REGULAR PAPER

Gill surface area provides a clue for the respiratory basis of brain size in the blacktip shark (*Carcharhinus limbatus*)

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Abstract

Brain size varies dramatically, both within and across species, and this variation is often believed to be the result of trade-offs between the cognitive benefits of having a large brain for a given body size and the energetic cost of sustaining neural tissue. One potential consequence of having a large brain is that organisms must also meet the associated high energetic demands. Thus, a key question is whether metabolic rate correlates with brain size. However, using metabolic rate to measure energetic demand yields a relatively instantaneous and dynamic measure of energy turnover, which is incompatible with the longer evolutionary timescale of changes in brain size within and across species. Morphological traits associated with oxygen consumption, specifically gill surface area, have been shown to be correlates of oxygen demand and energy use, and thus may serve as integrated correlates of these processes, allowing us to assess whether evolutionary changes in brain size correlate with changes in longer-term oxygen demand and energy use. We tested how brain size relates to gill surface area in the blacktip shark *Carcharhinus limbatus*. First, we examined whether the allometric slope of brain mass (*i.e.*, the rate that brain mass changes with body mass) is lower than the allometric slope of gill surface area across ontogeny. Second, we tested whether gill surface area explains variation in brain mass, after accounting for the effects of body mass on brain mass. We found that brain mass and gill surface area both had positive allometric slopes, with larger individuals having both larger brains and larger gill surface areas compared to smaller individuals. However, the allometric slope of brain mass was lower than the allometric slope of gill surface area, consistent with our prediction that the allometric slope of gill surface area could pose an upper limit to the allometric slope of brain mass. Finally, after accounting for body mass, individuals with larger brains tended to have larger gill surface areas. Together, our results provide clues as to how fishes may evolve and maintain large brains despite their high energetic cost, suggesting that *C. limbatus* individuals with a large gill surface area for their body mass may be able to support a higher energetic turnover, and, in turn, a larger brain for their body mass.

KEYWORDS
direct metabolic constraints, expensive brain, ontogenetic allometry, oxygen limitation, temperature-size rule

1 | INTRODUCTION

Animals must balance the selective benefits of greater behavioural complexity and cognitive function with the greater energy demands of having a large brain for a given body size (hereafter, large brain). Having a large brain has been associated with cognitive capability in a variety of vertebrate groups (Horschler *et al*., 2019; Maklakov *et al*., 2011; Sol *et al*., 2008), although some other studies challenge these findings (Fichtel *et al*., 2020; Turschwell & White, 2016). Although many factors likely influence the evolution of brain size, large brains have been linked to both habitat and social complexity across fish species; some studies suggest that that the cognitive requirements for living in spatially complex habitats such as coral reefs might have influenced the evolution of brain size, while others suggest that social behaviours and intra- and interspecific (*i.e*., within a species and across different species) interactions may also play a role (Bauchot *et al*., 1977; Gonzalez-Voyer *et al*., 2009; Yopak *et al*., 2007). Experimentally, guppies *Poecilia reticulata* Peters 1859 selected for large brains outperform small-brained individuals in both a numerical learning assay and a spatial learning task (Kotrschal *et al*., 2013, 2015). Consequently, there appear to be clear benefits of – and potentially a high selection pressure for – having a large brain. On the other hand, large brains come at an energetic cost. The brain requires considerably more energy per unit weight than most other organs, accounting for at least 2%–8% of resting oxygen consumption in vertebrates (Mink *et al*., 1981). For example, in the lizard *Cordylus jonesii* (Boulenger 1891), the metabolic rate of brain tissue was found to be around 2.3 times greater than that of liver tissue and 2.7 times greater than lung tissue (Wheeler, 1984). Additionally, fishes, reptiles, and amphibians have been documented to grow throughout their lives, exhibiting life-long neurogenesis and/or continual brain growth and thus must meet the costs of both the growth of new brain tissue and the maintenance of existing brain tissue (Leonard *et al*., 1978; Maruska *et al*., 2012; Zupanc, 2006). Therefore, despite the potential cognitive benefits of having a large brain, the energetic requirements of maintaining such a brain could constrain brain size evolution, particularly in ectotherms (Isler & van Schaik, 2009).

