RESEARCH ARTICLE

Metabolic proxy for cephalopods: Stable carbon isotope values recorded in different biogenic carbonates

Ming-Tsung Chung[1] 🟢 | Ching-Yi Chen[2] | Jen-Chieh Shiao[3] 🟢 | Kotaro Shirai[1] 🟢 | Chia-Hui Wang[2,4]

[1]Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwa, Japan

[2]Department of Environmental Biology and Fisheries Science, National Taiwan Ocean University, Keelung, Taiwan

[3]Institute of Oceanography, National Taiwan University, Taipei, Taiwan

[4]Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan

Correspondence
Chia-Hui Wang
Email: chwang99@mail.ntou.edu.tw

Abstract

1. Measuring the metabolic rate of marine animals in their natural environment is challenging, impeding our understanding of their physiological ecology. Recently, a novel metabolic proxy, namely the $\delta^{13}C$ values of biogenic carbonates (otoliths), was experimentally validated in teleost fishes. Cephalopods possess several types of biogenic carbonates, such as statolith, cuttlebone and other internal and external shells, which are all potential metabolic recorders, but few have been evaluated.

2. To test the feasibility of the $\delta^{13}C$ metabolic proxy in cephalopods, we conducted a temperature-controlled experiment with the pharaoh cuttlefish *Sepia pharaonis* to assess whether the proportion of metabolically derived carbon (C_{resp}) incorporated into statoliths and cuttlebones increases with metabolism in a warm environment. Moreover, we conducted multiple-species analysis using the published $\delta^{13}C$ values of various biogenic carbonates to evaluate the consistency of the proxy among cephalopod species and between cephalopods and teleost fishes.

3. In the temperature-controlled laboratory experiment, C_{resp} values calculated from statoliths and cuttlebones increased with an increase in environmental temperature. C_{resp} values did not differ between statoliths and cuttlebones. Moreover, the C_{resp} values of cephalopod biogenic carbonates had a similar range to those of fish otoliths and were correlated with metabolism-related factors, such as ambient temperature, body mass, ontogeny and functional behaviours, strengthening the feasibility of their use.

4. The $\delta^{13}C$ metabolic proxy reflects the total energy use in the natural environment, and it can be transformed into the oxygen consumption rate for a broader comparison with other species and for further evaluation with current theories related to metabolic ecology. However, experimental validation is highly recommended because the relationship between the oxygen consumption rate and C_{resp} values derived from biogenic carbonates of cephalopods might be structure- and species-specific. Our study revealed that the newly developed metabolic proxy

provides a valuable alternative approach to study ecophysiology in cephalopods, and further development might broaden its applicability.

1 | INTRODUCTION

The metabolic rate denotes the energy consumption of animals, reflecting their energy use for maintenance, growth, feeding and maturation (Pettersen et al., 2018; Treberg et al., 2016), and the rate varies with fitness components in response to environmental changes (Auer et al., 2018; Pettersen et al., 2016). In past decades, the metabolism of marine ectotherms has been influenced by the increasing pressure of climate change. Coleoid cephalopods exhibit rapid growth, a short life span and rapid movement ability through jet propulsion (Boyle & Rodhouse, 2005). These life-history traits are associated with high energy use and tend to be affected by environmental factors, reflecting population dynamics (Rodhouse et al., 2014). For example, high temperatures considerably increase the metabolic cost of early life for squids (Pimentel et al., 2012) and accelerate their life cycle, resulting in earlier hatching, smaller hatchlings and maturation at younger age (Pecl & Jackson, 2008). Within the optimal thermal window, warmer waters increase the population size of cephalopods due to higher individual growth and population turnover rates (Doubleday et al., 2016); however, outside the optimum thermal window, the aerobic scope is narrowed, leading to a decrease in predation activity (Rosa & Seibel, 2008).

Compared with their primary vertebrate competitors (fishes), cephalopods have higher metabolic rates and behave more sensitive to an increase in temperature (Seibel & Drazen, 2007). For example, the metabolic rate of oceanic squids is higher than that of scombrid fish at the same ambient temperature and body mass (Seibel & Drazen, 2007). Shallow-water cephalopod species have Q_{10} values ranging from 1.3 to 4.5 (thermal sensitivity criterion is expressed as a ratio of metabolic rates under a 10°C difference; Segawa, 1995; Boyle & Rodhouse, 2005; Birk et al., 2018), and the Q_{10} values of deep-water species can reach 7.9 (Seibel et al., 1997). The range of Q_{10} values for cephalopods is wider than that for teleost fishes (0.45–3.41; Clarke & Johnston, 1999). This indicates the importance of studying the physiological ecology of cephalopods; however, the influence of climate change on the metabolic rate of cephalopods under natural conditions remains unclear due to the difficulty of measuring their metabolic rate in the natural environment.

