© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 984-994 doi:10.1242/jeb.141440

RESEARCH ARTICLE

Interspecies comparison of the mechanical properties and biochemical composition of byssal threads

Zeineb Bouhlel[1], Bertrand Genard[2], Neilly Ibrahim[3], Emily Carrington[4], José M. F. Babarro[5], Aynur Lok[6], Augusto A. V. Flores[7], Christian Pellerin[3], Réjean Tremblay[1,*] and Isabelle Marcotte[2,*]

ABSTRACT

Several bivalve species produce byssus threads to provide attachment to substrates, with mechanical properties highly variable among species. Here, we examined the distal section of byssal threads produced by a range of bivalve species (*Mytilus edulis*, *Mytilus trossulus*, *Mytilus galloprovincialis*, *Mytilus californianus*, *Pinna nobilis*, *Perna perna*, *Xenostrobus securis*, *Brachidontes solisianus* and *Isognomon bicolor*) collected from different nearshore environments. Morphological and mechanical properties were measured, and biochemical analyses were performed. Multivariate redundancy analyses on mechanical properties revealed that byssal threads of *M. californianus*, *M. galloprovincialis* and *P. nobilis* have very distinct mechanical behaviours compared with the remaining species. Extensibility, strength and force were the main variables separating these species groups, which were highest for *M. californianus* and lowest for *P. nobilis*. Furthermore, the analysis of the amino acid composition revealed that *I. bicolor* and *P. nobilis* threads are significantly different from the other species, suggesting a different underlying structural strategy. Determination of metal contents showed that the individual concentration of inorganic elements varies, but that the dominant elements are conserved between species. Altogether, this bivalve species comparison suggests some molecular bases for the biomechanical characteristics of byssal fibres that may reflect phylogenetic limitations.

KEY WORDS: Byssus, Mechanical properties, Metals, Amino acids, Bivalves

INTRODUCTION

The colonization of wave-beaten shores by mussels depends on the production of a filamentous protective tissue, the byssus, providing attachment to rocky surfaces. This mechanism for avoiding dislodgement, however, comes at a cost. Babarro and Carrington (2013) suggested that mussel populations at wave-exposed habitats invest more energy in the production of protective tissues, such as shells and byssus, than in reproduction and growth. The byssus is a series of threads made of fibrous proteins with outstanding mechanical properties (Waite, 1985). In *Mytilus* species, each single fibre consists of an elastic, corrugated proximal section connected to a stiff, smooth distal section anchored to the substrate by the plaque (Benedict and Waite, 1986). The structure of each section is made of pepsin-resistant protein complexes called preCols, which are well adapted to providing a strong and flexible tether (Bell and Gosline, 1996; Benedict and Waite, 1986).

PreCols are well ordered and assembled in an axial gradient, with preCol-P in the proximal section transitioning to preCol-D in the distal section (Waite et al., 1998). A non-graded preCol (preCol-NG) is also present all along the thread. The collagen central domain of each preCol is flanked by regions similar to elastin in preCol-P, to silk in preCol-D, and to plant cell wall (PCW) proteins in preCol-NG (Coyne et al., 1997). Solid-state nuclear magnetic resonance (NMR) experiments showed that the collagen is highly ordered in the byssal threads, and identified the presence of β-sheet structures in the silk and PCW regions (Arnold et al., 2013). The flanking domains are tipped by histidine-rich domains generally cross-linked with divalent ions such as zinc and copper (Waite et al., 1998). Modified amino acids, namely DOPA (3,4-dihydroxyphenylalanine), are also involved in metal ion binding, particularly in preCol-D (Sun and Waite, 2005). The DOPA–metal links are generally associated with adhesion potential (Lin et al., 2007), but are also involved in the protection of byssus as DOPA is abundant in the main protein composing the protective sheath of the preCol-rich core where it principally complexes with iron (Sun and Waite, 2005). Therefore, the resistance of the cuticle should be directly related to the presence of such organometallic bonds (Harrington et al., 2010). These bonds are sacrificial and can be broken upon stretching and reform once the tension is removed.

There has been a growing interest in a better understanding of the relationship between the molecular composition and mechanical performance of each section of a byssal fibre. For instance, Waite (2002) reported that the stiffness (or initial modulus, E) of *M. galloprovincialis* threads is *ca* 500 and 50 MPa for the distal and proximal sections, respectively. This discrepancy in mechanical profiles could be due to biochemical composition. The presence of a dense collagen core combined with silk fibroin-like domains is believed to be partly responsible for the stiffness and toughness of the distal section (Waite et al., 2002). Variations in mechanical performance have also been observed among different mussel species (e.g. Bell and Gosline, 1996; Brazee and Carrington, 2006; Pearce and LaBarbera, 2009), and the biochemical structure of the byssal threads could potentially explain these interspecific differences (Waite et al., 2006). In particular, byssal fibres contain non-collagenous amino acid residues (Qin et al., 1997; Sagert and Waite, 2009) and metal ions (Tsukada et al., 1995) that could differ between species.

[1]Institut des Science de la Mer, Université du Québec à Rimouski, 310 allée des Ursulines, Rimouski, Québec, Canada, G5L 3A1. [2]Département de Chimie, Université du Québec à Montréal, C.P. 8888, Succursale Centre-Ville, Montréal, Québec, Canada, H3C 3P8. [3]Département de Chimie, Université de Montréal, C.P. 6128, Succursale Centre-Ville, Montréal, Québec, Canada, H3C 3J7. [4]Department of Biology and Friday Harbor Laboratories, University of Washington, 620 University Road, Friday Harbor, WA 98250, USA. [5]Department of Biotechnology and Aquaculture, Instituto de Investigaciones Marinas CSIC, Eduardo Cabello 6, Vigo 36208, Spain. [6]Aynur Lok, Ege University, Faculty of Fisheries, Genclik Caddesi No. 1235040 Bornova, Izmir, Turkey. [7]Centro de biologia marinha, Universidade de São Paulo, Rod, Maniel Hipólito, do Rego, São Sebastião, SP, 11600-000, Brazil.

*Authors for correspondence (rejean_tremblay@uqar.ca; marcotte.isabelle@uqam.ca)

I.M., 0000-0001-7467-7119

Received 5 April 2016; Accepted 19 December 2016

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 984-994 doi:10.1242/jeb.141440

The objectives of this work were to survey different byssus-producing bivalves and provide a deeper insight into the molecular composition of the threads, which could explain their different mechanical properties. We tested the hypothesis that the biochemical composition (amino acids and metal content) and diameter of a thread are important factors acting on its mechanical properties. We compared the distal section of the byssal thread, as it is the longest part of each fibre and is the most variable in mechanical properties among mussel species (Babarro and Reiriz, 2010; Bell and Gosline, 1997; Brazee and Carrington, 2006). We used threads from several species to encompass a broad range of biochemical and mechanical characteristics for our analyses. Because these threads came from species that vary in size, habitat and life history, all factors known to affect byssus (e.g. Bell and Gosline, 1997; Coombs and Keller, 1981; Moeser and Carrington, 2006), our ability to distinguish species-level differences is limited. Our approach instead provides a broader insight into the relationship between thread biochemical characteristics and mechanical properties.

