, the return of the MO_2 in unfed *S. verreauxi* from 6.3 h after CHX injection suggested that mitochondria might play a dominant role in protein synthesis in CHX-injected unfed *S. verreauxi* (Frankel 1970; Roberts & Orias 1974; Hallberg *et al.* 1985). The irreversible suppression of MO_2 in fed *S. verreauxi* following CHX injection was consistent with many other aquatic ectotherms where the post-prandial MO_2 was considerably suppressed after CHX administration (Brown & Cameron 1991b; Houlihan *et al.* 1995c; Thor 2000). Such irreversible decline of MO_2 suggested the adaptation mechanism of the reversibility of protein synthesis inhibition after administration of CHX might be dominant in unfed aquatic ectotherms where protein synthesis is low, while insignificant in fed aquatic ectotherms where protein synthesis is high.

2.6 Conclusions

This research for the first time investigated the contribution of protein synthesis to decapod crustacean metabolism under different nutritional conditions using a cytosolic protein synthesis inhibitor, cycloheximide. This study demonstrated that decapod crustacean metabolism downregulated with starvation, unfed decapod crustaceans expended little energy on protein synthesis, while fed decapod crustaceans expended most of the ingested post-prandial energy on protein synthesis. Protein synthesis in decapods is among the highest proportions of SDA in all aquatic ectotherms reported. The present study expanded the current knowledge of

crustacean nutritional physiology by providing previously restricted information on the contribution of protein synthesis to crustacean metabolism, which is crucial to explore the relationship between nutritional status and feed quality and how this affects growth potential in crustacean aquaculture. Our findings confirmed that decapod crustacean SDA is largely a post-absorptive process. The high correlation between SDA and protein synthesis suggested SDA is likely a useful indicator of growth potential of a feed and thus a useful tool to assess feed potential for aquaculture species. The present study also has some limitations considering the methodology to evaluate the stress influence of injection and handling. Therefore, further research in *S. verreauxi* can be performed to investigate whether stress influence from injection and handling is comparable among different nutritional status. Further studies may also investigate protein accretion and contribution of protein synthesis to SDA for juvenile *S. verreauxi* under different dietary protein to energy ratios in order to produce cost-effective feeds and to fully understand the inter-relationships between feed, SDA and long-term growth in decapod crustaceans. Moreover, further research may incorporate the full complement of key metabolic measurements (oxygen consumption, nitrogenous wastes excretion and carbon dioxide excretion), which will allow the use of a stoichiometric bioenergetic approach to predict SDA for aquaculture species more accurately. To better understand the contribution of protein synthesis to oxygen consumption in aquatic ectotherms, it would be worthwhile to investigate different decapod species. Attention should also be paid to the contribution of protein synthesis to oxygen consumption on decapods in terminal anecdysis and at the cellular level.

Chapter 3 Respiratory quotient and the stoichiometric approach to investigating metabolic energy substrate use in aquatic ectotherms

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3.1 Abstract

The respiratory quotient (RQ) has been used extensively as an index to evaluate metabolic energy expenditure in terrestrial animals including humans. In contrast, RQ use in understanding physiology and nutrition of aquatic ectotherms has been restricted due to technical challenges in measuring total CO_2 in water. With technical advances in measuring total CO_2 in water, RQ in aquatic ectotherms can be accurately determined and is potentially available as a valuable method. Here we provide a comprehensive review of studies on RQ and metabolic energy substrate use in aquatic ectotherms. Metabolic energy substrate use is evaluated by a reliable stoichiometric bioenergetic approach, based on measuring RQ and nitrogen quotient (NQ) simultaneously. Stoichiometry provides a non-destructive and unequivocal way to quantify the instantaneous oxidation of each major energy substrate (protein, lipid, or carbohydrate). This review aims to refine knowledge about bioenergetics of aquatic ectotherms under different conditions including nutritional aspects of sustainable aquaculture. Notably, stoichiometry provides a promising approach to optimize feeds and feeding regimes to realize sustainable aquaculture under differing conditions and with differing feed ingredients. It also provides an approach to consider climate change impacts and physiological adaptation mechanisms for survival and development in farmed environments

and natural ecosystems. Stoichiometric bioenergetics knowledge in aquatic ectotherms has relevance to commercial impacts in the face of overfishing and food security, and ecological significance in the face of environmental change scenarios. We suggest expanding the use of stoichiometry in future bioenergetic research in emerging aquaculture species.

3.2 Introduction

Metabolic energy sustains all life processes and for most animals is mainly derived from oxidation of major energy substrates (protein, lipid, carbohydrate) originating from ingested nutrients (Kleiber 1975; Blaxter 1989; Clarke 2019). A better understanding of metabolic energy substrate use is a key premise for the development of nutritionally balanced feeds and optimal feeding regimes to achieve more sustainable aquaculture under differing conditions and with differing feed ingredients (Cho & Kaushik 1990; Gelineau *et al.* 1998).

The respiratory quotient (RQ), determined from the ratio of excreted CO_2 to consumed O_2, is a non-destructive parameter used to assess metabolic energy substrate use without the need to sacrifice the animal (Barber & Blake 1985; Wood 2001; Li *et al.* 2017). In terrestrial animals, the RQ is the most common metabolic ratio (Dejours 1975; Hatcher 1991) and has been widely investigated in various classes including insects (Hahn & Denlinger 2011; Arnqvist *et al.* 2017; Levin *et al.* 2017), reptiles (Kleiber 1975; Wang *et al.* 2001; Malte *et al.* 2016), birds (Klaassen & Biebach 1994; Walsberg & Wolf 1995; Gerson *et al.* 2019) and mammals (Blaxter 1989; Jeanniard-du-Dot *et al.* 2017; Liu *et al.* 2019), and has also been broadly used in human clinical trials (Galgani & Castro-Sepulveda 2017; Mesquida *et al.* 2018; Murugesan *et al.* 2020). There have been recent reviews on the use of RQ to study nutrition and physiology in terrestrial animals (Butler *et al.* 2004; Arch *et al.* 2006) and humans (Compher *et al.* 2006; Miles-Chan *et al.* 2015). Compared with terrestrial animals and humans, there is a paucity of RQ literature in aquatic ectotherms (Brafield & Solomon 1972; Musisi

1984; Ferreira *et al.* 2019). The earliest RQ research in aquatic ectotherms dates back to the early 19th century (Humboldt & Provençal 1809), and studies before the 1960s have been well reviewed (Bosworth *et al.* 1936; Wolvekamp & Waterman 1960; Clifford & Brick 1983). However, the early RQ data in aquatic ectotherms can be questionable, largely because of the difficulty in accurately determining total CO_2 in water, which includes not only gaseous CO_2, but also carbonate, bicarbonate and carbonic acid (Brafield 1985; Mackenzie & Lerman 2006; Nelson 2016). In contrast, the measurement of aquatic oxygen consumption is generally considered accurate and convenient using oxygen sensors based on polarography or luminescence quenching (Clark 1956; Milburn & Beadle 1960; Nelson 2016). With technical advances in accurately determining total CO_2 in water (Kutty 1968; Harris 1982; Choi *et al.* 2002), RQ studies in aquatic ectotherms have increased (Kutty *et al.* 1971; Lauff & Wood 1996a; Wang *et al.* 2021).

Although the sole use of RQ can indicate which energy substrate is predominantly oxidized (Table 3. 1), the specific amount of each major energy substrate being oxidized cannot be determined (McCue & Welch 2016). In contrast, the use of a stoichiometric bioenergetic approach, based on the concurrent calculation of RQ and nitrogen quotient (NQ, used to quantify the fraction of aerobic energy substrate use supplied by protein), can unequivocally illustrate the oxidation of each major energy substrate at any one time, and has distinct merits compared with the traditional compositional approach (Ferrannini 1988; Kaushik & Médale 1994; Wood 2001). This is partially because the compositional approach is based on the assessment of changes in body weight and whole-body biochemical composition over a long period and is terminal as the animal is slaughtered (Lauff & Wood 1996b). In addition, endogenous energy substrates are likely interconverted before metabolic use or excreted without oxidation, which may affect the estimation accuracy of energy substrate oxidation (Brafield 1985; Lauff & Wood 1996b). In contrast, the stoichiometric approach examines

instantaneous fractional contributions of each major energy substrate to the support of oxygen consumption and is non-destructive (Brett 1995; Lauff & Wood 1996b; Ferreira *et al.* 2019), thus allowing repeated assessments of metabolic energy substrate use in the same animal (Kaushik *et al.* 1989; Lauff & Wood 1996b; De Boeck *et al.* 2001). The non-destructive feature is also vital for interpreting bioenergetics in the same animal over time and throughout their lifecycle, for use with endangered aquatic species and species with high commercial value, such as spiny lobsters (Wang *et al.* 2019a).

Traditional long-term growth experiments are instructive for quantifying specific nutrient requirements to optimize aquafeeds (Hauler & Carter 2001). However, long-term growth studies cannot provide detailed information about nutrient processing (Clifford & Brick 1979; Hewitt & Irving 1990). Alternatively, the estimation of metabolic use of each major energy substrate by stoichiometry sheds light on the nutritional and biochemical ability of an aquatic ectotherm to transform ingested nutrients for anabolic metabolism, crucial for efficiently describing nutrition and energy requirements to maximize survival and growth performance at the minimum energy expenditure, thus contributing to the development of sustainable aquaculture (Cho & Kaushik 1990; Carter & Brafield 1991). Currently, aquaculture supports increased market demands for aquatic food as capture fisheries are in decline partially due to overfishing (FAO 2018; Tacon *et al.* 2020) and is predicted to make an increasingly important contribution to global food security (Watson *et al.* 2016; Blanchard *et al.* 2017). In addition, stoichiometry is also applicable to research on the rich diversity of aquatic ectotherms inhabiting the world's coastal waters, many of which are harvested or farmed by humans for food (Tacon *et al.* 2020) or other biological materials (Watson *et al.* 2016). Aquatic ectotherms inhabiting coastal waters are usually subjected to changeable environments (Morash & Alter 2016; Twiname *et al.* 2019) and nutritional status mainly caused by frequent changes in feed availability (Norkko *et al.* 2005; Ohlberger 2013). A thorough understanding of RQ and

metabolic energy substrate use provides valuable information on bioenergetics in wild aquatic ectotherms, which can be of great ecological importance in unveiling physiological adaptations to environmental changes (McCue 2010; Birnie-Gauvin *et al.* 2017; Twiname *et al.* 2019).

This review aims to examine the effect of abiotic and biotic factors on RQ and stoichiometrically determined metabolic energy substrate use in aquatic ectotherms, to understand how the knowledge of stoichiometric bioenergetics can be used to improve feeding, nutrition and production variables for sustainable aquaculture and to interpret physiological mechanisms for survival and development of aquatic ectotherms in the wild. The review will summarize relevant literature in the field, explain the approaches to the determination of key metabolic parameters including total CO_2 and nitrogenous excretion, and describe the rationale behind the use of stoichiometry. Metabolic changes under various conditions and the potential use of stoichiometry are reviewed and discussed. Limitations of stoichiometry are illustrated and research directions relevant to RQ and metabolic energy substrate use that warrant further investigations are suggested.

Table 3. 1 Key equations showing stoichiometry and respiratory quotient (RQ)

Substrate	Metabolism	Equations	RQ	References
Glucose	Aerobic oxidation	$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$	1.0	Frayn (1983)
Standard protein where ammonia is the only nitrogenous end-product	Aerobic oxidation	$C_{4.42}H_7O_{1.44}N_{1.14} + 4.6\ O_2 \rightarrow 4.42\ CO_2 + 1.79\ H_2O + 1.14\ NH_3$	0.96	Brafield and Llewellyn (1982)
Standard protein where urea is the only nitrogenous end-product	Aerobic oxidation	$C_{4.42}H_7O_{1.44}N_{1.14} + 4.6\ O_2 \rightarrow 3.85\ CO_2 + 2.36\ H_2O + 0.57\ CO(NH_2)_2$	0.84	Brafield and Llewellyn (1982)
PSOG (palmitoyl-stearoyl-oleoyl-glycerol, a typical fat)	Aerobic oxidation	$C_{55}H_{104}O_6 + 78\ O_2 \rightarrow 55\ CO_2 + 52\ H_2O$	0.71	Frayn (1983)
A mix of major energy substrates	Aerobic oxidation	–	0.71-1.0	Elliott and Davison (1975)
Palmitic acid and alanine	Aerobic gluconeogenesis	$C_{16}H_{32}O_2 + 26\ C_3H_7O_2N + 23\ O_2 \rightarrow 13\ C_6H_{12}O_6 + 3\ CO_2 + 3\ H_2O + 13\ CO(NH_2)_2$	0.13	Frayn (1983)

Glucose	Aerobic lipogenesis	$83\ C_6H_{12}O_6 + 30\ O_2 \rightarrow 6\ C_{55}H_{104}O_6 + 168\ CO_2 + 186\ H_2O$	5.6	Frayn (1983)
Glucose	Aerobic lipogenesis	$4\ C_6H_{12}O_6 + O_2 \rightarrow C_{16}H_{32}O_2 + 8\ CO_2 + 8\ H_2O$	8.0	Lusk (1928)
A mix of major energy substrates	Anaerobic oxidation, mainly due to low dissolved oxygen, high-speed swimming, and aerial exposure	–	> 1.0	Peer and Kutty (1981) (low dissolved oxygen); Milligan (1996) (high-speed swimming); Morris and Oliver (1999) (aerial exposure)

3.3 Methods of article selection

Google Scholar and Scopus were searched from their inception to October 2020 using keywords including respiration, respiratory quotient, energy expenditure and (metabolic) fuel use, in addition to citation tracking and hand-searches. Peer-reviewed journals and book chapters that used RQ calculated from the accurate measurement of total CO_2 excretion and O_2 consumption in fish and aquatic invertebrates including arthropods, bryozoans, chordates, echinoderms and molluscs, published since 1968 were included. Articles published with incomplete descriptions for total CO_2 measurements were excluded. As a result, 65 articles including 46 research articles, 11 reviews, 6 book chapters and 2 doctoral dissertations were identified for the present review.

3.4 Determination of RQ and NQ

3.4.1 Definition of RQ and NQ

Respiratory quotient is defined as the ratio of the volume of excreted CO_2 (VCO_2) to the volume of consumed O_2 (VO_2) during respiration (Lampert 1984; Mayzaud *et al.* 2005). Based on the density and molar mass of CO_2 and O_2, VCO_2/VO_2 can be approximately converted to nCO_2/nO_2, where nCO_2 and nO_2 represent molar excreted CO_2 and consumed O_2, respectively (Ferreira *et al.* 2019; Wang *et al.* 2021). Nitrogen quotient is defined as the molar ratio of total nitrogenous excretion to oxygen consumption (Lauff & Wood 1996a; Ferreira *et al.* 2019).

3.4.2 Stoichiometric bioenergetic approach

Using stoichiometry to examine instantaneous metabolic energy substrate use is dependent on the simultaneous determination of NQ and substrate-specific aerobic RQ (Lauff & Wood 1996b; Wang *et al.* 2021). The potential use of stoichiometry in farmed and wild aquatic ectotherms is summarized in Figure 3. 1 and elaborated in the subsequent sections. The

rationale for the determination of the relative contributions of each major energy substrate to the support of oxygen consumption is presented in Figure 3. 2.

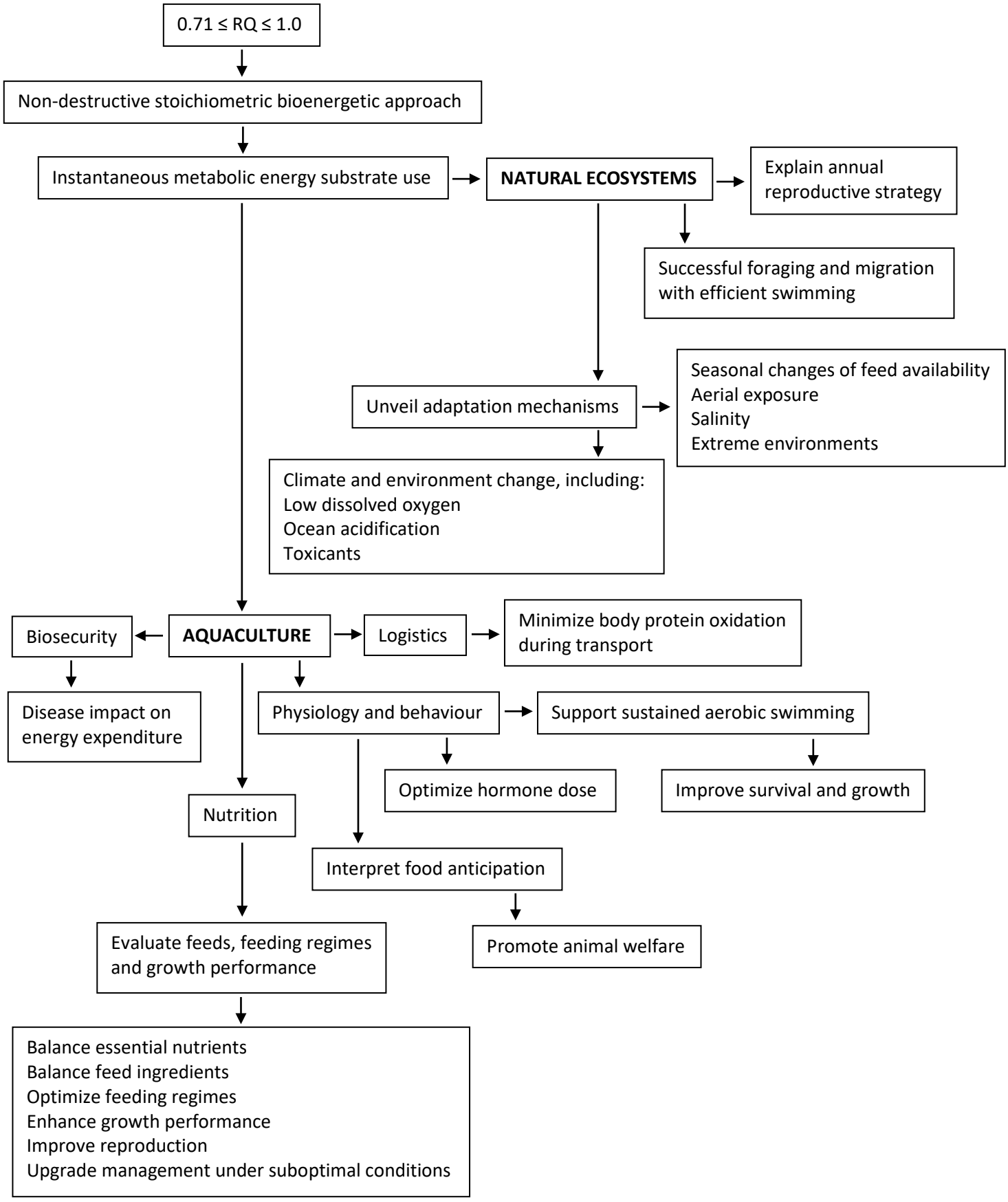
0.71 ≤ RQ ≤ 1.0
Non-destructive stoichiometric bioenergetic approach
Instantaneous metabolic energy substrate use
NATURAL ECOSYSTEMS
Explain annual reproductive strategy
Successful foraging and migration with efficient swimming
Unveil adaptation mechanisms
Seasonal changes of feed availability
Aerial exposure
Salinity
Extreme environments
Climate and environment change, including:
Low dissolved oxygen
Ocean acidification
Toxicants
Biosecurity
AQUACULTURE
Logistics
Minimize body protein oxidation during transport
Disease impact on energy expenditure
Physiology and behaviour
Support sustained aerobic swimming
Optimize hormone dose
Improve survival and growth
Nutrition
Interpret food anticipation
Promote animal welfare
Evaluate feeds, feeding regimes and growth performance
Balance essential nutrients
Balance feed ingredients
Optimize feeding regimes
Enhance growth performance
Improve reproduction
Upgrade management under suboptimal conditions

Figure 3. 1 An overview of some potential uses of the stoichiometric bioenergetic approach in aquaculture and natural ecosystems. The values of 0.71 and 1.0 are the minimum and maximum respiratory quotient (RQ) during aerobic oxidation, respectively (Frayn 1983; Lauff & Wood 1996b).

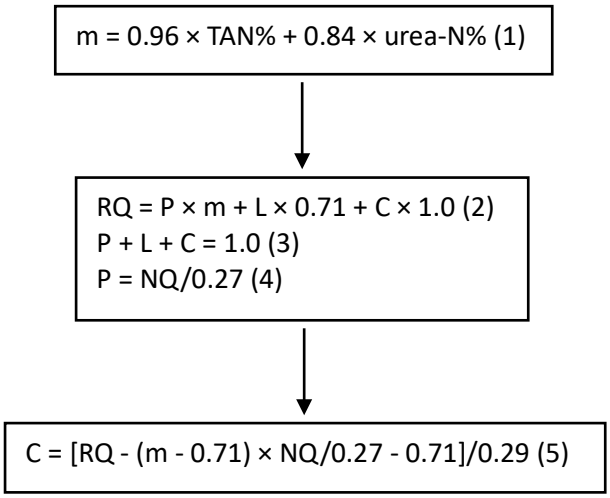

Figure 3. 2 Diagrammatic representation of the relationship among respiratory quotient (RQ), nitrogen quotient (NQ), and the stoichiometric bioenergetic approach in the determination of instantaneous metabolic energy substrate use in aquatic ectotherms. m is the aerobic RQ for protein oxidation, TAN% and urea-N% represent the contribution of total ammonia-N (NH_3-N + NH_4^+-N) and urea-N to total nitrogenous excretion, respectively. P, L, and C represent the fraction of aerobic energy substrate use supplied by protein, lipid, and carbohydrate, respectively. The values of 0.96 and 0.84 are the aerobic RQ for protein oxidation when ammonia (NH_3 + NH_4^+) and urea are the unique nitrogenous end-products, respectively (Brafield & Llewellyn 1982; Lauff & Wood 1996b). The values of 0.71 and 1.0 are the aerobic RQ for lipid and carbohydrate oxidation, respectively (Frayn 1983; Lauff & Wood 1996b). The value of 0.27 is the theoretical maximum NQ when protein is the only energy substrate being completely oxidized under aerobic conditions (Lauff & Wood 1996b). Equation (5) is derived from the combination of Equation (2), (3), and (4), where the percentage contribution of

carbohydrate to energy production is determined (Lauff & Wood 1996b; Wang *et al.* 2021). Thereafter, the lipid contribution can be determined from Equation (3).

3.4.3 Techniques to measure total CO_2 and nitrogenous excretion

Techniques used to quantify total CO_2 excretion in aquatic ectotherms are shown in Table 3. 2 and 3. 3. In detail, Kutty (1968) used a modified volumetric Van Slyke technique to estimate total CO_2 excretion in fish. The Van Slyke technique proved accurate to measure total CO_2 in water where samples were acidified so that all combined forms were converted to CO_2 gas and removed by a vacuum extraction system. Subsequently, the CO_2 gas was transferred without contact with the atmosphere to a standard volume of sodium hydroxide solution with a known electrical resistance at a standard temperature (Bosworth *et al.* 1936; Milburn & Beadle 1960; Oren 1981). Following the Van Slyke technique, a modified titrimetric Maros-Schulek technique was widely used in fish studies (Oren 1981; Sukumaran 1986). The Maros-Schulek technique is very accurate where distillation and subsequent titration are completed without the solutions coming into contact with the atmosphere (Kutty *et al.* 1971; Brafield 1985). Early RQ research in aquatic crustaceans used manometry (Moshiri *et al.* 1969; Wright & Wright 1976) or acid-base titration (Rakusa-Suszczewski *et al.* 1976; Clifford & Brick 1983). The use of manometry was based on the use of micro-volumetric respirometers, and the total CO_2 concentration was corrected from a titration curve (Scholander *et al.* 1952; Umbreit *et al.* 1964). The precision and accuracy of acid-base titration were verified with standard solutions of potassium or sodium bicarbonate (Clifford & Brick 1979; Harris 1982). With technical advances, potentiometry, conductometry, gas chromatography, colorimetry, coulometry, and infrared spectroscopy have been well developed for convenient total CO_2 determination (Edmond 1970; Harris 1982; Choi *et al.* 2002). The use of potentiometry is based on the measurement of pH, total alkalinity, salinity, temperature, and the apparent dissociation

constants of carbonic and boric acids (Edmond 1970; Mehrbach *et al.* 1973; Choi *et al.* 2002). The use of conductometry is dependent on the conversion of all combined forms to CO_2 gas by acidification, removal in a carrier gas stream, followed by absorption in an alkali solution and detection by differential conductivity (van den Thillart *et al.* 1983; Gelineau *et al.* 1998; Morris & Oliver 1999). Kieffer *et al.* (1998) and Alsop *et al.* (1999) used gas chromatography (Shimadzu GC-8A, Tokyo, Japan) with a calibration curve obtained from standard solutions of sodium bicarbonate. Dersjant-Li (2000) and Ozório *et al.* (2001) used a rapid flow autoanalyzer (Alpkem, Oregon, USA) based on colorimetry. Mayzaud *et al.* (2005) used coulometry to determine total CO_2 excretion in marine zooplankton and the data were corrected to Certified Reference Materials (Scripps Institution of Oceanography, USA) (Johnson *et al.* 1998; Chu *et al.* 2018). The non-dispersive infrared (NDIR) spectroscopy used to measure gas concentrations started in the late 1930s (Wong & Anderson 2012; Dinh *et al.* 2016) and can be used to accurately and precisely determine total CO_2 in water (Harter *et al.* 2017; Müller *et al.* 2017). When the water is acidified to pH less than 4, all combined forms are converted to CO_2 gas, then determined based on the energy absorption characteristics of CO_2 in the infrared region in the presence of N_2 as a carrier gas (Holopainen & Ranta 1977; Müller *et al.* 2017). However, the NDIR method has not been widely used in respiratory studies in aquatic ectotherms (McKenzie *et al.* 2007; Ferreira *et al.* 2019; Wang *et al.* 2021).

Compared with research examining total CO_2 excretion in aquatic ectotherms, there have been numerous studies measuring total nitrogenous excretion, including total ammonia-N (TAN = NH_3-N + NH_4^+-N) and urea-N excretion, with a variety of convenient and accurate methods, among which spectrophotometry is most commonly used (Wilkie *et al.* 2017; Zhu *et al.* 2019; Takeda *et al.* 2020). The concentration of TAN is usually determined using a salicylate-hypochlorite assay to measure a blue-coloured product formed by reacting ammonia with sodium salicylate and sodium hypochlorite, in the presence of sodium nitroprusside under

alkaline conditions (Bower & Holm-Hansen 1980; Wilkie *et al.* 2017). The concentration of urea-N is usually determined using a diacetyl monoxime assay to measure a red-coloured product formed by reacting urea with diacetyl monoxime under acid conditions (Alam *et al.* 2017; Wilkie *et al.* 2017).

3.4.4 Calculation of RQ and NQ

Theoretically, the completely aerobic oxidation of one mole of glucose requires 6 moles of O_2, yielding 6 moles of CO_2; thereby the RQ is 6/6, or 1.0 (Frayn 1983) (Table 3. 1). The RQ of protein oxidation assumes a standard protein (53% carbon (C), 7% hydrogen (H), 23% oxygen (O), 16% nitrogen (N), and 1% sulphur (S)) and depends on the contribution of TAN and urea-N to total N excretion (Elliott & Davison 1975; Brafield & Llewellyn 1982). Consequently, the RQ of completely aerobic protein oxidation is 0.96 when ammonia is the only nitrogenous end-product, and 0.84 when urea is the only nitrogenous end-product (Brafield & Llewellyn 1982) (Table 3. 1). The estimation of RQ of protein oxidation in Brafield and Llewellyn (1982) ignored the S due to the tiny amount and unclear excreted form. Their result is almost the same as that calculated by Elliott and Davison (1975), where the S is taken into account. Similar to protein oxidation, the completely aerobic oxidation of one mole of a representative fatty acid (palmitoyl-stearoyl-oleoyl-glycerol, PSOG), requires 78 moles of O_2, yielding 55 moles of CO_2; therefore the RQ is 0.71 (Frayn 1983) (Table 3. 1). Accordingly, a RQ of 1.0 indicates completely aerobic carbohydrate oxidation, a RQ of 0.71 indicates completely aerobic lipid oxidation, anything between 0.71 and 1.0 indicates an unknown mix of the major substrate oxidation (Elliott & Davison 1975; Brafield & Llewellyn 1982; Frayn 1983) (Table 3. 1). Kleiber (1975) provides a detailed account of relatively common metabolic circumstances that result in RQ values below or above the normal range of 0.71-1.0. In aquatic ectotherms, a RQ less than 0.71 could be due to endogenous gluconeogenesis (Frayn 1983) (Table 3. 1), CO_2

retention in the blood (Frayn 1983; van den Thillart *et al.* 1983), or shell calcification (calcium carbonate formation) (Boucher-Rodoni & Boucher 1993; Martin *et al.* 2006). In contrast, a RQ above 1 can be partly due to anaerobic metabolism where excreted CO_2 exceeds consumed O_2 (Sukumaran 1986; Lauff & Wood 1996b; Caciano *et al.* 2015). In aquatic ectotherms, high-speed swimming, aerial exposure and low dissolved oxygen may result in anaerobic metabolism (Peer & Kutty 1981; Milligan 1996; Sundt-Hansen *et al.* 2007). In addition, aquatic ectotherms under aerobic conditions may also experience transitory anaerobic metabolism caused by stress (Kutty 1968; Sukumaran 1986), short-term absence of steady state (Lauff & Wood 1996b), or periodically spontaneous bursts of working muscles (Lauff & Wood 1996b; Kieffer *et al.* 1998; Mayzaud *et al.* 2005). A RQ above 1 may also be a consequence of lipogenesis where endogenous carbohydrate converts to lipid (Lusk 1928; Frayn 1983; Barber & Blake 1985) (Table 3. 1), accumulation of proline during the Krebs cycle (Mayzaud *et al.* 2005), or shell decalcification (interaction of CO_2 with carbonate) (Bosworth *et al.* 1936; Wolvekamp & Waterman 1960; Boucher-Rodoni & Boucher 1993).

The theoretical maximum NQ is 0.27 when protein is the only energy substrate being completely oxidized under aerobic conditions (Lauff & Wood 1996b; Kieffer *et al.* 1998).

3.5 Factors influencing RQ and related metabolic energy substrate use

3.5.1 Abiotic factors

3.5.1.1 Temperature

Environmental temperature is the most important abiotic factor influencing aquatic ectotherms because their body temperature directly changes with the environmental temperature (Jobling 1981b; Katersky & Carter 2007; Pecl *et al.* 2017). One of the central physiological responses for aquatic ectotherms to adapt to changed environmental temperature is energy metabolism alteration (Elliott 1982; Jobling 1994; Brett 1995). To date, there have been few studies on the thermal effects on RQ and metabolic energy substrate use in aquatic ectotherms and the results are conflicting (Mohamed & Kutty 1986; Kieffer *et al.* 1998; Alsop *et al.* 1999). For example, in the tilapia, *Oreochromis mossambicus*, and the mullet, *Rhinomugil corsula*, both the routine RQ (Table 3. 2) and metabolic energy substrate use (Table 3. 4) remained stable at different temperatures, indicating temperature change within some range may not cause significant metabolic alterations (Mohamed 1982; Mohamed & Kutty 1986). Kutty and Mohamed (1975) also demonstrated that temperature elevation did not affect the routine RQ in *R. corsula* (Table 3. 2), however, the increase of temperature resulted in decreased lipid oxidation and increased carbohydrate oxidation, while protein oxidation remained minimal (Table 3. 4). Similarly, Kieffer *et al.* (1998) demonstrated that the routine RQ remained the same when rainbow trout (*Oncorhynchus mykiss*) acclimated at different temperatures (5 and 15 °C) (Table 3. 2) while the metabolic use of each major energy substrate differed (Table 3. 4). Cold-acclimated rainbow trout preferentially oxidized lipid and carbohydrate for energy production, and protein contribution was minimal. In contrast, warm-acclimated rainbow trout mainly oxidized lipid, followed by protein, and carbohydrate oxidation was minimal (Kieffer *et al.* 1998) (Table 3. 4). In some other fish species, both the routine RQ and metabolic energy substrate use may vary with temperature. For example, the routine RQ in Nile tilapia (*Oreochromis niloticus*)

increased from 0.86 to 0.93 when temperature decreased from 30 to 15 °C (Alsop *et al.* 1999) (Table 3. 2). Based on the stoichiometric bioenergetic approach, Alsop *et al.* (1999) suggested that warm-acclimated Nile tilapia mainly oxidized lipid, followed by protein, and carbohydrate oxidation was minimal; while cold-acclimated Nile tilapia mainly oxidized carbohydrate, followed by lipid, and protein oxidation was minimal (Table 3. 4). The optimum temperatures for rainbow trout and Nile tilapia growth are 15-18 and 26-30 °C, respectively (Hokanson *et al.* 1977; Azaza *et al.* 2008). Hence, the stoichiometric bioenergetic results from Kieffer *et al.* (1998) and Alsop *et al.* (1999) suggest that in one aquatic ectotherm the metabolic energy substrate use can vary with temperature, and that among different species the metabolic use can be similar when reared at optimum temperatures.

