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Climbing Waterfalls: How Metabolism and Behavior Impact Locomotor Performance of Tropical Climbing Gobies on Reunion Island

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Abstract :

The life cycle of gobies of the Sicydiinae subfamily depends on climbing waterfalls. Two sympatric sicydiines species from Reunion Island, Sicyopterus lagocephalus (SIL) and Cotylopus acutipinnis (COA), employ different climbing modes. SIL uses a steady "inching" mode interrupted by short rest periods, whereas COA exhibits short "power-burst" undulatory movements punctuated by longer rest periods. Consequently, we explored the relationship between climbing performance and metabolic activity in these two species. We demonstrated that the two climbing modes are supported by different ecophysiological profiles that promote the interspecific variability of locomotor performance. More specifically, SIL performed better than COA during a climbing experiment because of its inching climbing mode, supported by a generally greater metabolic capacity and a higher potential for oxidative metabolism. Interestingly, we did not detect any difference in metabolic fuel storage and lactate production during climbing in either species, suggesting that these species can maintain fuel reserves and limit lactate accumulation through extensive rest times. Overall, this study provides new insights into the ecophysiology of these two emblematic species and suggests that the better climbing capacity of SIL is supported by its muscular metabolic capacity.

Keywords : amphidromy, Cotylopus acutipinnis, enzymatic profile, metabolic fuel, migration, Sicyopterus lagocephalus

Introduction

Amphidromous gobies of the Sicydiinae subfamily display a remarkable locomotor style: the ability to climb waterfalls (Schoenfuss and Blob 2003). These species reproduce in freshwater (Teichert et al. 2013), their larvae immediately drift down to the sea (Lagarde et al. 2017) where they develop for months (Teichert et al. 2012, 2016) before returning to freshwater to grow and mature (Teichert et al. 2014a, 2014b). The rivers of the small tropical islands inhabited by sicydiines are punctuated by numerous waterfalls. Consequently, sicydiines often need to climb up vertical waterfalls to reach their spawning habitats (Schoenfuss et al. 2013). As most aquatic predators of sicydiines are not able to scale waterfalls, climbing to the upstream reach of these rivers greatly reduces the risk of predation (Diamond et al. 2019). Therefore, waterfalls climbing is fundamental for these species as their successful reproduction in freshwater greatly relies on it.

In Reunion Island (South Western Indian Ocean), two sicydiines species, *Sicyopterus lagocephalus* (SIL) and *Cotylopus acutipinnis* (COA), dominate the freshwater fish assemblage (Lagarde 2018) but use different climbing modes (Blob et al. 2019). SIL alternatively attaches to the substrate with its pelvic and oral suckers to climb up, with short resting periods when the pelvic sucker adheres to the substrate. This is analogous to the "inching" mode described in another *Sicyopterus* species in Hawaii (Schoenfuss and Blob 2003). In contrast, COA does not use its oral sucker but instead generates bursts of undulatory movements with its tail and pectoral fins. Similarly to SIL, COA rests between climbing bouts, but these climbing pauses are more frequent and last longer. This "powerburst" climbing has been described in other amphidromous gobies of the *Awaous* and *Lentipes* genuses in Hawaii (Schoenfuss and Blob 2003) and Sicydium genus in Dominica (Schoenfuss et al. 2011). Interestingly, the proportion of red muscle fibers is elevated in the inching *Sicyopterus stimpsoni* compared to the powerburst climbers *Lentipes concolor* and *Awaous*

60 *guamensis* (Cediel et al. 2008). Thus, it is possible that inching climbers may be more reliant
61 on oxidative metabolism than the powerburst climbers. In Reunion Island, a marked
62 difference of climbing performance has been observed, both experimentally and in the field,
63 as SIL is able to inch up steeper obstacles, much faster than the powerburst climber COA
64 (Lagarde et al. 2018, 2020; Blob et al. 2019).

65 We explored the relationship between climbing performance and metabolic activity in
66 the ecophysiological context of these two sympatric species. We proposed that SIL performs
67 better than COA due to its inching climbing mode supported by a generally greater metabolic
68 capacity and a higher potential for oxidative metabolism. Consequently, compared to the
69 sympatric powerburst climbing COA, the inch climbing SIL may rely on a higher supply of
70 metabolic fuels and have reduced lactate accumulation during a climbing effort.