MATERIALS AND METHODS

Sampling design

Byssal threads were collected from bivalve aggregations (beds) on shores not exposed to industrial effluent, during the summer. Nine different species of bivalve molluscs were selected for this study: *Mytilus edulis* (Linnaeus, 1758) and *Mytilus trossulus* (Gould, 1850) respectively from Magdalen Islands (47°25′N, 61°50′W) and Rivière-au-Renard (48°00′N, 65°20′W), Gulf of St Lawrence (Canada); *Mytilus californianus* (Conrad, 1837) from Cattle Point, San Juan Island (USA; 48°45′N, 122°96′W); *Mytilus galloprovincialis* (Lamarck, 1819) and *Xenostrobus securis* (Lamarck, 1819) from inner Ría de Vigo (NW Spain; 42°19′N, 8°37′W); *Pinna nobilis* (Linnaeus, 1758) from Karantina Island, Aegean Sea (Turkey; 38°22′N, 26°47′W); *Perna perna* (Linnaeus, 1758), *Isognomon bicolor* (Adams, 1845) and *Brachidontes solisianus* (d'Orbigny, 1846) near São Sebastião (Brazil; 23°45′S, 45°24′W). All collections were from intertidal populations except for *Pinna nobilis*, which was shallow subtidal. Note that all species belong to the Mytilidae taxonomic family, except for *I. bicolor*, an Isognomidae, and *P. nobilis*, a Pinnidae. *Brachidontes solisianus* (d'Orbigny, 1846) was placed under the genus *Mytilaster* by Scarabino (2003). However, recent work by Trovant et al. (2013) showed that further studies, including for *Brachidontes* species and related genera, are needed to resolve the full phylogeny of the group. In this context, the name *Brachidontes* was preserved herein. On Brazilian rocky shores, two species of mytilids generally dominate the intertidal zone, *Brachidontes darwinianus* and *B. solisianus*. *Brachidontes darwinianus* replaces *B. solisianus* at low salinity sites (Tanaka, 2005; Tanaka and Magalhães, 2002). In this study, *Brachidontes* specimens were collected in a full oceanic area, without any inputs of fresh water, and therefore the presence of *B. darwinianus* in samples was very unlikely. Guerra et al. (2013) have revised the genus *Xenostrobus* for *Limnoperna* and the correct name is apparently *Limnoperna securis*. However, to facilitate comparisons using black pygmy mussel, we used *Xenostrobus securis* in this study because it is also the accepted name in WoRMS (World Register of Marine Species) (Marshall et al., 2015).

For each species, at least 150 mg of thread was required for all analyses. Threads were obtained from 30 to 50 haphazardly selected individuals within a similar size range (less than 20% variation). Only mature threads that were golden in colour were selected; threads that were newly formed (milky white in colour) or showed signs of degradation were omitted (Carrington and Gosline, 2004; Moeser and Carrington, 2006). Under a focal stereomicroscope (Olympus America, Center Valley, PA, USA), distal sections were separated from the other parts of the thread with a razor blade. All samples were then rinsed three times with Nanopure water. Replicate pools of threads for each species were prepared for different analyses. Threads used for metal analysis were kept at 4°C in 2 ml Teflon tubes cleaned with HNO_3 (10 mol% and 1 mol%), and the remaining threads were kept in 4 ml cryogenic tubes stored at −20°C until biochemical and mechanical analyses were performed.

Morphometric characteristics

An optical microscope (Axioskop 40, Carl Zeiss, Göttingen, Germany) with a magnification of ×250 and associated image analysis software was used for dimension measurements as well as thickness of wet fibres. The cross-section of the thread was assumed to be circular, and the diameter was estimated as the average of 5–10 randomly selected measurements on each thread. The topography of the same thread was observed using a scanning electron microscope (SEM) (JSM-7600TFE FEG-SEM, JEOL, Japan). Samples were fixed on metal plates with graphite tape and covered with a fine layer of gold in a glazed-chamber Polaron SC502 sputter coater. The electron beam was operated at 2 kV in the secondary electrons emission detection mode with an Everhart-Thornley detector, typical for topographic images. In order to expose the core, the end of each thread was freeze-fractured using a razor blade.

Mechanical properties

Distal threads were kept at 4°C prior to the mechanical analyses. Tensile tests were conducted on an Instron 5465 mechanical testing frame (Norwood, MA, USA) equipped with a BioPuls bath filled with artificial seawater (pH 7.9) (Hagenau et al., 2011). The ends of the specimens were glued between two small square sheets (5×5 mm) of cellulose acetate using cyanoacrylate (Loctite Gel Control Super Glue, Henkel Consumer Adhesives, Avon, NY, USA) then placed between the grips of the testing frame. Each thread was extended at 5 mm min^{-1} until rupture. Threads that broke at the edge of the plastic sheets and those presenting unusual curve shapes due to inappropriate gluing were rejected. As described by Séguin-Heine et al. (2014), the ultimate force (F_{max}, in N) quantifies the maximum load, such as the one imposed by waves, that can be supported by the thread without rupture. It is not normalized to the specimen morphology (length, thickness) and is therefore the single-fibre analog to the common measurements of attachment force or 'strength' for the whole animal (Bell and Gosline, 1996). The stress (σ, in Pa) corresponds to the resistance of the thread material against the applied load and is obtained by dividing the force by the cross-sectional area (A, in m^2) calculated from the mean diameter of each thread, while the strain (ε, %) reflects the extensibility of the thread under tension and is calculated as change in length divided by initial length×100%. The maximum ultimate strength (σ_{max}) and ultimate strain (ε_{max}) were determined at the rupture point of each byssal thread. The Young's modulus (E, in Pa) is the initial stiffness of the thread and was obtained by calculating the slope of the stress–strain curve in the 5–10% strain range. The tensile tests were not performed on *X. securis* and *B. solisianus* because the threads of these species were too thin and fragile to be fixed between the jaws of the instrument.

Amino acid analyses

The amino acid composition of five pools of 10 distal threads from each bivalve species was analysed at the Hospital for Sick

Journal of Experimental Biology (2017) 220, 984-994 doi:10.1242/jeb.141440

Children's Advanced Protein Technology Centre, Department of Molecular Structure and Function (Toronto, Canada). The analysis was carried out using the Waters Acquity ultra-performance liquid chromatography (UPLC) system (Milford, MA, USA) equipped with a Waters Acquity UPLC BEH C18 column (2.1×100 mm). Data were assessed using Waters Empower 3 Chromatography software. Briefly, the byssal proteins were hydrolysed using 225 µl HCl (6 mol l^{-1} with 1% phenol) and 50 µl of norleucine internal standard under vacuum using the Pico-Tag Workstation at 110°C for 20–24 h. The hydrolysates were centrifuged at 11,000 ***g*** for 5 min. An aliquot was transferred to a glass culture tube, dried for 15 min under vacuum using a centrifugal evaporator (Tomy CC-181 centrifugal concentrator), hydrated with a methanol:water: triethylamine (2:2:1) solution, vortex-mixed and dried again under vacuum for 15 min. The sample was then derivatized for 20 min at room temperature with water:triethylamine: phenylisothiocyanate (PITC) (7:1:1), dried under vacuum for 15 min, washed with the hydration solution, vortex-shaked and vacuum-dried again for 15 min. Finally, the samples were dissolved in the sample diluent (~100 µl, pH 7.4), injected into the column and run on a modified PICO-TAG gradient at 48°C. The derivatized amino acids were detected at 254 nm. The concentrations of each amino acid were calculated in µg mg^{-1} and then expressed as relative concentration (% of total amino acids). The amino acid analysis does not allow discriminating between Asn/Asp, and Gln/Glu.