Currently, aquaculture in offshore and high energy locations has become increasingly important to support the growing demand for seafood (Oppedal *et al.* 2011; Klinger *et al.* 2017). However, offshore aquaculture can be constrained by changeable ocean temperatures, which are usually season-dependent and not easily controlled (Oppedal *et al.* 2011; Klinger *et al.* 2017). Using stoichiometry to quantify metabolic energy substrate use improves the understanding of physiological changes in cultured animals at different temperatures, critical for manipulation of nutrient compositions in response to ocean temperature change to maximize offshore aquaculture production. In addition, from an ecological perspective, the examination of stoichiometric bioenergetics in wild aquatic ectotherms in the annual cycle may uncover nutritional strategies to temperature-related seasonal changes of feed availability (Paloheimo & Dickie 1966; Jobling 1980; McCue 2010).

3.5.1.2 Aerial exposure

Many aquatic ectotherms such as intertidal fish, some decapod crustaceans, molluscs and polychaetes inhabiting intertidal zones, may regularly experience aerial exposure during their

natural life cycles (Innes & Taylor 1986; McMahon 1988; Martin & Lighton 1989). These animals when exposed to air are likely to experience hypoxia, resulting in partly anaerobic metabolism to provide energy without the need to increase metabolic rates (Brinkhoff *et al.* 1983; Martin 1993; Forgan *et al.* 2014). The RQ research in intertidal fish has been well reviewed by Martin (1993), where the RER (the ratio of excreted CO_2 at the respiratory surfaces to consumed O_2) is used to describe the respiratory gas exchange. In general, the aerial RER in intertidal fish is approximately 0.7-1.0 (Martin 1993) (Table 3. 2), comparable to that of the decapod crustaceans (Forgan *et al.* 2014) (Table 3. 3). Many freshwater bimodally air-breathing fish such as *Amia calva* have very low aerial RER, indicating ineffective CO_2 excretion through the air-breathing organs (Perry 1986; Martin 1993; Rankin & Jensen 1993). However, the combined RER of both the air-breathing organs and aquatic respiratory organs (gills) in freshwater air-breathing fish is approximately 0.7-1.0 (Martin 1993). In contrast, seawater air-breathing fish, such as *Xiphister mucosus* which temperately emerge in air only at low tide, and mudskippers such as *Periophthalmus vulgaris* which routinely emerge and are very active in air, can effectively exchange respiratory gases in air using the skin, gills, and buccopharyngeal mucosae (Martin & Lighton 1989; Martin 1993). Consequently, the aerial RER in seawater air-breathing fish is comparable to the combined RER in freshwater bimodally air-breathing fish (Martin 1993) (Table 3. 2). Richards (2011) suggested that the oxidation of glycogen and other fermentable energy substrates dominated in intertidal fish under aerial exposure to support metabolism.

Many aquaculture species with high commercial value, such as abalone and spiny lobsters, are usually transported and sold alive (Chiou *et al.* 2002; Forgan *et al.* 2014). These animals usually inhabit aquatic conditions but are subjected to aerial exposure up to several weeks during commercial transport (Whiteley & Taylor 1992; Forgan *et al.* 2014; Day *et al.* 2019). The RQ in these animals exposed to air is usually greater than 1 (Table 3. 3), and

arginine phosphate is demonstrated to be the major metabolic energy substrate (Donovan *et al.* 1999; Morris & Oliver 1999; Speed *et al.* 2001). However, Chiou *et al.* (2002) demonstrated that when the abalone *Haliotis diversicolor* were exposed to air, the oxidation of carbohydrate especially muscle glycogen dominated. The use of stoichiometry is promising to interpret nutrient oxidation in aquatic ectotherms under different durations of aerial exposure, vital for better nutritional manipulation before transport to enhance survival and minimize body protein oxidation during transport (Morris & Oliver 1999; Forgan *et al.* 2014; Wang *et al.* 2021). However, it should be noted that the use of stoichiometry is based on aerobic RQ values, indicating this approach is only valid when the RQ is less than 1, the theoretical maximum value under aerobic conditions (Lauff & Wood 1996a; Kieffer *et al.* 1998; Wang *et al.* 2021).

3.5.1.3 Dissolved oxygen

The dissolved oxygen (DO) is a key limiting factor for animals living in water (Brett 1979; Sundt-Hansen *et al.* 2007; Killen *et al.* 2012). Aquatic ectotherms are likely subjected to hypoxia when the DO is low enough that impairs individual performance and results in a RQ greater than 1 (Table 3. 2) (Peer & Kutty 1981; Sundt-Hansen *et al.* 2007; Killen *et al.* 2012). Hypoxia due to low DO can occur in both inland high-density aquaculture systems (Sundt-Hansen *et al.* 2007; Morash & Alter 2016; Li *et al.* 2018) and natural ecosystems, particularly in estuaries and coastal systems mainly caused by seasonal temperature change, eutrophication, climate change and anthropogenic nutrient loading (Pörtner & Knust 2007; Thomas *et al.* 2007; Li *et al.* 2018). The major energy substrates oxidized under hypoxia may vary with species and hypoxia duration. Peer and Kutty (1981) suggested that acute hypoxia may increase protein oxidation in *O. mossambicus*, critical for protecting acid-base balance and preventing potential acidosis. In contrast, other research suggested that lipid and carbohydrate were the fundamental metabolic energy substrates for fish subjected to acute hypoxia, and that lipid contribution

increased following long-term hypoxia, likely due to carbohydrate interconversion (Li *et al.* 2018; Abdel-Tawwab *et al.* 2019). Understanding metabolic energy substrate use under short-term hypoxia may elucidate whether and how quickly an aquatic ectotherm can regulate physiology and bioenergetics; and the description of the metabolic use under long-term hypoxia may uncover the evolution of hypoxia tolerance (Pörtner & Knust 2007; Richards 2011; Li *et al.* 2018).

3.5.1.4 pH

Water pH is critical for the survival and development of aquatic ectotherms (Whiteley 2011; Bolner *et al.* 2014; Wang *et al.* 2019b). Compared with preindustrial levels, the average surface ocean pH has dropped by 0.1 units, and is estimated to decline an additional 0.3 units by the end of the 21st century (Feely *et al.* 2009; Griffith *et al.* 2012; Boyd *et al.* 2016). The decrease in ocean pH is mainly caused by anthropogenic activities (Whiteley 2011; Griffith *et al.* 2012; Boyd *et al.* 2016) and predicted to have significant deleterious impacts on aquatic ectotherms, for example, slowing calcification in marine calcifiers, causing severe tissue damage, reducing growth and reproduction, disturbing acid-base balance, respiratory metabolism and energy allocation (Pörtner *et al.* 2004; Whiteley 2011; Frommel *et al.* 2012). In coho salmon (*O. kisutch*), when environmental pH declined sharply from 8.0 to 7.1, the routine RQ decreased from 0.62 to 0.21 (Table 3. 2), likely due to CO_2 retention in the body, confirmed by the decreased blood pH (van den Thillart *et al.* 1983). In contrast, Mingliang *et al.* (2011) demonstrated that in the scallop *Chlamys farreri*, when the environmental pH declined sharply from the optimal value of 8.1 to 7.3, the routine RQ remained stable at 0.87 (Table 3. 3), suggesting carbohydrate remained the major metabolic energy substrate and that environmental pH changed within some range might not affect energy metabolism considerably (Mingliang *et al.* 2011; Wang *et al.* 2018). However, for *C. farreri* when the environmental pH decreased

to 7.0 the routine RQ decreased significantly to 0.76 (Table 3. 3), suggesting protein became the primary metabolic energy substrate and that muscle was likely metabolized to compensate for the increased proton stress (Mingliang *et al.* 2011; Whiteley 2011). van den Thillart *et al.* (1983) and Mingliang *et al.* (2011) described the short-term (hours) effects of ocean acidification on stoichiometric bioenergetics in marine animals, however, the long-term (months to years) effects remain unknown. Aquatic ectotherms may have different physiological responses to short and long-term exposure to acidified water (Long *et al.* 2013; Meseck *et al.* 2016). Therefore, it is worthwhile to examine long-term impacts that ocean acidification may have on stoichiometric bioenergetics in wild aquatic ectotherms to understand their adaptations from short-term to evolutionary time scales.

3.5.1.5 Salinity

Salinity has a profound influence on the performance of aquatic ectotherms such as larval development, growth and respiratory metabolism (Boeuf & Payan 2001; Morash & Alter 2016; Rastrick *et al.* 2018). Many euryhaline ectotherms experience large salinity changes during their lifecycle (Boeuf & Payan 2001; Rastrick *et al.* 2018). For example, ectotherms inhabiting coastal estuaries and tide pools (Iverson *et al.* 1989; Boeuf & Payan 2001) and some special species, such as salmon and eel, whose lifecycle involves migration between freshwater and seawater environments (Boeuf & Payan 2001). Euryhaline ectotherms can tolerate wide salinity change mainly by osmoregulation, an energy-requiring process (Jobling 1994; Boeuf & Payan 2001; Li *et al.* 2007). Therefore, despite large tolerance ranges, salinity change may negatively affect growth performance in aquatic ectotherms because more energy is likely to be used for osmoregulation, thus limiting the energy partitioned towards growth (Jobling 1994; Li *et al.* 2017; Pourmozaffar *et al.* 2019). In the Pacific white shrimp (*Litopenaeus vannamei*), the world's most widely cultured crustacean species somewhat due to the broad range of

salinity tolerance (1-50‰) (Li *et al.* 2007), the growth performance decreased when the salinity declined from the optimal level of 20‰ to 3‰, while the routine metabolic rate (measured as the routine oxygen consumption rate) and RQ increased (Table 3. 3) (Li *et al.* 2007). To counter the negative effect on growth, commercial *L. vannamei* feeds have been developed to contain more energy, especially from carbohydrate, to compensate for the extra energy needed for osmoregulation and improve growth performance when reared at low salinity (Li *et al.* 2017).

Although there have been numerous studies examining energy expenditure and oxygen consumption in aquatic ectotherms subjected to acute or gradual salinity change (recent reviews: Ballantyne & Robinson 2010; Li *et al.* 2017; Pourmozaffar *et al.* 2019), metabolic energy substrate use has not been quantified. The quantification of the specific amount of each major energy substrate being oxidized may provide detailed information on the physiological adjustment to salinity change and the time required to complete the adjustment, which is essential for examining adjustment capacities and assessing nutrient requirements to optimize the management for aquaculture species reared at different salinities (Rosas *et al.* 2001; Bardera *et al.* 2019). In addition, the knowledge of stoichiometric bioenergetics is also of ecological importance as it can be used to explain how euryhaline ectotherms metabolize energy substrates to tolerate frequent salinity changes in the wild.

3.5.1.6 Toxicants

Environmental toxicants, especially persistent pollutants such as heavy metals, have become a severe threat to the survival and growth of aquatic ectotherms inhabiting the environment (Sokolova & Lannig 2008; Morris *et al.* 2019) and affect their respiratory and energy metabolism (Correa 1987; Spicer & Weber 1991). The examination of RQ and metabolic energy substrate use may indicate how aquatic ectotherms respond to acute toxicant exposure from bioenergetic perspectives and how they adapt to chronic exposure from evolutionary

perspectives. Vosloo *et al.* (2002) showed that the routine RQ in non-copper-exposed freshwater crab *Potamonautes warreni* was significantly higher compared to the sublethal copper-exposed group (Table 3. 3). Through stoichiometry Vosloo *et al.* (2002) found that non-copper-exposed *P. warreni* oxidized a mix of protein, lipid and carbohydrate, while sublethal copper-exposed *P. warreni* predominantly oxidized lipid (Table 3. 4), indicating increased energy requirements for maintenance and survival following copper exposure (Sokolova & Lannig 2008).

3.5.1.7 Extreme environments

Many aquatic ectotherms live in extreme environments with unique physiological and metabolic adaptations (Wells 1987; DeVries & Cheng 2005; Khripounoff *et al.* 2017). For example, many polar fish such as the Antarctic snailfish *Paraliparis devriesi* have evolved antifreeze proteins to reduce the freezing point of their body fluids below ambient temperature (Macdonald *et al.* 1988; DeVries & Cheng 2005). Although previous studies suggested that Antarctic fish adapt to the cold environment by increasing their metabolic rates (Wohlschlag 1964; Brett & Groves 1979), this metabolic strategy remains questionable (Wells 1987). To better understand how aquatic ectotherms survive in harsh conditions, it is essential to examine their metabolic energy substrate use as it will provide important information on nutritional and biochemical adaptations (Rakusa-Suszczewski *et al.* 1976; Khripounoff *et al.* 2017; Martinez & Torres 2017). Rakusa-Suszczewski *et al.* (1976) demonstrated a low RQ of 0.47 (Table 3. 3) and a high lipid content in the Antarctic copepod, *Rhincalanus gigas,* indicating that lipid oxidation dominated in *R. gigas*. Martinez and Torres (2017) measured oxygen consumption and ammonia excretion concurrently in the Antarctic Silverfish, *Pleuragramma antarctica,* and concluded that the major metabolic energy substrate is a mix of protein and lipid. Khripounoff *et al.* (2017) showed that most of the RQ values in bivalves living in hydrothermal vents were

above 1 (Table 3. 3), likely due to the “group effect” as these bivalves are symbiotic and the RQ measurement is based on a group of individuals in a respiration chamber, rather than a single individual as generally illustrated. Based on the concurrent quantification of oxygen consumption and ammonia excretion, Khripounoff *et al.* (2017) suggested preferential protein oxidation for bivalve metabolic use, however, the specific amount of each major energy substrate being oxidized remains unknown. Further research on this aspect in single individuals is necessary as it may provide a more complete understanding of their dietary composition and bioenergetics, representing ecological adjustments to extreme environments (Martinez & Torres 2017).

3.5.2 Biotic factors

3.5.2.1 Life stage

The change of RQ in aquatic ectotherms at different life stages reflects changes in energy metabolism mainly related to reproduction, indicating a nutritional strategy for population development in the wild (Wright & Wright 1976; Barber & Blake 1985). Based on a single determination of RQ, Wright and Wright (1976) indicated that lipid oxidation predominates in immature and mature amphipod *Gammarus pulex* because the RQ remained as 0.78 at different stages (Table 3. 3). In contrast, the RQ in the bay scallop, *Argopecten irradians concentricus*, varied considerably at different developmental stages (Table 3. 3), indicating different reproductive energy metabolism (Barber & Blake 1985). Based on the simultaneous calculation of RQ and NQ, Barber and Blake (1985) suggested that at the resting stage the bay scallop exclusively oxidizes lipid to provide energy, and that during early oogenesis carbohydrate replaces lipid to become the major metabolic energy substrate. During cytoplasmic growth, the RQ exceeds 1.0, suggesting the interconversion of carbohydrate to lipid in developing ova. When vitellogenesis occurs and spawning commences, the bay scallop exclusively oxidizes

protein (Barber & Blake 1985). Using stoichiometry to determine metabolic energy substrate use at different life stages is of great practical significance for aquaculture. For example, the information may contribute to optimizing broodstock feeds during gonad development to improve egg quality, and to advancing the knowledge of larval nutritional physiology for better manipulation of larval feeds to improve larval survival. In addition, better knowledge of stoichiometric bioenergetics is also helpful to fully understand the annual reproductive strategy in wild aquatic ectotherms.

3.5.2.2 Body weight

Research on body weight in aquatic ectotherms is of considerable commercial, ecological, and physiological significance (Clarke & Johnston 1999; Killen *et al.* 2010; Breck 2014). Body weight reflects body biochemical composition and energy density, associated with growth and feed efficiency (Breck 2014). Individuals of a cohort with higher body weight gain a survival advantage over lower conspecifics via improved starvation resistance, increased escape responses from predators, and better environmental tolerance (Sogard 1997), and indicate efficient energy allocation to growth (Priede 1985). Studies on rainbow trout showed that the routine RQ (Table 3. 2) and metabolic energy substrate use estimated by stoichiometry (Table 3. 4) did not vary with body weight (Lauff & Wood 1996b; McKenzie *et al.* 2007). In contrast, Mayzaud *et al.* (2005) showed that the routine RQ varied with body weights among wild crustaceans (Table 3. 3). Based on the measurements of RQ and unpublished NQ data, Mayzaud *et al.* (2005) suggested that protein oxidation predominates in these crustaceans. Hence, body weight likely has a species-specific impact on the routine RQ with little influence on metabolic energy substrate use in the same species.

3.5.2.3 Behaviour

Aquatic ectotherms possess various behavioural strategies, for example locomotion, to forage, avoid predators, migrate and find optimal physical places to adapt to changeable environments, where considerable energy is required (Brett 1964; Weihs 1973; Palstra & Planas 2011). Most locomotion-related RQ research in aquatic ectotherms investigates metabolic energy substrate use in active-swimming fish such as rainbow trout and tilapia, and there is limited research in species such as abalone and decapod crustaceans where crawling is the predominant locomotor mode (Table 3. 3). In fish, exhaustive swimming is primarily supported by anaerobic metabolism and results in a RQ greater than 1 (Table 3. 2) (Kutty 1968; Milligan 1996). The energy used for early stage exhaustive swimming is provided mainly by glycogen originating from white skeletal muscle (Kutty 1968; Weihs 1973; Milligan 1996). Following exhaustive swimming protein oxidation predominates, essential for maintaining acid-base balance to prevent acidosis (Peer & Kutty 1981). In contrast, fish RQ at rest or during aerobic swimming is usually below 1 (Table 3. 2) while the major energy substrate being oxidized can be controversial (Table 3. 4) (Mohamed 1982; van den Thillart 1986; Lauff & Wood 1997). Some literature suggests that resting and aerobic swimming fish is powered mainly by protein oxidation (Kutty 1972; Sukumaran 1986; van den Thillart 1986), while other research suggests lipid or carbohydrate oxidation can be considerable when the fish become more active but are still under aerobic conditions (Mohamed 1982; Lauff & Wood 1997; Richards *et al.* 2002).

It is worth noting that the interpretation of the major energy substrate being oxidized in one species may vary among studies although the RQ values are similar (Lauff & Wood 1996a). For instance, the RQ in resting and aerobic swimming rainbow trout in van den Thillart (1986) (0.91-0.94) was comparable to Kutty (1968) (0.96) (Table 3. 2). However, van den Thillart (1986) and Kutty (1968) suggested that protein and carbohydrate were the primary metabolic energy substrate, respectively. The use of stoichiometry avoids different interpretations of the

major energy substrate being oxidized in the same/comparable aquatic ectotherms with similar RQ values (Lauff & Wood 1996a; Ferreira *et al.* 2019). Based on the stoichiometric bioenergetic approach, Lauff and Wood (1996a) and Kieffer *et al.* (1998) suggested that lipid was the major metabolic energy substrate in resting rainbow trout, followed by protein, and carbohydrate contribution was minimal. Compared with resting rainbow trout, Lauff and Wood (1996a) and Kieffer *et al.* (1998) suggested that aerobic swimming individuals mainly oxidized lipid and that carbohydrate oxidation increased (Table 3. 4). In terms of protein oxidation, Lauff and Wood (1996a) suggested the contribution declined during aerobic swimming while Kieffer *et al.* (1998) suggested the contribution remained stable. This discrepancy was likely due to different experimental periods: Lauff and Wood (1996a) examined metabolic energy substrate use in a relatively long period of 72 h, while Kieffer *et al.* (1998) in a short period of 4 h.

In aquaculture, the induction of sustained aerobic swimming to fish at optimal speeds provides a non-destructive and economical way to reduce aggressive interactions and improve survival, growth performance, skeletal muscle mass, and flesh quality (Davison 1997; Palstra & Planas 2011; Magnoni *et al.* 2013). The adaptation to sustained aerobic swimming in fish is marked by the increased contractile capacity of red skeletal muscle, accompanied by the elevated metabolic energy expenditure (Richards *et al.* 2002; Palstra & Planas 2011). Previous research using the traditional compositional approach demonstrated that the predominant energy substrates oxidized during sustained aerobic swimming could be one of the three major energy substrates or a mixture (Richards *et al.* 2002; Palstra & Planas 2011; Li *et al.* 2015). Alternatively, stoichiometry is promising to be used to quantify the instantaneous oxidation of each major energy substrate that supports sustained aerobic swimming, crucial for optimizing the nutrient requirements for fish swimming at optimal speeds to increase fish production and achieve more sustainable aquaculture (Ozório 2008; Magnoni *et al.* 2013). On the other hand, fish may become active several hours before feeding, during which the locomotor activity can

be 3-100-fold higher than the normal baseline (Sánchez-Vázquez & Madrid 2001). This increased activity is termed feeding anticipatory behaviour, which has been used as a tool to evaluate a given feeding regime and as a stress indicator (Sánchez-Vázquez & Madrid 2001; Martins *et al.* 2012). For example, a poor feeding regime may cause increased anticipatory behaviour (Martins *et al.* 2012) and acute stress may result in reduced anticipatory behaviour representing poor animal welfare (Folkedal *et al.* 2012). However, no study has examined the stoichiometric bioenergetics relevant to anticipatory behaviour in aquaculture species. In future, it is worthwhile to examine the RQ during food anticipation and the metabolic energy substrate use that supports food anticipation to better understand feeding anticipatory behaviour to promote animal welfare in aquaculture (Folkedal *et al.* 2012; Martins *et al.* 2012). In addition, since swimming speeds in one fish species can vary at different life stages or due to feed availability, and the optimal swimming speeds can vary among species due to different migration strategies, it is necessary to investigate the RQ and detailed metabolic energy substrate use by stoichiometry to better understand their adaptations for efficient swimming (Weihs 1973; Ware 1975; van den Thillart 1986).

3.5.2.4 Nutrition-Nutritional status

Nutritional status of an aquatic ectotherm is defined as the physiological state including feeding, fasting and starvation, and is mainly influenced by nutrient intake and the use of absorbed nutrients (Gingerich *et al.* 2010). Nutritional status may have profound impacts on performance potential in aquatic ectotherms. For example, seasonality of feeding and nutritional status may lead to various strategies for wild aquatic ectotherms to survive starvation, including increased arginine phosphate oxidation and reduced metabolic rates (Jobling 1980; Hervant *et al.* 1999; McCue 2010). In addition, cycles of fasting and refeeding in cultured animals may result in compensatory growth partly due to hyperphagia (Hornick *et*

al. 2000; Ali *et al.* 2003; Mattila *et al.* 2009). There have been studies investigating the effects of fasting or feeding on stoichiometric bioenergetics in aquatic ectotherms (Clifford & Brick 1983; Rus *et al.* 2000; Ferreira *et al.* 2019), while the effects of cycles of fasting and refeeding remain unknown.

Rus *et al.* (2000) showed that the post-prandial RQ estimated over 24 h was 0.91 in goldfish (*Carassius auratus*) (Table 3. 2), fed a commercial feed (protein, lipid and carbohydrate compositions were 45%, 21% and 27%, respectively), and that carbohydrate oxidation dominated, followed by lipid, while protein oxidation was minimal (Table 3. 4). These findings indicate most of the ingested dietary protein in goldfish is likely to be used for improving protein synthesis retention efficiency and therefore growth, rather than for oxidation to provide metabolic energy (Gelineau *et al.* 1998; Carter & Houlihan 2001; Phumee *et al.* 2009). McKenzie *et al.* (2007) showed that the RQ at the early post-prandial stage in rainbow trout fed a commercial feed (protein, lipid and carbohydrate compositions were 48%, 23% and 11%, respectively) was comparable to individuals following starvation (Kaushik *et al.* 1989; Lauff & Wood 1996b) (Table 3. 2). Through stoichiometry McKenzie *et al.* (2007) found that rainbow trout at the early post-prandial stage mainly oxidized lipid, followed by protein, and carbohydrate was the minimum metabolic energy substrate (Table 3. 4). Following starvation, lipid remained the major metabolic energy substrate for rainbow trout and carbohydrate oxidation increased, while protein oxidation decreased (Lauff & Wood 1996b; McKenzie *et al.* 2007) (Table 3. 4).

The complexity of how ingested nutrients are used for metabolism and growth means it is impossible to describe metabolic energy substrate use from changes in the composition of growing animals in aquaculture. However, under starvation, animal growth halts and animals oxidize body composition to only maintain metabolic requirements, allowing the estimation of metabolic energy substate use using the traditional compositional approach and the

stoichiometric bioenergetic approach. Lauff and Wood (1996b) compared the two approaches using 15-day starved rainbow trout. The compositional estimate showed that the fractional contributions of protein, lipid and carbohydrate to metabolic use were 67%, 31% and 2%, respectively. In contrast, the stoichiometric estimate showed that the fractional contributions were 23%, 50% and 27%, respectively (Table 3. 4). The former reveals the net loss of energy substrates while the latter reveals the instantaneous oxidation (Lauff & Wood 1996b; Kieffer *et al.* 1998). Protein contribution determined by the compositional approach was likely overestimated as much of protein was assumed to be converted to lipid and carbohydrate before being oxidized or excreted without oxidation (Lauff & Wood 1996b). In addition, the compositional approach introduced greater experimental error compared to the stoichiometric bioenergetic approach (Brett 1995; Lauff & Wood 1996b; Kieffer *et al.* 1998). The compositional estimation was based on two different groups of fish at the beginning and end of starvation, which may introduce considerable variability as natural variation between individuals is inevitable (Cui & Liu 1990; Carter & Brafield 1991; Lauff & Wood 1996b). In contrast, the stoichiometric estimation was based on the same individuals (Lauff & Wood 1996b; Lauff & Wood 1996a). Furthermore, fish body weight measured by blot-drying was considered inaccurate by the authors due to their small size (Lauff & Wood 1996b). Consequently, Lauff and Wood (1996b) concluded that the stoichiometric bioenergetic approach is suitable for short-term physiological studies, while the compositional approach is preferred for long-term eco-physiological studies and large numbers of individuals are required to minimize variability.

Compared with rainbow trout, Ferreira *et al.* (2019) showed that in zebrafish (*Danio rerio*), fed a commercial feed (protein, lipid and carbohydrate compositions were 51%, 7% and 21%, respectively), the RQ at the early post-prandial stage was also comparable to starved individuals (Table 3. 2), and that the major metabolic energy substrate also changed following

starvation (Table 3. 4). At the early post-prandial stage, carbohydrate was the major energy substrate for zebrafish, and protein and lipid had similar secondary contributions. After 2-day starvation lipid and carbohydrate oxidation dominated, while protein oxidation decreased (Ferreira et al. 2019) (Table 3. 4), in accordance with other fish species (Wood 2001). Therefore, the RQ and major metabolic energy substrates likely vary among fish species and nutritional status.

Early crustacean literature showed an increase in RQ when nutritional status changed from starvation to feeding, indicating the increased carbohydrate oxidation (Wolvekamp & Waterman 1960; Schafer 1968). Clifford and Brick (1983) demonstrated that following starvation, protein and lipid oxidation in *Macrobrachium rosenbergii* increased and that the routine RQ (Table 3. 3) and carbohydrate oxidation declined, however, carbohydrate oxidation still dominated (Table 3. 4). Compared with *M. rosenbergii*, the routine RQ in 10-day starved spiny lobster *Sagmariasus verreauxi* was higher than the mean post-prandial RQ estimated over 36.5 h in individuals fed frozen squid *Nototodarus sloanii* (Wang *et al.* 2021) (Table 3. 3). In terms of metabolic energy substrate use, lipid oxidation dominated in starved *S. verreauxi* and protein oxidation dominated in fed *S. verreauxi*, while lipid and carbohydrate oxidation also provided non-neglectable energy at different post-prandial stages (Wang *et al.* 2021) (Table 3. 4). These findings suggest not only high-quality protein, but appropriate proportions of non-protein energy-yielding nutrients (lipid and carbohydrate) are also vital to the enhancement of dietary protein-sparing effects for growth (Hewitt & Irving 1990; Carter & Houlihan 2001).

Stoichiometry provides a powerful tool to describe the oxidation of each major energy substrate in response to different nutritional status and to quantify the optimal nutrient composition to enhance aquaculture production. In addition, the quantification of RQ and metabolic energy substrate use in different durations of fasting and refeeding may shed light

on the dynamics of compensatory growth and has great potential to define the optimal refeeding duration to develop an efficient compensatory growth strategy for better aquaculture management.

3.5.2.5 Nutrition-Feeding regimes

Feeding regimes include aspects of feed presentation including ration, time and frequency of feeding and are critical to the success of aquaculture (Gelineau *et al.* 1998; Carter *et al.* 2001; Hauler *et al.* 2007). Optimum feeding regimes should minimize feed wastage and enhance growth performance and feed utilization efficiency (Carter *et al.* 1994a; Imsland *et al.* 2003; Mente 2010). There have been studies using stoichiometry to justify feeding regimes in fish and crustaceans (Clifford & Brick 1979; Forsberg 1997; Gelineau *et al.* 1998). Forsberg (1997) showed that with the reduction of feeding rations in Atlantic salmon (*Salmo salar*), the post-prandial RQ estimated over 24 h decreased (Table 3. 2), and that lipid contribution to metabolic use increased while protein oxidation decreased with minimal carbohydrate oxidation (Table 3. 4). In rainbow trout fed at dawn (natural feeding time), the post-prandial RQ estimated over 24 h was low (Table 3. 2) and lipid was the major metabolic energy substrate, followed by protein with minimal carbohydrate contribution (Table 3. 4) (Gelineau *et al.* 1998). In contrast, in rainbow trout fed at midnight (the least unfavourable feeding time), the post-prandial RQ was high (Table 3. 2) and protein was the main metabolic energy substrate, followed by lipid, and carbohydrate contribution was minimal (Table 3. 4) (Gelineau *et al.* 1998). The findings from Gelineau *et al.* (1998) suggest that fish fed at the natural feeding time may spare absorbed dietary protein more efficiently, compared with individuals fed out of the natural feeding time (Gelineau *et al.* 1998; Verbeeten *et al.* 1999).

Clifford and Brick (1979) examined the RQ and metabolic energy substrate use in *M. rosenbergii* fed isoenergetic feeds (18 kJ g^{-1} dry matter) with different protein levels (15-40%)

and isoenergetic and isonitrogenous feeds (18 kJ g^{-1} dry matter, 25% protein) with different lipid to carbohydrate ratios. In *M. rosenbergii* fed isoenergetic and isonitrogenous feeds, the post-prandial RQ estimated over 8 h elevated proportionally with lipid to carbohydrate ratios (Table 3. 3), and protein and lipid oxidation decreased while carbohydrate oxidation increased (Table 3. 4), indicating that increased dietary lipid to carbohydrate ratios can result in improved oxidation of non-protein energy-yielding nutrients. Clifford and Brick (1979) also suggested dietary protein above 30% likely exceeded the optimum level as protein oxidization increased, and that 25% protein with a lipid to carbohydrate ratio of 1:4 was likely to result in the most cost-effective feed (Table 3. 4). Excessive dietary protein may stimulate both protein degradation and protein synthesis, leading to low protein synthesis retention efficiency in aquatic ectotherms (Carter & Houlihan 2001; Aragão *et al.* 2004; Carter & Mente 2014). In contrast, cost-effective aquafeeds containing suitable non-protein energy-yielding nutrients may improve the dietary protein-sparing effect so that assimilated protein enhances protein synthesis retention efficiency (Beamish & Medland 1986; De Silva *et al.* 1991; Carter *et al.* 1993). Clifford and Brick (1979) also found an unusual high post-prandial RQ in *M. rosenbergii* fed a low-protein feed (15% protein) (Table 3. 3), likely due to endogenous lipogenesis, and suggested that 15% protein was unlikely to produce an optimal feed for *M. rosenbergii* regardless of the lipid to carbohydrate ratio (Sargent 1976; Clifford & Brick 1979).

The current examinations on the relationship between feeding regimes and RQ and metabolic energy substrate use in aquaculture animals mainly concern integrated post-prandial metabolism (Clifford & Brick 1979; Forsberg 1997; Gelineau *et al.* 1998). In future, it would be beneficial to investigate RQ and metabolic energy substrate use on a fine level, for example, on an hourly level within a daily cycle to gain a more complete and detailed picture of the feeding plasticity and nutritional physiology to develop more efficient feeding regimes and cost-effective aquafeeds (Knights 1985; Jobling *et al.* 1995; Tacon *et al.* 2002).