71

Methods

Fish capture and experimental setup

Fish sampling and experimental design were approved under the permit N°16-019/DEAL/SEB/UPEMA issued by Direction de l'Environnement de l'Aménagement et du Logement de La Réunion. We collected more than 200 juveniles of SIL and COA in June 2016 (SIL: 10th of June; COA: 3rd of June) by electroshocking (Hans Grassel IG 200-2 electroshocker) immediately after their arrival at the mouth of the St Etienne River in South-Western Reunion Island (21.0°S-55.5°E, figure S1a-b, available online). Immediately after capture, we transported fish in aerated water to the laboratory where they were acclimated during a 48h period in the lower tank of the experimental arena before starting the experiment. Fish were not fed at any point during the experiment.

The detailed description of the experimental arena was described previously (Lagarde et al. 2018). Briefly, it consisted of a 2.40 m ramp angled at 45° linking two tanks (figure S1c, available online). The upper tank was fitted with a trapping device to prevent fish from falling back to the lower tank after successfully climbing the ramp. The ramp was roughened with sand paper to facilitate climbing and the temperature of the room was maintained at approximately 24°C in a 12:12h light/dark cycle.

Before commencing the climbing experiments, we randomly sampled 20 individuals of each species. They were then anesthetized in 0.3 ml·L^{-1} of clove oil solution (diluted at 30% in alcohol) and decapitated after they reached stage IV of anaesthesia (slow opercular movement, total loss of equilibrium) as recommended for surgery (Carter et al. 2011). After decapitation, we kept fish on dry ice before freezing at -80°C. The climbing experiment was completed during two consecutive days from 8:00 to 18:00. First, the climbing behaviour was stimulated by a 3 L·mn^{-1} discharge of stream water pumped from the lower tank to the

climbing ramp. The lowest 30 cm of the ramp was recorded for the duration of the experiment with a dorsal view of the climbing fish (Sony Handycam mini-DV camera; 30 frames·s^{-1}). Moreover, we continuously observed the upper part of the ramp and collected the fish that entered the trap in the upper tank. These fish were euthanized and preserved as described above.

Climbing performance analysis

We quantified climbing performance of the two species by measuring three variables: net climbing speed, bout climbing speed, and percentage of time spent in motion from videos sequences using the open access Tracker V. 4.11 software (Brown 2017). Net climbing speed corresponded to the total distance climbed by each individual divided by the total time spent in motion and resting. Bout climbing speed corresponded to the total distance climbed by each individual divided by the time spend in motion. Finally, percentage of time spend in motion corresponded to the time spend in motion divided by the total time recorded. We standardized climbing speeds to body length (BL·s^{-1}) to limit the influence of inter-specific and inter-individual size differences on climbing performance comparisons. We estimated BL (cm) using a Tracker measurement tool on each video sequence. We measured the distance travelled during one climbing bout as the difference of distance of the tip of the snout between the beginning and the end of the climbing bout. As some individuals failed to climb the lowest 30 cm of the ramp, measurements were repeated on three to 12 climbing bouts for each video sequence corresponding to a total distance climbed by the fish ranging from seven cm (i.e. approximately two BL) to 29 cm (approximately 10 BL).

Enzymatic profiles

To assess potential differences in metabolic profiles between the two species, we selected five metabolic enzymes to assay their maximal capacity in the axial muscles of both