Evolving a large brain may require a decrease in other energy requirements (as suggested by the energy trade-off hypothesis; Aiello & Wheeler, 1995), or an increase in overall energy turnover (as suggested by the direct metabolic constraints hypothesis; Armstrong, 1983; Hofman, 1983; Isler & van Schaik, 2009). Although data are scarce, large brains have been found in fish populations in environments with greater oxygen availability compared to populations in hypoxic conditions, and larger brains have been found in fish species with higher metabolic rates. For instance, a population of mormyrid fish in well-oxygenated waters had larger brains than another population in an oxygen-stressed environment (Chapman & Hulen, 2001). Within fish species, brain mass appears positively related to metabolic rate after controlling for body mass; however, this pattern was not statistically significant (Sukhum *et al*., 2019). On an interspecific scale, brain mass has been negatively correlated with water depth (a proxy for metabolic rate across fish species), although other studies have found no relationship between the two (Iglesias *et al*., 2015; Tsuboi *et al*., 2015). Additionally, brain mass correlates positively and significantly with resting metabolic rate across mormyrid fish species, even after controlling for evolutionary history and body mass (Sukhum *et al*., 2016). However, metabolic rate, that is, oxygen consumption per unit time, is a relatively instantaneous and dynamic measure of energy use, whereas brain size evolution is likely shaped by longer-term energetic conditions (Carlson *et al*., 2004). Thus, a morphological trait that serves as an integrated correlate of metabolic requirements on an appropriate timescale would improve our understanding of the energetic and oxygen requirements associated with having a large brain.

For most fishes, oxygen supply for aerobic metabolism is facilitated by the diffusion of oxygen across the gills, resulting in a close relationship between gill surface area and metabolic rate (Hughes, 1966; Wegner, 2011). Fick's second law of diffusion provides the framework for the role of gill surface area in metabolic oxygen supply capacity and shows that a larger respiratory surface area augments oxygen uptake (Fick, 1855 in Gillooly *et al*., 2016). Indeed, gill surface area is a metabolically important trait in fishes, and this has been shown on both intraspecific and interspecific scales (Bigman *et al*., 2018; Hughes, 1978; Pauly, 2021; Wegner *et al*., 2010). Intraspecific comparisons of ontogenetic allometries show that metabolic rate and gill surface area scale at similar rates with body mass (De Jager & Dekkers, 1975; Hughes, 1978; Wegner, 2016). Across species, fishes with higher metabolic rates have larger gill surface areas at a given body mass, with respiratory surface area and oxygen consumption also scaling at the same rate with body mass across fishes and other vertebrates (Bigman *et al*., 2021; Gillooly *et al*., 2016; Hughes, 1966). Thus, investigating gill surface area in tandem with brain size may improve our understanding of the energetic basis of brain size.

In addition to exhibiting lifelong neurogenesis, fishes grow throughout their lives (*i.e*., indeterminately), with both brain mass and gill surface area changing with body mass throughout ontogeny (Bigman *et al*., 2018; Laforest *et al*., 2020). Therefore, both brain mass and gill surface area must be studied in an allometric context and any comparison between brain mass and gill surface area throughout ontogeny must also account for body mass (Bigman *et al*., 2018; Laforest *et al*., 2020; Lisney *et al*., 2017). Although explanations for the rate at which brain mass changes with body mass (*i.e*., the allometric slope for brain mass) within a species and across ontogeny are rare, some explanations have been proposed for allometric slopes across species (*i.e*., evolutionary allometries). For example, because both brains and bodies are three-dimensional, brain mass may be expected to increase one-to-one with body mass (*i.e*., have an ontogenetic allometric slope of one) according to simple geometry, but comparisons both within and across species have suggested this is not the case (Harvey & Bennett, 1983; Tsuboi *et al*., 2018). For many years, the allometric slope of brain size was thought to be $^2/_3$ or 0.66 based on comparisons across both endothermic and ectothermic species, potentially due to brain mass innervating a two-dimensional body surface that increases with three-dimensional body mass (Jerison, 1973