Commonly, the metabolic rate of marine ectotherms is determined by directly measuring oxygen consumption through respirometry in laboratory experiments (Burford et al., 2019; Grigoriou & Richardson, 2009; Seibel et al., 1997; Spady et al., 2019). However, this method cannot be applied in the field to determine

their metabolic performance (Treberg et al., 2016). The cephalopod metabolic rate in the field has been investigated using several approaches (Aitken et al., 2005; O'Dor et al., 1995; Payne et al., 2011; Webber & O'Dor, 1986). For example, telemetered jet pressure (Aitken et al., 2005; O'Dor et al., 1995; Webber & O'Dor, 1986) or accelerometry (Payne et al., 2011) is used to monitor metabolism variation between resting and swimming states, reflecting the activity metabolic rates of cephalopods. Analysing enzyme activities associated with metabolism is also an alternative that can be used to study cephalopod ecology in difficult-to-monitor locations, including the deep sea (Seibel et al., 2000). Such approaches reveal an acute change in metabolic rates in a short period. Another novel metabolic proxy, namely the stable carbon isotope value (δ^{13}C) recorded in biogenic carbonates, has been used to infer the averaged field metabolic rate of marine ectotherms within a time frame (Chung et al., 2019; Chung et al., 2019; Martino et al., 2020). This approach has been evaluated and applied to teleost fishes but not yet to cephalopods.

The fundamental mechanism of δ^{13}C metabolic proxy is that carbon incorporated into biogenic carbonates is derived from two sources: water dissolved inorganic carbon (DIC) and dietary carbon; however, metabolism and ambient CO_2/O_2 ratios control the proportion of these two sources of carbon deposited on biogenic carbonate (Kalish, 1991; McConnaughey et al., 1997; McConnaughey & Gillikin, 2008). At a relatively stable CO_2/O_2 level, the proportion of dietary carbon varies with metabolic changes, and the percentage can be estimated when the stable carbon isotope values of these two sources and biogenic carbonates are known (McConnaughey & Gillikin, 2008; Schwarcz et al., 1998; Solomon et al., 2006). Moreover, the proportion of metabolically derived carbon as a metabolic proxy can be linked to the oxygen consumption rate, which represents the total energy use of organisms in the field (Chung, Trueman, Godiksen, Holmstrup, et al., 2019). Cephalopods possess several biogenic carbonate structures, such as external (*Nautilus* and argonaut shells) and internal shells (cuttlebones in cuttlefish and shells in ram's horn squid) as well as acoustic and vestibular apparatus (statoliths in coleoids; Boyle & Rodhouse, 2005), which are all potential metabolic recorders. Fossils of these carbonate hard structures also exist (Immenhauser et al., 2016); thus, the development of this metabolic proxy has value not only in contemporary ecology but also in the palaeontology of cephalopods.

Therefore, this study aimed to develop the δ^{13}C metabolic proxy among modern cephalopod species under a range of living temperatures and to evaluate the feasibility of the proxy. Two approaches

were designed: experimental evaluation and comparison with various species. (a) Through a temperature-controlled (20–30°C) experiment in cuttlefish *Sepia pharaonis*, we expect that the metabolic rate of cuttlefish increases with environmental temperature and reflects the increase in the proportion of metabolically derived carbon in two types of biogenic carbonates, namely cuttlebone and statolith. (b) The δ^{13}C values of biogenic carbonates among cephalopod species were extracted from the literature to examine the relationship of the δ^{13}C metabolic proxy with metabolism-related factors. In addition, the proxy of cephalopod biogenic carbonates was compared with that of fish otoliths, which have been substantially evaluated as metabolic recorders.

2 | MATERIALS AND METHODS

2.1 | Temperature-controlled laboratory experiments

The detailed experimental procedure has been described in the study of Chung, Chen, et al. (2020), and the summarised specimen information is provided in Table 1. Briefly, cuttlefish egg strings were maintained in temperature-controlled environments at 20, 25 and 30°C, and cuttlefish were reared for 35 days after hatching. During the experiment, cuttlefish were fed shrimps *Neocaridina denticulata* ad libitum twice per day. Tanks were equipped with a circulation system, in which a small amount of water was replaced every day and seawater was pumped from near the shore off the campus of National Taiwan Ocean University. The temperature of the water in which cuttlefish were reared was recorded regularly, with the recorded temperatures for the three groups were 21.4 ± 0.6, 25.0 ± 0.1 and 30.0 ± 0.1°C. The measured temperature values were used in the following statistical analyses.

Water samples were collected from each tank every 3 days and stored in amber glass bottles (Qorpak, A Division of Berlin Packaging), and saturated $HgCl_2$ solution was added for long-term preservation. Water samples from the beginning (3–6 days), mid-term (18–21 days) and end (27–35 days) periods of the experiment were analysed using the Delta V plus analyser (Thermo Fisher Scientific, Germany) equipped with a Finnigan GasBench II (Thermo Fisher Scientific) at the Atmosphere and Ocean Research Institute, The University of Tokyo, and the δ^{13}C value of DIC was measured. Long-term reproducibility was $\pm 0.1‰$. Isotope values were reported

based on the delta notation and were compared with the standard Vienna Pee Dee Belemnite as follows:

$$\delta^{13}C = \left(\frac{R_{Sample}}{R_{standard}} - 1 \right) \times 1,000‰,$$

where R is the ^{13}C/^{12}C ratio of the sample or standard.