3.5.2.6 Nutrition-Feed ingredients

Feed ingredients and how well they contribute to a nutritionally balanced feed are one of the crucial factors affecting growth and development of aquaculture species (Jobling 1994; Glencross *et al.* 2007; National Research Council 2011). There has been a considerable amount of research relevant to feed ingredients including replacement of dietary fishmeal with plant or insect meal to reduce the use of marine protein sources (Carter & Hauler 2000; Han *et al.* 2018; Rumbos *et al.* 2019), the inclusion of highly digestible and inexpensive carbohydrates into the feeds of carnivorous fish to develop cost-effective aquafeeds (Kaushik *et al.* 1989; Médale *et al.* 1999; Kamalam *et al.* 2017), supplementation of exogenous ingredients to enhance nutrient digestibility of plant-based feed and immunity and disease resistance (Li & Gatlin III 2006; Castillo & Gatlin III 2015), and the development of criteria to assess the applicability of specific ingredients (Glencross *et al.* 2007). In contrast, few studies have elucidated the relationship between feed ingredients and RQ and metabolic energy substrate use in aquaculture species (Kaushik *et al.* 1989; Dersjant-Li 2000; Ozório *et al.* 2001). Ozório *et al.* (2001) demonstrated that appropriate L-carnitine supplementation can improve the protein-sparing effect of dietary lipid for growth in African catfish (*Clarias gariepinus*), and lead to a drop of the post-prandial RQ estimated over 24 h (Table 3. 2). Dersjant-Li (2000) showed that high dietary cation-anion difference (DCAD) can improve the growth performance in African catfish, low energy expenditure to maintain acid-base balance and high post-prandial RQ estimated over 24 h (Table 3. 2). In contrast, low DCAD feeds can lead to reduced *C. gariepinus* growth performance, increased maintenance costs for homeostasis and reduced post-prandial RQ (Table 3. 2) (Dersjant-Li 2000).

In carnivorous rainbow trout fed feeds containing different carbohydrate levels, the RQ remained stable (Table 3. 2) while the metabolic energy substrate use differed considerably

(Table 3. 4) (Kaushik *et al.* 1989; McKenzie *et al.* 2007). The main metabolic energy substrate was carbohydrate when rainbow trout were fed high levels of carbohydrate, and shifted to protein/lipid when the dietary carbohydrate was reduced (Kaushik *et al.* 1989; McKenzie *et al.* 2007) (Table 3. 4). A study based on long-term feeding and short-term stoichiometry demonstrated that the inclusion of highly digestible carbohydrate in rainbow trout feeds can enhance the availability of dietary energy and does not adversely affect growth (Kaushik *et al.* 1989). Therefore, stoichiometry has potential as a tool to assess the availability of a feed ingredient in aquaculture.

3.5.2.7 Physiology

Cortisol and growth hormones regulate nutrient metabolism in aquatic ectotherms (Perez-Sanchez & Le Bail 1999; De Boeck *et al.* 2001). In fish, the increase in blood cortisol levels is usually followed by hyperglycaemia (De Boeck *et al.* 2001). Cortisol elevation may reduce fish appetite and result in reduced growth performance (Gregory & Wood 1999; De Boeck *et al.* 2001). De Boeck *et al.* (2001) demonstrated that the injection of cortisol elevated the RQ (Table 3. 2) and body carbohydrate content in 6-h starved rainbow trout, and that body protein content remained stable while body lipid content dropped. These findings suggest that lipid is likely to be the major metabolic energy substrate in rainbow trout when the cortisol level is increased (De Boeck *et al.* 2001). Growth hormones may enhance the metabolic use of digestible carbohydrate to improve growth performance (Higgs *et al.* 2009). de Celis *et al.* (2003) showed that the injection of growth hormones in European sea bass (*Dicentrarchus labrax*) led to a significant reduction in the routine RQ (Table 3. 2) and an increase in lipid oxidation. As a result, *D. labrax* may spare absorbed protein for growth (de Celis *et al.* 2003). The present examination of the effects of hormones on metabolic energy substrate use in aquatic ectotherms was based on the traditional compositional approach (De Boeck *et al.* 2001;

de Celis *et al.* 2003). Alternatively, stoichiometry may provide precise descriptions of the physiological mechanisms of hormone action to optimize the dose used in aquaculture.

3.5.2.8 Diseases

A variety of infectious diseases impact on aquaculture and can cause considerable reduction in production and profitability (Scarfe *et al.* 2008; Georgiades *et al.* 2016). Implementing biosecurity is important to minimize the risk of disease transmission, and better understanding nutritional physiology of cultured animals is key to effective biosecurity programs and disease management (Scarfe *et al.* 2008; Georgiades *et al.* 2016). Using the traditional compositional approach, Barber *et al.* (1988) demonstrated that in the oyster *Crassostrea virginica* infected with *Haplosporidium nelson*, energy consumption and glycogen oxidation increased significantly compared to uninfected oysters. Similar results were found in the scallop *Chlamys farreri* infected with *Vibrio anguillarum* (Wang *et al.* 2012b). It also has been suggested that feed quality has substantial impacts on disease resistance in aquaculture animals (Pruder 2004; Oliva-Teles 2012). Hence, it will be of great interest to examine RQ and metabolic energy substrate use by stoichiometry to understand how disease affects nutritional physiology and whether adjusting feed formulations provides a promising approach to assist infected animals in defending against pathogens.

Table 3. 2 Respiratory quotient (RQ) studies in fish (1968-2019) arranged by species, followed by abiotic and biotic variables

Species	Variable	Body weight (BW)	Nutritional status	Technique for CO_2 measurement	Temperature (°C)	RQ	References
Rhinomugil corsula	Temperature	41.2 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	30	0.91	Kutty and Mohamed (1975)
Rhinomugil corsula	Temperature	41.2 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	35	0.95	Kutty and Mohamed (1975)
Rhinomugil corsula	Temperature	41.2 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	30	0.93	Mohamed and Kutty (1986)
Rhinomugil corsula	Temperature	41.2 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	35	0.92	Mohamed and Kutty (1986)

Rhinomugil corsula	Aerial exposure	41.2 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	30	1.28	Mohamed and Kutty (1986)
Rhinomugil corsula	Aerial exposure	41.2 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	35	1.34	Mohamed and Kutty (1986)
Oreochromis mossambicus	Temperature	63.4 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	30	0.86	Mohamed (1982)
Oreochromis mossambicus	Temperature	64.1 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	35	0.87	Mohamed (1982)
Oreochromis mossambicus	DO	20 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	30	0.76 (DO 2.87 mg L^{-1}) -19.95	Kutty *et al.* (1971)

						(DO 0.48 mg L^{-1})	
Oreochromis mossambicus	DO	3.3-17 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	30	1.03 (DO > 2 mg L^{-1}) - $+\infty$ (DO < 1 mg L^{-1})	Kutty (1972)
Oreochromis mossambicus	DO	25.0-41.1 g	Unfed (After 1-day starvation)	Titrimetric Maros-Schulek technique	30, 35	0.5-2.5	Peer and Kutty (1981)
Oreochromis mossambicus	Forced swimming	6.5 g	Unfed (After 36-h starvation)	Titrimetric Maros-Schulek technique	30	1.2	Kutty (1972)
Oreochromis mossambicus	Forced swimming	33.9-53.1 g	Unfed (After 1-day starvation)	Titrimetric Maros-Schulek technique	30	0.6-2.0	Sukumaran (1986)

Oreochromis niloticus	Temperature	8-10 g	Unfed (After 4-day starvation)	Gas chromatography	15	0.93	Alsop *et al.* (1999)
Oreochromis niloticus	Temperature	8-10 g	Unfed (After 2-day starvation)	Gas chromatography	30	0.86	Alsop *et al.* (1999)
Intertidal fish†	Aerial exposure	1.79-69.05 g	Unfed	Infrared spectroscopy	15	0.69-1.05	Martin (1993)
Intertidal fish‡	Aerial exposure	4.49-175.43 g	Unfed	Infrared spectroscopy	23	0.89-0.98	Martin (1993)
Carassius auratus	DO < 50% air saturation	97 .9 g	Unfed (After 36-h starvation)	Volumetric Van Slyke technique	20	1.94	Kutty (1968)
Carassius auratus	DO > 50% air saturation	97 .9 g	Unfed	Volumetric Van Slyke technique	20	1.02	Kutty (1968)

			(After 36-h starvation)				
Carassius auratus	Forced swimming	111 g	Unfed (After 36-h starvation)	Volumetric Van Slyke technique	20	0.86-1.33	Kutty (1968)
Carassius auratus	During 1-day post-feeding	88 g	Fed	Conductometry	21	0.91	Rus *et al.* (2000)
Danio rerio	After 2-day starvation	0.6 g	Unfed	Infrared spectroscopy	21	0.89	Ferreira *et al.* (2019)
Danio rerio	During 10-h post-feeding	0.6 g	Fed	Infrared spectroscopy	21	0.94	Ferreira *et al.* (2019)
Clarias gariepinus	Dietary CAD	42.1 g	During 1-day post-feeding	Colorimetry	27	0.88 (CAD 146 mEq kg^{-1} DM) - 1.14	Dersjant-Li (2000)

						(CAD 713 mEq kg^{-1} DM)	
Clarias gariepinus	Dietary composition	77.5 g	During 1-day post-feeding	Colorimetry	27	0.83-1.0	Ozório *et al.* (2001)
Oncorhynchus mykiss	Temperature	16 g	Unfed (After 5-day starvation)	Gas chromatography	5	0.85	Kieffer *et al.* (1998)
Oncorhynchus mykiss	Temperature	16 g	Unfed (After 3-day starvation)	Gas chromatography	15	0.85	Kieffer *et al.* (1998)
Oncorhynchus mykiss	DO < 50% air saturation	80 g	Unfed (After 36-h starvation)	Volumetric Van Slyke technique	15	1.42	Kutty (1968)

Oncorhynchus mykiss	DO > 50% air saturation	80 g	Unfed (After 36-h starvation)	Volumetric Van Slyke technique	15	0.96	Kutty (1968)
Oncorhynchus mykiss	BW	77-182 g	Unfed (After 1-day starvation)	Infrared spectroscopy	14	0.83	McKenzie *et al.* (2007)
Oncorhynchus mykiss	BW	77-182 g	Fed (During 2-3-h post-feeding)	Infrared spectroscopy	14	0.82	McKenzie *et al.* (2007)
Oncorhynchus mykiss	Forced swimming	80 g	Unfed (After 36-h starvation)	Volumetric Van Slyke technique	15	0.71-1.09	Kutty (1968)
Oncorhynchus mykiss	Resting	330 g	Unfed (After 2-day starvation)	Conductometry	10	0.91	van den Thillart (1986)

Oncorhynchus mykiss	Swimming at 80% U_{crit}	330 g	Unfed (After 2-day starvation)	Conductometry	10	0.94	van den Thillart (1986)
Oncorhynchus mykiss	Swimming at 55-80% U_{crit}	17.5 g	Unfed (After 2-day starvation)	Gas chromatography	15	0.9	Lauff and Wood (1996a)
Oncorhynchus mykiss	Swimming at 55% U_{crit}	77.5 g	Unfed (After 2-day starvation)	Gas chromatography	15	0.85	Lauff and Wood (1997)
Oncorhynchus mykiss	During 1-15-day starvation	2-4 g	Unfed	Gas chromatography	15	0.85	Lauff and Wood (1996b)
Oncorhynchus mykiss	Feed ingredients (raw starch)	111-131 g	Unfed or during 8-h post-feeding	Conductometry	18	0.89 (After 5-day starvation	Kaushik *et al.* (1989)

						or during 8-h post-feeding)	
Oncorhynchus mykiss	Feed ingredients (extruded corn starch)	111-131 g	Unfed or during 8-h post-feeding	Conductometry	18	0.93 (After 5-day starvation), 0.87 (During 8-h post-feeding)	Kaushik *et al.* (1989)
Oncorhynchus mykiss	Feed ingredients (extruded whole corn)	111-131 g	Unfed or during 8-h post-feeding	Conductometry	18	0.90 (After 5-day starvation), 0.83	Kaushik *et al.* (1989)

						(During 8-h post-feeding)	
Oncorhynchus mykiss	Feed ingredients (extruded wheat starch)	111-131 g	Unfed or during 8-h post-feeding	Conductometry	18	0.90 (After 5-day starvation), 0.87 (During 8-h post-feeding)	Kaushik *et al.* (1989)
Oncorhynchus mykiss	Feed ingredients (extruded whole wheat)	111-131 g	Unfed or during 8-h post-feeding	Conductometry	18	0.87 (After 5-day starvation or during	Kaushik *et al.* (1989)

						8-h post-feeding)	
Oncorhynchus mykiss	Dawn feeding	78 g	During 1-day post-feeding	Conductometry	15	0.85	Gelineau *et al.* (1998)
Oncorhynchus mykiss	Midnight feeding	77 g	During 1-day post-feeding	Conductometry	15	0.87	Gelineau *et al.* (1998)
Oncorhynchus mykiss	Non-treated (Control)	14.6 g	Unfed (After 6-h starvation)	Gas chromatography	14	0.83	De Boeck *et al.* (2001)
Oncorhynchus mykiss	Coconut oil injection (10 μL g^{-1} BW)	14.6 g	Unfed (After 6-h starvation)	Gas chromatography	14	0.65-0.90	De Boeck *et al.* (2001)
Oncorhynchus mykiss	Cortisol-coconut oil	14.6 g	Unfed	Gas chromatography	14	0.90-1.4	De Boeck *et al.* (2001)

	injection (0.25 mg g^{-1} BW)		(After 6-h starvation)				
Salmo salar	After 10-day starvation	2 kg	Unfed	Potentiometry	8.5	0.7-0.74	Forsberg (1997)
Salmo salar	Feeding ration (0.15% BW day^{-1})	2 kg	During 1-day post-feeding	Potentiometry	8.8	0.75	Forsberg (1997)
Salmo salar	Feeding ration (0.3% BW day^{-1})	2 kg	During 1-day post-feeding	Potentiometry	8.5	0.78	Forsberg (1997)
Salmo salar	Feeding ration (0.6% BW day^{-1})	2 kg	During 1-day post-feeding	Potentiometry	8.5	0.8	Forsberg (1997)

Dicentrarchus labrax	Non-treated (Control)	200 g	Unfed (After 1-day starvation)	Conductometry	22	0.86	de Celis *et al.* (2003)
Dicentrarchus labrax	Growth hormone injection (0.1 μg g^{-1} BW)	200 g	Unfed (After 1-day starvation)	Conductometry	22	0.77-0.86	de Celis *et al.* (2003)
Oncorhynchus kisutch	pH	600 g	Unfed (After 2-day starvation)	Conductometry	13	0.21 (pH 7.1) - 0.62 (pH 7.95)	van den Thillart *et al.* (1983)

†Intertidal fish include *Anoplarchus purpurescens*, *Clinocottus globiceps*, *Girella nigricans*, *Gobiesox maeandricus*, *Leptocottus armatus*, *Oligocottus snyderi*, *Porichthys notatus*, *Xererpes fucorum*, *Xiphister atropurpureus*, and *X. mucosus*.

‡Intertidal fish include *Cebidichthys violaceus*, *Clinocottus recalvus*, and *Oligocottus snyderi*.

DO, dissolved oxygen; CAD, cation-anion difference; mEq kg^{-1} DM, milliequivalents per kilogram of dry matter; U_{crit}, critical swimming speed.

Table 3. 3 Respiratory quotient (RQ) studies in aquatic invertebrates (1969-2020) arranged in a taxonomy, followed by abiotic and biotic variables

Species	Variable	Body weight (BW)	Nutritional status	Technique for CO_2 measurement	Temperature (°C)	RQ	References
Echinodermata							
Ophiuroidea							
Ophiothrix fragilis	Temperature	1.01-2.35 g (ash-free DM)	Unfed (After 3 or 4-day starvation)	Potentiometry	7.7-15.4	0.55 (7.7 °C) - 0.78 (15.4 °C)	Migné and Davoult (1997)
Arthropoda							

Crustacea							
Leptodora kindtii	Temperature	NA	Unfed (After at least 3-h starvation)	Manometry	5-30	0.57 (5 °C) - 0.95 (30 °C)	Moshiri *et al.* (1969)
Diaptomus gracilis	Temperature	NA	Unfed (After 15-24-h starvation)	Potentiometry	12-20	0.74 (12 °C) - 1.25 (20 °C)	Kibby (1971)
Rhincalanus gigas	Extreme environment	97 mg	NA	Acid-base titration	-1.8	0.47	Rakusa-Suszczewski *et al.* (1976)
Copepods†	BW	0.004-1.58 mg (DM)	Unfed (After 10-15-h starvation)	Coulometry	13.9-17.8	0.61-0.95	Mayzaud *et al.* (2005)

Gammarus pulex	Reproduction	64.7-119.7 mg	NA	Manometry	10	0.72-0.86	Wright and Wright (1976)
Meganyctiphanes norvegica	BW	28.4-38.6 mg (DM)	Unfed (After 10-15-h starvation)	Coulometry	12.5-17.8	1.29-1.62	Mayzaud *et al.* (2005)
Potamonautes warreni	Non-copper-exposure (Control)	NA	Unfed	Infrared spectroscopy	23-25	0.8	Vosloo *et al.* (2002)
Potamonautes warreni	Copper exposure (1.0 mg Cu^{2+} L^{-1})	NA	Unfed	Infrared spectroscopy	23-25	0.7	Vosloo *et al.* (2002)
Litopenaeus vannamei	Salinity	0.69 g	Unfed (After 1-day starvation)	Acid-base titration	29	0.7 (Salinity 17-35‰) - 1.5	Li *et al.* (2007)

						(Salinity 3‰)	
Sergestes sp. and *Systellaspis debilis*	BW	48.0-68.8 mg (DM)	Unfed (After 10-15-h starvation)	Coulometry	12.5-13.9	1.4	Mayzaud *et al.* (2005)
Calunectes sapidus	After 2-day starvation	NA	Unfed	Conductometry	22	2.17	Henry and Cameron (1983)
Macrobrachium rosenbergii	After 4-day starvation	0.68 g (DM)	Unfed	Acid-base titration	29.5	0.93	Clifford and Brick (1983)
Macrobrachium rosenbergii	After 8-day starvation	0.68 g (DM)	Unfed	Acid-base titration	29.5	0.88	Clifford and Brick (1983)
Macrobrachium rosenbergii	After 4-day starvation	0.68 g (DM)	Unfed	Acid-base titration	29.5	0.93	Clifford and Brick (1979)

Macrobrachium rosenbergii	Nutrient composition (15% P, L : C = 1:2)	0.68 g (DM)	During 8-h post-feeding	Acid-base titration	29.5	1.2	Clifford and Brick (1979)
Macrobrachium rosenbergii	Nutrient composition (25% P, L : C = 1:1, 1:2, 1:3, 1:4)	0.68 g (DM)	During 8-h post-feeding	Acid-base titration	29.5	0.82 (25% P, L : C = 1:1)-0.94 (25% P, L : C = 1:4)	Clifford and Brick (1979)
Macrobrachium rosenbergii	Nutrient composition (35% P, L : C = 1:2)	0.68 g (DM)	During 8-h post-feeding	Acid-base titration	29.5	0.87	Clifford and Brick (1979)
Jasus edwardsii	Aerial exposure	400-700 g	Unfed (After 1-day starvation)	Conductometry	5	1.4 (10-h exposure)	Morris and Oliver (1999)

						-1.8 (5-h exposure)	
Jasus edwardsii	Aerial exposure	400-700 g	Unfed (After 1-day starvation)	Conductometry	18	3.9 (10-h exposure) -5.6 (5-h exposure)	Morris and Oliver (1999)
Jasus edwardsii	Aerial exposure	776 g	Unfed (After 3-day starvation)	Infrared spectroscopy	3.7	0.61 (0-h exposure) - 0.76 (1-day exposure)	Forgan *et al.* (2014)
Jasus edwardsii	Aerial exposure	739 g	Unfed (After 3-day starvation)	Infrared spectroscopy	7.5	0.79 (0-h exposure) -1.01 (1-	Forgan *et al.* (2014)

						day exposure)	
Jasus edwardsii	Aerial exposure	738 g	Unfed (After 3-day starvation)	Infrared spectroscopy	15	0.71 (0-h exposure) - 1.02 (1-day exposure)	Forgan *et al.* (2014)
Sagmariasus verreauxi	After 10-day starvation	350 g	Unfed	Infrared spectroscopy	21	1.2	Wang *et al.* (2021)
Sagmariasus verreauxi	After 2-day starvation	350 g	Unfed	Infrared spectroscopy	21	0.8-1.4	Wang *et al.* (2021)
Sagmariasus verreauxi	Feeding	350 g	During 36.5-h post-feeding	Infrared spectroscopy	21	0.8	Wang *et al.* (2021)

Insecta							
Aeshna spp. *and Anax junius*	BW	2.17 mg-1.46 g	Unfed	Infrared spectroscopy	22	0.73	Harter *et al.* (2017)
Mollusca							
Bivalvia							
Chlamys farreri	pH	21.78 g	Unfed	Infrared spectroscopy	16	0.76 (pH 6.96) - 0.87 (pH 8.08)	Mingliang *et al.* (2011)
Bivalves‡	Extreme environment	0.03-6.3 g (DM)	NA	Infrared spectroscopy	2-8	0.01-5.3	Khripounoff *et al.* (2017)
Argopecten irradians concentricus	Reproduction	0.97-2.03 g (DM)	Unfed (Overnight starvation)	Potentiometry	21.5-31.7	0.25 (After spawning)	Barber and Blake (1985)

						-1.31 (Early gametogenesis)	
Cephalopoda							
Nautilus macromphalus	BW	290-670 g	Unfed	Potentiometry	17-18	0.42 (290 g) - 1.07 (610 g)	Boucher-Rodoni and Boucher (1993)
Gastropoda							
Crepidula fornicata	Temperature and BW	0.02-0.33 g (ash-free DM)	NA	Infrared spectroscopy	8.5-18.7	0.7-0.8	Martin *et al.* (2006)
Haliotis roei	Starvation	125 g	Unfed (After at least 6-h starvation)	Conductometry	Ambient temperature	0.78	Hatcher (1989)

Thecosomes§	BW	1.3-333 mg (DM)	Unfed (After 10-15-h starvation)	Coulometry	13.9-17.8	0.80 (3 mg DM) - 1.31 (1.3 mg DM)	Mayzaud *et al.* (2005)
Polyplacophora							
Plaxiphora albida	Starvation	248 g	Unfed (After at least 6-h starvation)	Conductometry	Ambient temperature	0.68	Hatcher (1989)
Chordata							
Thaliacea							
Pegea sp. and *Thalia* sp.	BW	NA	Unfed (After 10-15-h starvation)	Coulometry	13.9	0.78-1.07	Mayzaud *et al.* (2005)
Ascidiacea							
Herdmania momus	Starvation	302 g	Unfed	Conductometry	Ambient temperature	0.78	Hatcher (1989)

			(After at least 6-h starvation)					
Bryozoa								
Gymnolaemata								
Triphyllozoon moniliferum	Starvation	47 g	Unfed (After at least 6-h starvation)	Conductometry	Ambient temperature	0.75	Hatcher (1989)	

†Copepods include *Acartia clausi*, *Calanus* sp., *Calanus gracilis*, *Candacia* spp., *Euchaeta* spp., *Euchirella rostrate*, *Oncaea* sp., and *Pleuromamma gracilis*.

‡Bivalves include *Abyssogena southwardae*, *Bathymodiolus azoricus*, *B. aff. boomerang*, *Branchipolynoe seepensis*, and *Christineconcha regab*.

§Thecosomes include *Cavolinia inflexa*, *Cymbulia peronii*, *Diacria trispinosa*, *Euclio pyramidata*, and *Limacina helicoides*.

DM, dry matter; NA, not available; P, protein; L, lipid; C, carbohydrate.

Table 3. 4 Studies of metabolic energy substrate use in aquatic ectotherms calculated by stoichiometry (1975-2020) arranged in a taxonomy, followed by abiotic and biotic variables

Species	Variable	Body weight (BW)	Nutritional status	Temperature (°C)	Metabolic energy substrate use			References
					P (%)	L (%)	C (%)	
Chordata								
Actinopterygii								
Rhinomugil corsula	Temperature	41.2 g	Unfed (After 36-h starvation)	30	14	42	44	Kutty and Mohamed (1975)
Rhinomugil corsula	Temperature	41.2 g	Unfed (After 36-h starvation)	35	15	30	55	Kutty and Mohamed (1975)
Rhinomugil corsula	Temperature	41.2 g	Unfed	30	48	18	34	Mohamed and Kutty (1986)

			(After 36-h starvation)					
Rhinomugil corsula	Temperature	41.2 g	Unfed (After 36-h starvation)	35	37	23	40	Mohamed and Kutty (1986)
Carassius auratus	Feeding	88 g	During 1-day post-feeding	21	14	27	59	Rus *et al.* (2000)
Danio rerio	After 2-day starvation	0.6 g	Unfed	21	48	40	12	Ferreira *et al.* (2019)
Danio rerio	During 10-h post-feeding	0.6 g	Fed	21	15	15	70	Ferreira *et al.* (2019)
Oreochromis mossambicus	Temperature	63.4 g	Unfed (After 36-h starvation)	30	37	43	20	Mohamed (1982)
Oreochromis mossambicus	Temperature	64.1 g	Unfed	35	39	39	22	Mohamed (1982)

			(After 36-h starvation)					
Oreochromis niloticus	Resting	8-10 g	Unfed	15	16	21	63	Alsop *et al.* (1999)
			(After 4-day starvation)					
Oreochromis niloticus	Resting	8-10 g	Unfed	30	42	31	27	Alsop *et al.* (1999)
			(After 2-day starvation)					
Oncorhynchus mykiss	BW	77-182 g	Unfed	14	15-17	53-60	23-32	McKenzie *et al.* (2007)
			(After 1-day starvation)					
Oncorhynchus mykiss	BW	77-182 g	Fed	14	35-44	49-57	8-9	McKenzie *et al.* (2007)
			(During 2-3-h post-feeding)					
Oncorhynchus mykiss	Resting	17.5 g	Unfed	15	30-45	43-50	5-23	Lauff and Wood (1996a)

			(After 2-day starvation)					
Oncorhynchus mykiss	Low-speed swimming (55% U_{crit})	17.5 g	Unfed (After 2-day starvation)	15	20-36	43-50	8-38	Lauff and Wood (1996a)
Oncorhynchus mykiss	High-speed swimming (80% U_{crit})	17.5 g	Unfed (After 2-day starvation)	15	20	43-54	29-38	Lauff and Wood (1996a)
Oncorhynchus mykiss	Low-speed swimming (55% U_{crit})	77.5 g	Unfed (After 2-day starvation)	15	17	51-60	21-34	Lauff and Wood (1997)
Oncorhynchus mykiss	Resting	16 g	Unfed (After 5-day starvation)	5	27	35	38	Kieffer *et al.* (1998)

Oncorhynchus mykiss	Low-speed swimming (45% U_{crit})	16 g	Unfed (After 5-day starvation)	5	15	40	45	Kieffer *et al.* (1998)
Oncorhynchus mykiss	High-speed swimming (75% U_{crit})	16 g	Unfed (After 5-day starvation)	5	17	42	41	Kieffer *et al.* (1998)
Oncorhynchus mykiss	Resting	16 g	Unfed (After 5-day starvation)	15	30	55	15	Kieffer *et al.* (1998)
Oncorhynchus mykiss	Low-speed swimming (45% U_{crit})	16 g	Unfed (After 5-day starvation)	15	30	55	15	Kieffer *et al.* (1998)
Oncorhynchus mykiss	High-speed swimming (75% U_{crit})	16 g	Unfed (After 5-day starvation)	15	30	45	25	Kieffer *et al.* (1998)

Oncorhynchus mykiss	After 1-day starvation	2-4 g	Unfed	15	12	68	20	Lauff and Wood (1996b)
Oncorhynchus mykiss	After 15-day starvation	2-4 g	Unfed	15	23	50	27	Lauff and Wood (1996b)
Oncorhynchus mykiss	Feed ingredients (raw starch)	111-131 g	Unfed (After 5-day starvation)	18	14	26	60	Kaushik *et al.* (1989)
Oncorhynchus mykiss	Feed ingredients (extruded corn starch)	111-131 g	Unfed (After 5-day starvation)	18	10	17	73	Kaushik *et al.* (1989)
Oncorhynchus mykiss	Feed ingredients (extruded whole corn)	111-131 g	Unfed (After 5-day starvation)	18	24	15	61	Kaushik *et al.* (1989)
Oncorhynchus mykiss	Feed ingredients (extruded wheat starch)	111-131 g	Unfed (After 5-day starvation)	18	18	19	63	Kaushik *et al.* (1989)

Oncorhynchus mykiss	Feed ingredients (extruded whole wheat)	111-131 g	Unfed (After 5-day starvation)	18	15	30	55	Kaushik *et al.* (1989)
Oncorhynchus mykiss	Dawn feeding	78 g	During 1-day post-feeding	15	42	47	11	Gelineau *et al.* (1998)
Oncorhynchus mykiss	Midnight feeding	77 g	During 1-day post-feeding	15	48	37	15	Gelineau *et al.* (1998)
Salmo salar	After 10-day starvation	2 kg	Unfed	8.5	14	86	0	Forsberg (1997)
Salmo salar	Feeding ration (0.15% BW day^{-1})	2 kg	During 1-day post-feeding	8.5	22	78	0	Forsberg (1997)
Salmo salar	Feeding ration (0.3% BW day^{-1})	2 kg	During 1-day post-feeding	8.5	28	71	1	Forsberg (1997)
Salmo salar	Feeding ration (0.6% BW day^{-1})	2 kg	During 1-day post-feeding	8.5	40	60	0	Forsberg (1997)
Arthropoda								

Crustacea								
Potamonautes warreni	Non-copper-exposure (Control)	NA	Unfed	23-25	30	41	29	Vosloo *et al.* (2002)
Potamonautes warreni	Copper exposure (1.0 mg Cu^{2+} L^{-1})	NA	Unfed	23-25	15	85	0	Vosloo *et al.* (2002)
Macrobrachium rosenbergii	After 4-day starvation	0.68 g (DM)	Unfed	29.5	8	18	74	Clifford and Brick (1979)
Macrobrachium rosenbergii	Nutrient composition (25% P, L : C = 1:1)	0.68 g (DM)	During 8-h post-feeding	29.5	36	43	21	Clifford and Brick (1979)
Macrobrachium rosenbergii	Nutrient composition (25% P, L : C = 1:2)	0.68 g (DM)	During 8-h post-feeding	29.5	37	17	46	Clifford and Brick (1979)

Macrobrachium rosenbergii	Nutrient composition (25% P, L : C = 1:3)	0.68 g (DM)	During 8-h post-feeding	29.5	18	9	73	Clifford and Brick (1979)
Macrobrachium rosenbergii	Nutrient composition (25% P, L : C = 1:4)	0.68 g (DM)	During 8-h post-feeding	29.5	15	12	73	Clifford and Brick (1979)
Macrobrachium rosenbergii	Nutrient composition (35% P, L : C = 1:2)	0.68 g (DM)	During 8-h post-feeding	29.5	39	22	39	Clifford and Brick (1979)
Macrobrachium rosenbergii	After 4-day starvation	0.68 g (DM)	Unfed	29.5	8	18	74	Clifford and Brick (1983)
Macrobrachium rosenbergii	After 8-day starvation	0.68 g (DM)	Unfed	29.5	15	34	51	Clifford and Brick (1983)

Sagmariasus verreauxi	After 2-day starvation	350 g	Unfed	21	65	35	0	Wang *et al.* (2021)
Sagmariasus verreauxi	Feeding	350 g	During 36.5-h post-feeding	21	>50	0-43	0-37	Wang *et al.* (2021)

P, protein; L, lipid; C, carbohydrate; Ucrit, critical swimming speed; NA, not available; DM, dry matter.

3.6 Conclusions

This review focused on incorporating the use of RQ into stoichiometric bioenergetic studies in aquatic ectotherms, this is timely because of the technical advances in measuring carbon dioxide and the benefits that the stoichiometric approach provides for understanding the energy metabolism under different conditions. During routine metabolism, the RQ may vary among fish species while lipid can be the major metabolic energy substrate when reared at optimum temperatures. In bivalves, the routine RQ and the principal role of carbohydrate oxidation may remain stable when environmental pH decreases within some range. With the decrease of pH, the RQ may decline and protein may replace lipid to become the major metabolic energy substrate. Exposure of crustaceans to heavy metals may result in a reduced RQ and the major metabolic energy substrate may shift from a mixture to lipid. Body weight likely has a species-specific impact on the routine RQ with little influence on metabolic energy substrate use in the same species. In both resting and aerobic swimming fish, the RQ and the principal role of lipid oxidation may remain stable. Using stoichiometry to evaluate metabolic use of each major energy substrate within a daily cycle holds great potential to support the continuous development and refinement of feeds and feeding regimes to maximize offshore aquaculture production, enhance broodstock reproductive performance and larval survival, and improve dietary protein-sparing effects. The examination of RQ and metabolic energy substrate use is also important for better understanding feeding anticipatory behaviour to improve the welfare of aquaculture species and evaluating the applicability of specific ingredients in aquafeeds.

3.7 Future research

In future, the effects of a specific environment such as low salinity, acidification, and extreme environments on RQ and metabolic energy substrate use in aquatic ectotherms need further investigations. This will allow a more holistic interpretation of behavioural, physiological, and

evolutionary adaptations to farmed environments or natural ecosystems. Factors including swimming speeds, cycles of fasting and refeeding, and biosecurity practices can be incorporated into experimental designs. The use of stoichiometry is suggested in future bioenergetic studies in emerging aquaculture species. However, it is important to note that the stoichiometric approach is not feasible in conditions where the RQ value is outside of the theoretical range (0.71-1.0). Therefore, additional studies are required to decipher the limitations and applicability of stoichiometry. Kleiber (1975) considered that other approaches, specifically the use of metabolic labels and tracers, would supersede RQ based stoichiometry, we contend that combining bioenergetic stoichiometry, as presented here, with other newer approaches including the use of tracers and "omics" will enhance the outcomes.