in Harvey & Bennett, 1983). However, findings of an evolutionary allometric slope of brain size of approximately ¾ or 0.75 across mammalian species led to the idea that the scaling of body mass and brain size matched the scaling of body mass and metabolic rate, which was also believed to scale with a slope of around ¾ (Armstrong, 1983; Hofman, 1983; Martin, 1981). More recent interspecific analyses that correct for shared evolutionary history, as well as intraspecific studies on the allometric scaling of brain mass (*i.e.*, both evolutionary and ontogenetic allometries), have found slopes much lower than these predictions, tending to range from 0.4 to 0.6, though there are few explanations as to why this might be (Bauchot *et al.*, 1976; Tsuboi *et al.*, 2018; Yopak *et al.*, 2019). Gill surface area is a roughly two-dimensional surface supplying oxygen to a three-dimensional body mass, and thus gill surface area is expected to change with body mass with a slope of $^2/_3$ to 1 (Bigman *et al.*, 2018; Wegner *et al.*, 2010). Thus, to meet metabolic demands for all other life activities, brain growth might be limited by oxygen supply over the gills. Theoretically, the allometric slope of brain mass could have an upper limit imposed by the energetic and oxygen requirements of brain growth and maintenance, necessitating a shallower allometric slope of brain mass than the allometric slope of gill surface area. This may explain the variation in allometric slopes seen both within and across species, as the allometric slope of brain mass would only have an upper limit (set by the allometric slope of gill surface area or metabolic rate) rather than exactly match a specific allometric slope value such as $^2/_3$ or $^3/_4$ (Karbowski, 2007).

Chondrichthyans present a valuable opportunity to study the allometric relationships between brain mass and gill surface area. First, ectothermic animals can shed light on the energetic costs of brains without the additional energetic costs of thermoregulation, yet studies of the energetic cost of brains in ectotherms are rare compared to those in endotherms (Armstrong, 1983; Hofman, 1983; Isler & van Schaik, 2006). Second, chondrichthyans possess relatively large brains for their size in comparison to other ectothermic vertebrates (Lisney & Collin, 2006; Myagkov, 1991; Tsuboi *et al.*, 2018). Third, chondrichthyans occupy a basal position on the evolutionary tree of jawed vertebrates (*i.e.*, gnathostomes) and are the first group to exhibit the fundamental and highly homologous vertebrate brain plan (Yopak, 2012). However, despite their basal place in vertebrate evolution, chondrichthyan brains have been poorly studied in comparison to other vertebrate groups, with brain mass data only available for about 16% of all currently described chondrichthyan species (Yopak, 2012), and even less is understood about intraspecific variation in brain size in this group (Laforest *et al.*, 2020; Lisney *et al.*, 2007, 2017). For these reasons, we chose to study the blacktip shark *Carcharhinus limbatus* (Valenciennes 1839) from the western Atlantic Ocean. Although there are many studies on the ecology of *C. limbatus*, to our knowledge there is only one estimate of their brain mass in the literature, likely from a single individual (Carlson *et al.*, 2006; Myagkov, 1991).

Here, we explore how brain mass relates to gill surface area in *C. limbatus*. We first ask whether the allometric slope of brain mass is lower than the allometric slope of gill surface area across ontogeny.

Second, we test whether *C. limbatus* individuals with a larger brain also have a larger gill surface area for their body mass. We predict that both brain mass and gill surface area will have positive allometric slopes, but that the rate that brain mass changes with body mass will be lower than the rate that gill surface area changes with body mass. Additionally, we predict that individuals with a larger than expected brain for their body mass will also have a larger gill surface area for their body mass.

2 | MATERIALS AND METHODS

2.1 | Sample collection

We received specimens of *C. limbatus* that were opportunistically collected in August 2017 from fisheries-independent and fisheries-dependent trawl and longline surveys (GULFSPAN and COASTSPAN) in the north-eastern Gulf of Mexico near Panama City, Florida ($n = 18$) and the southern Atlantic Bight, off South Carolina and Georgia ($n = 10$). For each specimen, fork length (cm) and total length (cm) were measured. When possible, body mass (g) was also measured, otherwise mass was estimated using previously published population-specific fork length–weight regression equations (SEDAR 29, 2012). After capture, the head was removed, the chondrocranium of each specimen was opened to allow the brain tissue to fix and specimens were placed in 10% neutral-buffered formalin for later processing. Following previous work, which assumes minimal shrinkage due to fixation, neither brain mass nor gill surface area were corrected for such shrinkage (Wootton *et al.*, 2015; Yopak *et al.*, 2007). This research was conducted with the approval of the University Animal Care Committee of Simon Fraser University, in accordance with the Canadian Council on Animal Care.

2.2 | Brain mass measurement

Total brain mass was measured in a manner consistent with previous methods following Yopak *et al.* (2007). After a period of post-fixation, each brain was excised from the chondrocranium and separated from the spinal cord caudal to the posterior tip of the fossa rhomboidea in the region of the first complete cervical spinal nerve (Lisney *et al.*, 2017; Yopak *et al.*, 2007). The meninges, blood vessels and connective tissue were removed, and the cranial and sensory nerves were transected to within 1 mm of their base. Each brain was blotted and weighed to the nearest 0.01 g (ScoutProScale).