SIL (n= 6-8) and COA (n= 6-7). Due to the small size of individuals, red and white muscle fiber types could not be separated resulting in mixed fibers samples. We measured the activity of two oxidative enzymes, the mitochondrial matrix citrate synthase (CS), and β-hydroxyacyl-CoA dehydrogenase (HOAD, a fatty acid oxidation enzyme). Conversely, we also assayed three enzymes, markers of anaerobic glycolytic capacity, pyruvate kinase (PK), hexokinase (HK), and lactate dehydrogenase (LDH). The part of the fish body posterior to the pelvic fins, primarily composed of axial musculature, was pulverized with a mortar and pestle in liquid nitrogen. Aliquots of powdered samples were then homogenized in 10 volumes of enzyme extraction buffer consisting of 20 mM HEPES pH 7.0, 1 mM EDTA, and 0.1% Triton X-100. Each enzymatic assay was run in triplicate with a SpectraMax Plus 384 spectrophotometer (Molecular Devices, San Jose, CA) as described previously (LeMoine et al. 2006, 2008). Briefly, the activity of HOAD was assayed on fresh homogenates at 340 nm in 20 mM imidazole, 0.1% Triton X-100, 0.15 mM NADH and 0.1 mM acetoacetyl CoA to start the reaction. Except where noted, subsequent enzymes assays were performed after one freeze/thaw cycle. The maximal activity of CS was measured at 412 nm in 20 mM Tris pH 8.0, 0.05% Triton X-100, 0.1 mM DTNB, 0.3 mM acetyl CoA, and 0.5 mM oxaloacetate. HK was assayed at 340 nm in 20 mM imidazole, 5 mM DTT and 5 mM MgCl2, 1 mM ATP, XU G-6-PDH and 0.5 mM NAD(P). The reaction was started with addition of 5 mM glucose (omitted for negative control). PK was measured in 50 mM Imidazole pH 7.4, with 5 mM ADP, 100 mM KCl, 10 mM MgCl2, 0.15 NADH, 10 uM fructose 1,6, P, 5 I LDH and +/- 5 mM PEP to start the reaction. Finally, LDH activity was assayed after the homogenate went through two freeze/thaw cycles, it was measured at 340 nm in 50 mM HEPES pH 7.0, 0.15 mM NADH, and 0.2 mM pyruvate-Na.

Evaluation of endogenous fuel supply and lactate production during climbing

We assayed tissue concentrations of two endogenous fuels (total lipids and glycogen) and a glycolytic metabolite (lactate) in triplicates for each individual. We measured total lipids tissue concentration on eight individuals of each species sampled before climbing, and expressed as the ratio of lipids content (mg) over the fish body mass (g). Lipids were extracted from pulverized axial musculature with chloroform-methanol. Their concentrations were estimated with the SpectraMax plus 384 spectrophotometer based on the optical density of vanillin reagent following the procedure described by Van Handel (1985). Similarly, we estimated glycogen and lactate tissue concentrations from 12 individuals of each species, five sampled before climbing and seven after climbing for COA, and six sampled before climbing and six after climbing for SIL. Tissue concentrations were measured in pulverized axial musculature using commercially available kits for lactate and glycogen (Sigma Aldrich, CA) following the manufacturer's instructions. Measurements of optical densities were made with the same spectrophotometer as described above.

Statistical analyses

When assays were run in triplicates (i.e. enzymatic activity; lipids, glycogen and lactate tissue concentration), the three values were generally consistent with each other (standard error, SE $\leq$ 15%). Consequently, the mean values of the triplicates were kept for further analyses.

For net climbing speed, percentage of time spend in motion, hexokinase concentration, the assumption of normality and homogeneity of variance were not respected and/or the sample size was relatively low (i.e. $\leq$ eight individuals). Accordingly, we performed non-parametric tests to compare variables among groups. We compared variables between two groups using a Wilcoxon rank test and variables between more than two groups using a Kruskal-Wallis test. We performed all statistical analyses with the open source R v. 3.5.1 software (R Development Core Team 2016).

Results

Climbing performance

We confirmed climbing performance differences for these species by recording the overall climbing characteristics of 136 wild-caught fish. In particular, we evaluated the climbing performance using 67 video sequences of climbing SIL (BL range: 2.6-4.2 cm), and 69 video sequences of climbing COA (BL range: 2.2-2.4 cm). SIL were able to climb three times faster (0.13 $BL \cdot s^{-1}$ or 0.52 $cm \cdot s^{-1}$, Figure 1) than their COA counterparts (0.04 $BL \cdot s^{-1}$ or 0.12 $cm \cdot s^{-1}$, Figure 1, Wilcoxon rank test, $W = 792$, $p < 0.001$). This difference was further evident as SIL spent five times longer in motion than COA (33% vs. 6.8%, Figure 1, Wilcoxon rank test, $W = 4689$, $p < 0.001$). In contrast, COA climbing bouts were two times faster than their SIL counterparts (0.65 $BL \cdot s^{-1}$ vs. 0.39 $BL \cdot s^{-1}$, Figure 1, Wilcoxon rank test, $W = 4431$, $p < 0.001$). COA faster climbing bouts were not sufficient to counterbalance their longer resting periods on the overall climbing performance compared to SIL.