Metal analyses

Metal analyses were performed on five pools of 20 mg of dry distal sections for each species. The numbers of threads vary from few tens to few hundreds, depending on species. An acid digestion was carried out in a mixture of concentrated HNO_3 (16%) and H_2O_2 (≥30%) at 90°C until complete digestion (i.e. for 2 h). The volume was adjusted to 5 ml with a 1% HNO_3 solution. The metal concentration was determined using an Agilent 7500c ICP-MS (New Castle, DE, USA) in normal mode equipped with a micro-nebulizer. This technique allowed the determination of the accurate concentrations of a large range of metals expressed in ng ml^{-1}, namely Be, B, Al, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Ag, Cd, Sn, Sb, Ba, Hg, TI, Pb, U as well as Mg, K, Ca, Rb, Sr, Mo, Cs and Ba. The concentrations were converted to nmol mg^{-1} considering the molecular weight of each analysed element, then expressed as relative concentration (% of total metal contents). Only metals with a proportion over 0.1% were considered for analysis.

Statistical analyses

One-way ANOVA was used to test for differences among species in (i) morphology (shell length and distal thread diameter), (ii) mechanical properties, (iii) total metal ion content, and (iv) amino acids and metals. Assumptions of homoscedasticity and normality were verified with Cochran's and Shapiro–Wilk's tests, respectively, and data were log+1 transformed if necessary. When ANOVA indicated a significant effect of species, *a posteriori* comparisons were performed using Tukey's honest significant difference (HSD) test. A redundancy analysis (RDA) was performed to estimate the differences ascribed to the species on mechanical properties of byssal threads using five parameters: ultimate stress, ultimate strain, modulus, ultimate force and diameter. Two other RDAs were applied to test the amino acid and metal contents of byssus from each species studied. This method allows an estimation of the fraction of variation in response variables (mechanical properties, metal or amino acid contents) attributable to the explanatory variables (species studied). It can be described as a series of multiple regressions followed by a principal component analysis where each response variable, Y, is regressed on the matrix corresponding to the explanatory variables, X. Matrices for species effects were coded using orthogonal dummy variables, and an ordination biplot (Z-plot type) was generated from the RDA results using the plotted RDA function (Legendre and Legendre, 2012). Finally, least-squares linear regression analyses were used to establish relationships between diameters of the byssal thread of species pooled together with mechanical properties (F_{max}, ε_{max}, σ_{max} and E) of these threads. ANOVAs were run using the SAS software system (version 8.1), regressions with Systat (version 12) and RDAs were tested using the R language package.

RESULTS

Morphometric characteristics

Table 1 shows that shell length differed among species ($F_{8,260}$=1582, P<0.001). Our sampling design does not allow us to distinguish between size and species effects on the morphological characteristics of the byssus, such as the diameter. Thus diameters will be used only in relation to the mechanical properties of the different byssal threads and not to discriminate trends in bivalve species. The diameter of distal byssal threads used in this experiment varied significantly ($F_{8,76}$=27, P<0.001; Table 1), ranging from 13 µm for *X. securis* to 200 µm in *M. edulis*, *M. californianus* and *P. perna*. Scanning electron microscopy (SEM) images (Fig. 1) show that the threads generally had a distinct cuticle, except for *I. bicolor* where the cuticle was barely detectable. SEM also suggests the absence of a morphological differentiation between the proximal and the distal section of threads in *I. bicolor*. All species seem to synthesize byssal filaments with longitudinally aligned microfibres, as shown by the regular lines on the cuticle, and the regular pattern in the core (Fig. 1).

Mechanical properties

Representative stress–strain curves showing the mechanical behaviour of the distal section of the byssal threads are presented in Fig. 2. Each mechanical property varies significantly among species: ultimate strain (ε_{max}; $F_{8,62}$=5, P=0.002), Young's modulus (E; $F_{8,62}$=21, P<0.001), ultimate strength (σ_{max}; $F_{8,62}$=16, P<0.001) and maximum force (F_{max}; $F_{8,62}$=68; P<0001). The stress–strain curves of byssal threads from all species (Fig. 2) show three phases with one yield point defining the limit of the elastic behaviour of the fibres at strains ranging from ~5% to ~20%, a plateau, and a strain hardening section that was more apparent in threads collected from *M. galloprovincialis*, *M. californianus*, *M. edulis* and *P. nobilis*. The yield points of *I. bicolor* and *P. nobilis* occur at a particularly low strain of ~5% and 7%, respectively. The mechanical properties of byssal threads obtained from *M. californianus* and *P. nobilis* present extreme differences compared with all other species. On one end, threads from *P. nobilis* are marked by low values for most of the parameters assessed (Fig. 2, Table 1), except for its modulus. At the opposite end, *M. californianus* byssal threads outperform those of all the remaining species in terms of E, σ_{max} and F_{max}, while maintaining a similar ε_{max} (Fig. 2, Table 1). Threads of *M. galloprovincialis* and *M. trossulus* can only withstand about 66% of the maximum stress (σ_{max}) that can be applied on *M. californianus* before breaking, while threads from *M. edulis* are ~50% weaker than for this species. Among the weakest, threads of *P. perna* and *I. bicolor* have about a third of the strength of *M. californianus* threads, while those of *P. nobilis* have about 25% of it. When further considering the small diameter of the byssus

Journal of Experimental Biology (2017) 220, 984-994 doi:10.1242/jeb.141440

Table 1. Summary of morphological and mechanical characteristics of bivalve shell and distal byssal threads

Bivalve species	Shell length (mm)	Thread diameter (µm)	Ultimate strain (%)	Young's modulus (MPa)	Ultimate strength (MPa)	Maximum force (N)
M. edulis	66±7^{c}	191±37^{a}	85±10^{a,b}	146±29^{b}	58±13^{b,c}	1.7±0.6^{b}
M. trossulus	57±4^{c}	99±23^{b}	101±30^{a}	198±49^{b}	84±29^{a,b}	0.6±0.2^{c}
M. galloprovincialis	35±2^{d}	90±19^{b,c}	65±10^{b}	361±123^{a}	81±23^{b}	0.5±0.2^{c}
M. californianus	81±5^{b}	210±39^{a}	81±10^{a,b}	396±113^{a}	121±29^{a}	4±2^{a}
P. nobilis	500±68^{a}	50±17^{c}	30±7^{c}	186±68^{b}	27±4^{d}	0.05±0.01^{d}
P. perna	56±4^{c}	211±29^{a}	87±20^{a,b}	132±45^{b}	43±13^{c,d}	1.5±0.3^{b}
I. bicolor	25±4^{e}	96±23^{b}	75±20^{a,b}	207±51^{b}	41±19^{c,d}	0.4±0.1^{c}
X. securis	34±2^{d}	13±3^{d}	n.e.	n.e.	n.e.	n.e.
B. solisianus	20±2^{e}	45±5^{c}	n.e.	n.e.	n.e.	n.e.

Values correspond to means and respective standard deviations. Different letters indicate significant differences between species (P<0.05). 'n.e.' indicates that these measurements could not be estimated.

of *P. nobilis*, its low strength leads to an ultimate load F_{max} as little as 1% of that measured for *M. californianus*.