2.2.1 | Gill surface area measurement

Total gill surface area was estimated according to Muir and Hughes (1969) and Hughes (1984):

$$A = L_{fil} \times 2n_{lam} \times A_{lam}$$

where A is gill surface area, L_{fil} is the total length of all gill filaments on both sides of the head, n_{lam} is the lamellar frequency (*i.e.*, the average number of lamellae per unit length on one side of a filament, doubled to account for both sides of the filament) and A_{lam} is the mean bilateral surface area of an individual lamella (Hughes, 1984; Muir & Hughes, 1969; Wegner, 2011). This standard method of measuring gill surface area was chosen to make our results comparable to other gill surface area estimates for elasmobranchs and other fishes (Bigman et al., 2018; Wegner et al., 2010; Wootton et al., 2015). Dissections were performed using a dissecting scope fitted with a digital camera (either Zeiss Stemi 2000-C with a Lumenera INFINITYLite camera or Meiji Stereo EMZ-8TR with a MoticCam 5+). Because gill surface area is symmetrical and either side can be used for measurements, we used either the right or left side depending on the condition of the filaments (Bigman et al., 2018; Wegner, 2011; Wootton et al., 2015). For all but one individual, gill surface area was measured on the right side of the head; for the remaining individual, gill arches were only available for the left side of head. For a more detailed description of the gill surface area measurement procedure see Bigman et al. (2018) and Vander Wright et al. (2020).

It was not possible to measure brain mass and gill surface area on all individuals. For 15 individuals, both brain mass and gill surface area were measured; for a further nine individuals, only brain mass was measured and for a further four individuals, only gill surface area was measured. Although this did not affect the body mass range over which gill surface area was measured (750–30,043 g), it resulted in a decreased body mass range for which brain mass could be measured (750–12,977 g; Table 1).

2.3 | Statistical analyses

2.3.1 | Is the allometric slope of brain mass lower than the allometric slope of gill surface area?

To test whether the allometric slope of brain mass was lower than the allometric slope of gill surface area, we fit two linear models, one for the relationship of brain mass and body mass ($n = 24$), and one for the relationship of gill surface area and body mass ($n = 19$). As samples came from two different locations, the Gulf of Mexico and the south Atlantic Bight, we assessed whether the allometric relationships of brain mass and gill surface area differed between the locations using location as a fixed factor and including an interaction with body mass (Supporting Information Table S1). This

parameterization allows for the estimation of location-specific allometric slopes and standardized intercepts, which were then compared between locations by assessing the overlap of the 95% confidence intervals (CIs). As we found little effect of location for this small and biased sample size, we pooled the data for subsequent analyses (Supporting Information Figure S1 and Table S2). To be consistent with previous work, we used linear regression on $\log_{10}$-transformed data for both brain mass and gill surface area allometric relationships (Bigman et al., 2018; Chapman & Hulen, 2001; Salas et al., 2015). On a $\log_{10}$-transformed scale, the intercept is estimated at 1 g of body mass, which lies far outside the range of body masses for the specimens in this study. To avoid this extrapolation of the intercept, which can lead to a correlation between intercepts and slope, we centred the body mass data around 2000 g (approximately the median of our specimen size range; Bigman et al., 2018; Quinn & Keough, 2002). Thus, the intercept is estimated at a more meaningful body mass (2000 g) and is termed the 'standardized intercept'. Importantly, the intercept can be centred on any value, and thus can be interpreted biologically as the gill surface area or brain mass at a given body mass (Bigman et al., 2018). All statistical analyses were performed in R v. 3.6.1 (R Core Team, 2019).

2.3.2 | Do individuals with larger brains for their body mass also have larger gill surface areas?

To determine whether individuals with a larger brain also have a larger gill surface area after accounting for body mass, we parameterized two linear models using data from individuals for which both gill surface area and brain mass were measured ($n = 15$). In both models, brain mass was the response variable. In the first model, only body mass was tested as an explanatory variable, while in the second model both gill surface area and body mass were explanatory variables. Both explanatory and response variables were $\log_{10}$-transformed, and body mass was centred to 2000 g, as above. To identify the model that provided the best fit to the data, we compared these two candidate models using the corrected Akaike information criteria (AICc) for small sample sizes, which penalizes models for their number of estimated parameters, with smaller AICc values indicating a better fitting model (Burnham & Anderson, 2002; Hurvich & Tsai, 1989). The weight of evidence for any given model out of those tested was measured by its Akaike weight (w_i), the relative likelihood of the model divided by the sum of the likelihoods of all other models.