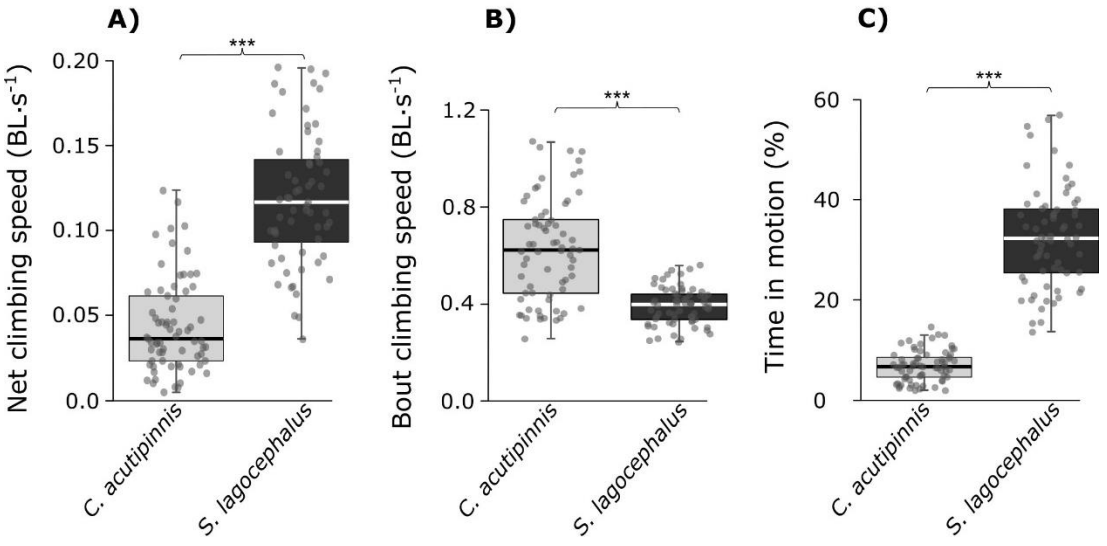


Figure 1 : Net climbing speed (A), bout climbing speed (B) and time spend in motion (C) for C. acutipinnis (N=69) and S. lagocephalus (N=67). Boxes are divided by quartiles, whiskers indicate the range of the data and grey dots the individual data points. The

187 significance of the difference between the two species was evaluated using a Wilcoxon rank
188 test. n.s : non-significant; * p <0.05; ** p<0.01; *** p<0.001.