Multivariate redundancy analysis (RDA) confirms significant differences in mechanical properties among species groups, with the 'species' factor explaining 74% of the total variance (Fig. 3). Regarding the similarity of mechanical properties of byssal threads, the RDA-1 axis, explaining 48% of the total variance, shows two non-clustered species, i.e. *M. californianus* having the threads with the highest mechanical properties and *P. nobilis* with the lowest values (Fig. 3, Table 1).

Inorganic content

The total concentration of inorganic elements (referred to as metals) and their composition in the byssal threads are shown in Table 2. A total of 31 variables were statistically tested, i.e. the proportion of each element (in %) and the absolute content (µmol g^{-1}). The absolute content of each element in µmol g^{-1} for the byssal threads of each species is given in Table S1. Overall, three groups of ions can be distinguished from Table 2. Group I includes the most abundant inorganic cations. Less abundant metal ions (<2%) are pooled into group II, whereas trace level ions (<0.01%) compose group III and are not considered in the comparison.

According to RDA results obtained from Table 2, the species factor explains 90% of the total variance (Fig. 4). Threads of *M. trossulus*, *M. galloprovincialis* and *X. securis* show a similar metal composition differing from other species by their higher abundance in Fe and Al (group I), Zn and Cu (group II), as well as their high total content in multivalent ions. Another distinct cluster is formed by *P. perna*, *B. solisianus*, *I. bicolor* and *P. nobilis* with notably higher proportions of Mg and K (group I), Sr and Sn (group II) as well as total divalent ions. *Mytilus edulis* is isolated in the RDA analysis, but shows some associations with *M. trossulus* and *M. galloprovincialis* based on proportions of Mg and Ca (group I) in their byssal threads. Threads from *M. californianus* are clearly distinct, revealing the highest abundance of B and elevated values of Ca and V.

The total metal concentration (µmol g^{-1}) of distal byssal threads varies among species ($F_{8,45}$=78, P<0.001) with values for *I. bicolor* over five times superior to the lowest values determined in *M. californianus*, *M. galloprovincialis* and *M. trossulus* threads. Finally, traces of metal (group III) considered as toxic, such as As, Cd, Pb, U and Hg (<0.01%), are observed in the distal threads of each species as detailed in Table 2.

Amino acid content

The amino acid composition of byssal threads varies strongly (P<0.0001) among species with *B. solisianus* and *M. galloprovincialis* containing the highest levels of L-DOPA (Table 3). Generally, the most abundant amino acid is Gly, representing between 26 and 41% of the total content, followed by Ala (8–16%) and Pro (6–11%). In *P. nobilis* threads, however, Gly and Pro are equally important (14%). The RDA results show that the species factor explains 97% of the total variance of the amino acids composition of the byssal threads (Fig. 5). All species are mostly clustered together, except *I. bicolor* and *P. nobilis*.

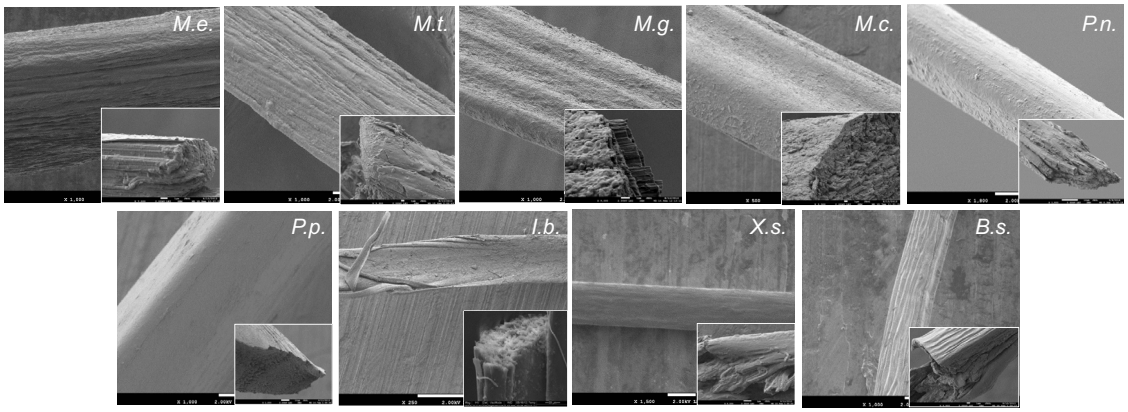

Fig. 1. Scanning electron microscopy images of the distal section of byssal threads of different species. Insets correspond to inner cores after longitudinal and cross-section cutting. *M.e.*, *Mytilus edulis*; *M.t.*, *Mytilus trossulus*; *M.g.*, *Mytilus galloprovincialis*; *M.c.*, *Mytilus californianus*; *P.n.*, *Pinna nobilis*; *P.p.*, *Perna perna*; *I.b.*, *Isognomon bicolor*; *X.s.*, *Xenostrobus secures*; *B.s.*, *Brachidontes solisianus*.

Journal of Experimental Biology (2017) 220, 984-994 doi:10.1242/jeb.141440

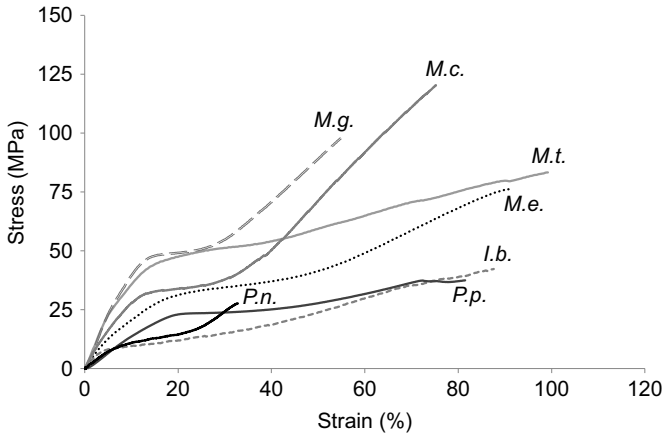

Fig. 2. Representative stress–strain curves of the distal section of byssal threads from different species. *M.e.*, *Mytilus edulis*; *M.t.*, *Mytilus trossulus*; *M.g.*, *Mytilus galloprovincialis*; *M.c.*, *Mytilus californianus*; *P.n.*, *Pinna nobilis*; *P.p.*, *Perna perna*; *I.b.*, *Isognomon bicolor*.

Indeed, *I. bicolor* threads have a particular amino acid composition characterized by remarkably high content of His, Pro, Lys and Gly, and lower proportions of most of the amino acid residues with polar or charged side chains, such as Thr, Arg, Ser, Glx and Asx (Table 3). Contrary to the other species, *P. nobilis* fibres are particularly rich in Asx, Pro, Tyr, Val, Met, Leu and Lys, but poor in Gly, Ala and L-DOPA.