Shrimps from the same batch were used as food sources for the 20 and 25°C groups, and another batch was used for the 30°C group. Before using the shrimps for feeding, a small number of shrimps were freeze-dried and crushed. Approximately 0.5 mg of powder samples was embedded in a tin cup for isotope analyses on the MAT 253 apparatus (Thermo Fisher Scientific) equipped with an elemental analyser (Flash EA; Thermo Fisher Scientific) at the Institute of Oceanography, National Taiwan University. The values were calibrated with the standard USGS40 (IAEA), and isotope values were reported based on the delta notation.

In each temperature group, nine hatchlings were randomly selected from among the individuals who had survived for 35 days, and their biogenic carbonates (cuttlebones and statoliths) were collected. Cuttlebones and statoliths were washed with 5% H_2O_2 and then cleaned with distilled water; cleaning was conducted per the procedure described by Chung, Chen, et al. (2020). Cuttlebone carbonate that formed after hatching and a whole left statolith were used for stable carbon isotope analyses on the Finnigan MAT 253 apparatus (Thermo Fisher Scientific) equipped with Kiel Carbonate IV (Thermo Fisher Scientific) at National Taiwan University. The long-term reproducibility of δ^{13}C and δ^{18}O was ± 0.05 and $\pm 0.08‰$ (standard deviations), respectively, according to the measurement of the NBS-19 standard.

According to a two-component mixing model (McConnaughey & Gillikin, 2008; Schwarcz et al., 1998; Solomon et al., 2006), the δ^{13}C value of biogenic carbonates is a weighted average of the isotopic composition of carbon from two main sources: DIC and dietary carbon. Therefore, it can be described using the following two-component mixing model:

$$\delta^{13}C = C_{resp} \times \delta^{13}C_{diet} + \left(1 - C_{resp}\right) \times \delta^{13}C_{DIC} + \varepsilon,$$

where $\delta^{13}C_{diet}$ and $\delta^{13}C_{DIC}$ are the average δ^{13}C values of the dietary carbon and DIC of seawater, respectively, and the term ε is the total net isotopic fractionation from the sources to biogenic carbonate. In this study, ε was set to 2.7 and 1.0 for aragonite (statoliths, cuttlebones

TABLE 1 Weight of cuttlefish and stable carbon isotope values of cuttlebone and statolith in the temperature-controlled experiment on pharaoh cuttlefish, *Sepia pharaonis*. All data are mean $\pm$ 1 *SD*. The term C_{resp} is the metabolic proxy of the weighted proportion of metabolically derived carbon incorporated into biogenic carbonates. *N* is numbers of sample size

			Statolith		Cuttlebone	
Mean temperature (°C)	N	Body weight (g)	δ^{13}C values (‰)	C_{resp}	δ^{13}C values (‰)	C_{resp}
21.4 ± 0.6	9	0.47 ± 0.03	-9.46 ± 0.41	0.39 ± 0.02	-9.64 ± 0.71	0.40 ± 0.03
25.0 ± 0.1	9	0.68 ± 0.06	-11.70 ± 0.31	0.46 ± 0.01	-11.73 ± 0.50	0.46 ± 0.02
30.0 ± 0.1	9	0.35 ± 0.05	-12.41 ± 0.37	0.50 ± 0.02	-12.28 ± 0.56	0.49 ± 0.02

and shells of *Nautilus* and *Spirula*) and calcite carbonates (shells of *Argonauta*), respectively, in accordance with the result from the inorganic precipitation of carbonate (Romanek et al., 1992). The term C_{resp} represents the proportion of metabolically derived carbon (from diets).

2.2 | Reported $\delta^{13}C$ and $\delta^{18}O$ values of biogenic carbonates of cephalopods in the literature

In all, 10 cephalopod species with $\delta^{13}C$ and $\delta^{18}O$ values from the literature were used in analyses of this study (Chung et al., 2021). $\delta^{13}C$ and $\delta^{18}O$ values were derived from external (*Nautilus* and *Argonauta*) and internal shells (ram's horn squids and cuttlefishes) as well as statoliths (squids). The experienced temperature was extracted from the same study. If recorded temperatures were absent in previous studies, we estimated temperatures from the reported $\delta^{18}O$ values using the temperature-dependant equations provided by Chung, Chen, et al. (2020).

The C_{resp} value was estimated based on the $\delta^{13}C$ values of biogenic carbonates using the two-component mixing model. In the model, the $\delta^{13}C_{DIC}$ values were based on published data in the same sampling area. The $\delta^{13}C_{diet}$ values were derived from the $\delta^{13}C$ values in other tissues/structures (muscle, beaks or organic matter in biogenic carbonate) minus assumed trophic enrichment factor 1, but for planktivores, the $\delta^{13}C_{diet}$ values were obtained from the $\delta^{13}C$ values of zooplankton in the sample areas according to published spatial ocean modelling (Chung et al., 2021).

2.3 | Reported $\delta^{13}C$ and $\delta^{18}O$ values of fish otoliths in the literature

The $\delta^{13}C$ and $\delta^{18}O$ values of fish otoliths were derived from the study of Chung, Trueman, Godiksen, Holmstrup, et al. (2019) and references therein, which also included simulated experienced temperature and C_{resp} values. In total, the $\delta^{13}C$ and $\delta^{18}O$ values of 76 fish species from 24 studies were used.