TABLE 1 Sample size (n), numbers of each sex (female, F; male, M) and ranges of body mass, fork length (FL), brain mass and gill surface area (GSA) for blacktip shark, *Carcharhinus limbatus*, samples for which we measured brain mass, gill surface area or both

Traits measured	n	Sex	Body mass range (g)	FL range (cm)	Brain mass range (g)	GSA range (cm²)
Brain mass	24	14 F, 10 M	750–12,977	45.5–101.0	6.02–21.23	
Gill surface area	19	12 F, 7 M	750–30,043	45.5–133.0		2,462.84–58,205.11
Both	15	10 F, 5 M	750–12,977	45.5–101.0	6.02–21.23	2,462.84–34,919.63

3 | RESULTS

Gill surface area estimates ranged from 2,463 to 58,205 cm^2 over the body mass range of 750 to 30,043 g ($n = 19$) while brain mass ranged from 6.02 to 21.23 g over the over the body mass range of 750 to 12,977 g ($n = 24$; Table 1).

3.1 | Is the allometric slope of brain mass lower than the allometric slope of gill surface area?

The allometric slope of brain mass was positive but lower than the allometric slope of gill surface area. The rate that brain mass changed with body mass was 0.45 (95% CI 0.41–0.49; Figure 1a) while the rate that gill surface area changed with body mass was 0.90 (95% CI 0.81–0.99; Figure 1b and Table 2). For a doubling (i.e., a 100% increase) in body mass from 2,000 to 4,000 g, brain mass increased by about 37% while gill surface area increased by about 86%. Thus, a 2000 g individual was predicted to have around an 8.41 g brain and a 5,282.82 cm^2 gill surface area, while a 4,000 g individual was predicted to have around an 11.49 g brain and a 9,848.31 cm^2 gill surface area.

3.2 | Do individuals with larger brains for their body mass also have larger gill surface areas?

Gill surface area explained some of the variation in brain mass after accounting for body mass, with individuals that had a large brain for their body mass also having a large gill surface area (Figure 1c). The model including both gill surface area and body mass as explanatory variables (AICc $= -58.8$, $w_i = 0.78$) fits the data slightly better than the model with just body mass (AICc $= -56.3$, $w_i = 0.22$) and had a greater weight of evidence out of the two candidate models.

4 | DISCUSSION

Overall, we found that the allometric slope of brain mass was lower than the allometric slope of gill surface area across *C. limbatus* individuals, and that individuals with a large brain for their body mass also had a larger gill surface area, as predicted. Next, we discuss (a) our results on the allometric relationships of brain mass and gill surface area in the context of the scientific literature, (b) our findings as they pertain to the relationship between brains, gills and energy demand, and (c) caveats of the study that should be considered and future directions of research.

Both brain mass and gill surface area increased ontogenetically with body mass, but the rate that brain mass changed with body mass (slope $= 0.45$) was lower than the rate that gill surface area changed with body mass (slope $= 0.90$). These allometric slopes show that larger *C. limbatus* individuals possess both larger brains and larger gill surface areas compared to smaller individuals, corroborating our predictions and the results of studies in other species (Bigman
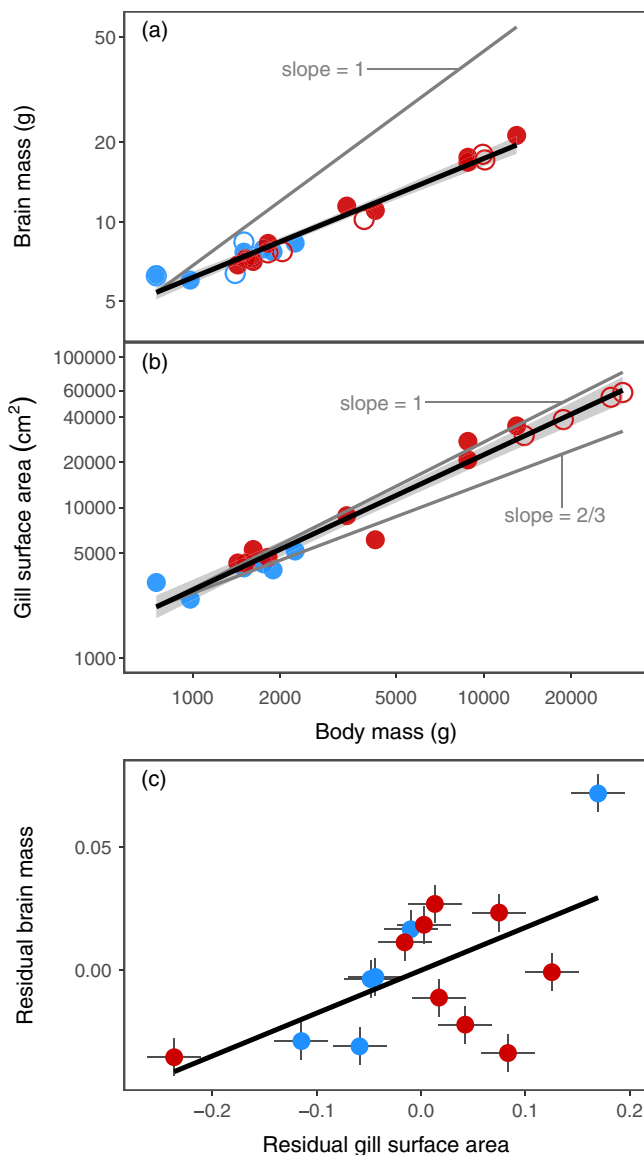