DISCUSSION

Mechanical properties versus morphology

The biomechanical evaluation of byssal threads produced by various bivalve species suggests that morphological characteristics of the fibres have an important impact on their mechanical properties. The diameter explained 62% of the ultimate force of the threads, independent of the species. As we explored a large variety of bivalve species, whose byssal threads diameters ranged from 30 to 240 µm, mechanical differences were expected. Multivariate redundancy analyses on mechanical properties revealed that threads of *M. californianus* and *P. nobilis* had a more distinguishable mechanical behaviour. *Mytilus californianus* fibres having diameter values of 200 µm had the highest strength, stiffness and ultimate force, whereas *P. nobilis* threads with diameters of only 50 µm were characterized by the lowest values among the pool. The byssus thread diameter depends on the anatomy of the pedal groove (Babarro and Lassudrie, 2011; Price, 1981), which is in turn dependent on the species and on the body size (Bell and Gosline, 1997). Mechanical properties of byssus threads may also be influenced by other factors that could not be evaluated in the field, such as the age and history of the threads as demonstrated by Carrington and Gosline (2004) in *M. californianus*, or by differential byssal thread degradation considering the large temperature differences between the coastal sites sampled (Moeser and Carrington, 2006).

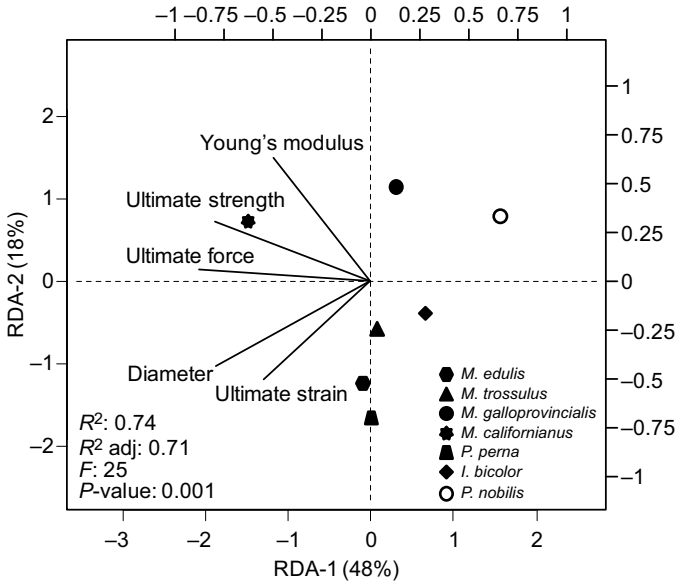

Fig. 3. Ordination biplot resulting from redundancy analysis (RDA) representing the significant effect of the factor species on the mechanical properties of byssus distal threads from different bivalve species. Lines represent response variables.

The impact of other morphological patterns on mechanical properties is difficult to determine, but the undistinguishable cuticle from *I. bicolor* could contribute to weak mechanical performance. In addition, *I. bicolor* sampled in a marine environment in Brazil produces homogenous filaments without distinction between the proximal and distal sections. Accordingly, the structural specialization of the two sections is likely to be specific for marine mytilid species, confirming the observations of Brazee and Carrington (2006) and Pearce and LaBarbera (2009).

Common mechanical properties in relation to metal ions

The mechanical behaviour, i.e. the general shape of the stress–strain curve, was conserved among species, suggesting common molecular features and organization. Although bivalves investigated in this work came from very different environments – from tropical to north-temperate areas – the same groups of metals were present in similar concentration ranges in all species. Inorganic ions such as K, Ca, Mg, Mn, Fe, B and Al were found in similar proportions, enabling us to pool these elements in a same group (I) for all nine bivalve species studied. A second group (II) of elements including Cu, Zn, Ti and V for example, were abundant in all the byssi investigated. This observation is consistent with the work of Jaworski et al. (2015) on *X. securis* and *M. galloprovincialis*, which showed that the metal profile in the byssus depends on the species more than the site.

Most of the elements from group I correspond to those reported to be coordinated with mussel foot proteins mfp-1 in the cuticle of the threads (Benedict and Waite, 1986; Sun and Waite, 2005; Taylor et al., 1996). This mainly includes iron (Holten-Andersen et al., 2011; Sun and Waite, 2005; Zeng et al., 2010), aluminium (Holten-Andersen et al., 2005; Holten-Andersen and Waite, 2008), and calcium (Holten-Andersen and Waite, 2008). DOPA residues are known to have a particular affinity for Fe^{3+} with which they form a tris-catechol in the byssus (Holten-Andersen et al., 2009a; Holten-Andersen et al., 2011; Sun and Waite, 2005). Copper and zinc in group II were detected at concentrations in the same range as those reported by Coombs and Keller (1981), and are known for their high affinity with histidine in the byssus (Harrington and Waite, 2007; Waite et al., 2004; Xu, 2013). Therefore the occurrence of an inorganic ion in byssus fibres does not only depend on its availability (Zhao and Waite, 2006) but also on the possibility of chemical interactions with amino acids – the biological ligands. Intrinsic properties such as oxidation state, coordination number and geometry are important parameters (Holm et al., 1996) when it comes to the selection of a metal ion cross-linking with DOPA in the

Table 2. Inorganic composition (% total inorganic element content) and total inorganic elements concentration of byssal threads for each species