2.4 | Statistical analyses

Shapiro–Wilk and Levene's tests were used to examine the normality of data and the equality of variances among the temperature groups. One-way analysis of variance (ANOVA) was used to evaluate the effect of temperature on $\delta^{13}C$ and C_{resp} values using biogenic carbonates in statoliths and cuttlebones. In addition, a paired t test was conducted to compare the difference in $\delta^{13}C$ and C_{resp} values between statoliths and cuttlebones.

A linear regression model was used to evaluate the relationship between $\delta^{13}C$ and $\delta^{18}O$ values extracted from previous studies and to examine the relationship between C_{resp} values and metabolism-related factors. All statistical analyses were performed and figures were composed using the R language (R Core Team, 2019).

3 | RESULTS

3.1 | Temperature-controlled laboratory experiments

$\delta^{13}C_{diet}$ values were −28.5 ± 0.1‰ (mean ± 1 *SD*) for the 20 and 25°C groups and −27.1 ± 0.8‰ for the 30°C group. Water $\delta^{13}C$ values of the 20, 25 and 30°C groups were −1.7 ± 1.5, −2.6 ± 0.9 and −3.3 ± 0.4‰, respectively. The mean dietary and water $\delta^{13}C$ values in each of the temperature-controlled groups were used to calculate the proportion of metabolically derived carbon of biogenic carbonates.

Statolith $\delta^{13}C$ values were significantly different among the temperature groups (ANOVA: $df = 2$, $F = 161$, $p < 0.01$), with the highest value of −9.46 ± 0.41‰ at 20°C followed by −11.70 ± 0.31 and −12.42 ± 0.37‰ at 25 and 30°C, respectively (Figure 1a). A similar trend was observed for cuttlebone $\delta^{13}C$ values (ANOVA: $df = 2$, $F = 48.8$, $p < 0.01$): −9.64 ± 0.71, −11.73 ± 0.50 and −12.28 ± 0.56‰ for the 20, 25 and 30°C groups, respectively (Figure 1a). According to the paired t test, no significant difference existed between statolith and cuttlebone $\delta^{13}C$ values ($df = 26$, $t = 0.20$, $p = 0.84$; Figure 1b).

The proportion of metabolically derived carbon (C_{resp}) increased when cuttlefish were reared in a warm environment. The calculated C_{resp} values for statolith were 0.39 ± 0.02,
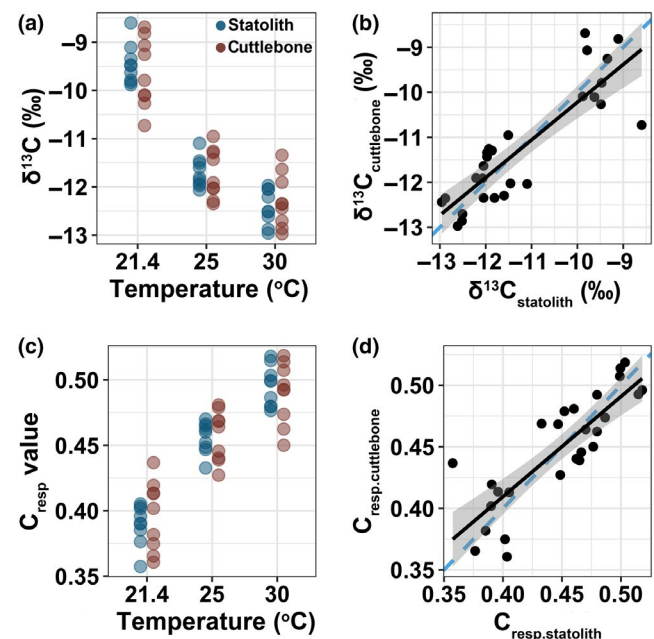

FIGURE 1 Stable carbon isotope values ($\delta^{13}C$) and the proportion of respired carbon (C_{resp} value) between cuttlebones and statoliths from temperature-controlled experiments. (a) The effect of temperature on the $\delta^{13}C$ value of statoliths and cuttlebones. (b) The relationship between the $\delta^{13}C$ value of statoliths and cuttlebones. (c) The effect of temperature on the C_{resp} value of statoliths and cuttlebones. (d) The relationship between the C_{resp} value of statoliths and cuttlebones. Blue dash lines are the 1:1 ratio

0.46 ± 0.01 and 0.50 ± 0.02 for the 20, 25 and 30°C groups, respectively, which showed a significant difference among the temperature groups (ANOVA: $df = 2$, $F = 126$, $p < 0.01$; Figure 1c). A similar finding was obtained for cuttlebone; C_{resp} values were 0.40 ± 0.02, 0.46 ± 0.02 and 0.49 ± 0.02 for the 20, 25 and 30°C groups, respectively, with significant differences found among the temperature groups (ANOVA: $df = 2$, $F = 37.2$, $p < 0.01$; Figure 1c). No significant difference was observed in the C_{resp} values of cuttlebone and statolith (paired t test: $df = 26$, $t = -0.15$, $p = 0.88$; Figure 1d).