FIGURE 1 The relationship of (a) brain mass (g) and (b) gill surface area (cm^2) to body mass (g) for blacktip shark, *Carcharhinus limbatus*, samples from the Gulf of Mexico (red) and Atlantic Bight (blue). Filled points represent individuals for which both brain mass and gill surface area were measured. The black fitted regression lines are from linear models of log$_{10}$-transformed gill surface area or log$_{10}$-transformed brain mass data as functions of log$_{10}$-transformed body mass. Shaded grey regions indicate the 95% confidence intervals. Grey lines depict theoretical allometric relationships with slopes of 1 (a), and ⅔ and 1 (b). Gill surface area is expected to change with body mass with a slope of ⅔ to 1 while brain mass is expected to change with body mass with a slope less than that of gill surface area. The relationship between residual brain mass (after correcting for body mass) and residual gill surface area is depicted in (c) with error bars showing the standard error of the mean (s.e.m.), although analyses were conducted using multiple regression to account for body mass rather than residuals

et al., 2018; Chapman & Hulen, 2001; Lisney et al., 2017). Brain mass likely changes with body mass to allow for neural control, sensation and regulation associated with a growing body (Leonard et al., 1978; Ngwenya et al., 2013). Since fishes (like other ectothermic

TABLE 2 Coefficients of the linear regressions for brain mass and body mass, and gill surface area and body mass for blacktip sharks, *Carcharhinus limbatus*

Model	Allometric slope	Standardized intercept
Brain mass~body mass	0.45 (0.41–0.49)	8.41 (8.12–8.71) g
Gill surface area~body mass	0.90 (0.81–0.99)	5,283.82 (4,705.57–5,933.13) cm^2

Note. Values in brackets are the 95% confidence intervals and standardized intercepts are the back-transformed estimates of gill surface area or brain mass for a 2,000 g individual. All variables were log$_{10}$-transformed.

vertebrates) grow indeterminately, their brains may exhibit lifelong neurogenesis and brain growth to match the neural demands of the body (Leonard *et al.*, 1978; Ngwenya *et al.*, 2013; Zupanc, 2006). For example, our estimate of the brain mass allometric slope was similar to those reported in various other intraspecific studies of fishes, which reported slopes of 0.48–0.57 (Bauchot *et al.*, 1976) and 0.5 (95% CI 0.46–0.54; Gonda *et al.*, 2011) for bony fishes and 0.427 (95% CI 0.374–0.480; Lisney *et al.*, 2017) and 0.46 (95% CI 0.43–0.49; Laforest *et al.*, 2020) for cartilaginous fishes. Our slope estimate was also similar to evolutionary allometric slopes across cartilaginous fishes broadly (slope = 0.43; Yopak *et al.*, 2019) and Carcharhiniformes specifically, where brain mass changes with body mass with a slope of 0.52 (Myagkov, 1991). However, this slope estimate was much lower than geometric expectations (slope = 1), or predictions based upon either body surface area (slope = $^2/_3$), or an exact match with metabolic scaling (slope = ¾; Armstrong, 1983; Hofman, 1983; Jerison, 1973 in Harvey & Bennett, 1983). Additionally, as gill surface area is a metabolically important trait that supports the oxygen diffusion necessary for aerobic metabolism in fishes, it fits that gill surface area would also change with body mass throughout ontogeny to support the energetic requirements of a larger body and potentially the energetic requirements of a growing brain (Bigman *et al.*, 2018; Hughes, 1978). The allometric slope of gill surface area found in this study corresponds to previous work on gills that found allometric slopes between $^2/_3$ and 1 (Bigman *et al.*, 2018; Wegner *et al.*, 2010; Wootton *et al.*, 2015), as well as an average of ontogenetic allometric slopes of metabolic rate in fishes (slope = 0.89; Jerde *et al.*, 2019). Although our results are correlational, the lower allometric slope of brain mass compared to gill surface area supports our prediction that gill surface area, and by extension oxygen supply capacity, may set an upper limit for the allometric slope of brain mass above which individuals would potentially reach a point where they had more neural tissue than they could supply oxygen to and energetically support (Karbowski, 2007). Therefore, these individuals would likely be selected against. Thus, examining the ontogenetic allometries of brain mass and gill surface area may provide some insight into the evolution of brain size.