Group	*M. edulis*	*M. trossulus*	*M. galloprovincialis*	*M. californianus*	*P. nobilis*	*P. perna*	*I. bicolor*	*X. securis*	*B. solisianus*
I									
B	2.2±0.2^{c,d}	2.0±0.5^{d}	9.2±0.9^{b}	18±2^{a}	1.2±0.2^{d,e}	3.5±0.4^{c}	0.63±0.2^{e,f}	0.6±0.2^{f}	0.30±0.05^{f}
Fe	18.2±0.5^{c,d}	24±4^{b}	21±2^{b,c}	12±2^{e}	24±2^{b}	7±1^{f}	13±1^{e}	48±7^{a}	13±1^{d,e}
Al	9±1^{e}	30±2^{a}	27±2^{a,b}	9±2^{e}	13±3^{d,e}	10±1^{e}	14±3^{c,d}	20±5^{c}	19±2^{b,c,d}
Mg	9±2^{e}	6.8±0.3^{e}	7±2^{e}	24±3^{d}	20±2^{d}	61±12^{a}	48±13^{b}	7±4^{e}	34.2±0.6^{c}
Ca	19±4b^{c,d}	11±3^{d}	19±2^{b,c}	25±7^{b}	38±2^{a}	12±3^{c,d}	17±6^{b,c}	17±3^{c,d}	17.2±0.4^{b,c,d}
Mn	37±5^{a}	15±2^{b}	2.4±0.4^{d}	4.0±0.7^{c}	0.09±0.02^{g}	0.26±0.02^{f}	1.9±0.3^{d}	0.3±0.1^{f}	0.95±0.05^{e}
K	1.8±0.2^{c}	2.7±0.2^{a,b,c}	6±4^{a,b}	2.5±0.5^{b,c}	0.9±0.2^{d}	5±1^{a,b}	4±1^{a,b,c}	3±1^{c}	12±5^{a}
II									
Ti	0.25±0.04^{e}	0.86±0.07^{a}	0.4±0.1^{c,d,e}	0.5±0.1^{c,d}	0.29±0.06^{d,e}	0.32±0.07^{d,e}	0.34±0.03^{c,d,e}	0.81±0.07^{a,b}	0.6±0.1^{b,c}
V	1.15±0.04^{c}	0.84±0.09^{c,d}	1.82±0.04^{b}	3.0±0.2^{a}	0.68±0.08^{d,e}	0.21±0.05^{f}	0.110±0.005^{f,g}	0.5±0.1^{e}	0.09±0.01^{g}
Cr	0.143±0.005^{b}	0.212±0.014^{a}	0.09±0.01^{c}	0.08±0.01^{cd}	0.041±0.009^{f,g}	0.034±0.006^{g}	0.05±0.01^{e,f}	0.064±0.007^{d,e}	0.055±0.006^{e,f,g}
Ni	1.12±0.02^{b}	1.6±0.1^{a}	0.24±0.02^{d}	0.45±0.04^{c}	0.25±0.07^{d}	0.037±0.006^{f}	0.12±0.02^{e}	0.26±0.04^{d}	0.388±0.007^{c}
Cu	0.2±0.02^{d}	3.8±0.4^{a}	1.2±0.7^{b,c}	0.2±0.1^{d}	1.4±0.3^{b}	0.2±0.1^{d}	0.6±0.1^{c}	1.3±0.3^{b}	0.82±0.03^{b,c}
Zn	0.42±0.02^{d,e}	1.4±0.1^{b}	2.5±0.3^{a}	0.44±0.06^{d,e}	0.5±0.1^{c,d,e}	0.14±0.02^{e}	0.7±0.1^{c}	1.6±0.3^{b}	0.86±0.05^{c,d}
Sn	0.008±0.006^{c,d}	0.025±0.003^{a,b,c,d}	0.06±0.05^{abc}	0.02±0.02^{d}	0.010±0.004^{b,c,d}	0.07±0.03^{a}	0.05±0.03^{a}	0.05±0.02^{a,b}	0.2±0.1^{a}
Sr	0.09±0.01^{c,d}	0.05±0.01^{d}	0.10±0.01^{d}	0.15±0.02^{c}	0.20±0.02^{b}	0.31±0.03^{a}	0.32±0.03^{a}	0.08±0.02^{d}	0.34±0.02^{a}
Mo	0.10±0.01^{b,c}	0.05±0.01^{c}	0.2±0.1^{b}	0.8±0.1^{a}	0.09±0.01^{bc}	0.009±0.001^{e}	(1.2±0.1)10^{-3g}	0.012±0.002^{d}	(1.7±0.1)10^{-3f}
Ba	0.052±0.003^{a,b}	0.054±0.006^{a}	0.04±0.02^{ab}	0.023±0.005^{c}	0.007±0.001^{f}	(1.5±0.1)10$^{-3\ e,f}$	0.002±0.001^{d,e}	(2.2±0.9)10$^{-3c,d}$	(3.8±0.2)10$^{-3b,c,d}$
III									
Be	(1.1±0.4)10$^{-3d,e,f}$	(0.7±0.2)10^{-3f}	0.011±0.002^{a}	(1.3±0.2)10$^{-3d,e}$	0.004±0.001^{b}	(9.7±0.2)10$^{-3\ e,f}$	(2.0±0.5) 10^{-3c}	0.008±0.002^{a}	(0.18±0.01)10$^{-3\ c,d}$
Co	0.0270±0.006^{c}	0.063±0.007^{a,b}	0.08±0.04^{a}	0.027±0.007^{c}	0.014±0.003^{d}	(4.4±0.2)10$^{-3\ e}$	0.013±0.001^{b}	0.038±0.006^{b,c}	0.074±0.008^{a,b}
As	(11.2±0.8)10^{-3c}	0.023±0.002^{b}	0.032±0.006^{a,b}	(9.1±0.9)10$^{-3\ c,d}$	0.04±0.01$^{3\ a}$	(6.9±0.1)10$^{-3\ e}$	0.009±0.001^{c}	0.033±0.006^{a,b}	(6.8±0.9)10$^{-3d,e}$
Se	(7.5±0.7)10^{-3d}	0.033±0.006^{c}	0.22±0.06^{a}	0.095±0.009^{b}	n.e.	0.027±0.005^{c}	0.026±0.001^{c}	0.07±0.01^{b}	(21.1±0.2) 10^{-3c}
Ag	0.014±0.007^{b}	(3.7±0.6)10$^{-3\ d}$	0.005±0.002^{c,d}	0.042±0.005^{a}	(5.8±0.8) 10$^{-3b,c,d}$	0.004±0.001^{d}	0.008±0.001^{b,c}	(6.3±0.9)10$^{-3\ b,c,d}$	0.007±0.002^{b,c,d}
Cd	(1.4±0.1)10$^{-3d,e}$	(2.4±0.4)10^{-3d}	(4.7±0.4)10^{-3c}	0.040±0.004^{a}	(0.7±0.7)10$^{-3\ f}$	(1.4±0.3)10$^{-3\ d,e}$	(8±2)10^{-4ef}	(1.7±0.3)10$^{-3d,e}$	(12.1±0.7)10^{-3b}
Sb	(3.2±0.1)10$^{-3b,c}$	(3.3±0.2)10$^{-3\ a,b,c}$	(3.4±0.2)10^{-3b}	(3.9±0.4)10$^{-3\ a,b}$	(5.2±0.9)10$^{-3\ a}$	(0.6±0.1)10$^{-3\ e}$	(1.6±0.2)10$^{-3\ d}$	(4.2±0.8) 10$^{-3a,b}$	(1.85±0.04)10$^{-3\ c,d}$
Pb	(1.0±0.6)10^{-3d}	0.013±0.001^{c}	0.074±0.008^{a}	0.002±0.002^{d}	0.08±0.02^{a}	0.010±0.003^{c}	0.010±0.002^{c}	0.047±0.005^{b}	0.012±0.004^{c}
U	0.034±0.001^{b,c}	0.029±0.003^{b,c}	0.045±0.003^{b}	(0.10±0.01)10^{-3a}	0.029±0.008^{b,c}	(8.9±0.6) 10$^{-3d,e}$	(7.4±0.5)10^{-3e}	0.04±0.01^{b}	0.018±0.002^{c,d}
Rb	(4.4±0.5)10$^{-3c,d}$	(2±1)10$^{-3d,e}$	n.e.	(3.1±0.5)10$^{-3\ c,d,e}$	0.005±0.001^{c}	0.004±0.001^{c,d,e}	0.009±0.002^{b}	0.002±0.001^{e}	0.011±0.002^{a}
Cs	n.e.	n.e.	n.e.	n.e.	(7±2)10^{-4a}	(2.4±0.2)10$^{-4\ b}$	(5±1)10$^{-4\ a}$	n.e.	(4.01±0.01)10$^{-4\ a}$
Tl	(4±1)10$^{-5\ b}$	n.e.	n.e.	(12±3)10^{-5a}	(2.4±0.5)10$^{-5\ b,c}$	n.e.	(3±1)10$^{-5\ b}$	n.e.	n.e.
Hg	(12±8)10$^{-5d,e}$	(17±1)10$^{-5c,d}$	(25±3)10$^{-5\ b,c}$	(6±2)10$^{-5\ e}$	(61±9)10^{-5a}	(17±2)10$^{-5c,d}$	(28±2)10^{-5b}	(34±5)10^{-5b}	(33±2)10$^{-5b,c}$
Total (μmol g^{-1})	123±7^{c,d}	98±4^{d,e}	81±4^{d,e}	62±9^{e}	184±20^{b}	205±35^{b}	384±97^{a}	167±23^{b,c}	182±8^{b,c}

Values correspond to means and respective standard deviations. Among-species contrasts were tested with an ANOVA. Different letters indicate significant differences between species (P<0.05). 'n.e.' indicates that these measurements could not be estimated.