3.2 | Comparisons between cephalopods and teleost fishes

The number of published $\delta^{13}C$ and $\delta^{18}O$ values of biogenic carbonate was much lower for cephalopods than for teleost fishes; however, the slope of the relationship of $\delta^{13}C$ and $\delta^{18}O$ values was not significantly different (two-way ANOVA, interactions between $\delta^{18}O$ and animal groups: $df = 1$, mean square = 1.5, $F = 0.46$, $p = 0.50$; Figure 2). Although the slopes were similar, the intercepts showed relatively positive $\delta^{13}C$ values for cephalopods (analysis of covariance, intercepts between cephalopods and fishes: $df = 1$, mean square = 180, $F = 56.7$, $p < 0.01$; Figure 2). This indicates that demersal or less active cephalopod species (cuttlefishes and nautiluses) have been more studied than pelagic and active species (squids) have. After the transformation of $\delta^{13}C$ and $\delta^{18}O$ values to the C_{resp} value and experienced temperature, respectively, cephalopods showed a range of C_{resp} values from 0.09 to 0.50 and of experienced temperature from 9.1 to 23.3°C (Chung et al., 2021). Fishes had C_{resp} values between 0 and 0.50 but a wider range of experienced temperature between 0.77 and 32.5°C.
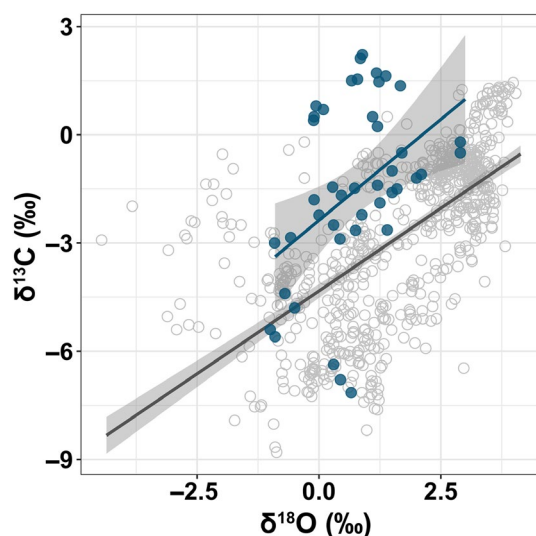

FIGURE 2 Stable oxygen and carbon isotopes of biogenic carbonates reported in the literature. Grey circle: teleost fishes; blue dot: cephalopods

4 | DISCUSSION

Our study is the first to provide an estimate of the proportion of respired carbon (C_{resp}) in biogenic carbonates of cephalopods based on a temperature-controlled laboratory experiment, and the results revealed that the metabolic proxy of cuttlebones and statoliths was comparable. In a further evaluation, the metabolic proxy recorded in different types of biogenic carbonates among cephalopod species also varied in a reasonable range and showed potential use when compared with fish otoliths. To strengthen the feasibility of using the metabolic proxy for cephalopods, we systematically assessed C_{resp} values in relation to different metabolism-related factors.

4.1 | Temperature

Temperature strongly influences the metabolism of ectotherms. According to our temperature-controlled laboratory experiments, C_{resp} values as a metabolic proxy showed a positive trend with increased temperature, which is consistent with the results of two recent temperature-controlled studies on fish otoliths (Chung, Trueman, Godiksen, Holmstrup, et al., 2019; Martino et al., 2019), but C_{resp} values were higher in pharaoh cuttlefish hatchlings than in Atlantic cod and Australasian snapper juveniles (Figure 3a). With a 10°C increase in temperature, the C_{resp} values of pharaoh cuttlefish increased by approximately 0.1, and the thermal sensitivity of C_{resp} values (slope) decreased with an increase in temperature. The proxy revealed that the metabolism of pharaoh cuttlefish hatchlings responds to temperature changes more rapidly at 20–25°C than at 25–30°C.

In multiple-species analysis of fishes, C_{resp} values increased by 0.01 per 1°C increase (Figure 3b). However, the same analysis cannot provide a convincing result for cephalopods because of their limited data and the great variation in values. Instead, we regressed the C_{resp} value according to the temperature for each species and found that for most of the cephalopod species, C_{resp} values increased with temperature (increase from 0.001 to 0.02 per 1°C increase; Figure 3c). However, temperature exerted less influence or a negative effect on the C_{resp} values of *Illex illecebrosus* and *Nautilus*. Body mass may be the main driving force of metabolic changes in *I. illecebrosus* because smaller individuals captured in colder environments exhibit higher C_{resp} values than do larger ones captured in warmer waters. The body size of nautiluses is not recorded in the literature, but according to the ontogenetic decrease in C_{resp} values with septum numbers (see details in the next section), body mass probably influences the C_{resp} values of nautiluses more than ambient temperature does.