Our results also suggest that the ability to acquire the oxygen required for metabolism may explain some of the variation in brain size in this species. Using gill surface area as a proxy for oxygen consumption and energetic demand on evolutionary time scales, we found that *C. limbatus* individuals with a large gill surface area for their body mass may be able to support a higher energetic turnover and, in turn, a larger brain for their body mass. However, brain size is highly variable at large body sizes in cartilaginous fishes (Laforest *et al.*, 2020). Thus, although our results suggest that individuals with a large brain for a given body mass may also have a large gill surface area, more data is needed spanning the full range of body sizes in *C. limbatus*. Our results are consistent with the direct metabolic constraints hypothesis, which suggests that the cost of evolving a larger brain can be met through the evolution of increased energy intake (Armstrong, 1983; Hofman, 1983; Isler & van Schaik, 2009). However, this strategy is likely to be risky, given that individuals would be more vulnerable to unexpected shortages of energy supply, such as hypoxic events or food shortages (Deaner *et al.*, 2003; Isler & van Schaik, 2009). To our knowledge, evidence for the direct metabolic constraints hypothesis has not previously been presented within wild individuals of a species, since studies usually use interspecific trends or laboratory experiments to demonstrate a connection between metabolism and brain size (Iglesias *et al.*, 2015; Isler & van Schaik, 2006; Kotrschal *et al.*, 2013).

Our results show a relationship between brain mass and gill surface area, a metabolically important morphological trait that may portray a more integrated estimate of oxygen demand and energy use compared to shorter-term measures like metabolic rate (Bigman *et al.*, 2021; Gillooly *et al.*, 2016). However, the energetic requirements of maintaining a large brain could also require compromises that affect other organs or processes, in addition to an increase in energy turnover (Aiello & Wheeler, 1995; Isler & van Schaik, 2009). Indeed, brain size has been shown to trade off with other energetically expensive organs or activities that are beyond the scope of this study. For example, across species, brain size has been negatively correlated with gut length in fishes and anurans (Kotrschal *et al.*, 2013; Liao *et al.*, 2016; Tsuboi *et al.*, 2015), gonad size in bats (Pitnick *et al.*, 2006) and pectoral muscle size in birds (Isler & van Schaik, 2006). Interspecific analyses of sharks, cichlids and frogs, respectively, reveal that species with 'slower' life histories tend to have a larger brain for their body size (Mull *et al.*, 2011; Tsuboi *et al.*, 2015; Yu *et al.*, 2018). Additionally, an intraspecific study of *P. reticulata* revealed that individuals selected for larger brains have fewer offspring (Kotrschal *et al.*, 2013). Accounting for whether trade-offs occur with other energetically expensive organs or activities will strengthen our understanding of the energetic basis of variation in brain size. Hence, investigating the energetic 'budgets' (*i.e.*, energy uptake, allocation and use) of *C. limbatus* would be an interesting avenue for future study.