Journal of Experimental Biology (2017) 220, 984-994 doi:10.1242/jeb.141440

Journal of Experimental Biology (2017) 220, 984-994 doi:10.1242/jeb.141440

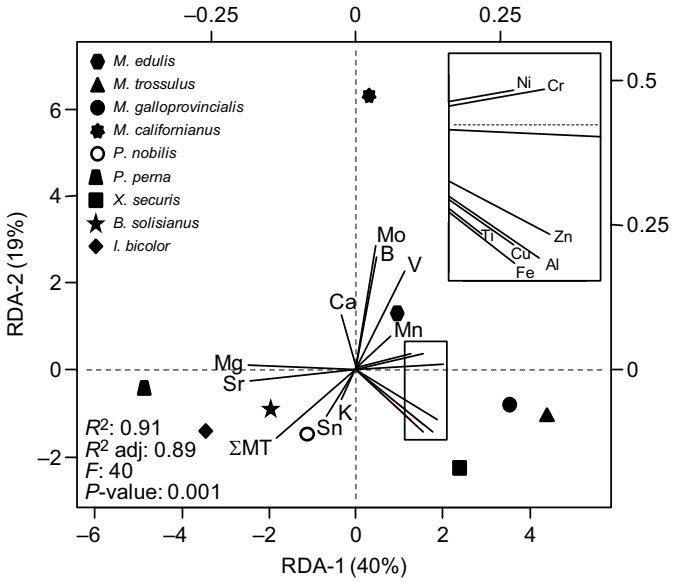

Fig. 4. Ordination biplot resulting from redundancy analysis (RDA) representing the significant effect of the factor species on the metal composition of byssal distal threads from different bivalve species. Lines represent response variables.

cuticle or histidine in the core of the fibre. Yet the uptake and trafficking of specific metal ions in molluscs is controlled through complex physiological pathways (Marigomez et al., 2002). Indeed, a radiolabelling study by George et al. (1976) on *M. edulis* showed that the iron present in the threads first passes through the soft tissue, while Schmitt et al. (2015) showed that *M. californianus* can opportunistically use some different metal ions to stabilize the byssus cuticle.

All species used in this study seem to rely on the metal cross-linking strategy for byssal thread biosynthesis. The relative abundance of the inorganic ions varies from one species to another. Traces of metal considered as toxic, such as As, Cd, Pb, U and Hg (<0.01%) have been observed in the distal threads of each species. As metal concentrations were reported to be higher in byssal threads in comparison with soft tissues, these results seem to confirm the hypothesis of Yap (2012), suggesting that byssus production may be a means of detoxification.

Common mechanical properties in relation to amino acid composition

In general, for all species studied, glycine, alanine and proline are the most abundant amino acid residues in native byssal threads. Their relative proportion is similar to that found in type 1 collagen which typically contains 35% Gly, 11% Ala and 12% Pro (Zhao and Chi, 2009), suggesting the presence of collagen in the fibres studied, in agreement with other work on mytilids (Arnold et al., 2013; Lucas et al., 2002; Qin and Waite, 1995). Glycine of the collagen-like domains is generally considered as being involved in strengthening the protein complexes of the distal section (Waite et al., 2002). An exception is found in *P. nobilis*, which produces anchoring threads containing 14% Gly, 4% Ala and 14% Pro (Table 2).

The amino acid profile in the noble pen shell *P. nobilis* suggests a paucity of collagen components in its byssus. The low proportion of DOPA and histidine, and the elevated proportion of Asx, Glx and Lys that is similar to PTMP1 (thread matrix protein from the proximal region of *M. edulis* byssal threads which is believed to

Table 3. Amino acid composition (% total amino acid content) of native byssal threads for each species

	M. edulis	*M. trossulus*	*M. galloprovincialis*	*M. californianus*	*P. nobilis*	*P. perna*	*I. bicolor*	*X. securis*	*B. solisianus*
Ala	14.9±0.07^{a}	11.18±0.07^{c}	12.5±0.2^{b}	16.0±0.4^{a}	4.41±0.03^{g}	15.4±0.4^{a}	9.4±0.2^{e}	10.3±0.3^{d}	8.1±0.2^{f}
Arg	4.75±0.015^{b}	5.25±0.02^{a}	4.37±0.02^{c}	4.7±0.2^{b,c}	3.43±0.07^{d}	4.80±0.09^{b}	1.78±0.09^{e}	4.38±0.03^{c}	5.2±0.2^{a}
Asx	4.35±0.05^{e}	6.2±0.2^{d}	5.7±0.4^{d}	6.7±0.6^{c,d}	9.9±0.2^{a}	5.8±0.4^{d}	2.5±0.4^{f}	8.10±0.07^{b}	7.08±0.05^{c}
Cys	0.14±0.02^{c,d}	0.52±0.02^{b}	0.18±0.08^{c}	0.41±0.07^{b}	0.4±0.1^{b}	0.38±0.04^{b}	0.75±0.04^{a}	0.020±0.004^{d}	0.51±0.04^{b}
L-DOPA	0.52±0.03^{b,c,d}	0.63±0.03^{b,c}	0.95±0.02^{a}	0.4±0.2^{c,d,e}	0.27±0.05^{e}	0.67±0.08^{b}	0.28±0.03^{d,e}	0.05±0.04^{f}	1.17±0.03^{a}
Glx	5.99±0.07^{b,c}	6.29±0.08^{b}	4.71±0.05^{e}	6.0±0.3^{b,c}	5.29±0.03^{d}	5.88±0.07^{c}	1.9±0.2^{f}	7.32±0.07^{a}	5.0±0.1^{d,e}
Gly	37.7±0.8^{a}	30.6±0.4^{a,b,c}	34.4±0.4^{a,b}	28±4^{c,d}	13.93±0.07^{e}	28±1^{c,d}	41±1^{a}	30±1^{b,c,d}	26.0±0.7^{d}
His	2.87±0.04^{c}	2.61±0.04^{d}	3.31±0.04^{b}	2.2±0.2^{e,f}	1.79±0.03^{g}	2.43±0.04^{d,e}	6.2±0.3^{a}	2.10±0.03^{f}	2.43±0.09^{d,e}
Ile	1.22±0.01^{f}	2.13±0.01^{c,d}	1.95±0.08^{d}	2.5±0.2^{c}	3.07±0.02^{b}	1.92±0.08^{d}	3.6±0.2^{a,b}	1.68±0.08^{e}	6±1^{a}
Leu	2.82±0.07^{e}	3.35±0.05^{c}	2.22±0.04^{f}	2.9±0.3^{d,e}	5.68±0.04^{a}	3.1±0.1^{c,d}	1.7±0.2^{g}	2.0±0.1^{f}	3.7±0.2^{b}
Lys	3.45±0.05^{d}	4.2±0.1^{c}	4.23±0.06^{c}	4.6±0.5^{c}	9.2±0.1^{a}	2.9±0.1^{e}	6.2±0.2^{b}	6.1±0.8^{b}	3.89±0.07^{c,d}
Met	0.87±0.02^{d,e}	1.07±0.02^{c}	0.91±0.03^{d,e}	0.68±0.02^{f,g}	2.50±0.05^{a}	0.59±0.06^{g}	0.83±0.08^{e,f}	0.94±0.06^{c,d}	1.52±0.08^{b}
Phe	1.37±0.03^{d}	1.99±0.04^{c}	1.44±0.04^{d}	2.0±0.2^{c}	2.58±0.04^{b}	2.4±0.1^{b}	1.3±0.2^{d}	1.8±0.1^{c}	3.1±0.1^{a}
Pro	6.7±0.1^{d}	7.0±0.1^{d}	5.9±0.2^{e}	6.8±0.2^{d}	14.1±0.2^{a}	8.45±0.05^{c}	11.26±0.07^{b}	7.3±0.2^{d}	8.1±0.1^{c}
Ser	5.6±0.1^{f}	7.5±0.1^{c}	6.82±0.04^{d}	6.0±0.3^{f}	6.5±0.1^{e}	7.32±0.07^{c}	2.0±0.3^{g}	7.8±0.2^{b}	8.44±0.04^{a}
Thr	2.93±0.05^{e}	3.93±0.04^{b,c}	4.06±0.02^{b}	3.94±0.06^{b,c}	3.5±0.1^{d}	3.1±0.1^{e}	1.57±0.09^{f}	4.7±0.1^{a}	3.6±0.2^{c,d}
Tyr	1.13±0.05^{f}	1.74±0.04^{e}	3.1±0.2^{b,c}	2.7±0.2^{d}	5.6±0.2^{a}	2.75±0.07^{c,d}	3.5±0.1^{b}	1.8±0.1^{e}	2.48±0.08^{d}
Val	2.65±0.05^{f}	3.6±0.1^{d}	3.3±0.1^{e}	3.8±0.2^{c,d}	7.78±0.04^{a}	4.0±0.1^{b,c}	4.5±0.2^{b}	3.38±0.09^{d,e}	3.8±0.4^{c,d,e}