4.2 | Body mass and ontogeny

Small body size leads to a high mass-specific metabolic rate. The multiple-species analysis of fishes demonstrated that C_{resp} values decreased by approximately 0.04 per log-transformed body mass (Figure 3d). However, most studies on the $\delta^{13}C$ and $\delta^{18}O$ values of
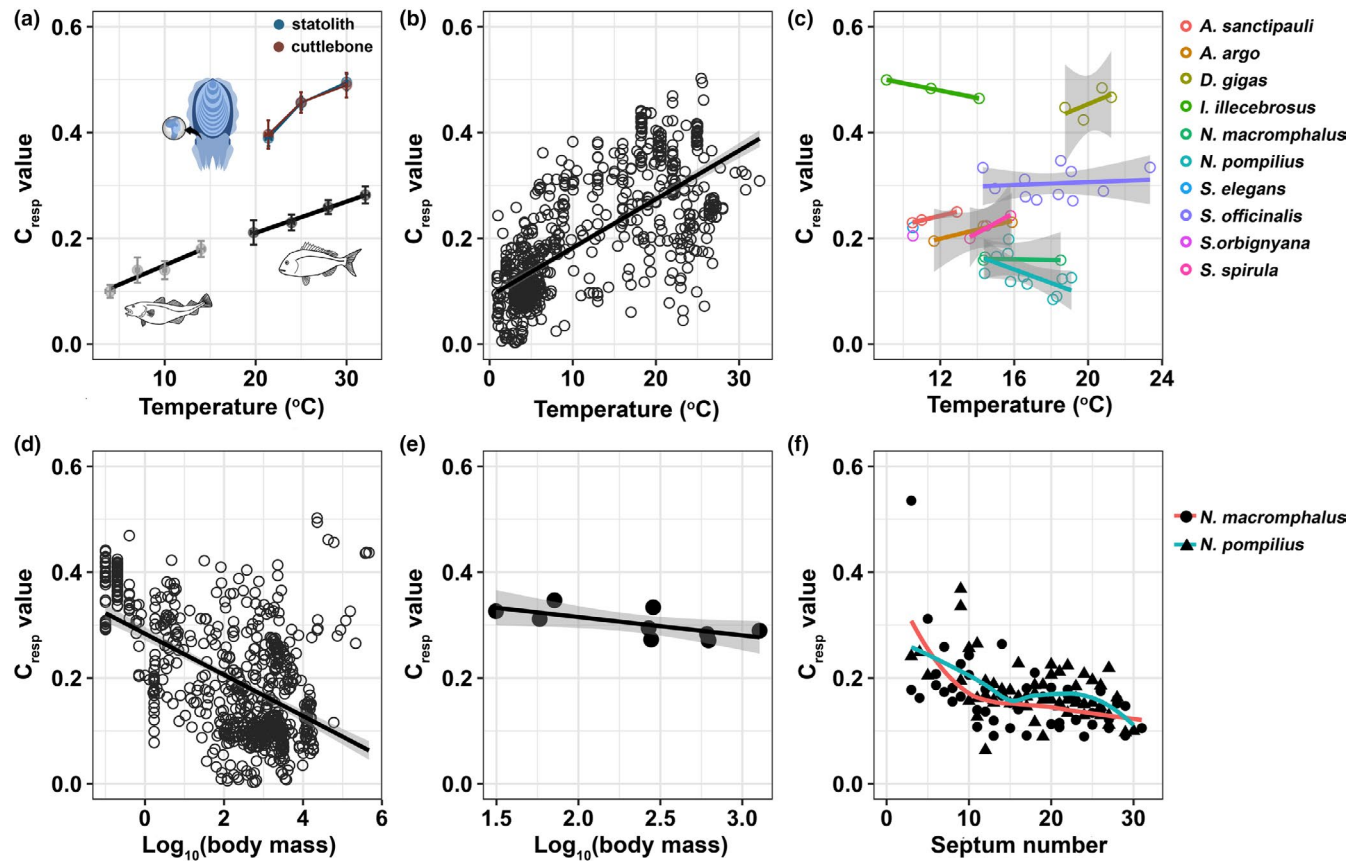
FIGURE 3 The proportion of respired carbon (C_{resp} value) recorded in biogenic carbonates with varying temperature (a–c), body mass and ontogeny (d–f). (a) Experimental validation of C_{resp} values with temperature between cephalopods (this study) and fishes (Chung, Trueman, Godiksen, Holmstrup, et al., 2019; Martino et al., 2019). (b) Reported C_{resp} values with temperature in teleost fish species. The figure is derived from figure 2c in Chung, Trueman, Godiksen, Holmstrup, et al. (2019). (c) Reconstructed C_{resp} values with temperature among cephalopod species. (d) Reported C_{resp} values with body mass in teleost fish species. The figure is derived from figure 2b in Chung, Trueman, Godiksen, Holmstrup, et al. (2019). (e) Reconstructed C_{resp} values with temperature in the European common cuttlefish *Sepia officinalis*. (f) The ontogenetic trend of C_{resp} values in two *Nautilus* species

biogenic carbonates in cephalopods have not recorded individual body mass; thus, we analysed the $\delta^{13}C$ and $\delta^{18}O$ values of only nine cuttlefish extracted from the study of Bettencourt and Guerra (1999). These cuttlefish were collected at the same time and location, and their $\delta^{13}C$ and $\delta^{18}O$ values recorded in the outermost portion of cuttlebone (the newly formed septum) represent the same environmental conditions within the same time frame. The temperature estimated from $\delta^{18}O$ values showed no significant difference among individuals and no significant influence on C_{resp} values, thus excluding the influence of temperature on metabolism. The body mass of these cuttlefish ranged from 31.4 to 1,275 g, and C_{resp} values decreased by approximately 0.035 per log-transformed body mass, which was close to the value obtained from fishes (Figure 3e).