Chapter 4 The use of stoichiometric bioenergetics to elucidate metabolic energy substrate use and specific dynamic action in cultured juvenile spiny lobsters (*Sagmariasus verreauxi*) of different nutritional status

Part of the research contained within this chapter has been published as Wang S, Carter CG, Fitzgibbon QP, Smith GG (2021) The use of stoichiometric bioenergetics to elucidate metabolic energy substrate use and specific dynamic action in cultured juvenile spiny lobsters (*Sagmariasus verreauxi*) of different nutritional status. *Aquaculture* **532**: 736021. https://doi.org/10.1016/j.aquaculture.2020.736021

4.1 Abstract

Simultaneous measurements of nitrogenous (ammonia and urea) excretion and respiratory gas (O_2 and CO_2) exchange provide a non-destructive stoichiometric bioenergetic approach to elucidate metabolic energy substrate use which has rarely been used with aquatic ectotherms due to previous difficulties in measuring total CO_2 excretion. This study examined metabolic energy substrate use and specific dynamic action (SDA) in cultured spiny lobster, *Sagmariasus verreauxi*, of different nutritional status. SDA magnitude calculated by stoichiometry was compared to a traditional composite oxycalorific coefficient approach. Protein synthesis can account for a large part of SDA in aquatic ectotherms. This study used a protein synthesis inhibitor cycloheximide to investigate the contribution of cycloheximide-sensitive protein synthesis to SDA and the effect of cycloheximide on nitrogenous and CO_2 excretion. Lobsters were subjected to five treatments: 2-day fasted juveniles sham injected with saline (FS treatment); 2-day fasted juveniles injected with cycloheximide (FC treatment); 10-day starved juveniles injected with cycloheximide (SC treatment); post-prandial juveniles fed squid *Nototodarus sloanii* (FED treatment) and; post-prandial juveniles injected with cycloheximide (FEDC treatment). Protein was the primary energy substrate (65% of oxygen consumption) for 2-day fasted juveniles, with lipid accounting for the remainder (35%). After 10-day starvation lipid became the main substrate, indicating lipid oxidation increased with extended fasting. Following feeding, protein contribution remained above

50%, while lipid (0-43%) and carbohydrate (0-37%) provided significant energy at different time periods, indicating besides protein appropriate proportions of non-protein ingredients are also essential to lobsters. SDA magnitude in FED and FEDC treatments estimated by the traditional approach was 10.5 and 0.4 J g^{-1}, respectively, indicating *S. verreauxi* expended 96% of post-prandial energy on protein synthesis and that decapod protein synthesis can account for one of the highest proportions of SDA in aquatic ectotherms. SDA magnitude in the FED treatment evaluated by stoichiometry (12.6 J g^{-1}) was comparable to the traditional approach. Interestingly, stoichiometry was not applicable in the FEDC treatment as the respiratory quotient exceeded the theoretical maximum under aerobic conditions. Cycloheximide did not affect CO_2 excretion among all treatments or nitrogenous excretion among unfed treatments, while the post-treatment total nitrogenous excretion decreased in fed lobsters. The combined measurement of substrate use and SDA potentially helps optimize the feed to achieve sustainable aquaculture. However, more research is required to decipher limitations and the applicability of stoichiometry for crustaceans.

4.2 Introduction

Specific dynamic action (SDA) is the increment in metabolic rates related to feeding, representing energetic costs from ingestion, digestion, absorption and metabolic processing of major energy substrates (protein/amino acid, lipid, and carbohydrate) (Whiteley *et al.* 2001a; Secor 2009). Specific dynamic action mainly represents post-absorptive metabolic costs, particularly increased protein synthesis, and reflects the balance of available nutrients (Whiteley *et al.* 2001a; Carter & Mente 2014). Imbalanced aquafeeds where the digestible protein (amino acid) to energy (DP/DE) ratio or amino acid balance is outside of the optimum range will either stimulate protein synthesis or cause excess amino acids to be immediately deaminated and oxidized, with a resultant elevation of SDA (Carter *et al.* 1993). In contrast, aquafeeds with optimum DP/DE ratios and amino acid balances can minimize amino acid catabolism and maximize protein synthesis retention efficiency, growth performance and

reduce energy loss via SDA (Carter & Houlihan 2001; Eliason *et al.* 2007; Hu *et al.* 2008). Investigating metabolic energy substrate use and SDA improves understanding of nutrient processing including anabolic and catabolic processes to provide the physiological basis of growth, potentially helping develop nutritionally balanced and cost-effective feeds to achieve more sustainable aquaculture (Clifford & Brick 1979; National Research Council 2011; Carter & Mente 2014).

Metabolic energy substrate use in aquatic ectotherms can be illustrated by atomic oxygen consumption to ammonia-N excretion (O/N) ratios or respiratory quotient (RQ) (Clifford & Brick 1983). However, O/N or RQ only provides information on the type of substrates being oxidized, while the balance of substrate oxidation cannot be determined. In contrast, a non-destructive stoichiometric bioenergetic approach can be used to investigate the balance at any one time during aerobic metabolism by concurrently determining nitrogen quotient (NQ) and RQ (Ferreira *et al.* 2019). This approach allows repeated assessments of substrate oxidation on the same individuals, thus providing precise measurements on substrate use under different feeding conditions (Clifford & Brick 1979). In addition, non-destructive stoichiometry can also be useful for understanding the nutritional bioenergetics of aquaculture species with high commercial value and sold as live products, such as spiny lobster (Wang *et al.* 2019a). However, this approach has not been widely used in aquatic ectotherms, mainly due to previous difficulties in accurately determining total CO_2 concentrations in water (Nelson 2016; Ferreira *et al.* 2019). In aquatic invertebrates, stoichiometry has only been used twice in the freshwater shrimp *Macrobrachium rosenbergii* (Clifford & Brick 1979; Clifford & Brick 1983) and not tested in any marine species.

Total metabolic energy expended during SDA (SDA magnitude) in aquatic ectotherms can be determined by simplified traditional approaches based on an empirical or composite oxycalorific coefficient (Q_{ox}), or by stoichiometric bioenergetic approach based on the full

complement of key metabolic parameters, including respiratory gas (O_2 and CO_2) exchange and nitrogenous (ammonia and urea) excretion (Brafield & Llewellyn 1982). Theoretically, the stoichiometric estimation is more reliable (Cho *et al.* 1982; Brafield 1985). However, stoichiometry has only been used to examine SDA magnitude in fish (Musisi 1984; Meyer-Burgdorff & Rosenow 1995; Gelineau *et al.* 1998) and not examined in crustaceans.

Sagmariasus verreauxi is the largest of the spiny lobster (Palinuridae) species and an important commercial seafood product in the Southern Hemisphere (Nguyen *et al.* 2018). Successful cultivation from eggs in Australia has made this species an emerging candidate for closed-cycle aquaculture (Fitzgibbon & Battaglene 2012a). Our previous research on the use of a protein synthesis inhibitor cycloheximide demonstrated that energy expenditure associated with post-prandial protein synthesis in *S. verreauxi* accounted for a large proportion of SDA (Wang *et al.* 2019a). However, Wang *et al.* (2019a) did not examine metabolic energy substrate use and the SDA magnitude was estimated only based on the empirical Q_{ox}. Consideration of different aspects of SDA, including protein turnover, has the potential to provide insight into formulating cost-effective aquafeeds (Carter & Mente 2014; Wang *et al.* 2019a). In the present study, carbon dioxide and nitrogenous excretion data are presented for the first time and combined with oxygen consumption data from Wang *et al.* (2019a) to investigate metabolic energy substrate use, re-calculate SDA magnitude by stoichiometry and re-calculate the contribution of protein synthesis to SDA using cycloheximide in *S. verreauxi* of different nutritional status; fed, 2-day fasted, and 10-day starved. Cycloheximide inhibits cytosolic protein synthesis and does not affect protein degradation (Pestka 1977; Rastrick & Whiteley 2017), therefore the reduced oxygen consumption (representing reduced metabolic energy expenditure) following cycloheximide administration in aquatic ectotherms represents the oxygen consumed and energy expended on cycloheximide-sensitive protein synthesis (Pannevis & Houlihan 1992; Houlihan *et al.* 1995c; Rastrick & Whiteley 2017). To our

knowledge, the effect of protein synthesis inhibitors on whole-body carbon dioxide and nitrogenous excretion in aquatic ectotherms has only been examined in the crab *Calunectes sapidus* and *Gecarcinus lateraus* (Henry & Cameron 1983; Gilles & Gilles-Baillien 1985) and channel catfish, *Ictalurus punctatus* (Brown & Cameron 1991a), respectively.

Fasting and starvation reflect different status of feed limitation where fasting is generally short-term and part of a routine cycle, while starvation is prolonged fasting and is detrimental, even fatal (McCue 2012; Sugumar *et al.* 2013). Many aquaculture and fishery species such as lobsters, are subjected to medium starvation (up to several weeks) before commercial live transport (Whiteley & Taylor 1986; Day *et al.* 2019). Starvation may reduce lobster quality as lobsters should oxidize body composition for example protein (amino acid) to maintain metabolism (McLeod *et al.* 2004; Sánchez-Paz *et al.* 2006). Since metabolic energy substrate use may vary with recent feeding history (Regnault 1981; National Research Council 2011), the investigation of substrate use in lobsters subjected to different feeds and feeding regimes and followed by starvation may help achieve better nutritional manipulation to minimize body protein (amino acid) oxidation and maximize lobster quality during transport (Fotedar & Evans 2011). In addition, quantification of the effects of cycles of fasting and refeeding on substrate use in aquaculture animals may provide valuable information about physiological and bioenergetic mechanisms underlying compensatory growth (McCue 2010; Stumpf *et al.* 2019).

4.3 Materials and methods

4.3.1 Experimental lobsters and acclimation

The present research was undertaken at the Institute for Marine and Antarctic Studies (IMAS) in Hobart, Australia. It used the same lobsters and was conducted simultaneously with Wang *et al.* (2019a). In brief, hatchery-reared *S. verreauxi* juveniles from the same batch were

maintained in a 4000-L fiberglass tank supplied with flow-through filtered seawater at 21 ± 0.2 °C, salinity 35 ± 0.1 ppt, dissolved oxygen 100 ± 10% air saturation, pH 8.1 ± 0.1. Lobsters were acclimated to constant dim light for 4 weeks before the experiment to avoid the influence of circadian rhythms. During acclimation, *S. verreauxi* were fed frozen squid *Nototodarus sloanii* and fresh blue mussels *Mytilus galloprovincialis* twice a week *ad libitum* (Wang *et al.* 2019a). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (Wang *et al.* 2019a).

4.3.2 Experimental treatments

After acclimation, 30 intermoult juvenile *S. verreauxi* [body weight, BW, 350 ± 14 g (mean ± standard error (SE)), range 229-460 g, male : female sex ratio = 1:1] were randomly allocated among five treatments (N=6) as described by Wang *et al.* (2019a). FS: 2-day fasted followed by lobster saline injection treatment (BW, 342 ± 36 g). FC: 2-day fasted followed by cycloheximide injection treatment (BW, 314 ± 22 g). SC: 10-day starved followed by cycloheximide injection treatment (BW, 404 ± 30 g). FED: fed treatment (BW, 340 ± 24 g). FEDC: fed followed by cycloheximide injection treatment (BW, 354 ± 38 g). Lobsters in FC and SC treatments were injected with cycloheximide dissolved in lobster saline at 2.0 mg kg^{-1} BW (Wang *et al.* 2019a). Lobsters in the FS treatment were sham injected with the same volume of lobster saline as that of injected cycloheximide in the other treatments. Lobsters in FED and FEDC treatments ate frozen squid at a 3% ration level, thereafter the lobsters in the FEDC treatment were injected with cycloheximide within 10 min after the lobster consumed all the squid. The body weight among treatments was not statistically different and the sex ratio within treatments was 1:1.

4.3.3 Oxygen consumption rate and seawater sampling

The oxygen consumption rate (MO_2, μg g^{-1} h^{-1}) used in the present study to calculate O/N ratios, RQ, NQ, metabolic energy substrate use and SDA magnitude were derived from Wang *et al.* (2019a), and presented in the attached *Supplementary research data*. In brief, an intermittent flow-through respirometer system including a 3.55-L gas-tight acrylic respiration chamber was used and set to a 10-min on- and a 10-min off-cycle allowing one MO_2 measurement every 20 min during the off-cycle and complete water exchange during the on-cycle (Jensen *et al.* 2013b; Wang *et al.* 2019a).

Seawater samples were taken to determine total CO_2 and nitrogenous excretion after correction for background levels, determined in blank respirometers without a lobster for 2 h after each respiratory experiment (Jensen *et al.* 2013b; Jensen *et al.* 2013c; Wang *et al.* 2019a). In brief, at the completion of MO_2 measurement, seawater samples from the respiration chamber were collected via the connected Tygon E-3603 tubing (internal diameter = 7.9 mm, thickness = 2.4 mm, Saint-Gobain Performance Plastics, Charny, France), and directly dispensed into a 20 mL disposable plastic vial until overflowing for 3 s. Fourteen milliliters of the collected seawater was immediately dispensed into a 12 mL precooled glass vial (Labco Limited, Lampeter, UK) until overflowing to minimize air-water gas exchange. Thereafter the 12 mL seawater was disinfected with 3.6 μL of saturated mercuric chloride (Mayzaud *et al.* 2005; Bockmon & Dickson 2015). The seawater sampling and disinfection were completed within 2 min. The glass vial was subsequently capped and kept at room temperature before total CO_2 analysis. The remaining 6 mL sample was used to evaluate nitrogenous excretion, with 3 mL sealed in a 10 mL disposable plastic vial to determine total ammonia-N (TAN) excretion, disinfected with 10% chloroform to prevent bacterial activity (Jensen *et al.* 2013b; Jensen *et al.* 2013c), and the remaining 3 mL sealed in another 10 mL vial to determine urea-N excretion. Seawater samples for the determination of TAN and urea-N excretion were frozen

at −20 °C within 20 min after sampling and thawed at room temperature before analysis (Fellman *et al.* 2008; Chen *et al.* 2015).

Seawater samples taken after the measurement of the pre-treatment (routine) metabolic rate (Wang *et al.* 2019a) were defined as routine samplings. After routine samplings, lobsters were subjected to one of the five experimental treatments, and the post-treatment metabolic rate was measured after 24 h in unfed (FS, FC, and SC) treatments and 36.5 h in fed (FED and FEDC) treatments (Wang *et al.* 2019a). Seawater samples taken after 24 h in unfed treatments and 36.5 h in fed treatments were defined as post-treatment samplings. During routine and post-treatment samplings, the respirometer off-cycle was extended to 20 min to intensify respiratory gas and nitrogenous end-product accumulation (Jensen *et al.* 2013b; Jensen *et al.* 2013c). For unfed treatments, only routine and post-treatment samples were taken during the experimental period. For fed treatments, further to routine and post-treatment samplings, additional six-time post-prandial samplings were performed. The 1st sample was taken at 0.5 h after feeding then every 4 h for the first 12 h. The 5th and 6th samples were taken at the 24th and 30th h, respectively. These six-time samplings were performed after a 10-min off-cycle due to heightened post-prandial metabolic demands. The respirometer experienced three on-off-on cycles per hour, samplings were performed at the 2nd cycle and the time of sampling was precisely recorded for further simultaneously investigating respiratory gas exchange and nitrogenous excretion.

4.3.4 Total CO_2 and nitrogenous excretion analysis

The total CO_2 (including gaseous CO_2, carbonate, bicarbonate and carbonic acid) concentration was measured using a recently well-developed high-precision non-dispersive infrared CO_2 analyzer (AS-C3, Apollo SciTech, Newark, USA) (Bockmon & Dickson 2015; Müller *et al.* 2017; van der Loos *et al.* 2019). The analyzer, calibrated with Certified Reference Materials (Scripps Institution of Oceanography, USA) (Chu *et al.* 2018), determined the molar

concentration of total dissolved inorganic carbon (DIC) (Bockmon & Dickson 2015; Chu *et al.* 2018), which was then converted to the mass concentration of total CO_2.

Nitrogenous concentrations were colorimetrically measured using a Synergy HT Multi-detection Microplate Reader (BioTek Instruments, Winooski, VT, USA). The concentration of TAN was determined by the salicylate-hypochlorite method (Bower & Holm-Hansen 1980; Wilkie *et al.* 2017). The concentration of urea-N was determined by the diacetyl monoxime method (Chen *et al.* 2015) with modification to increase sensitivity (Alam *et al.* 2017). The squares of the correlation coefficient of the linear calibration curves in DIC, TAN and urea-N determination were higher than 0.99. The duration (h) of DIC, TAN, urea-N, and total nitrogenous (TN, the sum of TAN and urea-N) excretion was determined as the time when post-prandial excretion rates returned to routine levels.

4.3.5 Atomic O/N ratio, nitrogen quotient and respiratory quotient calculation

The atomic O/N ratio was calculated as (MO$_2$/16)/(MTAN/14), where 16 and 14 are the atomic masses of O and N, respectively; MTAN (μg g^{-1} h^{-1}) is the rate of TAN excretion (Quetin *et al.* 1980; Mayzaud & Conover 1988). The Ex_{TN}/In_{-N} (%) represented the contribution of accumulated TN excretion during SDA (Ex_{TN}, μg g^{-1}) to total nitrogen intake (In_{-N}, μg g^{-1}). The In_{-N} was calculated as ingested protein divided by 6.25 (the nitrogen-to-protein conversion factor) (Hu *et al.* 2008; Luo & Xie 2009). The NQ was calculated as (MTN/14)/(MO$_2$/32), and the RQ was calculated as (MDIC/12)/(MO$_2$/32), where 14, 32 and 12 are the atomic masses of N, O_2, and C, respectively; MTN (μg g^{-1} h^{-1}) and MDIC (μg g^{-1} h^{-1}) represent the rate of total nitrogenous excretion (MTAN plus Murea-N) and total dissolved inorganic carbon excretion, respectively (Lauff & Wood 1996b; Ferreira *et al.* 2019).

4.3.6 Instantaneous metabolic energy substrate use calculation

Instantaneous metabolic energy substrate use, regarding fractional contributions of protein (amino acid), lipid and carbohydrate to the support of MO_2, was determined using substrate-specific aerobic RQ (Lauff & Wood 1996b; Ferreira *et al.* 2019):

$RQ = P \times m + L \times 0.71 + C \times 1.0$ (1)

where P, L, and C represent the percentage of energy substrates supporting MO_2 from protein, lipid and carbohydrate, respectively; m is the aerobic RQ for protein oxidation, determined by $0.96 \times TAN\% + 0.83 \times$ urea-N%, where 0.96 and 0.83 are the aerobic RQ for protein oxidation when ammonia and urea are the unique nitrogenous end-products, respectively; TAN% and urea-N% represent the contribution of *M*TAN and *M*urea-N to *M*TN, respectively (Elliott & Davison 1975; Lauff & Wood 1996b). The values of 0.71 and 1.0 are the aerobic RQ for lipid and carbohydrate oxidation, respectively (Frayn 1983; Lauff & Wood 1996b).

$P + L + C = 1.0$ (2)

$P = NQ/0.27$ (3)

where 0.27 is the theoretical maximum NQ when protein is the only substrate being oxidized under aerobic conditions (Guillaume *et al.* 2001).

From (1), (2) and (3),

$RQ = (m - 0.71) \times NQ/0.27 + 0.29 \times C + 0.71$ (4)

4.3.7 SDA magnitude and protein synthesis-related energy expenditure calculation

The calculation of a composite oxycalorific coefficient (Q_{ox}, J mg^{-1} O_2) was based on the method outlined by Brafield (1985), assuming energy substrates were oxidized in the same proportions as they were absorbed, and dependent on the biochemical composition of the squid *N. sloanii* (Protein 16.6%, lipid 1.0%, carbohydrate 0%. Doxa *et al.* 2013). Theoretical Q_{ox} of 13.36, 13.60, 13.72 and 14.76 J mg^{-1} O_2 for the oxidation of protein (to ammonia), protein (to

urea), lipid and carbohydrate, respectively, was used (Brafield & Llewellyn 1982). The SDA magnitude (J g^{-1}) was calculated by multiplying the composite Q_{ox} by the accumulated MO_2 (TMO_2, mg g^{-1}) during SDA (Brafield 1985).

The original equation used to determine SDA magnitude using the stoichiometric bioenergetic approach from Weir (1949) and Brafield (1985) was further modified to include urea excretion: SDA magnitude (J g^{-1}) = 11 × TMO_2 + 2.6 × TMCO_2 - 9.5 × TMNH_3 - 2.44 × T*M*urea. In the formula, TMO_2 (mg g^{-1}) was derived from Wang *et al.* (2019a), TMCO_2 (accumulated CO_2 excretion, mg g^{-1}), TMNH_3 (accumulated ammonia excretion, mg g^{-1}), and T*M*urea (accumulated urea excretion, mg g^{-1}) were calculated based on the magnitude of *M*DIC, *M*TAN and *M*urea-N during SDA, respectively.

In this study, the difference in the SDA magnitude between FED and FEDC treatments represented the energy expended on cycloheximide-sensitive protein synthesis (Houlihan *et al.* 1995c).

4.3.8 Data analysis

All statistical analysis was performed using SPSS Software (Version 24, IBM Corporation, New York, USA). Data of $P < 0.05$ were considered significant. Normality tests were carried out before statistical analysis via Kolmogorov-Smirnov test, followed by the verification of homogeneity of variances via Bartlett's test. Homogeneous data were compared using t-tests and one-way analysis of variance (ANOVA), heterogeneous data were compared using the Kruskal-Wallis test. All data were expressed as mean ± SE, except the data of the instantaneous metabolic energy substrate use in the FED treatment presented as mean values (Lauff & Wood 1996b; Ferreira *et al.* 2019)**.** Figures were plotted using SigmaPlot (Version 12.5, Systat Software, San Jose, USA).

4.4 Results

4.4.1 Routine nitrogenous excretion and respiratory gas exchange

There were no differences in the routine *M*TAN among FS, FC, SC and FEDC treatments, or between FED and FEDC treatments. However, the routine *M*TAN in the FED treatment was significantly higher compared to FS, FC and SC treatments (Table 4. 1). The routine *M*urea-N did not differ among FS, FC, SC and FEDC treatments, or between FC and FED treatments. However, the routine *M*urea-N in the FED treatment was significantly higher compared to FS, SC and FEDC treatments (Table 4. 1). The routine *M*TN in the FED treatment was significantly higher (P = 0.001) compared to the SC treatment, but did not differ among other treatments (Table 4. 1). There were no differences in any of the other routine metabolic parameters among treatments (Table 4. 1).

Table 4. 1 Summary of routine nitrogenous excretion and respiratory gas exchange for *Sagmariasus verreauxi* of different nutritional status

	Nutritional status				
	FS	**FC**	**SC**	**FED**	**FEDC**
Routine *M*TAN (μg g^{-1} h^{-1})	2.6 ± 1.0^{a}	2.5 ± 0.6^{a}	1.0 ± 0.2^{a}	5.0 ± 0.9^{b}	3.1 ± 0.7^{ab}
Routine *M*urea-N (μg g^{-1} h^{-1})	0.2 ± 0.1^{a}	0.5 ± 0.1^{ab}	0.2 ± 0.1^{a}	0.6 ± 0.2^{b}	0.3 ± 0.1^{a}
Routine *M*TN (μg g^{-1} h^{-1})	2.8 ± 1.0^{ab}	3.0 ± 0.6^{ab}	1.2 ± 0.2^{a}	5.6 ± 0.9^{b}	3.4 ± 0.7^{ab}
Routine *M*TAN/*M*TN (%)	89.3 ± 4.6	83.2 ± 4.5	86.2 ± 1.6	89.0 ± 1.9	91.8 ± 2.0
Routine *M*urea-N/*M*TN (%)	10.7 ± 4.6	16.8 ± 4.5	13.8 ± 1.6	11.0 ± 1.9	8.2 ± 2.0
Routine O/N ratio	45.1 ± 13.8	36.3 ± 12.3	48.1 ± 6.7	16.2 ± 3.3	26.2 ± 6.2
Routine NQ	0.1 ± 0.05	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.04	0.1 ± 0.05
Routine *M*DIC (μg g^{-1} h^{-1})	34.2 ± 5.9	30.1 ± 2.0	23.5 ± 2.5	22.2 ± 4.6	28.5 ± 4.1
Routine RQ	1.4 ± 0.3	1.1 ± 0.1	1.2 ± 0.1	0.8 ± 0.3	1.1 ± 0.2

All data represent mean ± standard error (SE) of 6 individuals. Different superscripts (a, b) in each row indicate significant differences among treatments (One-way ANOVA, $P < 0.05$).

FS, 2-day fasted and saline sham injected; FC, 2-day fasted and cycloheximide injected; SC, 10-day starved and cycloheximide injected; FED, fed lobsters with no further treatment; FEDC, fed lobsters injected with cycloheximide. *M*TAN, the rate of total ammonia-N excretion; *M*urea-N, the rate of urea-N excretion; *M*TN, the rate of total nitrogenous excretion; O/N ratio, oxygen consumption/ammonia-N excretion atomic ratio; NQ, nitrogen quotient; *M*DIC, the rate of total dissolved inorganic carbon excretion; RQ, respiratory quotient.

4.4.2 Post-treatment nitrogenous excretion and respiratory gas exchange

There were no differences between routine and post-treatment metabolic parameters in any treatment (Paired t-tests) (Table 4. 1 and 4. 2, Table 4. 1 and 4. 3). There were no differences in post-treatment nitrogenous excretion or respiratory gas exchange among FS, FC and SC treatments (Table 4. 2). Post-treatment *M*TN in the FED treatment was significantly higher ($P = 0.045$) compared to the FEDC treatment (Table 4. 3). Post-treatment RQ in the FEDC treatment was significantly higher ($P = 0.016$) compared to the FED treatment (Table 4. 3). There were no differences in any of the other post-treatment metabolic parameters between FED and FEDC treatments (Table 4. 3).

Table 4. 2 Summary of nitrogenous excretion and respiratory gas exchange for 2-day fasted and saline sham injected (FS), 2-day fasted and cycloheximide injected (FC), and 10-day starved and cycloheximide injected (SC) *Sagmariasus verreauxi* at 24 h after treatment

	Nutritional status		
	FS	**FC**	**SC**
Post-treatment *M*TAN (μg g^{-1} h^{-1})	2.3 ± 0.5	2.6 ± 0.6	1.8 ± 0.6
Post-treatment *M*urea-N (μg g^{-1} h^{-1})	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.1
Post-treatment *M*TN (μg g^{-1} h^{-1})	2.7 ± 0.5	3.1 ± 0.7	2.3 ± 0.6
Post-treatment *M*TAN/*M*TN (%)	85.9 ± 4.2	80.5 ± 5.9	76.0 ± 8.4
Post-treatment *M*urea-N/*M*TN (%)	14.1 ± 4.2	19.5 ± 5.9	24.0 ± 8.4
Post-treatment O/N ratio	39.2 ± 9.0	36.8 ± 10.2	59.6 ± 18.0
Post-treatment NQ	0.08 ± 0.02	0.1 ± 0.02	0.08 ± 0.02
Post-treatment *M*DIC (μg g^{-1} h^{-1})	32.8 ± 3.7	29.5 ± 1.8	25.9 ± 2.4
Post-treatment RQ	1.1 ± 0.2	1.1 ± 0.1	1.1 ± 0.1

All data represent mean ± standard error (SE) of 6 individuals. *M*TAN, the rate of total ammonia-N excretion; *M*urea-N, the rate of urea-N excretion; *M*TN, the rate of total nitrogenous excretion; O/N ratio, oxygen consumption/ammonia-N excretion atomic ratio; NQ, nitrogen quotient; *M*DIC, the rate of total dissolved inorganic carbon excretion; RQ, respiratory quotient.

Table 4. 3 Summary of nitrogenous excretion and respiratory gas exchange for *Sagmariasus verreauxi* fed squid at 3% body weight at 36.5 h post-feeding

	Nutritional status	
	FED	**FEDC**
Post-treatment *M*TAN (μg g^{-1} h^{-1})	5.6 ± 0.9	3.6 ± 0.5
Post-treatment *M*urea-N (μg g^{-1} h^{-1})	0.8 ± 0.6	0.5 ± 0.4
Post-treatment *M*TN (μg g^{-1} h^{-1})	6.4 ± 0.9*	4.0 ± 0.6
Post-treatment *M*TAN/*M*TN (%)	86.6 ± 11.1	89.3 ± 6.1
Post-treatment *M*urea-N/*M*TN (%)	13.5 ± 4.5	10.7 ± 2.5
Post-treatment O/N ratio	14.9 ± 2.9	19.6 ± 3.9
Post-treatment NQ	0.2 ± 0.03	0.1 ± 0.02
Post-treatment *M*DIC (μg g^{-1} h^{-1})	26.3 ± 3.6	27.3 ± 2.8
Post-treatment RQ	0.8 ± 0.1	1.0 ± 0.1*

All data represent mean ± standard error (SE) of 6 individuals. The asterisk (*) in each row indicates significant differences between FED and FEDC treatments (Independent samples t-test, $P < 0.05$). FED, fed lobsters with no further treatment; FEDC, fed lobsters injected with cycloheximide. *M*TAN, the rate of total ammonia-N excretion; *M*urea-N, the rate of urea-N excretion; *M*TN, the rate of total nitrogenous excretion; O/N ratio, oxygen consumption/ammonia-N excretion atomic ratio; NQ, nitrogen quotient; *M*DIC, the rate of total dissolved inorganic carbon excretion; RQ, respiratory quotient.

4.4.3 Post-prandial nitrogenous excretion and respiratory gas exchange

In the FED treatment, there were double peaks in both post-prandial *M*TAN and *M*TN, and the first peaks were significantly higher compared to the second and the routine values (Table 4. 4). In the FEDC treatment, there were double peaks in both *M*TAN and *M*TN after feeding and cycloheximide injection, while the first and second peaks were not different from each other. The first *M*TAN peak in the FEDC treatment was significantly higher (Paired t-tests, $P = 0.016$) than the routine *M*TAN, and the second was comparable to the routine value. Both the first and second *M*TN peaks in the FEDC treatment were significantly higher than the routine *M*TN (Table 4. 4).

Following feeding, *M*urea-N in the FED treatment increased and reached a peak at 10.5 ± 1.4 h, significantly higher (Paired t-tests, $P = 0.015$) than the routine *M*urea-N. Subsequently, *M*urea-N dropped and was not different from the routine value at 27.8 ± 5.7 h (Table 4. 4). Following feeding, *M*DIC in the FED treatment increased and reached a peak at 11.2 ± 3.2 h, significantly higher (Paired t-tests, $P = 0.023$) than the routine *M*DIC. Thereafter, *M*DIC decreased and was not different from the routine value at 22.4 ± 5.8 h (Table 4. 4). Following feeding and cycloheximide injection, *M*DIC in the FEDC treatment increased and reached a peak at 7.2 ± 3.7 h, significantly higher (Paired t-tests, $P = 0.041$) than the routine *M*DIC. Thereafter, *M*DIC declined and was not different from the routine value at 31.5 ± 2.4 h (Table 4. 4). In the FEDC treatment, both the mean post-prandial *M*urea-N and *M*DIC were significantly higher compared to the respective routine values (Paired t-tests, $P = 0.037$ and 0.010, respectively) (Table 4. 4). There were no differences between other mean post-prandial and routine metabolic parameters in the FED or FEDC treatment (Table 4. 4).