189

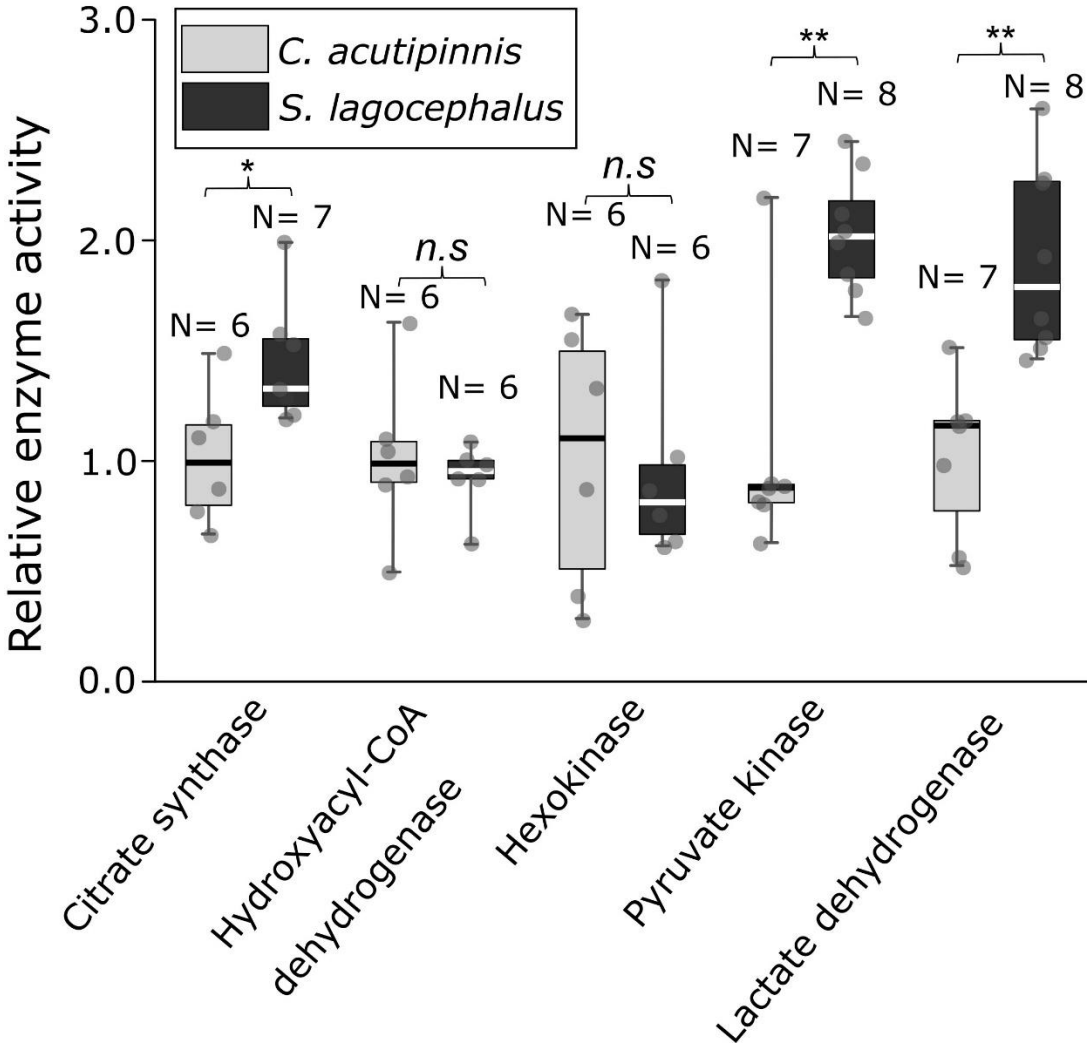
190 **Table 1 :** Mean (±SE) enzymatic activity of Citrate Synthase (CS), Hydroxyacyl-CoA-
191 deshydrogenase (HOAD), Hexokinase (HK), Pyruvate Kinase (PK) and Lactate
192 Deshydrogenase (LD) expressed in tissue concentration ($U.g^{-1}$).

	S. lagocephalus		*C. acutipinnis*	
	N	Tissue concentration	N	Tissue concentration
CS	7	14.68 (1.10) $U.g^{-1}$	6	10.27 (1.28) $U.g^{-1}$
HOAD	6	0.87 (0.06) $U.g^{-1}$	6	0.96 (0.14) $U.g^{-1}$
HK	6	0.06 (0.01) $U.g^{-1}$	6	0.07 (0.01) $U.g^{-1}$
PK	8	45.80 (2.22) $U.g^{-1}$	7	22.75 (4.53) $U.g^{-1}$
LDH	8	163.79 (13.14) $U.g^{-1}$	7	86.6 (11.79) $U.g^{-1}$

193

194 *Enzymatic profile*

195 The two species had different enzymatic profiles with SIL having generally higher
196 enzymatic capacity, with a 1.4-fold higher CS activity (Wilcoxon rank test, $W = 38$, $p = 0.014$, Table 1, Figure 2), a 1.9-fold higher PK activity (Wilcoxon rank test, $W = 50$, $p = 0.009$, Table 1, Figure 2) and a 2.0-fold higher LDH activity (Wilcoxon rank test, $W = 54$, $p = 0.001$, Table 1, Figure 2). In contrast, HOAD and HK showed no differences between the two
200 species (Wilcoxon rank test, $W \leq 17$, $p \geq 0.70$, Table 1, Figure 2).



201

202 **Figure 2 :** Enzymatic activity of S. lagocephalus relatively to the mean concentration
203 of the same enzymes measured in C. acutipinnis. Boxes are divided by quartiles, whiskers
204 indicate the range of the data and greys dots the individual data points. The number of
205 individuals (N) for which enzymatic activity was measured is specified. The significance of
206 the difference between the two species was evaluated using a Wilcoxon rank test. n.s : non-
207 significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

208

209 *Energetic fuel storage and lactate production*

210 In order to assess interspecies differences in metabolic fuels and metabolites post-
211 climbing, we evaluated total lipids, glycogen and lactate concentrations in the axial

musculature of SIL (n =6-8) and COA (n= 6-8). Sampled tissues mass ranged from 6.6 to 26.4 mg and the total lipids concentration averaged 7.0 $mg \cdot g^{-1}$ for SIL and 8.7 $mg \cdot g^{-1}$ for COA (Figure 3) with no significant differences between the two species (Wilcoxon rank test, W = 40, p = 0.19).