The ontogenetic decrease in C_{resp} values also reflects the influence of body mass increase (Chung, Trueman, Godiksen, Holmstrup, et al., 2019). In this study, the ontogenetic decrease in the C_{resp} values of *Nautilus* was obvious throughout life (Figure 3f). Because *Nautilus* eggs hatch around septum 7–10 (Landman et al., 1994; Zakharov et al., 2006), the C_{resp} values before septum 10 are biased due to uncertain $\delta^{13}C_{diet}$ and $\delta^{13}C_{DIC}$ values,

rendering the evaluation of ontogenetic variation in metabolism in the egg stage difficult. After hatching, *Nautilus* individuals showed a continual decrease in C_{resp} values from 0.26 to 0.09, which indicates decreased metabolism with growth. The reconstruction of ontogenetic variation in C_{resp} values is informative that reveals energy-dependent biological processes by providing the allometric scaling exponent among species (Chung, Trueman, Godiksen, Holmstrup, et al., 2019).

4.3 | Functional groups and species

Functional behaviours can shape the metabolic demands of marine animals. In fishes, the morphology of the caudal fin reflects their swimming capability and is correlated with C_{resp} variations (Solomon et al., 2006). The C_{resp} value of active swimmers, such as tuna and billfish, is higher (up to ~0.5) than that of benthopelagic fishes (<0.25; Figure 4). The same observation has been made for cephalopods. In the current study, C_{resp} values were the highest for oceanic squids (close to 0.5), such as *I. illecebrosus*
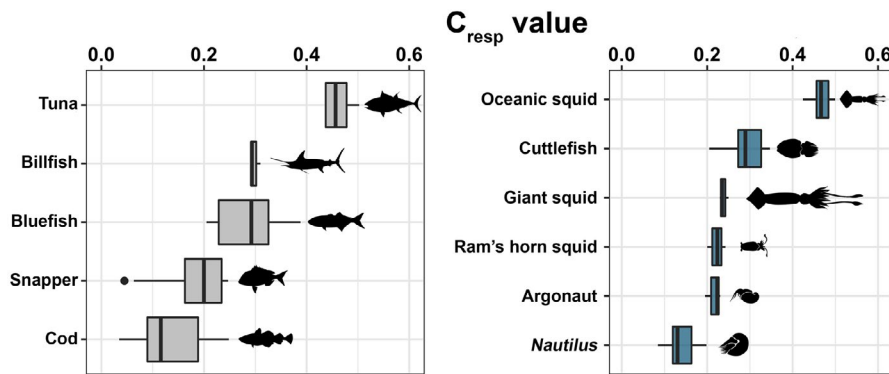
FIGURE 4 C_{resp} values are compared between teleost fishes and cephalopods. The C_{resp} values also relate to functional behaviours that pelagic predators have higher C_{resp} values than the less active predators

and *Dosidicus gigas*, followed by the values for benthopelagic species (such as cuttlefish; Figure 4). Giant squid, ram's horn squid and argonaut had similar C_{resp} values, but the values were lower than those of other squid and cuttlefish species. The lowest C_{resp} value in our study was found for *Nautilus*, and the feeding strategies for conserving energy may explain the low metabolic demand (Dunstan et al., 2011; O'Dor et al., 1993). The low metabolism also favours the longevity of some cephalopods, such as nautiluses (with life spans of approximately 10–12 years; Landman & Cochran, 2010) and giant squids (with life spans of up to 14 years; Landman et al., 2004).

4.4 | Transforming C_{resp} values to oxygen consumption rate

To make the metabolic proxy comparable to conventional approaches used in measuring animal metabolism, the C_{resp} values of the otoliths of Atlantic cod and Australasian snapper were successfully converted to metabolic rates expressed as oxygen consumption rates (Chung, Trueman, Godiksen, Holmstrup, et al., 2019; Martino et al., 2020). Our study did not record the oxygen consumption of cuttlefish; thus, temperature and body mass were fitted to a previously validated model (Melzner et al., 2007). Then, the relationship between routine metabolic rates and C_{resp} values was evaluated according to the increasing form of the exponent decay model, as reported by Chung, Trueman, Godiksen, Holmstrup, et al. (2019), as follows:

$$C_{resp.statolith} = 0.513 \pm 0.008 \left(1 - e^{-0.077 \pm 0.004(\text{oxygen consumption})}\right),$$

$$C_{resp.cuttlebone} = 0.503 \pm 0.011 \left(1 - e^{-0.084 \pm 0.007(\text{oxygen consumption})}\right),$$

where the unit of oxygen consumption is μmol O_2 hr^{-1} g^{-1}. The upper-bound C_{resp} values of statoliths and cuttlebones were 0.513 and 0.503, respectively, which are close to the upper limit of observational values in fish otoliths (Chung, Trueman, Godiksen, & Grønkjær, 2019) but are much higher than the theoretically predicted average of 0.1 in the shells of aquatic invertebrates (McConnaughey & Gillikin, 2008). Compared with the equation established using juvenile Atlantic cod

otoliths (0.243; Chung, Trueman, Godiksen, Holmstrup, et al., 2019), the upper limit of the C_{resp} value was much higher in pharaoh cuttlefish hatchlings (0.26–0.27 higher than that of Atlantic cod juveniles).