Table 4. 4 Comparison between routine and post-prandial nitrogenous excretion, and between routine and respiratory gas exchange for *Sagmariasus verreauxi* fed squid at 3% body weight

	Nutritional status	
	FED	**FEDC**
Routine *M*TAN (μg g^{-1} h^{-1})	5.0 ± 0.9[a]	3.1 ± 0.7[a]
Mean post-prandial *M*TAN (μg g^{-1} h^{-1})	6.3 ± 1.0	5.1 ± 1.0
First *M*TAN peak (μg g^{-1} h^{-1})	11.9 ± 1.5[b]	8.0 ± 1.4[b]
Second *M*TAN peak (μg g^{-1} h^{-1})	7.4 ± 1.4[a]	5.9 ± 1.2[ab]
Time to the first *M*TAN peak (h)	8.2 ± 4.8	2.5 ± 1.4
Time to the second *M*TAN peak (h)	21.2 ± 4.4	23.5 ± 4.8
TAN duration (h)	24.1 ± 6.7	28.2 ± 5.9
TAN magnitude (μg g^{-1})	76.2 ± 28.4	81.1 ± 27.3
TAN magnitude integrated over 24 h (μg g^{-1})	34.0 ± 13.9	57.4 ± 15.9
Routine *M*urea-N (μg g^{-1} h^{-1})	0.6 ± 0.2	0.3 ± 0.1
Mean post-prandial *M*urea-N (μg g^{-1} h^{-1})	1.1 ± 0.1	1.0 ± 0.2[*]
*M*urea-N peak (μg g^{-1} h^{-1})	1.8 ± 0.2[*]	2.9 ± 1.3
Time to *M*urea-N peak (h)	10.5 ± 1.4	13.2 ± 2.6
Urea-N duration (h)	27.8 ± 5.7	29.5 ± 3.9
Urea-N magnitude (μg g^{-1})	20.1 ± 5.8	23.9 ± 5.8
Urea-N magnitude integrated over 24 h (μg g^{-1})	14.8 ± 4.5	23.9 ± 9.3
Routine *M*TN (μg g^{-1} h^{-1})	5.6 ± 0.9[a]	3.4 ± 0.7[a]
Mean post-prandial *M*TN (μg g^{-1} h^{-1})	7.4 ± 1.0	6.1 ± 1.1
First *M*TN peak (μg g^{-1} h^{-1})	12.7 ± 1.4[b]	10.1 ± 1.4[b]
Second *M*TN peak (μg g^{-1} h^{-1})	8.3 ± 1.5[a]	7.3 ± 1.3[b]
Time to the first *M*TN peak (h)	7.5 ± 4.8	5.2 ± 3.9

Time to the second *M*TN peak (h)	24.8 ± 3.8	23.5 ± 4.8
TN duration (h)	24.6 ± 6.7	28.6 ± 5.9
TN magnitude (μg g^{-1})	86.3 ± 27.9	108.1 ± 32.4
TN magnitude integrated over 24 h (μg g^{-1})	76.0 ± 11.6	74.2 ± 19.6
Mean post-prandial Ex_{TN}/In_{-N} (%)	8.3 ± 3.1	12.2 ± 3.7
Routine *M*TAN/*M*TN (%)	89.0 ± 1.9	91.8 ± 2.0
Mean post-prandial *M*TAN/*M*TN (%)	82.2 ± 2.4	84.6 ± 2.4
Routine *M*urea-N/*M*TN (%)	11.0 ± 1.9	8.2 ± 2.0
Mean post-prandial *M*urea-N/*M*TN (%)	17.8 ± 2.4	15.4 ± 2.4
Routine O/N ratio	16.2 ± 3.3	26.2 ± 6.2
Mean post-prandial O/N ratio	21.6 ± 4.6	20.8 ± 3.5
Routine NQ	0.2 ± 0.04	0.1 ± 0.05
Mean post-prandial NQ	0.2 ± 0.03	0.2 ± 0.02
Routine *M*DIC (μg g^{-1} h^{-1})	22.2 ± 4.6	28.5 ± 4.1
Mean post-prandial *M*DIC (μg g^{-1} h^{-1})	32.8 ± 4.6	50.4 ± 7.3*
*M*DIC peak (μg g^{-1} h^{-1})	44.5 ± 4.9*	105.3 ± 28.7*
Time to *M*DIC peak (h)	11.2 ± 3.2	7.2 ± 3.7
DIC duration (h)	22.4 ± 5.8	31.5 ± 2.4
DIC magnitude (μg g^{-1})	518.2 ± 187.7	636.0 ± 182.2
DIC magnitude integrated over 24 h (μg g^{-1})	412.2 ± 144.1	498.5 ± 142.7
Routine RQ	0.8 ± 0.3	1.1 ± 0.2
Mean post-prandial RQ	0.88 ± 0.1	1.5 ± 0.1^{+}
SDA magnitude (J g^{-1}) calculated by the composite Q_{ox} approach	10.5 ± 1.5	0.4 ± 0.1^{+}

SDA magnitude (J g^{-1}) calculated by stoichiometry	12.6 ± 3.0	NA

All data represent mean ± standard error (SE) of 6 individuals. Different superscripts (a, b) in each column indicate significant differences among routine and two peak values in each treatment (One-way ANOVA, $P < 0.05$). The asterisk (*) in each column indicates significant differences between routine and peak values or between routine and mean post-prandial values (Paired t-tests, $P < 0.05$). The plus (+) in each row indicates significant differences between FED and FEDC treatments (Independent samples t-test, $P < 0.05$). FED, fed lobsters with no further treatment; FEDC, fed lobsters injected with cycloheximide. *M*TAN, the rate of total ammonia-N excretion; *M*urea-N, the rate of urea-N excretion; *M*TN, the rate of total nitrogenous excretion; NQ, nitrogen quotient; Ex_{TN}/In_{-N} (%), the ratio of total TN excretion to total N intake (In_{-N}); *M*DIC, the rate of total dissolved inorganic carbon excretion; RQ, respiratory quotient; SDA, specific dynamic action; Q_{ox}, oxycalorific coefficient; NA, not available.

The post-prandial O/N ratio (Figure 4. 1), NQ (Figure 4. 2) and RQ (Figure 4. 3) at different time periods were not different from the respective routine levels (Paired t-tests) in the FED or FEDC treatment. The mean post-prandial RQ in the FEDC treatment was significantly higher (P = 0.006) compared to the FED treatment, while other mean post-prandial metabolic parameters did not differ between FED and FEDC treatments (Table 4. 4).

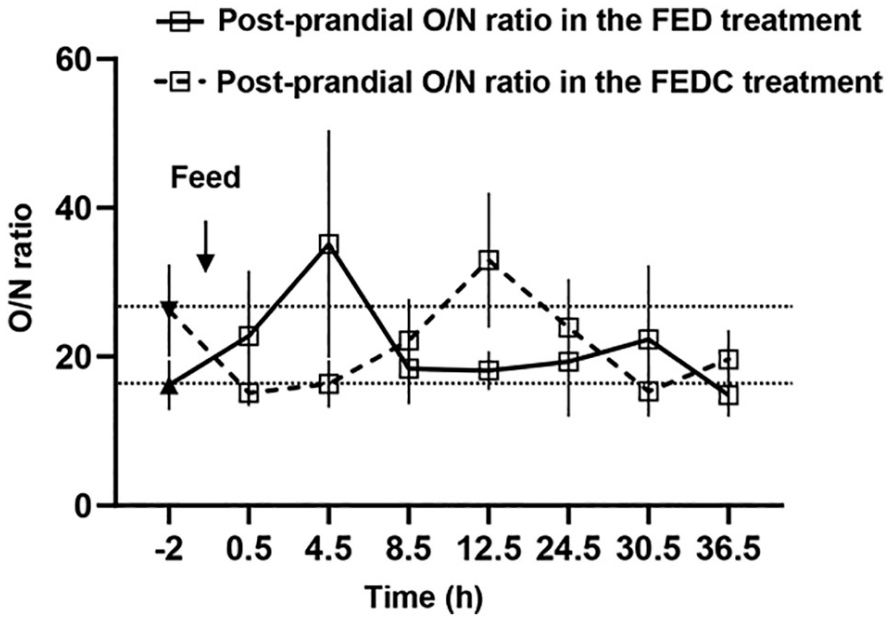

Figure 4. 1 Oxygen consumption/ammonia-N excretion atomic (O/N) ratio for *Sagmariasus verreauxi* reared at 21 °C and fed squid at 3% body weight. The upward triangle and the dotted horizontal line indicate the routine O/N ratio in the FED treatment. The downward triangle and the dotted horizontal line indicate the routine O/N ratio in the FEDC treatment. FED, fed lobsters with no further treatment; FEDC, fed lobsters injected with cycloheximide within 10 min after the lobsters consumed all the squid. All data represent mean ± standard error (SE) of 6 individuals.

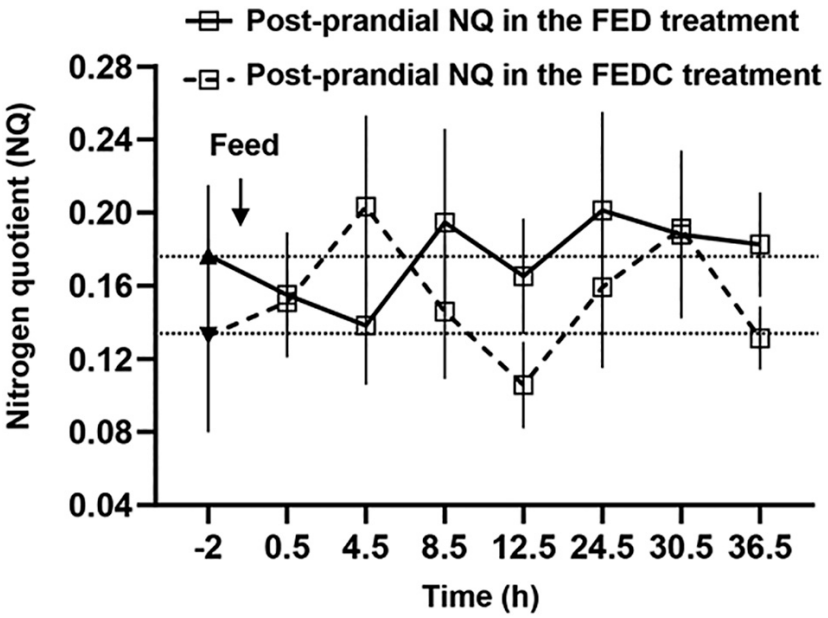

Figure 4. 2 Nitrogen quotient (NQ) for *Sagmariasus verreauxi* reared at 21 °C and fed squid at 3% body weight. The upward triangle and the dotted horizontal line indicate the routine NQ in the FED treatment. The downward triangle and the dotted horizontal line indicate the routine NQ in the FEDC treatment. FED, fed lobsters with no further treatment; FEDC, fed lobsters injected with cycloheximide within 10 min after the lobsters consumed all the squid. All data represent mean ± standard error (SE) of 6 individuals.

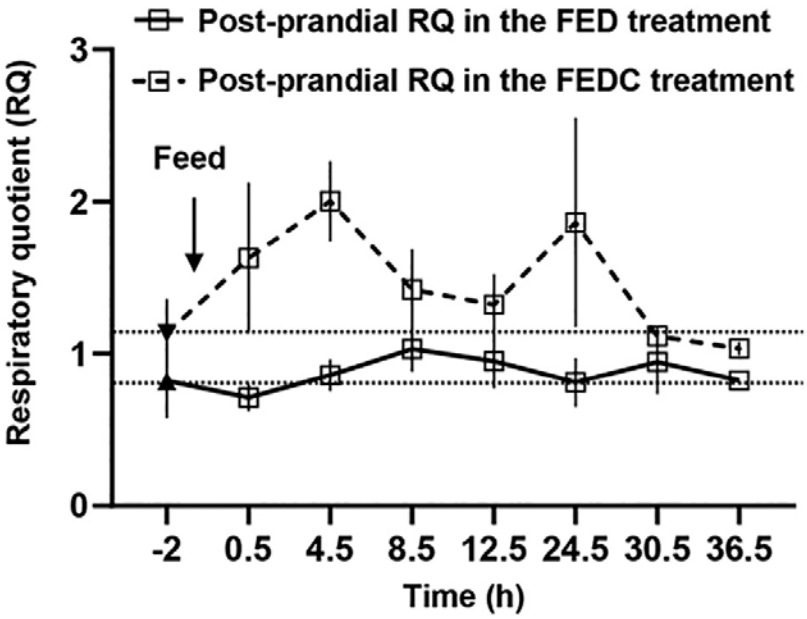

Figure 4. 3 Respiratory quotient (RQ) for *Sagmariasus verreauxi* reared at 21 °C and fed squid at 3% body weight. The upward triangle and the dotted horizontal line indicate the routine RQ in the FED treatment. The downward triangle and the dotted horizontal line indicate the routine RQ in the FEDC treatment. FED, fed lobsters with no further treatment; FEDC, fed lobsters injected with cycloheximide within 10 min after the lobsters consumed all the squid. All data represent mean ± standard error (SE) of 6 individuals.

4.4.4 Instantaneous metabolic energy substrate use

Instantaneous energy substrate use during aerobic metabolism in the FED treatment varied at different time periods after feeding, but amino acid oxidation always dominated, contributing over 50% of oxygen consumption, and therefore of total energy substrate oxidation (Figure 4. 4). During routine metabolism and in the first 0.5 h after feeding, lipid was the secondary energy substrate (35-43%), and the contribution from carbohydrate was 0%. From 0.5 to 8.5 h after feeding, lipid contribution decreased from 43% to 0%, then increased in the next 16 h to 25%. From 0.5 to 12.5 h after feeding, the contribution from carbohydrate increased and reached a peak of 37% at 12.5 h, then decreased in the next 12 h to 0%. From 24.5 to 30.5 h after feeding, lipid oxidation decreased while carbohydrate oxidation increased. From 30.5 to 36.5 h after feeding, lipid oxidation increased to 32% while carbohydrate contribution to metabolic use decreased to 0% (Figure 4. 4).

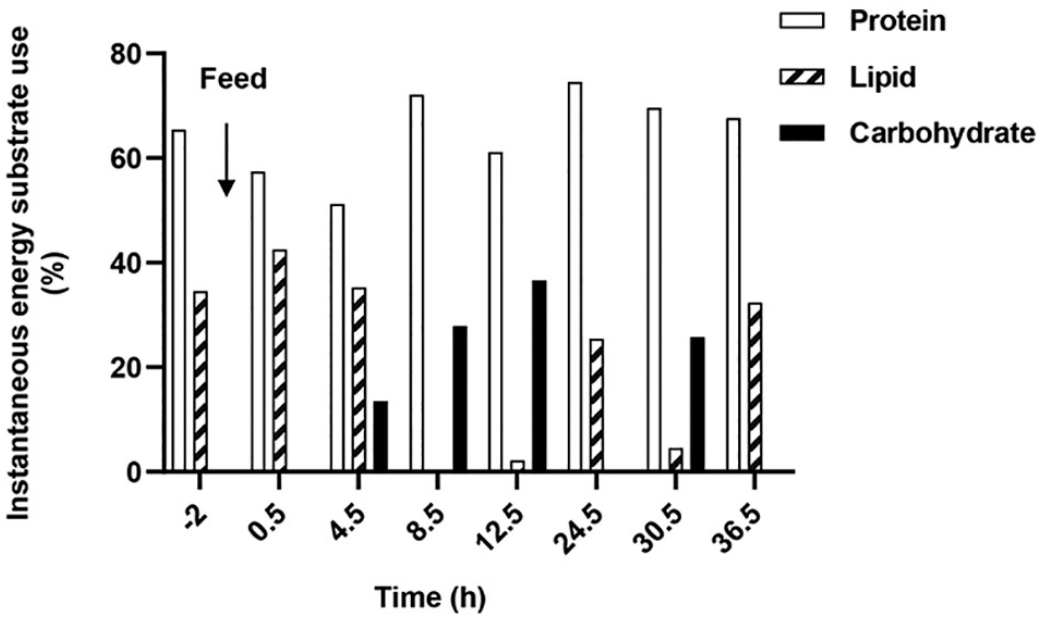

Figure 4. 4 Instantaneous energy substrate use for *Sagmariasus verreauxi* in the FED treatment. Lobsters were reared at 21 °C and fed squid at 3% body weight with no further treatment. All data represent mean values of 6 individuals.

4.4.5 SDA magnitude

The SDA magnitude, calculated by the composite oxycalorific coefficient (Q_{ox}) approach, in the FED treatment was significantly higher ($P = 0.0001$) than that in the FEDC treatment (Table 4. 4). Following cycloheximide injection, the SDA magnitude was reduced by 96% (Table 4. 4). In the FED treatment, there was no difference between the SDA magnitude evaluated by the composite Q_{ox} approach and the stoichiometric bioenergetic approach (Table 4. 4).

4.5 Discussion

This study used a stoichiometric bioenergetic approach to examine metabolic energy substrate use and SDA in lobsters of different nutritional status, and is one of only a limited number of complete datasets for aquatic ectotherms that simultaneously measured O_2 consumption, CO_2 and nitrogenous excretion rates (Clifford & Brick 1983; Musisi 1984; Ferreira *et al.* 2019). This study also examined SDA magnitude using a composite oxycalorific coefficient (Q_{ox}) approach and estimated the contribution of protein synthesis to SDA by injection of cycloheximide. Protein was the main energy substrate for 2-day fasted *S. verreauxi*, lipid provided the remainder and carbohydrate was not used. After 10-day starvation lipid became the major energy substrate. Following feeding, protein (amino acid) remained the dominant energy substrate, while lipid and carbohydrate both provided significant energy at different time periods. The SDA magnitude in the FED treatment evaluated by stoichiometry was comparable to the composite Q_{ox} approach. The SDA magnitude determined using the composite Q_{ox} approach was reduced by 96% after cycloheximide injection. Interestingly, stoichiometry was not applicable in the FEDC treatment because the post-prandial RQ exceeded 1, the theoretical maximum under aerobic conditions (Frayn 1983). A comprehensive understanding of metabolic energy substrate use, SDA, and protein metabolism advances the

knowledge of the relationship of amino acid flux to energy metabolism and the nutritional bioenergetics in aquaculture animals on a fine level, which potentially assists in developing more sustainable aquaculture (Clifford & Brick 1979; Carter & Houlihan 2001; National Research Council 2011). For example, the investigation of substrate use at an hourly level within a 24-h cycle of feeding and SDA magnitude by stoichiometry may help understand physiological mechanisms underpinning long-term growth by considering anabolic and catabolic processes in more detail, efficiently evaluate feed effectiveness including protein-sparing effects and optimize feeding regimes (Clifford & Brick 1979; Carter & Houlihan 2001; Anderson *et al.* 2020). In the present study, we took the approach to only examine an injection control for one treatment, the 2-day fasted lobster (FS) treatment. A control was not required for all treatments because the control was for the process of an injection rather than for the combination of the treatment and the injection. In addition, our results showed that there were no differences between routine and 24 h post-treatment rates of CO_2 or nitrogenous excretion in the FS treatment. Further, previous research in other decapods also demonstrated that the rates of CO_2 excretion (Henry & Cameron 1983) and nitrogenous excretion (Yoganandhan *et al.* 2003; Jacobo *et al.* 2016) at different time periods following a single saline injection did not differ from the routine levels, indicating the stress influence of injection on decapod CO_2 and nitrogenous excretion rates could be negligible.

4.5.1 Instantaneous metabolic energy substrate use

Two-day fasted *S. verreauxi* in the present study oxidized 65% of protein (amino acid) and 35% of lipid, whereas carbohydrate contribution was 0% (Figure 4. 4). This is comparable with other short-term fasted carnivorous decapods, where protein (amino acid) was the primary metabolic energy substrate, followed by lipid, and carbohydrate oxidation played a minor role, if at all; however, the balance of substrate oxidation was not determined (Castell & Budson

1974; Regnault 1981; Dall & Smith 1986). The use of stoichiometry to examine metabolic energy substrate use in aquatic invertebrates has only been reported by Clifford and Brick (1979, 1983), where the 4-8-day fasted freshwater shrimp *Macrobrachium rosenbergii* oxidized 8-15% protein (amino acid), 18-34% lipid and 51-74% carbohydrate, indicating the predominance of carbohydrate oxidation. The preferential use of major energy substrates in unfed decapods can be species-specific (Hervant *et al.* 1999; Sánchez-Paz *et al.* 2006). For example, during short-term fasting *Astacus astacus* and *M. rosenbergii* mainly oxidize carbohydrate (Clifford & Brick 1983; Sánchez-Paz *et al.* 2006), while *Hemigrapsus nudus* and *Cancer payurus* mainly oxidize protein (amino acid) (Clifford & Brick 1983). The present study did not evaluate the proportion of each substrate being oxidized in 10-day starved *S. verreauxi* because the routine RQ exceeded 1 (see below).

As with other decapod crustaceans (Hewitt & Irving 1990; Radford *et al.* 2004), amino acid oxidation in the FED treatment was the major energy pathway during SDA. Protein synthesis accounts for a large part of SDA in crustaceans (Whiteley *et al.* 2001a; Wang *et al.* 2019a). Therefore, the major amino acid oxidation in fed *S. verreauxi* indicated that the absorbed amino acids derived from the high-protein feed both provided substrates for protein synthesis and stimulated catabolic pathways via deamination: excessive amino acids in free pools are likely deaminated and oxidized providing energy including for protein synthesis (Carter & Houlihan 2001; Jiang *et al.* 2013). Deamination is imperative because free amino acid accumulation can be toxic, even fatal to crustaceans (Weihrauch & O'Donnell 2017). Ultimately, excessive amino acid deamination and oxidation lead to the production of nitrogenous end-products (Jiang *et al.* 2013; Weihrauch & O'Donnell 2017). This study also showed lipid and carbohydrate oxidation following feeding in the FED treatment, comparable with other studies in decapods fed high-protein feeds (Regnault 1981; Rosas *et al.* 1995). In the shrimp *Crangon crangon* fed crab *Carcinus maenas* meat, Regnault (1981) showed that

carbohydrate was primarily oxidized in the first 3-4 days after feeding, followed by protein and lipid. In the shrimp *Penaeus setiferus* fed squid *Loligo brevis* meat, Rosas *et al.* (1995) demonstrated the hemolymph glucose concentration increased 1 h after feeding and that glucose was a major energy substrate in 24 h following feeding. The investigation of metabolic energy substrate use does not provide information about metabolic interconversions which may occur before substrate oxidation (Gelineau *et al.* 1998). It has been demonstrated that some carbon skeletons of deaminated amino acids can be used for gluconeogenesis and lipogenesis in decapod fed high-protein feeds (Rosas *et al.* 1995; Jimenez & Kinsey 2015). Therefore, the carbohydrate (and lipid) oxidized in this study could be from ingested protein (amino acid) interconversion. As protein is the major and one of the most expensive components in decapod feeds (Ward *et al.* 2003; National Research Council 2011), appropriate proportions of high-quality protein and non-protein energy-yielding nutrients including lipid and carbohydrate are imperative to develop cost-effective feeds and improve dietary protein-sparing effects so that assimilated protein (amino acid) enhances protein synthesis retention efficiency and therefore growth (Carter & Houlihan 2001; Ward *et al.* 2003).

4.5.2 SDA magnitude and protein synthesis-related energy expenditure

The SDA magnitude in *S. verreauxi* in FED and FEDC treatments calculated based on the composite oxycalorific coefficient (Q_{ox}) was similar to the values calculated based on the empirical Q_{ox}, respectively (Wang *et al.* 2019a). Though the composite Q_{ox} was calculated based on the published biochemical composition of the squid *N. sloanii* (Doxa *et al.* 2013), the result is accurate because the *N. sloanii* biochemical composition is relatively stable (protein 17-19%, lipid 1.0-1.7%, carbohydrate 0-0.2%, moisture 78-81%), resulting in similar composite Q_{ox} calculated based on the biochemical composition (13.45, 13.45 and 13.43 J mg^{-1} O_2, respectively) (Vlieg 1984; Krzynowek & Murphy 1987; Doxa *et al.* 2013, respectively).

Following cycloheximide injection, the SDA magnitude in *S. verreauxi* estimated based on the composite Q_{ox} was reduced by 96% (Table 4. 4), in accordance with Wang *et al.* (2019a) estimating the reduction based on the empirical Q_{ox}. The reduction in the SDA magnitude by 96% demonstrated that in juvenile *S. verreauxi* most of the SDA was attributable to cycloheximide-sensitive protein synthesis; protein synthesis in *S. verreauxi* accounted for one of the highest proportions of SDA measured in an aquatic ectotherm (Pannevis & Houlihan 1992; Thor 2000; Whiteley *et al.* 2001a).

In the FED treatment, there was no difference between the SDA magnitude estimated by the traditional Q_{ox} approach and the stoichiometric bioenergetic approach. Similar comparisons are limited to Musisi (1984) and Gelineau *et al.* (1998) who also demonstrated no differences between approaches in calculating SDA magnitude in fish. The present study did not evaluate the SDA magnitude by stoichiometry in the FEDC treatment because the post-prandial RQ exceeded 1 (see below).

4.5.3 Total dissolved inorganic carbon excretion

Previously reported routine MDIC in crustaceans (Mayzaud *et al.* 2005; Li *et al.* 2007) were 2-20 times higher than that in *S. verreauxi* (22-34 $\mu g\ g^{-1}\ h^{-1}$) in the present study. Three reasons could explain the large discrepancy. First, Mayzaud *et al.* (2005) used coulometric titration and Li *et al.* (2007) used acid-base titration, while the present study used highly precise infrared detection (Müller *et al.* 2017; Ferreira *et al.* 2019; van der Loos *et al.* 2019). Second, Mayzaud *et al.* (2005) examined wild crustaceans from the Atlantic Ocean, while Li *et al.* (2007) and the present study examined cultured species. The nutritional status between wild and cultured crustaceans would vary by feed availability and composition (Montaño & Navarro 1996; Mente *et al.* 2011; Carter & Mente 2014). Third, the body weight of *S. verreauxi* in the present study was over 1000 times higher than that in Mayzaud *et al.* (2005) and Li *et al.* (2007), which likely

affects mass-specific routine metabolism across different taxa (Ikeda & Mitchell 1982; McCue 2006; Glazier 2015). There were no differences in the routine *M*DIC among treatments in this study, comparable with Lauff and Wood (1996b), suggesting starvation is unlikely to affect the routine *M*DIC in aquatic ectotherms. There were no differences between the routine and post-treatment *M*DIC following cycloheximide injection, consistent with the only other study on aquatic ectotherms (Henry & Cameron 1983). This study provided, for the first time, detailed post-prandial *M*DIC in decapods. The mean post-prandial *M*DIC in the FED treatment (32.8 $\mu g\ g^{-1}\ h^{-1}$) was comparable to a study on Atlantic salmon, *Salmo salar* (14.4-32.4 $\mu g\ g^{-1}\ h^{-1}$) and there was no difference between the mean and routine values (Forsberg 1997).

4.5.4 Respiratory quotient

The routine RQ values among treatments in this study were not statistically different, indicating starvation may not affect routine RQ in aquatic ectotherms (Lauff & Wood 1996b). However, the routine RQ in the FED treatment was below 1, while in other treatments was above 1, ranging from 1.1 to 1.4. Inaccuracies in data collection are unlikely to explain the high RQ values as this study used well-proven MO_2 and MCO_2 analytical techniques (Briceño *et al.* 2018; Ferreira *et al.* 2019) and non-gas permeable connective tubing to reduce the potential for gas leaks (Spicer & McMahon 1992; Roy *et al.* 2007; Crispin & White 2013). On the other hand, despite the theoretical constrains, routine RQ higher than 1 is not uncommon in crustaceans and has been previously observed in marine species including the crab *Carcinus maenas* (1.2, Wolvekamp & Waterman 1960) and *Calunectes sapidus* (2.17, Henry & Cameron 1983), the American lobster *Homarus americanus* (1.4, Wolvekamp & Waterman 1960), the shrimp *Sergestes* sp. and *Systellaspis debilis* (1.4, Mayzaud *et al.* 2005) and the krill *Meganyctiphanes norvegica* (1.3-1.6, Mayzaud *et al.* 2005). There are several possible explanations, including anaerobic metabolism (Mayzaud *et al.* 2005), muscular development

(Mayzaud *et al.* 2005), and interaction of excreted CO_2 with carbonate in calcified exoskeletons (Wolvekamp & Waterman 1960; Schafer 1968). Anaerobic metabolism can be due to hypoxia (Craig & Crowder 2005) or spontaneous activity (McGaw & Penney 2014). However, hypoxia was unlikely in the present study where oxygen tension was above 70% air saturation (Jensen *et al.* 2013c). Spontaneous activity is common during respirometry of decapod crustaceans (Zimmer-Faust *et al.* 1996; McGaw & Penney 2014; Briceño *et al.* 2018) and can cause transitory anaerobic metabolism, resulting in high RQ values (Lauff & Wood 1996b; Mayzaud *et al.* 2005). The high abdominal muscular levels of free amino acids in decapods are precursors for energy substrates (Shirai *et al.* 1996; Mente *et al.* 2002). Free amino acids turn over rapidly through the Krebs cycle and may result in a high RQ due to an increase of CO_2 excretion without corresponding O_2 consumption (Mayzaud *et al.* 2005). In addition, crustaceans such as spiny lobsters have strongly calcified exoskeletons (George 2005; Frisch & Hobbs 2007), where the interaction of CO_2 with carbonate (decalcification) may also lead to an increase of CO_2 excretion without corresponding O_2 consumption (Bosworth *et al.* 1936; Wolvekamp & Waterman 1960). In this study, there were no differences between the routine and post-treatment RQ following cycloheximide injection, in line with the only other study in aquatic ectotherms, suggesting decapod RQ does not change following cycloheximide injection (Henry & Cameron 1983). When using stoichiometry to examine substrate use and SDA magnitude under aerobic conditions, RQ higher than 1 should be discarded because the value has exceeded the theoretical maximum (Lauff & Wood 1996b). Since various biotic factors may contribute to RQ values exceeding 1 in decapods, more research is required to examine the limitations and the applicability of stoichiometry in decapods, for example to investigate the use of stoichiometry in examining the metabolic energy substrate use at different lifecycle stages.

4.5.5 Nitrogenous excretion

In the present study, *M*TAN/*M*TN (76-92%) was comparable to other decapod studies (Binns & Peterson 1969; Jawed 1969; Mayzaud 1973) and *M*urea-N/*M*TN (8-24%) also comparable to other decapod studies (Zoutendyk 1987; Crear & Forteath 2002; Weihrauch *et al.* 2009). Post-prandial *M*TAN in the FED treatment showed double peaks, and the time to the first peak (8 h post-feeding) was comparable with other decapods (2-7 h post-feeding) (Zoutendyk 1987; Crear & Forteath 2002; Kemp *et al.* 2009a). The first and larger peak likely originated from protein metabolism, and the later and smaller peak probably resulted from urine (Crear & Forteath 2002). Post-prandial *M*urea-N in the FED treatment occurred as a single peak at 10.5 h, about 2 h later than the appearance of the first *M*TAN peak, consistent with the only other crustacean study in the Cape rock lobster, *Jasus lalandii* (Zoutendyk 1987) and a study on turbot, *Scophthalmus maximus* (Dosdat *et al.* 1996). However, this post-prandial *M*urea-N pattern differed from other aquatic ectotherms. For example, the post-prandial *M*urea-N did not change following feeding in the sockeye salmon, *Oncorhynchus nerka* and Atlantic halibut, *Hippoglossus hippoglossus* (Brett & Zala 1975; Fraser *et al.* 1998), while the post-prandial flounder *Rhombosolea tapirina* reached the *M*urea-N and *M*TAN peaks synchronously (Verbeeten *et al.* 1999). These findings suggest post-prandial *M*urea-N and *M*TAN patterns in aquatic ectotherms can be species-specific.

There were no differences in ammonia excretion between FED and FEDC treatments. Comparisons are scarce, however, Brown and Cameron (1991a) also demonstrated that ammonia excretion in channel catfish following the infusion of cycloheximide and amino acids did not differ from that in channel catfish only infused with amino acids. The present study and Wang *et al.* (2019a) demonstrated that SDA in the FEDC treatment was almost completely inhibited following cycloheximide injection, indicating there was no mechanism for removing absorbed amino acids from free pools via protein synthesis. Lobsters in FED and FEDC

treatments consumed the same ration of high-protein squid and should have resulted in excessive amino acids in free pools. The similar amount of ammonia excreted between FED and FEDC treatments suggested excessive amino acids likely remained in free pools and that the deamination pathway most likely was saturated by the large amount of amino acids (Brown & Cameron 1991a). Another explanation for the similar amount of excreted ammonia could be that part of produced ammonia in the FEDC treatment may accumulate in the haemolymph and other tissues (Whiteley *et al.* 2001b; Weihrauch & O'Donnell 2017). Most of the produced ammonia in crustaceans is actively transported by gill Na^+/K^+-ATPase (Whiteley *et al.* 2001b; Weihrauch *et al.* 2004), which can be inhibited by cycloheximide (Miner Jr *et al.* 1980). In the present study, ammonia excretion across the gill in the FEDC treatment may be partially inhibited following cycloheximide injection, leading to ammonia accumulating in the haemolymph and other tissues (Huang & Chen 2001; Jensen *et al.* 2013c).

In the present study, urea was assumed to be only produced from protein metabolism. Previous decapod research showed that most urea is likely produced through uricolysis or argininolysis, especially through the uricolysis of purines derived from nonessential amino acids (Regnault 1987; Weihrauch & O'Donnell 2017). In addition, most previous research in aquatic ectotherms measured the sum of ammonia-N and urea-N excretion as the total excreted N to calculate the NQ and metabolic energy substrate use (Lauff & Wood 1996b; Ferreira *et al.* 2019). Consequently, this study follows the accepted practice in using the sum of ammonia-N and urea-N excretion to calculate NQ, metabolic energy substrate use and SDA magnitude. Further, the proportion of N excreted as urea-N was extremely low with a correspondingly low influence on any errors associated with non-protein sources (Regnault 1987; Carter & Brafield 1992a).