The links between energy demand and oxygen availability, and between brains and gills specifically, provide a rich area for future research. First, investigating the allometric relationships of separate regions of the brain – and not just total brain mass – may provide further clues about the various pressures acting on these metabolically important organs and may be particularly important for fishes, given

their indeterminate brain growth. Several studies have documented intraspecific variability in patterns of brain organization, both between populations and throughout ontogeny. For instance, individuals from marine populations of nine-spined sticklebacks *Pungitius pungitius* L. had a larger olfactory bulb and telencephalon (after accounting for both body size and total brain size) compared to individuals from pond populations, likely due to differences in habitat complexity between the environments (Gonda *et al.*, 2009). Additionally, ontogenetic shifts in the sizes of major brain regions compared to the whole brain in the bluespotted stingray *Neotrygon kuhlii* (Müller & Henle 1841), pouched lamprey *Geotria australis* Grey 1851 and Atlantic sharpnose shark *Rhizoprionodon terraenovae* (Richardson 1836) have been associated with shifts in diet, sensory specialization, habitat use and activity patterns (Laforest *et al.*, 2020; Lisney *et al.*, 2007, 2017; Salas *et al.*, 2015). Ontogenetic studies that focus on various brain regions may thus shed light on some of the external pressures that play a role in selecting brain size. Second, since this is a correlative study comparing brain mass and gill surface area, there may be other factors affecting these traits that we were unable to account for, including the cost of other potentially energetically expensive organs and processes such as the gut, liver and reproductive investment. Further studies that are able to combine both *in situ* population studies and experimental manipulations that specifically include multiple generations (*i.e.*, selection experiments) and other expensive organs could both control for factors not investigated here and also help tease apart phenotypic plasticity from adaptive selection, a central challenge in evolutionary biology and ecology (Chapman *et al.*, 2000; Gonda *et al.*, 2011). For instance, Crispo and Chapman (2010) conducted a laboratory-rearing experiment using broods from multiple populations of an African cichlid fish *Pseudocrenilabrus multicolor* (Schöller 1903) under high- and low-oxygen treatment. They found that variation in *P. multicolor* brain mass and gill surface area was partially due to plasticity, such that individuals in the low-oxygen treatment had smaller brains and larger gills for their body size than those in the high-oxygen treatment, regardless of the population that they originally came from. However, they also found population variation in brain mass within treatments, suggesting that there are also genetic effects on *P. multicolor* brain mass (Crispo & Chapman, 2010).

As ectothermic metabolic rate, and thus oxygen demand, increases with temperature, future studies could also investigate the interrelationships between temperature, oxygen availability and metabolically important traits like gill surface area and brain mass. The Gulf of Mexico and Atlantic Bight differ environmentally, with the Gulf of Mexico tending to have warmer, more oxygen-stressed waters (Belkin, 2009; Rabalais & Turner, 2001). Additionally, *C. limbatus* from the Gulf of Mexico and Atlantic Bight are considered to be two separate populations, as they are genetically and geographically distinct, with individuals from the Gulf of Mexico tending to mature earlier and grow to a smaller size (Carlson *et al.*, 2006; Keeney *et al.*, 2003, 2005). Studies on the differences in metabolically important traits like brain mass and gill surface area between individuals from these two populations could thus help identify bases for temperature-body-size patterns and further our understanding of how temperature and oxygen availability may affect metabolically important morphological traits. Comparisons of traits among distinct populations fill an important gap between large-scale, interspecific comparisons across species and studies on individual variation, thus helping to improve our understanding of the evolution, and functional significance, of variation in key traits (Gonda *et al.*, 2013). Insight into these interrelationships could also shed light on drivers of life history variation and explanations for temperature-body-size patterns.

In conclusion, our results indicate that both brain mass and gill surface area increase allometrically with body mass in *C. limbatus* throughout ontogeny, but that brain mass changes with body mass at a lower rate than gill surface area does. Additionally, our results suggest that *C. limbatus* individuals with a larger brain for their body mass may also have a larger gill surface area. As with many other organismal traits, brain size is likely the result of multiple trade-offs between energetic costs and benefits. By showing that both brain mass and gill surface area increase with body mass, and that one measure of the capacity for oxygen diffusion (*i.e.*, gill surface area) explains some of the variation in brain mass throughout ontogeny, this study provides clues as to one potential mechanism through which fishes may have evolved and maintained large brains, despite their high energetic cost. This work on the relationship between these two metabolically important traits, as well as future studies on how allometric relationships vary between and among populations, could provide clues about the evolution of brain size. Both this work and further studies may also help us predict the effects of future challenges on fishes, such as increasing water temperature and severe hypoxic events, which may change energetic demands and oxygen availabilities.

AUTHOR CONTRIBUTIONS

S.W., J.S.B. and N.K.D. designed the study. S.W. and J.S.B. conducted sample collection and gill surface area measurements. S.W. conducted brain mass measurements with the help of K.E.Y. K.E.Y and N.K.D. provided laboratory space and resources. S.W. performed all statistical analyses and data visualizations, and wrote the first draft of the manuscript. All authors contributed to the final version of the manuscript.

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