The reconstructed C_{resp} values of various cephalopod species were further applied to the exponential decay model to estimate their oxygen consumption rates. We used the equation derived from cuttlebone and applied it to the shells of *nautiluses*, argonauts and ram's horn squids. A comparison between reconstructed and reported oxygen consumption (Table S1) revealed a crucial finding: The equation may be structure-specific because the reconstructed oxygen consumption rate was much closer to the reported values in *S. officinalis* and *I. illecebrosus* than to those in nautiluses and argonauts (Figure S1). The equation derived from cuttlebone seemed to be unsuitable for application to the outer shells of nautiluses and argonauts. This study provides a preliminary evaluation for transforming the δ^{13}C metabolic proxy to the oxygen consumption rate; further development and assessment of the transformation of oxygen consumption among species are required. In particular, some deep-water species, such as *Nautilus*, develop strategies for living in a low-oxygen environment, and they show low metabolism (O'Dor et al., 1993; Tajika et al., 2020); these factors could further affect the relationship between the δ^{13}C metabolic proxy and oxygen consumption rate.

As a metabolic proxy, the δ^{13}C values of biogenic carbonates have great potential in the investigation of the metabolic rate of cephalopods in the field. (a) The feature of the continuous increase in biogenic carbonates along with δ^{13}C values indicates ontogenetic variation in metabolism, improving our understanding of the fitness traits of cephalopods. (b) Cuttlebone can provide metabolic information at a near daily resolution because the structural unit, lamella, is formed approximately every 1.7 days (Chung & Wang, 2013). Although statoliths are small, a newly developed method of micro-volume isotope analyses can enhance the sampling resolution in tiny carbonates (Sakamoto et al., 2019). In addition, the advanced analytical technique of using secondary ion mass spectrometry can provide high temporal resolution with high precision in both stable oxygen and carbon isotope analyses (Linzmeier et al., 2018; Weidel et al., 2007); this enables the study of tiny samples such as statoliths. (c) Combined with other chemical signals, such as growth rate proxy of Li/Ca ratios (Chung, Huang, et al., 2020) or temperature

proxy of δ^{18}O (Chung, Chen, et al., 2020), the effects of temperature and the growth rate on metabolic rates can be evaluated. (d) To estimate the metabolic proxy, stable carbon isotope values from the diet are needed. $\delta^{13}C_{diet}$ values can be obtained from the gladius of squids (Ruiz-Cooley et al., 2010), beaks of most cephalopod species (Cherel et al., 2019), and intercrystalline and intracrystalline organic matter of carbonate shells (Ohkouchi et al., 2013). These hard structures provide lifelong dietary and trophic isotope signals; thus, the ontogenetic variation of the trophic level and metabolism can be reconstructed. (e) The δ^{13}C metabolic proxy is also valuable in palaeoecological and palaeontological studies through the analysis of fossil samples or the historical collection of biogenic carbonates. Well-preserved external shells from ancient species, such as orthoconic and coiled nautiloids, ammonoids and belemnoids (Immenhauser et al., 2016), or statoliths from coleoids (Clarke & Fitch, 1975) provide clues to the evolutionary history of cephalopods. The elemental ratios or isotopic values recorded in fossil shells reveal the environmental conditions and their physiological traits (Immenhauser et al., 2016; Seuss et al., 2012). For example, the δ^{13}C metabolic proxy of ammonites approximately ranges from 0.17 to 0.37 (Landman et al., 2018; Tobin & Ward, 2015), which is close to the value of the modern cuttlefish species at the adult stage. Moreover, the organic matrix can be found in fossil mollusc shells (Clark, 1999), and their isotope values provide trophic information in the past (Lueders-Dumont et al., 2018). Based on these developed proxies, we can develop modelling frameworks with Bayesian methods, which take account uncertainties caused by the variation in parameters, such as salinity and $\delta^{13}C_{DIC}$ values varying across spatial scales through geologic time. Such frameworks would enhance data explanation for gaining a more precise understanding of life and metabolic history between ancient and modern species (Bowen et al., 2020). We believe that the approach is valuable in cephalopod ecology, especially in relation to their behavioural traits and population dynamics. Moreover, the metabolic proxy is comparable with teleost fishes for answering a wide range of physiological, ecological and evolutionary questions.

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AUTHORS' CONTRIBUTIONS

M.-T.C. and C.-H.W. conceived the ideas and designed the methodology; C.-Y.C. collected the data; J.-C.S. and K.S. conducted the isotope analyses. All authors contributed critically to the manuscript preparation.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The results and data from the rearing experiment are present in the manuscript and the data extracted from the literature are deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.hqbzkh1g5 (Chung et al., 2021).

ORCID

Ming-Tsung Chung https://orcid.org/0000-0003-3305-3339
Jen-Chieh Shiao https://orcid.org/0000-0002-3824-5738
Kotaro Shirai https://orcid.org/0000-0003-3922-5971

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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