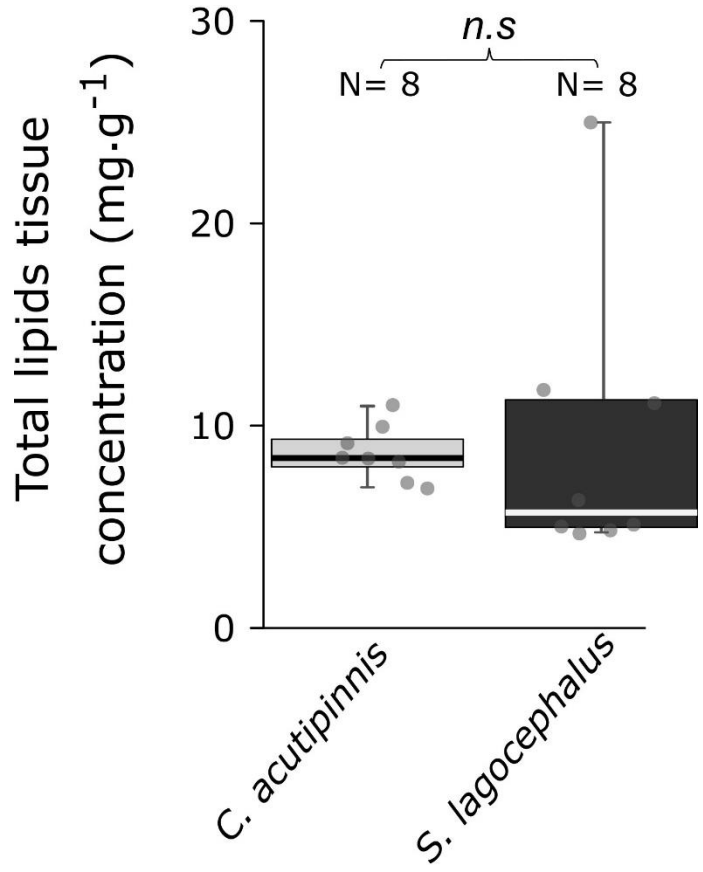


Figure 3 : Total lipids tissue concentration measured in C. acutipinnis and S. lagocephalus. Boxes are divided by quartiles, whiskers indicate the range of the data and grey dots the individual data points. The number of individuals (N) for which lipids tissue concentration was measured is specified. The significance of the difference between the two species was evaluated using a Wilcoxon rank test. n.s : non-significant; * p <0.05; ** p<0.01; *** p<0.001.

Glycogen concentrations did not differ between the two species or before and after climbing within each species (Kruskal-Wallis, $Chi^2 = 0.79$, df = 3, p = 0.85, Figure 4). These concentrations were 1.3 and 1.2 µmol·g^{-1} for SIL and 1.5 and 1.5 µmol·g^{-1} for COA before and after climbing, respectively. On average, SIL and COA had lactate concentrations of 1.8 and 1.7 µmol·g^{-1} before climbing and 2.2 and 2.1 µmol·g^{-1} after climbing, respectively (Figure 4), though none of these concentrations were significantly different (Kruskal-Wallis, $Chi^2 = 3.5$, df = 3, p = 0.32).