4.5.6 Atomic O/N ratio

The atomic O/N ratio in 2-day fasted *S. verreauxi* (16-45) was comparable with other studies in short-term fasting decapod crustaceans (Robertson *et al.* 2001a; Robertson *et al.* 2001b; Radford *et al.* 2004). Complete oxidation of only protein, protein and some lipid, equivalent amounts of protein and lipid, and lipid and carbohydrate results in O/N ratios of 3-16, 17-50, 50-60, and above 60, respectively (Mayzaud & Conover 1988). Therefore, the O/N ratio in 2-day fasted *S. verreauxi* indicated that *S. verreauxi* oxidized a mix of protein and lipid to provide energy (Schafer 1968; Radford *et al.* 2004; Jensen *et al.* 2013b), comparable with the measured metabolic energy substrate use by stoichiometry. The O/N ratio of 48 in 10-day starved juvenile *S. verreauxi* was comparable with 10-day starved *S. verreauxi* pueruli (Fitzgibbon *et al.* 2014a), 5-d starved adult shrimp *Crangon crangon* (Regnault 1981), and 12-day starved adult king crab *Paralomis granulosa* (Comoglio *et al.* 2005), demonstrating lipid could be the primary energy substrate during long-term fasting (McLeod *et al.* 2004; Fitzgibbon *et al.* 2014a; Simon *et al.* 2015). In contrast, some other decapod research showed low O/N ratios (7-8) after 15-day starvation (Regnault 1981; Dall & Smith 1986), indicating predominant protein oxidation (Neiland & Scheer 1953; Barclay *et al.* 1983). These results suggest the O/N ratio and major energy substrates in unfed decapods may vary among species.

There is limited information on daily post-prandial O/N ratios in juvenile decapods (Radford *et al.* 2004; Perera *et al.* 2005). Juvenile spiny lobster *Jasus edwardsii* (16 g) fed squid showed a significant decrease of post-prandial O/N ratios within 6 h, thereafter the ratio increased gradually towards the routine value, suggesting protein was the only energy substrate in the early post-prandial stage, then the major energy substrate changed to a mix of protein and lipid, and that protein oxidation still dominated (Radford *et al.* 2004). In contrast, juvenile Caribbean spiny lobster (*Panulirus argus*) (120 g) fed high-quality fish meal and squid showed a similar pattern of post-prandial O/N ratios to the FED treatment in the present study, where

the ratio ranged from 15 to 35 and was not different from the routine value, suggesting both protein and lipid were oxidized following feeding, and that protein was the primary energy substrate (Perera *et al.* 2005).

4.6 Conclusions

This study used a non-destructive stoichiometric approach to investigate metabolic energy substrate use and SDA magnitude in crustaceans of different nutritional status. This study also examined SDA magnitude using a traditional composite oxycalorific coefficient approach and estimated the contribution of protein synthesis to SDA using a protein synthesis inhibitor cycloheximide. Lipid oxidation in *S. verreauxi* increased with the extent of fasting. During the 36.5-h SDA process (Wang *et al.* 2019a), protein (amino acid) remained the major energy substrate, while lipid and carbohydrate both provided significant energy at different time periods, suggesting suitable amounts of high-quality protein and non-protein major energy-yielding nutrients are essential for developing cost-effective aquafeeds. The magnitude of SDA evaluated by the stoichiometric bioenergetic approach and the traditional approach was similar. Cycloheximide-sensitive protein synthesis accounted for 96% of SDA in *S. verreauxi*, demonstrating protein synthesis in this decapod can account for one of the highest proportions of SDA measured in an aquatic ectotherm, suggesting SDA may be a functional indicator in assessing growth potential of aquafeeds. The determination of metabolic energy substrate use, with an hourly level of resolution and within a 24-h cycle of feeding, in combination with SDA magnitude by stoichiometry potentially helps optimize feed formulations and manage feeding regimes to improve dietary protein-sparing effects and growth performance in aquaculture animals. In addition, stoichiometry can be a useful tool to explore the relationship between SDA and protein synthesis in aquatic ectotherms at different dietary protein levels, in order to thoroughly assess the appropriateness of potential feeds. However, it is important to note that

the use of stoichiometry requires a RQ value less than 1; given that various factors may cause a RQ above 1 in aquatic ectotherms, more research is required to decipher the limitations and the applicability of stoichiometry. In addition, ammonia concentrations in the haemolymph and other tissues should be measured in future to fully understand the effects of cycloheximide on protein (amino acid) metabolism in crustaceans.

Chapter 5 Effect of dietary protein on energy metabolism including protein synthesis in the spiny lobster *Sagmariasus verreauxi*

Part of the research contained within this chapter has been prepared as Wang S, Carter CG, Fitzgibbon QP, Codabaccus BM, Smith GG. Effect of dietary protein on energy metabolism including protein synthesis in the spiny lobster *Sagmariasus verreauxi*. *Scientific Reports*. Under review.

5.1 Abstract

This is the first study in an aquatic ectotherm to combine a stoichiometric bioenergetic approach with an endpoint stochastic model to predict an optimum dietary macronutrient content. The combination of measuring respiratory gas (O_2 and CO_2) exchange, nitrogenous (ammonia and urea) excretion, specific dynamic action (SDA), metabolic energy substrate use, and whole-body protein synthesis in spiny lobster, *Sagmariasus verreauxi*, was examined in relation to dietary protein. Three isoenergetic feeds containing 1% ^{15}N-labeled *Spirulina* were formulated and manufactured with three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively. Total CO_2 and ammonia excretion, SDA magnitude and coefficient, and protein synthesis in the CP60 treatment were higher compared to the CP40 treatment. These differences demonstrate dietary protein influences post-prandial energy metabolism. During 72 h post-feeding, metabolic use of each major energy substrate varied at different times, indicating suitable amounts of high-quality protein with major non-protein energy-yielding nutrients, lipid and carbohydrate, are critical for lobsters. The average contribution of protein oxidation was lowest in the CP_{50} treatment, suggesting the mechanisms underlying the most efficient retention of dietary protein and the optimum dietary inclusion. This study advances understanding of how deficient and surplus

dietary protein affects energy metabolism and provides approaches for fine-scale feed evaluation to support sustainable aquaculture.

5.2 Introduction

The spiny lobster *Sagmariasus verreauxi* is the largest spiny lobster (Palinuridae) species and an important commercial seafood product in the Southern Hemisphere (Phillips 2013). The recent closure of the life-cycle of *S. verreauxi* from eggs to adult in captivity has improved the pathway to sustainable aquaculture for this species (Fitzgibbon & Battaglene 2012a). A plethora of nutritional and physiological knowledge is imperative to achieve sustainable aquaculture (Halver & Hardy 2003). Compared with other decapod crustaceans, the information of the effect of dietary protein on *S. verreauxi* nutritional physiology, and other spiny lobsters in general, is limited (Glencross *et al.* 2001; Ward *et al.* 2003; Wang *et al.* 2021).

Specific dynamic action (SDA) is the increment in metabolism following feeding, representing energetic costs from ingestion, digestion, absorption and metabolic processing of energy substrates (Whiteley *et al.* 2001a; Wang *et al.* 2019a). Specific dynamic action mainly represents post-absorptive metabolic costs, especially increased protein synthesis, and reflects the balance of available nutrients (Carter & Houlihan 2001; Whiteley *et al.* 2001a). Aquafeeds with optimum digestible protein (amino acid) to energy (DP/DE) ratios and amino acid balances can efficiently spare dietary protein to maximize protein synthesis retention efficiency, growth performance and reduce energy loss via SDA (Carter & Brafield 1992b; Eliason *et al.* 2007; Hu *et al.* 2008). In contrast, imbalanced aquafeeds where the DP/DE ratio or amino acid balance is outside of the optimum range will stimulate mechanisms for regulating excess amino acids via protein synthesis or deamination and oxidation with a resultant elevation of SDA (Carter & Mente 2014). Therefore, understanding metabolic energy substrate use and SDA is essential to explore physiological mechanisms of growth, potentially helping

formulate cost-effective feeds and optimizing feeding regimes (Clifford & Brick 1979; Hewitt & Irving 1990; Wang *et al.* 2021).

A non-destructive stoichiometric bioenergetic approach offers great potential as it can be used to examine the balance of metabolic energy substrate use in an aquatic ectotherm at any time, thus providing precise measurements on metabolic energy substrate use under different feeding conditions (Ferreira *et al.* 2019; Wang *et al.* 2021). The use of the stoichiometric bioenergetic approach is based on the simultaneous investigation of respiratory gas (O_2 and CO_2) exchange and nitrogenous (ammonia and urea) excretion, allowing repeated assessments of substrate oxidation on the same individuals (Ferreira *et al.* 2019; Wang *et al.* 2021). However, this approach has not been widely used in aquatic ectotherms, mainly due to previous technical difficulty in accurately determining total CO_2 concentrations in water (Brafield 1985; Nelson 2016; Ferreira *et al.* 2019).

Protein synthesis is central to aquatic animal growth as growth occurs when whole-body protein synthesis (WBPS) exceeds protein degradation (Hawkins 1985; Carter & Houlihan 2001; Fraser & Rogers 2007). Research on protein synthesis and degradation in aquatic ectotherms has focused on fish and is less on invertebrates (Hawkins 1985; Whiteley *et al.* 2001a; Moltschaniwskyj & Carter 2010). Investigation of WBPS in aquaculture animals provides a sensitive way to examine dietary protein (amino acid) efficiency to achieve long-term growth (Hewitt 1992; Fraser & Rogers 2007; Carter *et al.* 2012). The development of endpoint stochastic models enables the measurement of WBPS in aquatic ectotherms in a non-destructive way (Hawkins 1985; Fraser *et al.* 1998; McCarthy *et al.* 2016). This method allows WBPS determinations in a complete daily cycle to reduce the variation among different times of a day due to feeding and/or natural circadian rhythms, thus ensuring an integrated description of protein metabolism in aquatic ectotherms (Carter & Houlihan 2001). However, the endpoint stochastic model has not yet been tested in any crustacean species.

This study aimed to examine the effects of dietary protein on SDA, metabolic energy substrate use, and WBPS in juvenile *S. verreauxi* using a stoichiometric bioenergetic approach and an endpoint stochastic model. Spiny lobsters were kept in individual tanks during the WBPS measurement without transferring into another tank to minimize the possible handling stress (Carter & Bransden 2001; McCarthy *et al.* 2016). The results improve the understanding of how dietary protein affects energy metabolism including protein synthesis in aquatic ectotherms, which provides a physiological basis of growth and is essential to optimize feeds and feeding regimes in aquaculture.

5.3 Materials and methods

5.3.1 Lobster husbandry

Sagmariasus verreauxi were hatchery reared from eggs at the Institute for Marine and Antarctic Studies (IMAS), Hobart, Australia (Fitzgibbon & Battaglene 2012b). Thirty juvenile lobsters [body weight (BW), 993 ± 23 g (mean ± standard error (SE)), range 750-1150 g] were evenly and randomly divided into three identical 200 L rectangular cages (A, B, C) made of oyster mesh (5 mm mesh size), which were floating in a 4000-L fiberglass tank supplied with flow-through filtered seawater and aerated by an air-stone connected to a central air supply. The male-female sex ratio was 1:1 in each cage. The tank was covered with black plastic to decrease lobster visual disturbance. Seawater quality was maintained at temperature 21 ± 0.2 °C, salinity 35 ± 0.1 ppt, pH 8.1 ± 0.1, dissolved oxygen 100 ± 10.0% saturation. To avoid interference from circadian rhythms, lobsters were acclimated to constant dim light for 4 weeks before experimentation (Wang *et al.* 2019a; Wang *et al.* 2021). During acclimation, lobsters were fed *ad libitum* fresh blue mussels *Mytilus galloprovincialis* twice a week at 08:00. Intermolt lobsters were used during the whole study, including an adjunct experiment to evaluate the

apparent digestibility (AD) of *Spirulina* protein, followed by an energy metabolism experiment including WBPS determination.

5.3.2 Feed formulation and manufacturing

5.3.2.1 Spirulina protein apparent digestibility experiment

A reference feed and a test feed were formulated and manufactured to determine the AD of *Spirulina* protein (for feed formulation, see Table 5. 1). All chemicals used in this study were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) unless noted otherwise. Yttrium oxide (Y_2O_3) (1 g kg^{-1}) as an inert digestibility marker was added into the feeds at the start of feed preparation (Sugiura *et al.* 1998). Briefly, all finely ground dry ingredients were thoroughly mixed with a mixer (Premier Chef KMC510, Kenwood, London, UK) for 20 min, after which krill oil, lecithin and de-ionized water (40 °C) were added to form a soft dough. The dough was then cold extruded through a 10 mm die using a Dolly Pasta Machine (LaMonferrina, Italy). Thereafter, the pellets were cut to 5 cm in length appropriate for the experimental lobsters and kept in air-tight containers at 4 °C overnight to complete the enzymatic binding process, then placed into a fan-ventilated oven (Steridium, Australia) at 40 °C for an hour to attain a moisture content less than 30% (Glencross *et al.* 2001; Ward *et al.* 2003) and cooled for 15 min. The cooled pellets (feeds) were packed in sealed bags and stored at -20 °C until used.

Table 5. 1 Ingredient and chemical composition of the reference and test feeds (g 100 g^{-1} dry matter)

Ingredient	Reference feed	Test feed
Sodium caseinate	48.5	33.9
Defatted GSM[a] meal	23.8	16.6

Krill oil	14.3	10.0
Lecithin	1.0	0.7
Cholesterol	0.5	0.4
Carophyll pink	1.0	0.7
Choline chloride	1.0	0.7
Vitamin C	0.5	0.4
Betaine	1.0	0.7
Vitamin premix[b]	1.3	0.9
Mineral premix[c]	1.3	0.9
Transglutaminase	5.7	4.0
Spirulina	-	30
Yttrium oxide (Y_2O_3)	0.1	0.1
Total (g)	100	100
Chemical composition		
Dry matter (DM, %)	94.3	95.1
Crude protein (%)	63.6	63.1
Total lipid (%)	15.7	12.4
Carbohydrate (%)[d]	12.5	16.3
Ash (%)	8.2	8.2
Gross energy (kJ g^{-1} DM)	24.0	23.3

5.3.2.2 Energy metabolism including protein synthesis experiment

Three isoenergetic experimental feeds containing 1% ^{15}N-labeled *Spirulina* (^{15}N enrichment > 98 atom% ^{15}N) were formulated and manufactured with three crude protein (CP) levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively (for feed formulation, see Table 5. 2). Another three feeds used in a 1-week acclimation phase before the experiment, as described below, were also formulated. The chemical composition was the same as that in the experimental feeds, except that 1% normal *Spirulina* (^{14}N-labeled), instead of ^{15}N-labeled *Spirulina* was added. All feeds were manufactured using the same procedure and dimensions as the adjunct experiment measuring the AD of *Spirulina* protein.

Table 5. 2 Formulation and chemical composition of experimental feeds (g 100 g^{-1} dry matter), made at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively

	CP_{40}	CP_{50}	CP_{60}
Sodium caseinate	28.3	37.3	46.2
Defatted GSM[a] meal	12.8	16.6	20.5
Krill oil	14.3	14.3	14.3
Lecithin	1.0	1.0	1.0
Cholesterol	0.5	0.5	0.5
Carophyll pink	1.0	1.0	1.0
Choline chloride	1.0	1.0	1.0
Vitamin C	0.5	0.5	0.5
Betaine	1.0	1.0	1.0
Vitamin premix[b]	1.3	1.3	1.3
Mineral premix[c]	1.3	1.3	1.3

Transglutaminase	5.7	5.7	5.7
^{15}N-labeled *Spirulina*	1.0	1.0	1.0
Diatomaceous earth	0	2.5	4.7
Corn starch	30.3	15.0	0
Total	100	100	100
Chemical composition			
Dry matter (DM, %)	94.2	93.7	94.0
Crude protein (%)	40.0	50.0	59.9
Total lipid (%)	15.4	15.4	15.5
Carbohydrate (%)[d]	38.8	25.3	11.9
Ash (%)	5.8	9.3	12.7
Gross energy (kJ g^{-1} DM)	22.6	22.7	22.8

[a] GSM: green shell mussel.

[b] Vitamin premix supplied per 100 g of feed: cholecalciferol 0.04 mg, vitamin B12 0.18 mg, retinol acetate 1.59 mg, D-biotin 1.76 mg, folic acid 8.80 mg, menadione sodium bisulfite 8.80 mg, pyridoxine HCl 64.48 mg, nicotinic acid 70.46 mg, calcium D-pantothenate 76.57 mg, DL alpha tocopherol acetate 88.01 mg, thiamin HCl 98.93 mg, riboflavin 88.01 mg, myo-inositol 352.17 mg (Sigma Aldrich, Castle Hill, NSW, Australia) and Rovimix Stay-C 440.18 mg (Shu-Chien *et al.* 2017).

[c] Mineral premix supplied per 100 g of feed: $CoSO_4$ $7H_2O$ 0.004 mg, Na_2SeO_3 0.03 mg, KI 0.12 mg, $CrK(SO_4)_2$ $12H_2O$ 0.66 mg, $FeSO_4$ $7H_2O$ 2.74 mg, $MnSO_4$ H_2O 2.75 mg, $CuSO_4$ $5H_2O$ 6.04 mg, $ZnSO_4$ $7H_2O$ 30.42 mg, $MgSO_4$ $7H_2O$ 55.25 mg, NaCl 101.53 mg, KH_2PO_4 198.25 mg, $CaCO_3$ 278.33 mg and $Ca(H_2PO_4)_2$ H_2O 624.00 mg (Sigma Aldrich, Castle Hill, NSW, Australia) (Shu-Chien *et al.* 2017).

[d] Carbohydrate (%) was calculated by 100 - (Crude protein + Total lipid + Ash) (Codabaccus *et al.* 2020).

5.3.3 Apparent digestibility of Spirulina protein

After the 4-week acclimation, two lobsters from each cage were randomly chosen, weighed and transferred individually by a net into 30 L identical blue polyethylene tanks (width, 30 cm; length, 40 cm; height, 25 cm; N = 6) containing 16 L of running (flow rate: 80 L min^{-1}) and aerated seawater. The six lobsters (BW, 1017 ± 60 g, range 750-1150 g, male : female sex ratio = 1:1) were reared under the same conditions as acclimation for 3 weeks. Artificial shelters made of oyster mesh were placed on the bottom to provide the lobsters with substrates to hold to minimize stress. Three of the lobsters were randomly chosen and fed the reference feed twice a day by hand at 1% BW at 08:00 and 2% BW at 18:00 for a week, and the remaining three lobsters were fed the test feed using the same method. Lobsters were allowed 30 min to consume the feeds followed by siphoning of feed residues. Faeces were siphon-collected onto a 500-μm mesh screen hourly from 09:00 to 21:00 to determine the peak time point of egestion. Preliminary observations showed that lobsters fed the reference and test feeds egested the largest amount of faeces at 4-7 h and 5-8 h post-feeding, respectively. Thereafter, lobsters were fed following the same method for a further 2 weeks to measure the AD of *Spirulina* protein, used to determine the assimilated dose of ^{15}N-labeled *Spirulina* protein to calculate WBPS (Fraser *et al.* 1998). Fresh faeces were siphon-collected within the peak time point of egestion (4-8 h post-feeding) onto a 500-μm mesh screen every 0.5 h to minimize the possible leaching. Collected faeces were washed immediately with de-ionized water for 5 seconds (Wang *et al.* 2018). Rinsed faeces from each lobster were pooled over the 2 weeks, frozen and stored at -20 °C until chemical analysis.

Apparent digestibility of the reference and test feeds was calculated based on Bureau *et al.* (1999):

AD_{DM} (%) = $(1 - Y_{Feed}/Y_{Faeces}) \times 100$

AD_{CP} (%) = $[1 - (X_{Faeces}/X_{Feed}) \times (Y_{Feed}/Y_{Faeces})] \times 100$

Where AD_{DM} represents the AD of DM in the feed; Y_{Feed} and Y_{Faeces} signify the proportion of the marker ($\%Y_2O_3$) in the feed and faeces, respectively; AD_{CP} represents the AD of CP in the feed; X_{Feed} and X_{Faeces} signify the proportion (%) of CP in the feed and faeces, respectively.

Apparent digestibility of CP in the test ingredient *Spirulina* (ADI_{CP}) was calculated based on Bureau *et al.* (1999):

ADI_{CP} (%) = $AD_{CP\text{-}TD} + [(AD_{CP\text{-}TD} - AD_{CP\text{-}RD}) \times (0.7 \times X_{RD})/(0.3 \times X_{IN})]$

Where $AD_{CP\text{-}TD}$ and $AD_{CP\text{-}RD}$ represent the AD of CP in the test and reference feeds, respectively; X_{RD} and X_{IN} signify the proportion (%) of CP in the reference feed and the test ingredient, respectively.

5.3.4 Lobster acclimation to energy metabolism including protein synthesis feeds

After the experiment determining the AD of *Spirulina* protein, the remaining lobsters in Cage A, B and C, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively, were fed *ad libitum* isoenergetic feeds containing 1% normal *Spirulina* as described above at 08:00 daily for a week. Thereafter, one lobster from each cage was randomly chosen, marked with a waterproof label adhered to the carapace (Wang *et al.* 2019a), fasted for three days, then weighed and anaesthetized by immersion in 0 °C seawater (Oellermann *et al.* 2020). These three lobsters (BW, 1044 ± 121 g, range 812-1220 g, male : female sex ratio = 2:1) were subsequently stored at -20 °C for whole-body chemical analysis as described below.

5.3.5 Experimental lobsters

After 1-week acclimation, 6 lobsters from each of the three treatments (BW, 985 ± 30 g, range 793-1150 g, N = 18) were evenly and randomly chosen for energy metabolism experiments including WBPS determination. The BW among treatments was not statistically different and the sex ratio within treatments was 1:1. Each time two lobsters were weighed and transferred individually into two identical polyethylene tanks. The tank, seawater volume, flow rate and other experimental conditions were the same as that of the AD determination experiment, and the seawater was aerated by an air-stone connected to the central air supply. The lobsters were fasted for three days to ensure they were at the same post-absorptive status (Jensen *et al.* 2013b; Lee *et al.* 2015). Before transfer, seawater levels of 16 and 20 L were marked inside of the tank using a marker pen. Thereafter, the seawater level was dropped to 16 L and a lobster (body volume, BV, L) was transferred. The seawater level was again marked to account for 16 L + BV. Then, 4 L of freshly filtered seawater was added and a seawater level of 20 L + BV was marked. The seawater level was kept at 16 L + BV during the fasting period and elevated to 20 L + BV during the feeding period, during which the lobster was fed a ^{15}N-labeled experimental feed at 1.5% BW. Lobsters that did not consume the entire feed within 30 min were excluded for further analysis so that feed intake was known for each lobster.

5.3.6 Oxygen consumption rate

The oxygen consumption rate (MO_2, μmol g^{-1} h^{-1}) was determined according to methods and equipment that have been validated and used consistently in numerous experiments (Jensen *et al.* 2013b; Fitzgibbon *et al.* 2014a; Wang *et al.* 2019a). During the last 2 h during the 3-day fasting period, the routine metabolic rate (RMR) in each lobster was determined (started from 07:00) based on the measurement of MO_2 (Hewitt & Irving 1990; Radford *et al.* 2008; McGaw & Curtis 2013). In brief, MO_2 was calculated from the decline in dissolved oxygen

concentrations in the tank during the experiment measured with a luminescent dissolved oxygen optode (Hach LDO, HQ40d, Hach Company, USA), fixed to a corner of the tank and housed in a separate net to avoid scratches from the lobster. The oxygen optode was calibrated before the experiment and logged dissolved oxygen recorded every 30 s (Wang *et al.* 2019a). Experimental tanks were equipped with a submersible aquarium pump (101 Maxi Pump Power Head 400 L h^{-1}, Aqua One, Wallington, Australia) to ensure seawater was well-mixed. Aeration and seawater flow were manually halted for 20 min (MO_2 measurement period), then restarted for 10 min (re-oxygenation period), allowing one MO_2 value recorded per 30 min. Oxygen contents never fell below 70% saturation. A blue transparent solar pool cover (Intex Development Co., Ltd., Hong Kong) was floated on the surface of the seawater during the measuring period to avoid air-seawater gas exchange (Gelineau *et al.* 1998). During re-oxygenation, the pool cover was removed, and aeration and seawater flow restarted. The halt-restart process was repeated three times and the RMR was determined as the mean of the three MO_2 measurements, where the background MO_2 for each lobster was subtracted (Hewitt & Irving 1990; Radford *et al.* 2008; Wang *et al.* 2019a). Background MO_2 was determined in each tank by the same process described above for 2 h before the lobster was stocked.

When the lobster consumed all the feed, the seawater level in the tank was dropped to 16 L + BV by siphoning 4 L of seawater into a 5-L plastic flask via Tygon E-3603 tubing (Saint-Gobain Performance Plastics, Charny, France). Thereafter, the lobster was subjected to the 20-min seawater and airflow halt and 10-min restart cycles (11 times in total) at 2 h intervals for the first 12 h, thereafter every 12 h up to 48 h and at 72 h to provide 11 post-prandial MO_2 measurements for the determination of SDA (Wang *et al.* 2021).

5.3.7 Seawater sampling

During the RMR determination, a 20-mL seawater sample was collected within 3 seconds at the start and end of each measuring period via a 20 mL syringe (Terumo Co., Ltd., Japan) to determine routine excretion rates of total dissolved inorganic carbon (*M*DIC, μmol g^{-1} h^{-1}), total ammonia-N (*M*TAN, μmol g^{-1} h^{-1}) and urea-N (*M*urea-N, nmol g^{-1} h^{-1}), after correction for background levels. The methods used for seawater sampling were based on Wang *et al.* (2021). Briefly, 14 mL seawater from the collected 20 mL was dispensed into a 12-mL precooled glass vial (Labco Limited, Lampeter, UK) until overflow to minimize air-water gas exchange and disinfected with 3.6 μL of saturated mercuric chloride (Wang *et al.* 2021). The dispensation and disinfection were completed within 1 min. The vial was subsequently capped and kept at room temperature until *M*DIC measurement. The remaining 6 mL was used to determine nitrogenous excretion, with 3 mL sealed in a 10 mL disposable plastic vial for *M*TAN measurement, disinfected with 10% chloroform to prevent bacterial activity, and the remaining 3 mL sealed in another 10 mL vial for *M*urea-N measurement. Seawater samples for *M*TAN and *M*urea-N measurement were frozen at −20 °C within 20 min after sampling and thawed at room temperature before analysis (Wang *et al.* 2021).

After feeding, the siphon-collected 4-L seawater sample was immediately acidified with 10 mL of 4 M HCl and transferred and stored at 4 °C in a 5-L round bottom glass flask (Schott Duran, Mainz, Germany) for the determination of the initial ^{15}N concentration (Fraser *et al.* 1998; McCarthy *et al.* 2016). A 20-mL seawater sample was collected at the start and end of each measuring period to determine the post-prandial *M*DIC, *M*TAN and *M*urea-N. At 12, 24, 48 and 72 h post-feeding, faeces were pipetted onto a 500-μm mesh screen using a plastic pipette following the 20-mL seawater sampling. Pipetting was completed within 1 min. The mesh screen was set on top of the tank to avoid seawater loss from the tank during faecal collection. Subsequently, 4 L of seawater was collected and stored for the later determination

of ^{15}N enrichment of ammonia (Carter *et al.* 1994b; Martin *et al.* 2003). Then 4 L of freshly filtered seawater was added into the tank. The reduction of 20 mL after each sampling was taken into account in post-prandial parameter calculation. After adding fresh seawater for the last time at 72 h, the lobster was taken out and weighed. The MO_2, *M*DIC, *M*TAN and *M*urea-N in the tank were measured for 20 min to identify if there were differences in background parameters before and after each experiment, and the results showed no differences.

5.3.8 Parameters in SDA

Seven variables were investigated: (1) RMR; (2) peak post-prandial MO_2 (SDA_{peak}, μmol g^{-1} h^{-1}); (3) time to SDA_{peak} (h); (4) time when the post-prandial MO_2 returns to RMR (SDA duration, h), determined as two or three consecutive post-prandial MO_2 falling within 1 RMR ± 1 SE; (5) SDA magnitude (TMO_2, μmol O_2 g^{-1}), calculated by total post-prandial rise of MO_2 above the RMR (TMO_2) (McGaw & Curtis 2013); (6) E_{SDA} (energetic cost of SDA, J g^{-1}), where SDA magnitude was converted to energy; (7) SDA coefficient (C_{SDA}, %), calculated by dividing E_{SDA} by the energy in the ingested feed (J g^{-1}) (Wang *et al.* 2019a).

Sagmariasus verreauxi E_{SDA} was investigated using three indirect calorimetric approaches (Brafield 1985). The first two are simplified traditional approaches that multiply measured TMO_2 (mg g^{-1}) and an empirical oxycalorific coefficient (Q_{ox}) of 13.84 J mg^{-1} O_2, or a composite Q_{ox} calculated based on the method outlined by Brafield (1985) and Wang *et al.* (2021), and dependent on the feed chemical composition (Carter & Brafield 1991). The third is the stoichiometric bioenergetic approach, where E_{SDA} (J g^{-1}) = 11 × TMO_2 + 2.6 × $TMCO_2$ - 9.5 × $TMNH_3$ - 2.44 × T*M*urea, where $TMCO_2$, $TMNH_3$ and T*M*urea represent the accumulated CO_2, NH_3 and urea excretion during SDA, respectively, and are expressed as mg g^{-1} (Gelineau *et al.* 1998; Wang *et al.* 2021).

5.3.9 Instantaneous metabolic energy substrate use calculation

The calculation of instantaneous metabolic energy substrate use has been detailed by Wang *et al.* (2021). Briefly, the fraction of aerobic energy substrate use supplied by protein (amino acid) (P), lipid (L) and carbohydrate (C) was calculated as:

$P = NQ/0.27$ (1)

$P + L + C = 1.0$ (2)

$RQ = (m - 0.71) \times NQ/0.27 + 0.29 \times C + 0.71$ (3)

Where 0.27 is the theoretical maximum nitrogen quotient (NQ) when protein (amino acid) is the only substrate being completely oxidized under aerobic conditions; m is the aerobic respiratory quotient (RQ) for protein (amino acid) oxidation, determined by 0.96 × TAN% + 0.83 × urea-N%, where 0.96 and 0.83 are the aerobic RQ for protein (amino acid) oxidation when ammonia and urea are the unique nitrogenous end-products, respectively; TAN% and urea-N% represent the contribution of *M*TAN and *M*urea-N to *M*TN (total nitrogenous excretion), respectively. The NQ was calculated as *M*TN/*M*O_2, and the RQ calculated as *M*DIC/*M*O_2, where *M*TN, *M*O_2 and *M*DIC were expressed as μmol g^{-1} h^{-1} (Wang *et al.* 2021).

5.3.10 Whole-body protein synthesis

Ammonia from the 4 L collected seawater was distilled into boric acid to form ammonium borate. Full details of ammonia distillation were given by Carter *et al.* (1994b) with modification. Briefly, the acidified sample was distilled with 20 anti-bump granules and 80 mL mixture of 8 M NaOH and 0.1 M EDTA. Following distillation, ammonia was trapped as ammonium borate into 10 mL of 1 M boric acid. The ammonium borate was stored at -20 °C and lyophilized (freeze-dried, FD) using a freeze dryer (FDA5508, Ilshin Lab Co., Ltd., Korea) at -37 °C for 5 days to constant weight. The FD ammonium borate samples were used to determine ^{15}N enrichment (expressed as atom percent excess, APE) of ammonia. Whole-body

protein synthesis (WBPS, mg g^{-1} day^{-1}) was calculated based on the ^{15}N enrichment of ammonia, using the endpoint stochastic model (Carter *et al.* 1994b; McCarthy *et al.* 2016). Following the WBPS determination, the whole-body fractional protein synthesis rate (k_s, % day^{-1}) was determined using the whole-body protein content data as described by Fraser *et al.* (1998).

5.3.11 Chemical analysis

5.3.11.1 Analysis of chemical composition in feeds, Spirulina, faeces, and whole-body lobster

Feeds, test ingredient *Spirulina*, and faecal samples were FD to constant weight before analysis. Whole-body lobsters were homogenized using a silent cutter (MSK 760-II, Mado, Germany) and three subsamples from each lobster were FD to constant weight. Each FD sample was then finely ground to homogenous powders using a mortar and pestle. For feed and whole-body lobster samples, the content of dry matter (DM), ash, CP, total lipid (TL) and carbohydrate (Carb), and gross energy (GE) were determined. For *Spirulina* and faecal samples, DM and CP were determined.