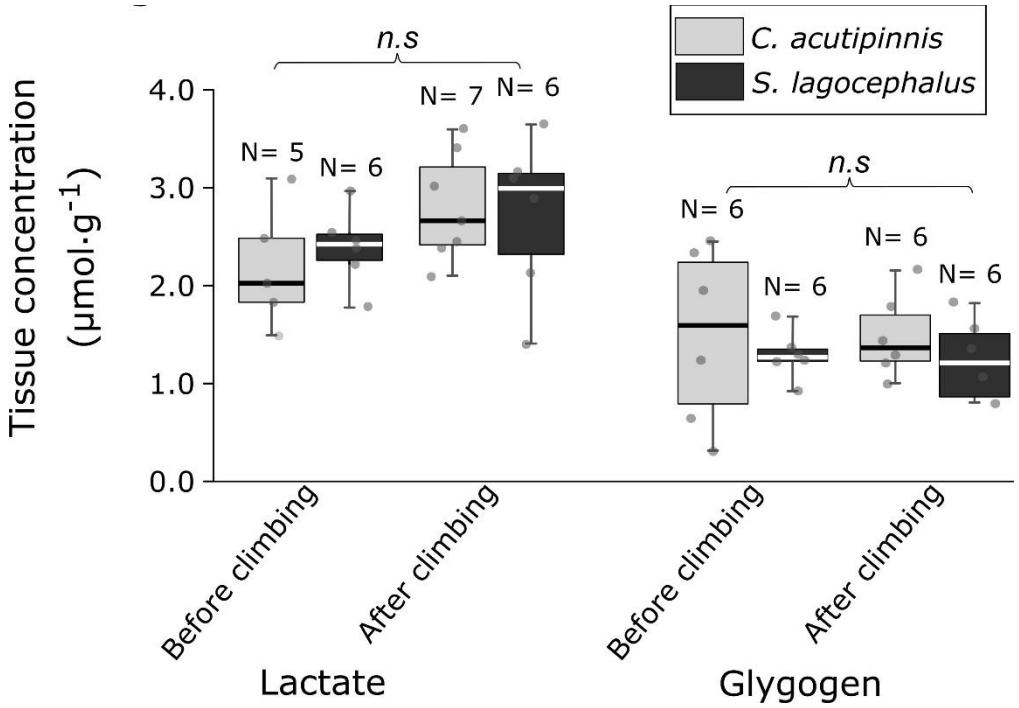


Figure 4 : Lactate and glycogen tissue concentration measured in C. acutipinnis (COA) and S. lagocephalus (SIL). For each species these concentrations are compared between individuals sampled before starting the climbing experiment ("Before climbing") and those sample immediately after they successfully climbed the experimental ramp ("After climbing"). Boxes are divided by quartiles, whiskers indicate the range of the data and grey dots the individual data points. The number of individuals (N) for which lactate and glycogen tissue concentrations were measured is specified. A Kruskal-Wallis test revealed a non-

significant difference (n.s) of lactate and glycogen tissue concentrations between each groups (COA and SIL sampled before and after climbing).

Discussion

Our data support the differential climbing performance of young individuals of both species previously observed, as SIL performed better than COA (Blob et al. 2019). SIL is metabolically better poised than COA, with generally higher aerobic and anaerobic enzyme capacities. The two species do not differ in their fuel reserves or metabolite built-up after climbing.

Climbing performance

COA is typically a "powerbust" climber with fast and short climbing bouts interspersed by extended resting phases (Figure 1; Blob et al. (2019)), however, the "incher" SIL could scale an obstacle faster (Figure 1; Blob et al. (2019); Lagarde et al. (2020)). These results corroborate previous observations, though with slight discrepancy in bout climbing speed and time spend in motion. Indeed, we observed a higher bout climbing speeds (0.39 vs. 0.17 $BL \cdot s^{-1}$ for SIL and 0.65 vs. 0.14 $BL \cdot s^{-1}$ for COA) and less time spent in motion (33 vs. 47% for SIL and 7 vs. 14% for COA) than previously reported (Blob et al. (2019). This discrepancy may be related to difference of climbing arenas, as the ramp was angled at 45° compared to 70° in Blob et al. (2019). On a global scale, the two Reunionese species show climbing speeds comparable to other gobies with similar locomotor style. Both Hawaiian powerbust climbers *Awaous guamensis and Lentipes concolor* scale steep obstacles at ~0.15 $BL \cdot s^{-1}$ which is faster than the climbing speed observed for COA (0. 06-0.04 $BL \cdot s^{-1}$; Schoenfuss and Blob (2003); this study). In contrast, SIL moves at speeds similar to those of to *Sicyopterus stimpsoni*, its Hawaiian incher counterpart (~0.10 vs. 0.11-0.13 $BL \cdot s^{-1}$; Schoenfuss and Blob (2003); this study).