The DM content of the FD samples was measured gravimetrically following drying at 105 °C for 24 h (AOAC 1995). Ash content was determined after incineration at 600 °C for 2 h with a combustion oven (AOAC 1995). All remaining composition analysis was performed based on the FD samples and corrected for DM. Crude protein content was determined after measuring the elemental nitrogen (N) composition in the FD samples using flash combustion isotope ratio mass spectrometry (FCIRMS) (Vario PYRO cube coupled to Isoprime100 mass spectrometer, Elementar Analysensysteme GmbH, Hanau, Germany) at the Central Science Laboratory (CSL), University of Tasmania. A nitrogen-to-protein conversion factor of 6.25 × N was used to calculate the CP content (Codabaccus *et al.* 2020). Total lipid in the FD samples was extracted in a mix of dichloromethane, methanol and Milli-Q water at a volume ratio of

1:1:0.9. The extracted TL was then dried under a stream of nitrogen gas. Thereafter, the TL content was measured gravimetrically (Codabaccus *et al.* 2020). Carbohydrate content in the FD samples was calculated by 100 - (CP + TL + ash) (Codabaccus *et al.* 2020). Gross energy in the FD samples was determined using an oxygen-bomb calorimeter (Parr 6400, Parr Instrument Company, Moline Illinois, USA) (Codabaccus *et al.* 2020).

5.3.11.2 Analysis of ^{15}N enrichment

The ^{15}N enrichment in the experimental feeds and ammonium borate samples was measured by running samples of known nitrogen enrichment alongside the experimental samples using FCIRMS (Carter *et al.* 1994b; Fraser *et al.* 1998; Martin *et al.* 2003). Briefly, 1 mg of FD experimental feeds or 5 mg of FD ammonium borate were packed into tin capsules (8 mm × 5 mm, Europa Scientific Ltd., Crewe, UK), then closed and compressed with tweezers. The ^{15}N enrichment in the experimental feeds in CP_{40}, CP_{50} and CP_{60} treatments was 1.3, 1.1 and 0.89 APE, respectively.

5.3.11.3 Analysis of the proportion of the inert digestibility marker

The proportion of Y_2O_3 in the FD samples (feeds and faeces) was determined by digestion. Briefly, 0.5 g FD samples were digested with 2 mL of nitric acid (70%; w/v) overnight at room temperature, then heated to 100 °C for 2 h and left to cool. Thereafter, 1 mL of hydrogen peroxide (30%; w/v) was added and reheated to 100 °C for 2 h. Digested samples were diluted with milli-Q water to a total volume of 5 mL, and then analyzed using a high-resolution inductively coupled plasma mass spectrometer (Thermo Fisher Scientific ELEMENT 2 HR-ICP-MS, MA, USA) at the CSL (Codabaccus *et al.* 2020).

5.3.11.4 Analysis of nitrogenous and total dissolved inorganic carbon excretion

The salicylate-hypochlorite method was used for *M*TAN determination (Bower & Holm-Hansen 1980; Wilkie *et al.* 2017). Aeration did not cause ammonia loss from the tank (Carter *et al.* 1994b; Fraser *et al.* 1998). The diacetyl monoxime method (Chen *et al.* 2015) with modification to increase sensitivity (Alam *et al.* 2017) was used to determine *M*urea-N. An infrared detection method using a DIC analyzer (AS-C3, Apollo SciTech, Newark, USA) was used to determine *M*DIC (Müller *et al.* 2017; Wang *et al.* 2021).

5.3.12 Data analysis

All figures were plotted using SigmaPlot (Version 12.5, Systat Software, San Jose, USA). All statistical analysis was performed using SPSS Statistics Software (Version 24, IBM Corporation, New York, USA). Before statistical analyses normality tests were carried out via Kolmogorov-Smirnov test, followed by the verification of homogeneity of variances via Bartlett's test. Homogeneous data were compared using t-tests and one-way analysis of variance (ANOVA), heterogeneous data were compared using the Kruskal-Wallis test. The relationship between the cumulative rate of ^{15}N-labeled ammonia excretion after feeding (ce*, %, expressed as the percentage of ^{15}N in the assimilated feed) and time (t) was estimated using a nonlinear regression model and described by $ce^* = a(1-e^{-bt})$, where a and b are constants (Carter *et al.* 1994b; McCarthy *et al.* 2016). Differences in ce* over time in each treatment were examined using a one-way repeated-measures ANOVA to identify the period of constant ^{15}N-labeled ammonia excretion, followed by post-hoc Tukey HSD test to enable the WBPS calculation to be made with the least overestimation (Fraser *et al.* 1998; McCarthy *et al.* 2016). A probability of $P < 0.05$ was considered significant in all analyses. All data were expressed as mean ± SE, except that the chemical composition, the AD of *Spirulina* protein, and the fractional data of instantaneous metabolic energy substrate use were presented as mean values.

The first step to calculate metabolic energy substrate use was to filter the outliers of MO_2, *M*DIC, *M*TAN and *M*urea-N, possibly due to lobster spontaneous activity (Zimmer-Faust *et al.* 1996; Briceño *et al.* 2018), using the interquartile range (IQR), the most commonly used outlier-resistant method in aquatic ectotherms (Helsel & Hirsch 1992; Pinkiewicz *et al.* 2011; Hu *et al.* 2019).

5.4 Results

5.4.1 Spirulina and lobster chemical composition and apparent digestibility of Spirulina protein

The dry matter, crude protein, total lipid, carbohydrate, ash and gross energy in *Spirulina* were 97.0%, 70.7%, 7.0%, 13.8%, 8.5% and 21.1 kJ g^{-1} DM, respectively; and 28.4%, 58.4%, 10.4%, 7.9%, 23.3% and 17.5 kJ g^{-1} DM, respectively, in whole-body *S. verreauxi*. The apparent digestibility of *Spirulina* protein was 52.9%.

5.4.2 Parameters in SDA

There were no differences in RMR, SDA_{peak}, time to SDA_{peak}, or SDA duration among treatments (Table 5. 3, Figure 5. 1). The E_{SDA} evaluated by different approaches was similar in each treatment (Table 5. 3). The SDA magnitude, E_{SDA} and C_{SDA} in the CP_{60} treatment were higher ($P < 0.05$) compared to the CP_{40} treatment, and there were no differences between CP_{40} and CP_{50} or between CP_{50} and CP_{60} treatments (Table 5. 3).

Table 5. 3 Characteristics of the specific dynamic action (SDA) response in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively

Parameters	CP_{40}	CP_{50}	CP_{60}
RMR (μmol g^{-1} h^{-1})	0.88 ± 0.20	0.89 ± 0.16	0.86 ± 0.10
SDA_{peak} (μmol g^{-1} h^{-1})	2.63 ± 0.52	2.24 ± 0.23	2.91 ± 0.22
Time to SDA_{peak} (h)	6 ± 1.86	10.33 ± 2.99	11.67 ± 2.70
SDA duration (h)	64 ± 8	72 ± 0	66 ± 6
SDA magnitude (μmol g^{-1})	12.82 ± 1.05^{a}	17.32 ± 1.79^{ab}	18.60 ± 2.00^{b}
E_{SDA} (J g^{-1}) calculated by the empirical Q_{ox} approach	5.68 ± 0.46^{a}	7.67 ± 0.79^{ab}	8.24 ± 0.89^{b}
E_{SDA} (J g^{-1}) calculated by the composite Q_{ox} approach	5.74 ± 0.47^{a}	7.65 ± 0.79^{ab}	8.10 ± 0.87^{b}
E_{SDA} (J g^{-1}) calculated by the stoichiometric approach	5.81 ± 0.40^{a}	7.54 ± 0.73^{ab}	8.53 ± 0.87^{b}
C_{SDA} (%)	1.80 ± 0.15^{a}	2.45± 0.25^{ab}	2.59 ± 0.28^{b}

RMR, routine metabolic rate; E_{SDA}, SDA magnitude converted to energy; Q_{ox}, oxycalorific coefficient; C_{SDA}, SDA coefficient. All data represent mean ± standard error (SE) of 6 individuals. Different superscripts (a, b) in each row indicate significant differences among treatments (One-way ANOVA, $P < 0.05$)

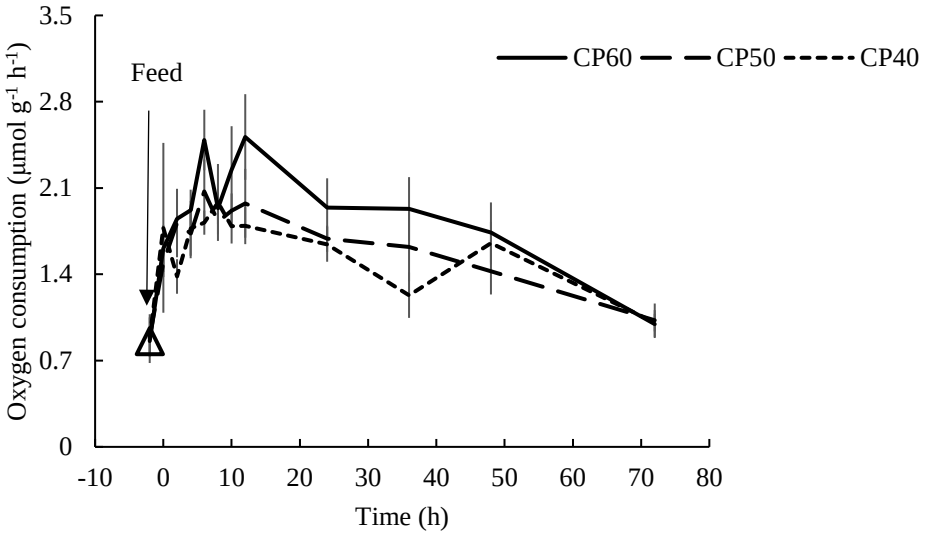

Figure 5. 1 Oxygen consumption (MO_2) in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively. The triangle indicates the routine metabolic rate (RMR). The first post-prandial MO_2 was recorded at 0 h. All data represent mean ± standard error (SE) of 6 individuals.

5.4.3 Nitrogenous excretion and nitrogen quotient

There were no differences in routine *M*TAN, *M*TAN_{peak}, time to *M*TAN_{peak}, or *M*TAN duration among treatments (Table 5. 4, Figure 5. 2). The *M*TAN magnitude in the CP_{60} treatment during SDA was higher ($P < 0.05$) compared to the CP_{40} treatment, and there were no differences between CP_{40} and CP_{50} or between CP_{50} and CP_{60} treatments (Table 5. 4). There were no differences in routine *M*urea-N, *M*urea-N_{peak}, time to *M*urea-N_{peak}, or *M*urea-N duration among treatments (Table 5. 4, Figure 5. 3). The *M*urea-N magnitude in CP_{50} and CP_{60} treatments during SDA was higher ($P < 0.05$) compared to the CP_{40} treatment (Table 5. 4).

Table 5. 4 Characteristics of total ammonia-N (*M*TAN) and urea-N (*M*urea-N) excretion in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively

Parameters	CP_{40}	CP_{50}	CP_{60}
Routine *M*TAN (μmol g^{-1} h^{-1})	0.048 ± 0.01	0.035 ± 0.01	0.065 ± 0.01
$MTAN_{peak}$ (μmol g^{-1} h^{-1})	0.31 ± 0.05	0.28 ± 0.06	0.35 ± 0.08
Time to $MTAN_{peak}$ (h)	28 ± 4	19.33 ± 5.23	25 ± 6.96
*M*TAN duration (h)	62 ± 6.51	46 ± 5.73	54 ± 6
*M*TAN magnitude (μmol g^{-1})	1.62 ± 0.14^{a}	1.88 ± 0.15^{ab}	2.37 ± 0.21^{b}
Routine *M*urea-N (nmol g^{-1} h^{-1})	0.0015 ± 0.00032	0.0011 ± 0.00030	0.0012 ± 0.00036
*M*urea-N_{peak} (nmol g^{-1} h^{-1})	0.0042 ± 0.00064	0.0060 ± 0.0017	0.0077 ± 0.0014
Time to *M*urea-N_{peak} (h)	12.33 ± 4.01	7.67 ± 5.74	13 ± 2.41
*M*urea-N duration (h)	22 ± 4.82	24 ± 6.20	34 ± 3.69
*M*urea-N magnitude (nmol g^{-1})	0.0075 ± 0.00042^{a}	0.013 ± 0.0025^{b}	0.022 ± 0.0030^{b}

All data represent mean ± standard error (SE) of 6 individuals. Different superscripts (a, b) in each row indicate significant differences among treatments (One-way ANOVA, $P < 0.05$)

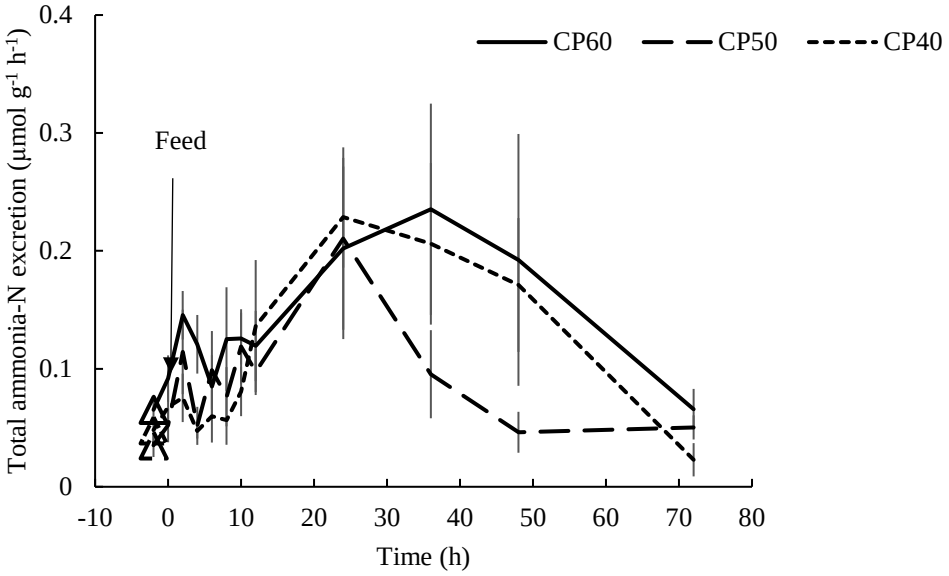

Figure 5. 2 Total ammonia-N excretion (*M*TAN) in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively. The triangle indicates the routine *M*TAN. The first post-prandial *M*TAN was recorded at 0 h. All data represent mean ± standard error (SE) of 6 individuals.

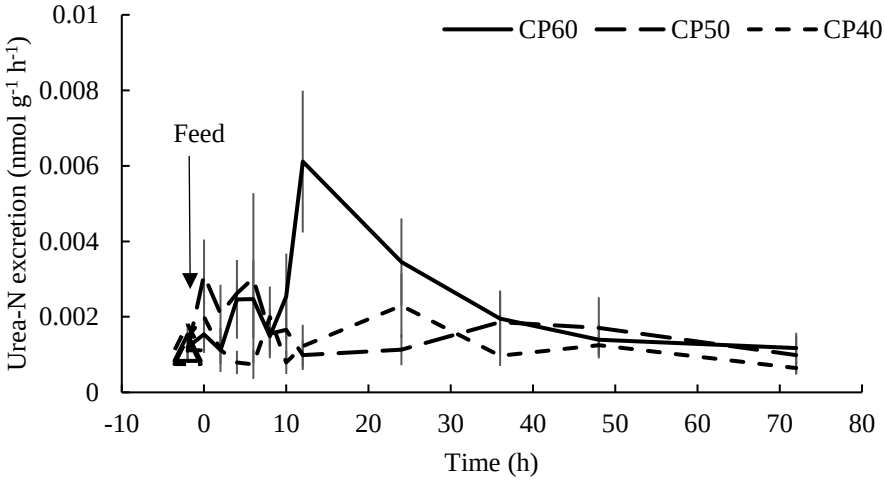

Figure 5. 3 Urea-N excretion (*M*urea-N) in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively. The triangle indicates the routine *M*urea-N. The first post-prandial *M*urea-N was recorded at 0 h. All data represent mean ± standard error (SE) of 6 individuals.

In the CP_{60} treatment, the routine NQ was higher (Paired t-tests, $P = 0.024$) than that at 6 h post-feeding, and there were no differences between the routine and other post-prandial NQ (Figure 5. 4). There were no differences between the routine and post-prandial NQ in the CP_{50} treatment at different time periods (Figure 5. 4). In the CP_{40} treatment, the routine NQ was higher (Paired t-tests, $P = 0.032$) than that at 6 h post-feeding, and lower than that at 24 and 36 h post-feeding (Paired t-tests, $P = 0.012$ and 0.047, respectively) (Figure 5. 4).

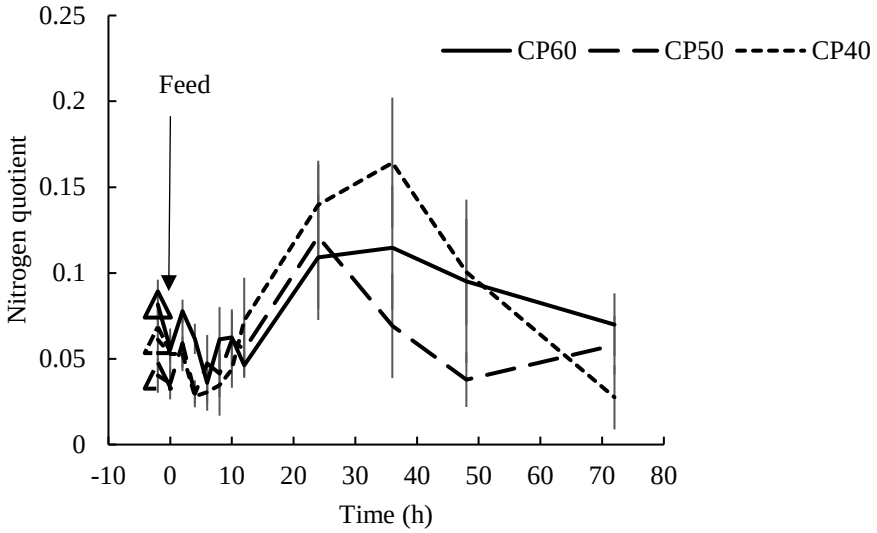

Figure 5. 4 Nitrogen quotient (NQ) in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively. The triangle indicates

the routine NQ. The first post-prandial NQ was recorded at 0 h. All data represent mean ± standard error (SE) of 6 individuals.

5.4.4 Total dissolved inorganic carbon excretion and respiratory quotient

There were no differences in routine *M*DIC, $MDIC_{peak}$, time to $MDIC_{peak}$, or *M*DIC duration among treatments (Table 5. 5, Figure 5. 5). The *M*DIC magnitude in the CP_{60} treatment during SDA was higher ($P < 0.05$) compared to the CP_{40} treatment, and there were no differences between CP_{40} and CP_{50} or between CP_{50} and CP_{60} treatments (Table 5. 5). There were no differences between routine and post-prandial RQ at different time periods in all treatments (Figure 5. 6).

Table 5. 5 Characteristics of total dissolved inorganic carbon excretion (*M*DIC) in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively

Parameters	CP_{40}	CP_{50}	CP_{60}
Routine *M*DIC (μmol g^{-1} h^{-1})	0.66 ± 0.13	0.69 ± 0.11	0. 71 ± 0.12
$MDIC_{peak}$ (μmol g^{-1} h^{-1})	2.15 ± 0.20	2.61 ± 0.43	2.50 ± 0.23
Time to $MDIC_{peak}$ (h)	17.67 ± 6.60	14 ± 6.85	18.33 ± 5.64
*M*DIC duration (h)	50 ± 7.85	46 ± 6.51	62 ± 6.51
*M*DIC magnitude (μmol g^{-1})	13.25 ± 1.25^{a}	14.84 ± 2.66^{ab}	20.12 ± 2.31^{b}

All data represent mean ± standard error (SE) of 6 individuals. Different superscripts (a, b) in each row indicate significant differences among treatments (One-way ANOVA, $P < 0.05$)

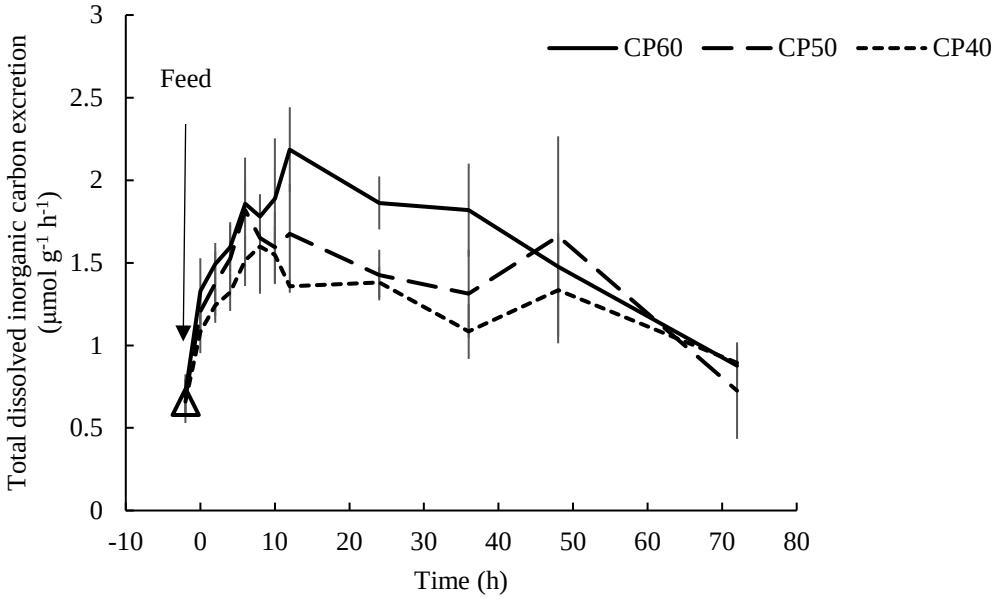

Figure 5. 5 Total dissolved inorganic carbon excretion (*M*DIC) in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively. The triangle indicates the routine *M*DIC. The first post-prandial *M*DIC was recorded at 0 h. All data represent mean ± standard error (SE) of 6 individuals.

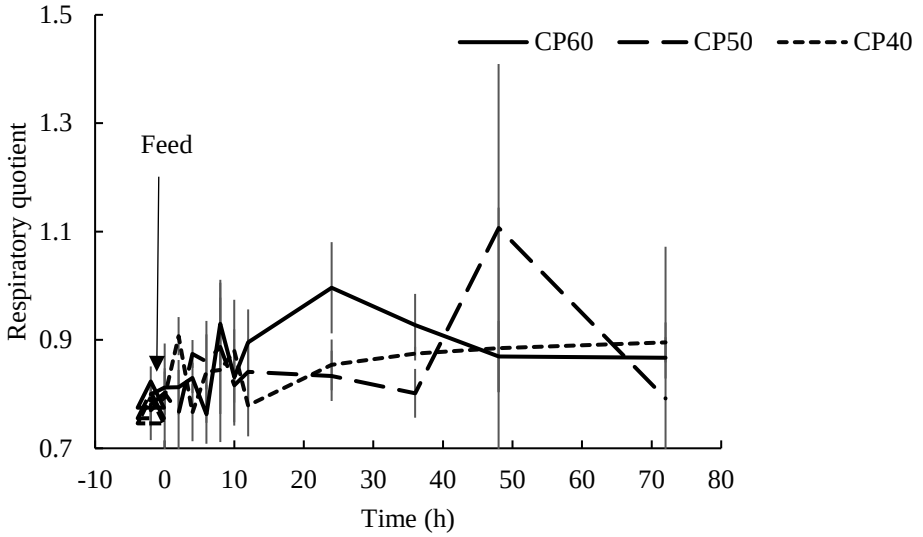

Figure 5. 6 Respiratory quotient (RQ) in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively. The triangle indicates the routine RQ. The first post-prandial RQ was recorded at 0 h. All data represent mean ± standard error (SE) of 6 individuals.

5.4.5 Instantaneous metabolic energy substrate use

In the CP_{60} treatment, lipid was the main metabolic energy substrate in 3-day fasted lobsters, followed by protein (amino acid), and carbohydrate oxidation played a minor role (Figure 5. 7). During the 72 h post-feeding, the average fractional contributions of protein (amino acid), lipid and carbohydrate to metabolic use were 26.7%, 39.6% and 33.7%, respectively. In the first 36 h after feeding, although there was some fluctuation among time-blocks there was a trend for increased protein (amino acid) and carbohydrate oxidation and decreased lipid oxidation. From 36 to 48 h post-feeding, protein (amino acid) and carbohydrate oxidation decreased and lipid oxidation increased. From 48 to 72 h post-feeding, protein (amino acid) and lipid oxidation decreased and carbohydrate oxidation increased (Figure 5. 7).

In the CP_{50} treatment, lipid was preferentially oxidized for energy production in 3-day fasted lobsters, followed by protein (amino acid), and carbohydrate oxidation played a minor role (Figure 5. 7). During the 72 h post-feeding, the average fractional contributions of protein (amino acid), lipid and carbohydrate to metabolic use were 19.6%, 54.8% and 25.6%, respectively. In the first 12 h after feeding, protein (amino acid) oxidation remained stable, lipid oxidation decreased and carbohydrate oxidation increased. From 12 to 24 h post-feeding, protein (amino acid) and lipid oxidation increased and carbohydrate oxidation decreased. From 24 to 72 h post-feeding, protein (amino acid) oxidation decreased, lipid oxidation increased and carbohydrate oxidation remained low (Figure 5. 7).

In the CP_{40} treatment, carbohydrate was the predominant metabolic energy substrate in 3-day fasted lobsters, followed by protein (amino acid), and lipid oxidation played a minor role (Figure 5. 7). During the 72 h post-feeding, the average fractional contributions of protein (amino acid), lipid and carbohydrate to metabolic use were 22.4%, 44.8% and 32.8%, respectively. Protein (amino acid) contribution decreased in the first 8 h after feeding, then increased from 8 to 36 h post-feeding, and thereafter decreased in 36-72 h post-feeding. Lipid contribution fluctuated during the SDA process. The lowest lipid contribution (0.9%) was at 2 h post-feeding and the highest (81.4%) at 12 h. Carbohydrate contribution fluctuated in 0-8 h post-feeding, then decreased from 8 to 24 h post-feeding, and thereafter increased in 24-72 h post-feeding (Figure 5. 7).

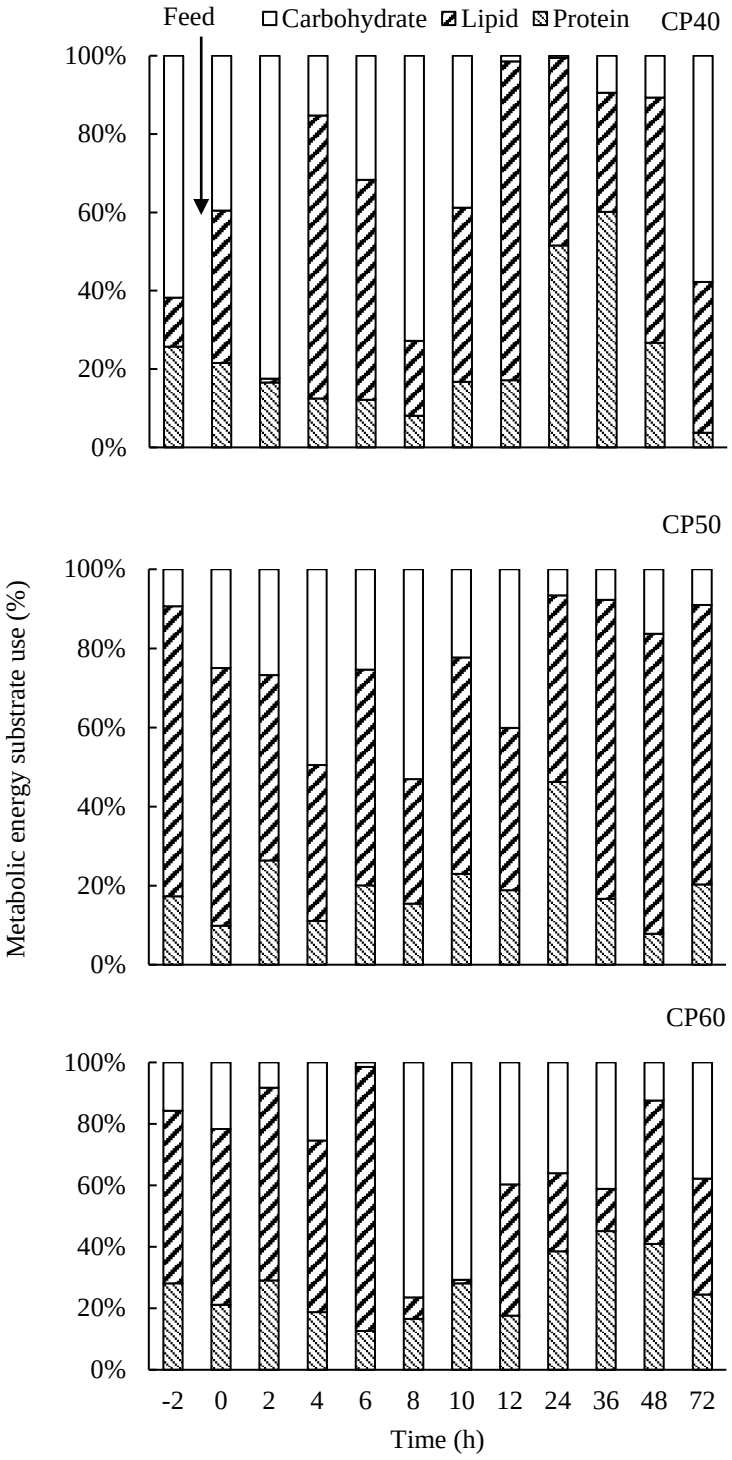
Feed
Carbohydrate
Lipid
Protein
CP40
CP50
CP60
Metabolic energy substrate use (%)
100%
80%
60%
40%
20%
0%
-2
0
2
4
6
8
10
12
24
36
48
72
Time (h)

Figure 5. 7 Instantaneous metabolic energy substrate use in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively. All data represent mean values of 6 individuals.

5.4.6 Whole-body protein synthesis

The cumulative rate of ^{15}N-labeled ammonia excretion after feeding (ce*) in all treatments had significant negative exponential regressions over time (t) (Figure 5. 8). In all treatments, ce* was different among 12-72 h post-feeding (one-way repeated-measures ANOVA, $P < 0.05$) and similar between 48 and 72 h post-feeding (Tukey HSD test, $P > 0.05$). Therefore, the whole-body protein synthesis (WBPS) and fractional protein synthesis rate (k_s) were estimated using data collected over 48 h post-feeding, during which the WBPS and k_s in CP_{60} and CP_{50} treatments were higher ($P < 0.05$) compared to the CP_{40} treatment (Table 5. 6 and 5. 7). Over 24 h post-feeding, the WBPS and k_s in CP_{60} and CP_{50} treatments were also higher ($P < 0.05$) than that in the CP_{40} treatment. In all treatments, the WBPS and k_s estimated over 24 h post-feeding in all treatments were two times higher (Paired t-tests, $P < 0.05$) than that over 48 h post-feeding (Table 5. 6 and 5. 7).

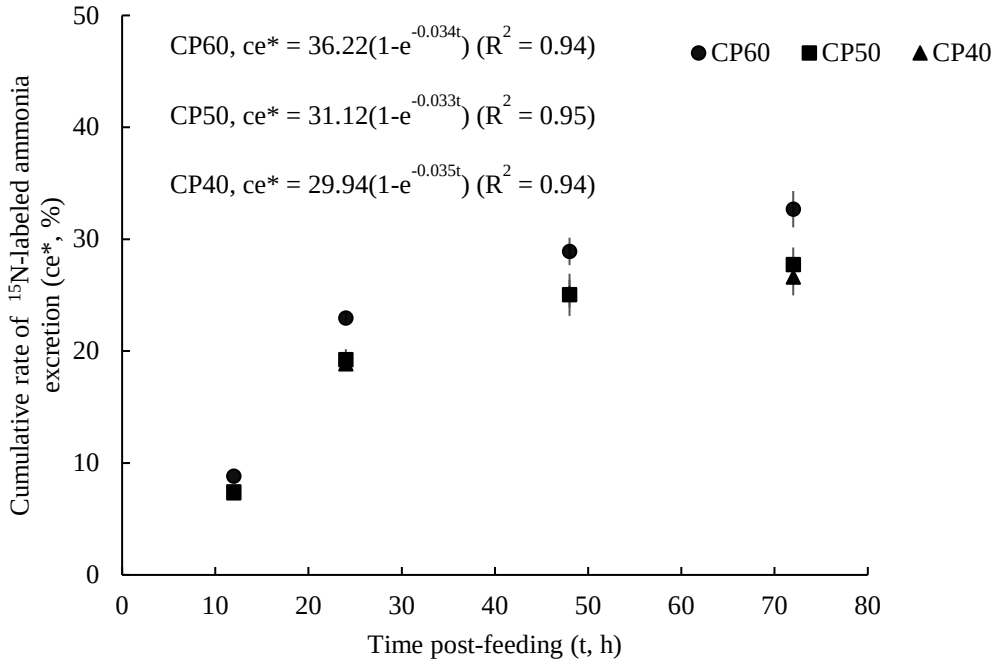

Figure 5. 8 Cumulative rate of ^{15}N-labeled ammonia excretion (ce*, %, expressed as the percentage of ^{15}N in the assimilated feed) for *Sagmariasus verreauxi* fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively. All data represent mean ± standard error (SE) of 6 individuals.