Metabolic profiles

As originally hypothesized, enzymatic activity was higher in SIL than in COA as CS maximal activity, a mitochondrial marker (Bystriansky et al. 2007), was on average 1.4-fold higher in SIL compared to COA. HOAD, involved in lipid β-oxidation, showed no interspecific difference but glycolytic enzymes, PK and LDH (Willmott et al. 2005), were both significantly more elevated in SIL. SIL is equipped with a higher aerobic metabolic capacity, to use carbohydrates rather than lipids, and a higher anaerobic metabolic capacity to power its climbing. This is consistent with the lack of any apparent difference in endogenous lipids contents between the two species (Fig. 3). Even if our study concerned two species (Garland and Adolph, 1994), they are the only species displaying the waterfall climbing behaviour in Reunion Island and for which climbing performance influences competitive interactions. Moreover, despite our different approach from Cediel et al. (2008) who described a higher proportion of red muscle fibers in the inching *S. stimpsoni* compared to the powerburst *L. concolor* and *A. guamensis* the conclusions of the two studies are consistent and concern two inching and three powerburst species.

If SIL were mostly adapted to use carbohydrates during exercise, the fish would need greater accessible reserves (i.e., glycogen) to mobilize during climbing. These reserves should be depleted after a strenuous climbing effort, though surprisingly, glycogen contents were not affected by climbing in either species (Fig. 4). The two species exhibited almost identical glycogen levels despite having presumably quite different metabolic capacities as suggested by the enzyme profiles. Thus it appears that these fish may be using other sources of fuels, such as circulating glucose or glycogen reserves in the liver, it is unlikely, however, that these species would be able to climb several hundred meters high obstacles solely on endogenous reserves. Indeed, both SIL and COA are observed above quasi-vertical waterfalls as high as 200 m in Reunion Island (Lagarde 2018), thus with an approximate climbing speed of

0.5 $cm \cdot s^{-1}$ for SIL and 0.1 $cm \cdot s^{-1}$ for COA, climbing would last between one and five months. Given the relatively low quantity of metabolic fuels, it is very unlikely that they can sustain several weeks of climbing effort without feeding. Considering this, it is probable that sicydiines species can feed while climbing, at least in nature. Indeed, sicydiines are known to feed on periphyton (Bielsa et al. 2003) and more specifically on diatoms (Julius et al. 2005). As both species climb in areas which are permanently wetted by a few millimetres thick water layer, they encounter patches of periphyton during climbing.

A slow and sustained type of exercise must be powered by aerobic pathways, while fast and explosive activities must rely on anaerobic pathways. After climbing, we would expect COA, and not SIL, to show an accumulation of lactic acid, the glycolytic end product under anaerobic conditions but neither species showed increased lactate after climbing (Fig. 4). While it could be argued that the climbing effort was not sufficient to elicit changes in metabolites owing to a relatively low slope (45°) of a short ramp, under similar conditions 80% of the COA failed to climb the ramp (Lagarde et al. 2018). Thus, at least for COA, climbing the experimental ramp constitutes a challenge. Furthermore, for both species the climbing effort took at least an hour. It is likely that these results are better explained by the two different climbing strategies, as COA spent over 90% of its climbing time resting. This could allow COA to metabolize lactate and avoid excessive accumulation. Similarly, SIL spent close to two-thirds of its time resting, providing ample time for lactate clearance. Furthermore, a presumably higher oxidative capacity of SIL could not only prevent lactate production by reducing reliance on anaerobic metabolism, but also mitigate lactate accumulation through its oxidation.

Conclusion

Overall, SIL climbed faster and demonstrated a higher aerobic and anaerobic capacity than COA. However, resting phases associated with both climbing modes could mitigate the influence of metabolic profiles on climbing performance. Despite its behaviour being seemingly appropriated to its climbing mode, the climbing failure rate of COA was higher compared to SIL (Lagarde et al. 2018). This suggests that a greater proportion of SIL migrating individuals are successful at reaching habitats upstream of waterfalls. Because SIL and COA juveniles arrive simultaneously and year round in Reunionese rivers (Teichert et al. 2012, 2016), and COA migration is slower than SIL (Lagarde et al. 2020), SIL likely reaches upstream habitats before COA. Therefore, the higher climbing success and migration speed of SIL both likely represent a strong competitive advantage for this species.

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