Table 5. 6 Whole-body protein synthesis (WBPS, mg g^{-1} day^{-1}) in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively

Time	CP_{40}	CP_{50}	CP_{60}
WBPS estimated over 24 h post-feeding	0.47 ± 0.007^{a}	0.53 ± 0.028^{b}	0.54 ± 0.011^{b}
WBPS estimated over 48 h post-feeding	$0.23 \pm 0.004^{a*}$	$0.26 \pm 0.007^{b*}$	$0.26 \pm 0.006^{b*}$

All data represent mean ± standard error (SE) of 6 individuals. Different superscripts (a, b) in each row indicate significant differences among treatments (One-way ANOVA, $P < 0.05$).

The asterisk (*) in each column indicates significant differences in each treatment (Paired t-tests, $P < 0.05$)

Table 5. 7 Whole-body fractional protein synthesis rate (k_s, % day^{-1}) in juvenile *Sagmariasus verreauxi*. Lobsters were reared at 21 °C and fed ^{15}N-labeled feeds at 1.5% body weight at three crude protein levels: 40%, 50% and 60%, corresponding to CP_{40}, CP_{50} and CP_{60} treatments, respectively

Time	CP_{40}	CP_{50}	CP_{60}
k_s estimated over 24 h post-feeding	0.34 ± 0.005^{a}	0.38 ± 0.019^{b}	0.39 ± 0.007^{b}
k_s estimated over 48 h post-feeding	$0.17 \pm 0.003^{a*}$	$0.19 \pm 0.005^{b*}$	$0.19 \pm 0.004^{b*}$

All data represent mean ± standard error (SE) of 6 individuals. Different superscripts (a, b) in each row indicate significant differences among treatments (One-way ANOVA, $P < 0.05$). The asterisk (*) in each column indicates significant differences in each treatment (Paired t-tests, $P < 0.05$)

5.5 Discussion

This study for the first time in an aquatic ectotherm successfully combined a stoichiometric bioenergetic approach used to evaluate metabolic substrate use and SDA with an endpoint stochastic model used to assess whole-body protein synthesis (WBPS). Protein synthesis and SDA magnitude increased with dietary protein. The metabolic use of each major energy substrate varied at different time periods during the SDA process, and the average contribution of protein (amino acid) oxidation was lowest in the treatment with 50% dietary protein. The approaches and findings from this comprehensive study extend the understanding of nutritional physiology and bioenergetics in crustaceans to support the continuous development and refinement of sustainable and cost-effective aquafeeds (Clifford & Brick 1979; National Research Council 2011; Carter & Mente 2014).

5.5.1 Respiratory gas exchange and nitrogenous excretion

The energetic cost of SDA (E_{SDA}) estimated by three different approaches (two simplified traditional approaches and one stoichiometric bioenergetic approach) within every treatment was similar and consistent with other studies in aquatic ectotherms (e.g. Musisi 1984; Gelineau *et al.* 1998). These findings suggest that traditional approaches would provide adequate information on E_{SDA}; however, more studies on a variety of aquatic ectotherms are required to further identify whether the estimation is comparable among approaches (Brafield 1985). As with other decapods fed isoenergetic feeds (Clifford & Brick 1978; Rosas *et al.* 1996; Taboada *et al.* 1998), lobster SDA magnitude increased with dietary protein. A growing body of evidence suggests that the major part of SDA in aquatic ectotherms is attributable to WBPS (Brown & Cameron 1991a; Robertson *et al.* 2001a; Wang *et al.* 2019a). Hence, the increased SDA magnitude with increased dietary protein indicates increased WBPS.

The SDA coefficient (C_{SDA}) is the most informative parameter in SDA, reflecting the metabolic use of digestible energy and dietary protein (Robertson *et al.* 2001a; Radford *et al.* 2004; Secor 2009). For example, Ai and Xie (2006) showed that the C_{SDA} in carnivorous southern catfish, *Silurus meridionalis*, increased with increasing dietary soybean protein while the growth performance decreased, suggesting that imbalanced amino acids due to the replacement of fish meal by soybean meal may result in more energy used for metabolism. Carter and Houlihan (2001) presented a model to explain how different levels of dietary protein and essential amino acid supply, ranging from deficiency to excess, influence the stimulation of protein synthesis, amino acid oxidation and energy expenditure, and ultimately protein retention efficiency. Therefore, the combination of C_{SDA} with nitrogenous excretion further improves the understanding of dietary protein use (Hewitt & Irving 1990). For example, increased C_{SDA} and nitrogenous excretion indicates increased protein metabolism and decreased efficiency of transformation of ingested nutrients to growth (Rosas *et al.* 1996; Taboada *et al.* 1998; Palafox *et al.* 2017). In contrast, optimal dietary protein may result in low C_{SDA} and nitrogenous excretion (Hewitt & Irving 1990). Rosas *et al.* (1996) found the C_{SDA} and nitrogenous excretion in the postlarvae of the shrimp *Penaeus notiatis* increased when dietary protein increased from 40% to 65%, concluding that 40% dietary protein may meet the postlarval requirement. Taboada *et al.* (1998) showed that the C_{SDA} and nitrogenous excretion in juvenile *P. setiferus* were lowest when dietary protein was 30% and increased when dietary protein was below or above 30%, concluding that 30% dietary protein may spare dietary protein more efficiently. Similarly, Palafox *et al.* (2017) found the C_{SDA} and nitrogenous excretion in the subadult white shrimp, *Litopenaeus vannamei*, increased when dietary protein increased from 20% to 50%, concluding that 20% dietary protein may spare dietary protein more efficiently. As with Rosas *et al.* (1996) and Palafox *et al.* (2017) fed decapods isoenergetic feeds, C_{SDA} and post-prandial ammonia excretion in the present study increased with dietary

protein. In contrast, compared with previous research in the same or related spiny lobster species, *Jasus edwardsii*, fed squid rich in protein (Crear & Forteath 2000; Radford *et al.* 2004; Wang *et al.* 2019a), the C_{SDA} in the present study was low. Since C_{SDA} is independent of body weight (Secor 2009; McGaw & Curtis 2013), the low C_{SDA} in the present study indicates efficient use of the provided formulated feeds in comparison to squid feeds (Rosas *et al.* 1996; Taboada *et al.* 1998; Ai & Xie 2006).

In the present study, ammonia excretion was similar to other decapod crustaceans (Regnault 1987; Koshio *et al.* 1993). Ammonia was the major nitrogenous end-product and urea contribution was negligible and there was a large 4-8-fold increase following feeding (Wickins 1985; Zoutendyk 1987; Radford *et al.* 2004). The magnitude of nitrogenous and CO_2 excretion during SDA in the present study increased with dietary protein, indicating high-protein feeds provided substrates for both protein synthesis and energy to fuel protein synthesis (Hewitt & Irving 1990; Koshio *et al.* 1993; Carter & Houlihan 2001).

5.5.2 Metabolic energy substrate use

The investigation of instantaneous metabolic energy substrate use helps understand how aquatic ectotherms oxidize energy substrates to provide energy at different nutritional status and evaluate the dietary protein-sparing effect (Ferreira *et al.* 2019; Wang *et al.* 2021). The major energy substrates oxidized in 3-day fasted lobsters was lipid in CP_{60} and CP_{50} treatments and was carbohydrate in the CP_{40} treatment, differing from Wang *et al.* (2021) where protein (amino acid) oxidation predominated. This discrepancy could be due to the variation in previous nutritional history (Houlihan *et al.* 1990; Houlihan 1991; Carter & Mente 2014). Wang *et al.* (2021) fed *S. verreauxi* high-protein natural feed before experimentation and the present study fed formulated feeds at different dietary protein before short-term fasting. In all treatments, ingested protein (amino acid), lipid and carbohydrate were all oxidized at different

proportions at different time periods over 72 h post-feeding, indicating that suitable amounts of high-quality protein with both major non-protein energy-yielding nutrients, lipid and carbohydrate, are critical for the formulation of balanced and cost-effective feeds to spiny lobsters (Nelson *et al.* 2006; Wang *et al.* 2021). The average fractional contribution of protein (amino acid) oxidation to total energy production during the SDA process was lowest in the CP_{50} treatment and highest in the CP_{60} treatment, suggesting that the feed containing 50% crude protein, 15% total lipid and 25% carbohydrate may spare dietary protein more efficiently. There is little information on protein requirements in spiny lobsters (Glencross *et al.* 2001; Ward *et al.* 2003; Nelson *et al.* 2006) and the optimum dietary protein in juvenile *S. verreauxi* is still unknown. Long-term growth experiments by Johnston *et al.* (2003) with *J. edwardsii* showed that a feed containing 32% crude protein, 14% total lipid and 27% carbohydrate was suitable for juvenile lobster growth. In contrast, Ward *et al.* (2003) suggested that a feed containing 40% crude protein and 9% total lipid (carbohydrate content was not given) was more suitable for *J. edwardsii* growth. The higher protein requirement recorded in the present study compared with Johnston *et al.* (2003) and Ward *et al.* (2003) could be due to different dietary protein sources. The present study used casein and mussel at a ratio of 2:1, while Johnston *et al.* (2003) and Ward *et al.* (2003) used fish meal as the main protein sources, which may result in different dietary amino acid profiles (Mente *et al.* 2002). However, as with Johnston *et al.* (2003), the total lipid to carbohydrate ratio in the most efficient feed in the present study was 1:2. Hence, the stoichiometric estimation of metabolic energy substrate use suggests that the stoichiometric bioenergetic approach may provide a promising and efficient alternative to evaluate nutrient compositions.

5.5.3 Whole-body protein synthesis

The investigation of WBPS is the key to understanding daily protein-nitrogen flux in aquatic ectotherms, critical for optimizing dietary protein to formulate cost-effective aquafeeds (Hawkins 1985; Hewitt 1992; Carter & Bransden 2001). The present study, for the first time, used an endpoint stochastic model to measure WBPS in crustaceans. To determine *S. verreauxi* WBPS, the apparent digestibility of *Spirulina* protein was evaluated. As with the prawn *Macrobrachium tenellum* (Montoya-Martínez *et al.* 2018), the apparent digestibility of *Spirulina* protein for *S. verreauxi* was 53%.

Using the endpoint stochastic model to validly determine WBPS requires ^{15}N-labeled nitrogen appearing in a single major nitrogenous end-product (Hawkins 1985; Fraser *et al.* 1998; McCarthy *et al.* 2016). Most previous studies in aquatic ectotherms used ^{15}N-labeled ammonia to calculate WBPS as urea excretion was low (Hawkins 1985; Martin *et al.* 2003; McCarthy *et al.* 2016). In the present study, urea excretion was negligible. Therefore, the use of ^{15}N-labeled ammonia to calculate WBPS in *S. verreauxi* is acceptable. The validation data in the present study showed that as with other aquatic ectotherms (Hawkins 1985; Carter *et al.* 1998; Fraser *et al.* 1998), the cumulative ^{15}N-labeled ammonia excretion rates in *S. verreauxi* were constant at 48 h post-feeding.

In the present study, the WBPS estimated over 24 h post-feeding in all treatments was two times higher than that over 48 h post-feeding (0.23-0.26 mg g^{-1} day^{-1}). The decline in protein synthesis was not unexpected since the lobsters were not re-fed after receiving the ^{15}N-labeled feed (Carter *et al.* 1994b). There has been little work on post-prandial WBPS in crustaceans (Houlihan *et al.* 1990; Robertson *et al.* 2001a; Robertson *et al.* 2001b). Houlihan *et al.* (1990) showed that in the crab *Carcinus maenas* (15-18 °C, 54 g) fed at 3% BW, the WBPS ranged from 0.8 to 1.6 mg g^{-1} day^{-1} at different post-prandial times. In the isopod *Glyptonotus antarcticus* (33 g) fed at 5% BW at 0 °C, the WBPS at the SDA_{peak} was 0.13 mg

g^{-1} day^{-1} (Robertson *et al.* 2001a). In the isopod *Saduria entomon* (1 g) fed at 5% BW at 4 °C and 13°C, the WBPS at the SDA_{peak} was 0.6 and 1.0 mg g^{-1} day^{-1}, respectively (Robertson *et al.* 2001b). The large WBPS variation can be due to different experimental temperature, body weight, and feeding regimes (Carter & Bransden 2001; Mente *et al.* 2001; Robertson *et al.* 2001b). Moreover, different species may also result in various WBPS (Carter & Mente 2014). For example, the routine WBPS in *S. entomon* (Robertson *et al.* 2001b) was three times higher than that in *G. antarticus* at 4 °C (Robertson *et al.* 2001a). The whole-body protein content estimated in the present study was in line with *S. verreauxi* puerulus (Jensen *et al.* 2013a) and juvenile *J. edwardsii* (Crear & Forteath 2000; Johnston *et al.* 2003; Ward *et al.* 2003). The change of the whole-body fractional protein synthesis rate (k_s) was comparable to WBPS change among treatments.

This study was the first to examine the relationship between WBPS and dietary protein in a crustacean species. As with other aquatic ectotherms (Clifford & Brick 1978; Jobling 1983; Houlihan *et al.* 1990), *S. verreauxi* WBPS increased with dietary protein. In combination with the findings in the SDA magnitude and metabolic energy substrate use, the present study suggests that a formulated feed containing 50% crude protein can satisfy *S. verreauxi* protein requirement.

5.6 Conclusions

This study comprehensively examined the effects of dietary protein on all major components of whole-animal metabolism: respiratory gas exchange, nitrogenous excretion, SDA, metabolic energy substrate use, and whole-body protein synthesis in *S. verreauxi*, with the use of a stoichiometric bioenergetic approach and an endpoint stochastic model. Dietary protein had significant influence on routine and post-prandial nutritional physiology and bioenergetics. A balanced feed containing suitable protein (amino acid), lipid and carbohydrate is essential to

spare dietary protein for spiny lobsters, and a feed containing 50% crude protein appears to best satisfy *S. verreauxi* protein requirement. The comprehensive results advance the knowledge of how deficient and surplus dietary protein affects energy metabolism and provide approaches for fine-scale feed evaluation, critical for achieving more sustainable aquaculture through refinement of feeds with an emphasis on maximizing the use of dietary protein for growth. In future, using different indirect calorimetric approaches to estimate the energetic cost of SDA should be expanded to assess the availability of simplified traditional approaches.

Chapter 6 General discussion and conclusions

The examination of protein synthesis, SDA, and metabolic energy substrate use advances the current knowledge of animal nutritional physiology. The combined information has great applications for the evaluation of feeds and feeding regimes to support more sustainable aquaculture, contributing to achieve food security. In addition, the combined measurement is also of ecological importance in unveiling bioenergetic adaptations of wild animals to natural or anthropogenic environmental change.

This thesis presents some highly novel research in crustacean nutritional physiology, including:

- Examination of the contribution of protein synthesis to energy metabolism by using a protein synthesis inhibitor (Chapter 2 and 4);
- Determination of metabolic energy substrate use and SDA through stoichiometry (Chapter 4 and 5);
- Estimation of protein synthesis using an endpoint stochastic model and description of the relationships between SDA, protein synthesis, and dietary protein at different nutritional status (Chapter 5).

Additionally, this thesis provides a comprehensive overview of the effects of abiotic and biotic factors on RQ and stoichiometrically determined metabolic energy substrate use in fish and aquatic invertebrates and the potential use of stoichiometry in aquatic ectotherms (Chapter 3). Limitations of stoichiometry (Chapter 3, 4 and 5) and research directions relevant to protein synthesis (Chapter 2, 4 and 5) and stoichiometry (Chapter 3, 4 and 5) that warrant further investigations are also illustrated. This chapter aims to synthesize the key findings from Chapter 2 to 5, discuss how the knowledge of protein synthesis, SDA, and metabolic energy substrate use can be integrated into current research in optimizing feeds and feeding regimes

in farmed animals and unveiling physiological adaptations to the environment, and provide future directions relevant to nutrition and physiology in aquatic ectotherms.

6.1 The relationship between protein synthesis and energy metabolism

Protein synthesis is central to animal life and is energetically expensive (Whiteley *et al.* 2001a; Carter & Mente 2014). Understanding the relationship between protein synthesis and energy metabolism is ecologically important because it uncovers the metabolic adaptations to extreme environments (Marsh *et al.* 2001; Pace & Manahan 2007). For example, the embryos and larvae of Antarctic sea urchin *Sterechinus neumayeri* have high energy efficiency with low energetic costs of protein synthesis that allows them to adapt to the extreme-cold environments (Marsh *et al.* 2001; Pace & Manahan 2007). A better understanding of the relationship between protein synthesis and energy metabolism is also commercially important as it may help formulate nutritionally balanced and cost-effective feeds to enhance dietary protein-sparing effects, so that the majority of assimilated protein (amino acid) is used to improve protein synthesis retention efficiency (SRE) and therefore growth, rather than for oxidation to provide metabolic energy (Carter & Houlihan 2001). Protein synthesis and animal growth are both closely correlated with feed intake (Jobling 1983; Kaushik & Seiliez 2010; Carter & Mente 2014). Ingested energy exceeding maintenance requirements will be converted into growth, and the efficiency whereby ingested energy surpasses maintenance requirements can be determined by SDA, with the proviso that the feed is nutritionally balanced (Kiørboe *et al.* 1987; Carter & Houlihan 2001). Evidence that SDA is positively associated with growth is increasing (Jobling 1985; Carter & Brafield 1992; Pedersen 1997). As protein synthesis underpins growth in aquatic ectotherms (Houlihan *et al.* 1995a; Pedersen 1997; Kaushik & Seiliez 2010), understanding the relationship between protein synthesis and energy metabolism is central to

assessing growth potential of a feed and determining dietary protein (amino acid) requirements (Jobling 1985; Houlihan *et al.* 1993a; Carter & Mente 2014).

The use of a protein synthesis inhibitor cycloheximide provides an efficient way to investigate the relationship between protein synthesis and energy metabolism (Brown & Cameron 1991b; Houlihan 1991; Rastrick & Whiteley 2017). The decreased oxygen consumption after cycloheximide treatment represents the energetic cost of cycloheximide-sensitive protein synthesis (Pannevis & Houlihan 1992; Secor 2009). As with other crustaceans (Whiteley *et al.* 1996; Robertson *et al.* 2001b; Rastrick & Whiteley 2017), the contribution of cycloheximide-sensitive protein synthesis to *S. verreauxi* routine metabolism was low (13-28%, Chapter 2), indicating that unfed aquatic ectotherms spend less energy on protein synthesis (Jobling 1983; Carter *et al.* 1993; Sacristán *et al.* 2016). In contrast, cycloheximide-sensitive protein synthesis in fed *S. verreauxi* accounted for 96% of the SDA magnitude (Chapter 2 and 4), comparable with other ectotherms (71-100%, Brown & Cameron 1991b; Thor 2000; McCue *et al.* 2005). For example, the channel catfish *Ictalurus punctatus* showed a significant SDA response after infusion of a meal of essential amino acids, while cycloheximide-treated individuals did not show an SDA response, demonstrating a cause-and-effect relationship between protein synthesis and SDA (Brown & Cameron 1991b). Thor (2000) incubated copepods *Acartia tonsa* and *Calanus finmarchicus* with cycloheximide and showed that the SDA magnitude was reduced by 93% in *A. tonsa* and 88% in *C. finmarchicus* compared with non-cycloheximide-treated individuals. Similarly, McCue *et al.* (2005) showed that the inhibition of protein synthesis with cycloheximide caused a 71% decrease in SDA in python *Python molurus*. The high contribution of cycloheximide-sensitive protein synthesis to SDA indicates that SDA is primarily a post-absorptive process (Jobling & Davies 1980; Lurman *et al.* 2013), and that protein synthesis is one of the most energetically expensive cellular processes (Whiteley *et al.* 2001a; Mente *et al.* 2003). These estimations of the contribution of

cycloheximide-sensitive protein synthesis to SDA were higher compared with other post-prandial aquatic ectotherms where the contribution (20-44%) was estimated based on the theoretical minimum cost of peptide bond formation and determined rates of protein synthesis and total oxygen consumption (Houlihan *et al.* 1990; Lyndon *et al.* 1992; Carter *et al.* 1993). It has been suggested that the use of the theoretical cost has many concomitant sources of error as this method is dependent on many assumptions (Reeds *et al.* 1985; Houlihan *et al.* 1995b; Fraser & Rogers 2007). For example, the measurement assumes that 1 molecule of consumed oxygen is linked to the synthesis of 6 ATP molecules, 5 ATP molecules are required to incorporate one amino acid into protein to form one peptide bond, and that the mean molecular weight of one amino acid is 110 daltons. These assumptions result in an estimated theoretical minimum cost of 8.3 mmol O_2 per gram of protein synthesized (Reeds *et al.* 1985). However, the theoretical minimum cost may vary because of the differences in amino acid composition and mean molecular weight (Pace & Manahan 2007; Rastrick & Whiteley 2017). Another reason for the different contributions can be that the estimated 71-100% contribution was calculated based on the increased oxygen consumption after feeding (SDA) (Brown & Cameron 1991b; Thor 2000; McCue *et al.* 2005), while the estimated 20-44% contribution was calculated based on the total oxygen consumption after feeding (Houlihan *et al.* 1990; Lyndon *et al.* 1992; Carter *et al.* 1993). Therefore, the energetic cost of protein synthesis in the latter studies would be higher than 20-44% if SDA is used (Houlihan *et al.* 1990; Lyndon *et al.* 1992; Carter *et al.* 1993). Regarding the large disparities in reported energetic costs of protein synthesis in aquatic ectotherms, carefully validated studies in future are necessary to resolve these inconsistencies (Fraser & Rogers 2007).

There has been little research on post-prandial whole-body protein synthesis in crustaceans (0.13-1.6 mg g^{-1} day^{-1}, Houlihan *et al.* 1990; Robertson *et al.* 2001a; Robertson *et al.* 2001b). The post-prandial whole-body protein synthesis in *S. verreauxi* estimated by the

endpoint stochastic model (0.23-0.26 mg g^{-1} day^{-1}, Chapter 5) fell within the range and at the lower end. The large range can partly be explained by the combination of factors in each experiment including environmental temperature, body weight, feeding regimes and sampling time after feeding (Mente *et al.* 2001; Whiteley *et al.* 2001a; Carter & Mente 2014). For example, at the SDA peak the whole-body protein synthesis in the Baltic isopod *Saduria entomon* reared at 13 °C was two times higher compared to *S. entomon* at 4 °C (Robertson *et al.* 2001b). In addition, species variation may also result in different protein synthesis (Carter & Mente 2014). For example, the routine whole-body protein synthesis in *S. entomon* (Robertson *et al.* 2001b) was three times higher than that in *G. antarticus* at 4 °C (Robertson *et al.* 2001a). This study was the first to use the endpoint stochastic model to assess protein synthesis over a complete daily cycle. This method reduces the variation among different times of a day due to feeding and/or natural circadian rhythms, thus ensuring an integrated description of protein metabolism in aquatic ectotherms (Houlihan 1991; Carter & Houlihan 2001).

As with other aquatic ectotherms (Clifford & Brick 1978; Jobling 1983; Houlihan *et al.* 1990), the whole-body protein synthesis and SDA magnitude in *S. verreauxi* increased with dietary protein (Chapter 5). However, it should be noted that a variety of nutritional conditions may stimulate or decrease rates of protein synthesis, and nutritionally imbalanced feeds may stimulate protein synthesis and result in an elevation of SDA (Carter *et al.* 1993; Eliason *et al.* 2007; Jiang *et al.* 2013). Consequently, only part of synthesized protein, expressed as SRE, is retained as growth because both protein synthesis and degradation may elevate after a meal (Reeds & Fuller 1983; Carter & Houlihan 2001). Therefore, the measurement of protein degradation and metabolic energy substrate use, combined with the measurement of protein synthesis and SDA, would provide new information about nutrient processing and nutritional requirements, contributing to the development of nutritionally balanced and cost-effective feeds (Clifford & Brick 1979; National Research Council 2011; Carter & Mente 2014). The

quantification of protein degradation is difficult as little is known about the degradation mechanisms (Houlihan *et al.* 1995a; Carter & Houlihan 2001). In general, protein degradation is estimated from the difference between protein synthesis (measured in short-term, hours to days) and protein accretion (measured in long-term, weeks to months) (Houlihan *et al.* 1986; Moltschaniwskyj & Carter 2010). To minimize potential errors using this method, animals should be acclimated to a nutritional regime with constant protein accretion and protein synthesis is measured over a complete daily cycle (Carter & Houlihan 2001; Fraser & Rogers 2007). The estimation of protein degradation in crustaceans through growth experiments is challenging, partly due to the intermittent growth related to moulting (Hartnoll 1978; Carter & Houlihan 2001; Whiteley *et al.* 2001a). Viarengo *et al.* (1992) developed a method to determine protein degradation in the digestive gland of mussel *Mytilus galloprovincialis*, by bathing the mussel in seawater containing ^{14}C-labeled leucine for 24 hours, then transferring and bathing in seawater containing a high concentration of unlabelled leucine to minimize the re-incorporation of ^{14}C-labeled leucine released from protein degradation. Subsequently, protein degradation could be quantified from the decreased radioactivity of the digestive gland with time. However, Viarengo *et al.* (1992) only provided the half-life (1.2 days) of the digestive gland cytosolic protein while the value of protein degradation was not given. Hence, it is worthwhile to test whether isotope-labeled protein/amino acid can be used to measure protein degradation in crustaceans.

6.2 The stoichiometric bioenergetic approach to determine SDA magnitude and metabolic energy substrate use

Stoichiometry has been widely used to study bioenergetics in human beings and terrestrial farm animals (Blaxter 1989) and can be used to predict long-term weight changes (Jequier & Schutz 1983; Ravussin & Gautier 1999; Hall 2010). Stoichiometry provides reliable measurements of

SDA magnitude (Brafield 1985) and metabolic energy substrate use (Lauff & Wood 1996b) in aquatic ectotherms; this is mainly because the use of stoichiometry is based on simultaneous and repeated determinations of all key metabolic parameters including oxygen consumption, nitrogenous and carbon dioxide excretion, and the resultant NQ and RQ in single individuals (Brafield 1985). However, stoichiometry has not been widely used in aquatic ectotherms largely due to the difficulty in accurately measuring total CO_2 in water (Brafield 1985; Nelson 2016; Ferreira *et al.* 2019). As with limited studies in fish (Musisi 1984; Gelineau *et al.* 1998), the SDA magnitude determined in the present study by stoichiometry and simplified traditional approaches was similar (Chapter 4 and 5). This indicates that simplified traditional approaches are likely to provide adequate information on the energetic costs of SDA in aquatic ectotherms (Brafield 1985). However, the comparison among different approaches should be extended to a variety of species to further identify whether the estimation is comparable among approaches (Brafield 1985). Advanced technologies that allow accurate, automatic and integrated determinations of respiratory gas exchange and nitrogenous excretion will greatly contribute to the expanded stoichiometric studies (Gelineau *et al.* 1998; Rus *et al.* 2000; Ferreira *et al.* 2019).

The examination of instantaneous metabolic energy substrate use by stoichiometry may assist our understanding of how animals oxidize energy substrates to provide energy under different nutritional and environmental conditions (Clifford & Brick 1979; Ferreira *et al.* 2019). As with other short-term fasted carnivorous decapods (Castell & Budson 1974; Regnault 1981; Dall & Smith 1986), protein (amino acid) oxidation dominated in 2-day fasted *S. verreauxi*, followed by lipid with carbohydrate providing minimal contribution (Chapter 4); however, the balance of substrate oxidation was not determined in these previous studies. In contrast, lipid was the main metabolic energy substrate in 3-day fasted lobsters in CP_{60} and CP_{50} treatments, and carbohydrate oxidation predominated in the CP_{40} treatment (Chapter 5). Using

stoichiometry to evaluate metabolic energy substrate use in aquatic invertebrates was only found in 4-8-day fasted freshwater shrimp *Macrobrachium rosenbergii*, where carbohydrate was the major metabolic energy substrate, followed by lipid and protein (amino acid) had minimal contribution (Clifford & Brick 1979, 1983). The results in unfed *S. verreauxi* and *M. rosenbergii* suggest that the preferential use of major metabolic energy substrates is likely to be species-specific (Hervant *et al.* 1999; Sánchez-Paz *et al.* 2006), and can be related to recent feeding history (Houlihan *et al.* 1990; Carter & Mente 2014). During SDA ingested protein (amino acid), lipid and carbohydrate were all oxidized at different proportions at different times (Chapter 4 and 5), and the average fractional contribution of protein (amino acid) oxidation was lowest in the feed containing 50% crude protein (Chapter 5). This indicates the *S. verreauxi* feed containing 50% crude protein may spare dietary protein efficiently and that appropriate amounts of high-quality protein with both major non-protein energy-yielding nutrients, lipid and carbohydrate, are essential to developing nutritionally balanced and cost-effective feeds to spiny lobsters (Clifford & Brick 1979; Hewitt & Irving 1990; Carter & Houlihan 2001). Hewitt and Irving (1990) showed that the SDA magnitude, nitrogenous excretion and protein (amino acid) oxidation over 24 h in the prawn *Penaeus esculentus* were lowest at 40% dietary protein, compared to 30% and 50% dietary protein, concluding 40% dietary protein is optimal for *P. esculentus* growth performance. This conclusion agreed with a long-term growth study demonstrating 40% dietary protein resulted in the highest *P. esculentus* growth rate (Hewitt 1992). Gelineau *et al.* (1998) showed that rainbow trout fed at dawn (natural feeding time) spared dietary protein more efficiently compared to rainbow trout fed at midnight, in agreement with a long-term growth study demonstrating rainbow trout fed at dawn had better growth (Gélineau *et al.* 1996). Therefore, short-term stoichiometry provides a promising and efficient tool to justify feeds and feeding regimes to maximize growth and dietary protein-sparing effects to achieve more sustainable aquaculture. However, some conditions should be considered when

using stoichiometry in aquatic ectotherms. For example, the animals should have been acclimated to the feeds or feeding regimes as recent feeding history may affect metabolic energy substrate use (Houlihan *et al.* 1990; National Research Council 2011; Carter & Mente 2014). The major shifts in life-history should also be paid attention to, for example, aquatic ectotherms at maturity or during the moult cycle may have different metabolic strategies (Barber & Blake 1985; Carter & Mente 2014). Furthermore, the use of stoichiometry in aquatic ectotherms has limitations, as the RQ may exceed 1 on many occasions in various aquatic ectotherms (Wolvekamp & Waterman 1960; Kleiber 1975; Barber & Blake 1985) (Chapter 3), including some *S. verreauxi* in this thesis (Chapter 4 and 5). The RQ higher than 1 should be discarded when using stoichiometry because the value has exceeded the theoretical maximum (Lauff & Wood 1996b). Therefore, more research is necessary to decipher the limitations and applicability of stoichiometry in aquatic ectotherms, including lobsters.

6.3 Conclusions

To the best of my knowledge this thesis is the first in an aquatic ectotherm to combine the measurement of metabolic energy substrate use and SDA using a stoichiometric bioenergetic approach with the assessment of whole-body protein synthesis using an endpoint stochastic model. The outcomes from this thesis contribute to a better understanding of nutritional physiology that extends from humans and terrestrial mammals (Lusk 1912; Kleiber 1975; Blaxter 1989) to aquatic ectotherms (Keys 1930; Waterman 1960; Tytler & Calow 1985) by investigating amino acid flux and the metabolic use of dietary macro-nutrients and energy at a fine scale. The stoichiometric approach provides an instantaneous and non-destructive way to unequivocally describe the specific amount of each major energy substrate being oxidized, which has advantages around examining bioenergetic changes within a daily cycle at a whole animal level compared to molecular approaches, and allowing repeated measures in the same

animals to better understand system biology. The endpoint stochastic model enables measurements of protein synthesis in aquatic ectotherms in a non-destructive way, which has great potential to explore physiological mechanisms of individual variation in growth efficiency (Carter & Houlihan 2001; McCarthy *et al.* 2016). The combined results in this thesis have commercial impacts to the ongoing and continuous development and refinement of feeds and feeding regimes to improve aquaculture production. Currently, to achieve more sustainable aquaculture, it is necessary to replace part of traditional feed ingredients such as fish meal and fish oil with plant products (Tacon & Metian 2008; Hardy 2010) or other non-traditional ingredients such as microbial flocs and insects (Turchini *et al.* 2019). However, alternative ingredients can differ from the traditional ingredients regarding nutrient composition and may contain compounds and antinutritional factors, negatively affecting the feed efficiency and animal growth (Halver & Hardy 2003). The investigation of SDA, metabolic energy substrate use and protein metabolism provides a promising tool to uncover the effect of different ingredients on physiology and metabolism in aquaculture animals, essential to optimize new feeds to satisfy nutrient requirements at different life stages to improve the survival, growth performance and final quality of aquaculture animals. In addition, understanding stoichiometric bioenergetics in wild aquatic ectotherms is also ecologically important to interpret the adaptations to environmental change. Overall, the techniques used in this thesis are still highly relevant today and advanced technology including tracer labelling and “omics” can also be included in future research to better understand physiological and metabolic mechanisms involved in aquatic animal nutrition (Kleiber 1975; Claydon *et al.* 2012; Alfaro & Young 2018).

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