Values correspond to means and respective standard deviations. Among-species contrasts were tested with an ANOVA. Different letters indicate significant differences between species (P<0.05). Ala, alanine; Arg, arginine; Asx, aspartic acid/asparginine; Cys, cysteine; L-DOPA, 3,4-dihydroxyphenylalanine; Glx, glutamic acid/glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

Journal of Experimental Biology (2017) 220, 984-994 doi:10.1242/jeb.141440

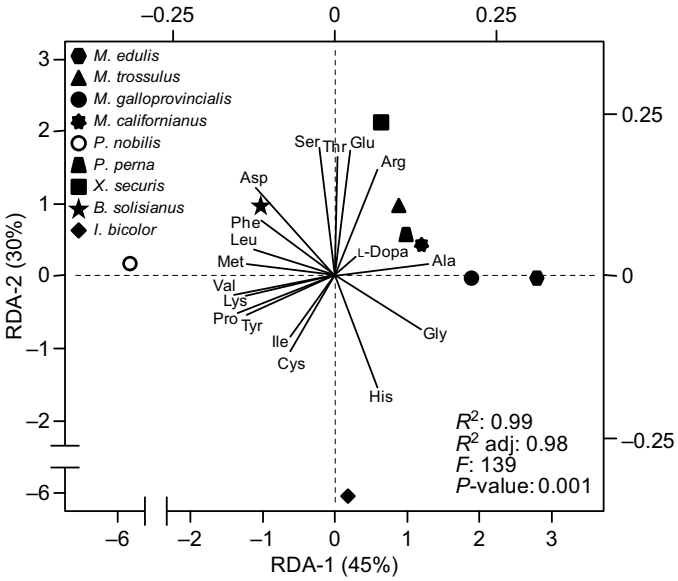

Fig. 5. Ordination biplot resulting from redundancy analysis (RDA) representing the significant effect of the factor species on amino acid composition of byssal distal threads from different bivalve species. Lines represent response variables.

cross-bind collagen without metal) (Sun et al., 2002), suggest that the byssus protein assembly could be principally driven by electrostatic interactions. This would explain the lowest mechanical performances of *P. nobilis* threads. Indeed, materials made from byssus protein hydrolysate self-assembled through electrostatic interactions showed much lower mechanical properties when compared with the native *M. edulis* byssus fibres (Byette et al., 2014). Although the byssus of *P. nobilis* is a dense bundle of long threads, single fibres were characterized by a low diameter (50 µm in average), and the lowest extensibility and toughness (as revealed by the area under the stress–strain curves shown in Fig. 2). These observations suggest that *P. nobilis* is probably relying on thread quantity over thread quality – a behaviour similar to *X. secures*, which secretes up to 1000 thin and weak threads (Babarro and Lassudrie, 2011). For *P. nobilis*, this byssal thread production strategy may be an advantageous means to achieve attachment in quiescent sandy habitats of the Mediterranean Sea, where this species is commonly found (García-March et al., 2007). We suggest that, collectively, all those fibres entrap sand in three dimensions and essentially make a ball-shaped anchor in the sand.

The amino acid composition of *I. bicolor* threads was distinguished by high proportions in lysine and histidine compared with the other species, in addition to an overall lower content in DOPA and charged or potentially charged residues Arg, Asx, Glx and Lys (total of 12%). However, the relative percentages of Gly, Ala and Pro (41:9:11) are comparable to the byssus of the other mussels, suggesting the presence of collagen in the fibre, with a slightly higher content in proline residues. Overall, the amino acid profile of *I. bicolor* threads suggests a macromolecular organization consisting of collagen and histidine-rich domains, as for mytilids (Arnold et al., 2013; Waite et al., 2004). Indeed, the high abundance of lysine seems to be correlated to the high proportion of histidine, as Lys is present in the His-rich domains of both preCol-NG and preCol-D (Qin et al., 1997). Moreover, the metal analysis suggests that this species preferentially selects divalent elements such as Ca, Mg, Cu and Zn in its byssal threads compared with *P. perna* and *B. solisianus* sampled in the same environment. Waite et al. (2004) have shown that the content of divalent transition metals such as Cu and Zn correlated to the abundance of histidine residues. A high His content could provide the highest potential for building metal cross-linking strategies (Waite et al., 1998) as the proportion of DOPA is low.

In terms of mechanical properties, *I. bicolor* threads were as extensible as those of mytilid species, although much weaker. They were also as stiff as those of *P. perna*, *M. edulis* and *M. trossulus*. However, *I. bicolor* threads yielded at a relatively low strain (5%) and stress, which reduces thread toughness as well the collective strength of a byssus consisting of numerous threads (Bell and Gosline, 1996). The yield point also represents the limit beyond which permanent plastic deformation and molecular changes occur in a material, such that a specimen cannot support a subsequent load cycle in the same manner as the previous one. However, plastic deformation in the byssal threads of *Mytilus* species is not permanent; threads show a fascinating self-healing capability and can gradually recover their original mechanical properties with time – a clear advantage for survival in environments swept by repetitive waves (Carrington and Gosline, 2004; Harrington et al., 2009; Harrington and Waite, 2007; Krauss et al., 2013; Vaccaro and Waite, 2001). Hagenau et al. (2009) have explained the plateau section following the yield point as a consequence of molecular conformational relaxation after the breakage of hydrogen bonds in the byssus fibres. The role of sacrificial bonds and hidden length in the flanking and histidine-rich domains has recently been suggested to explain mechanical performance of byssus (Harrington et al., 2009; Holten-Andersen and Waite, 2009a; Krauss et al., 2013). The reversible nature of these various non-covalent interactions helps explain the self-healing behaviour of byssal threads. A similar recovery of the mechanical properties was recently reported for films derived from the byssus of *M. edulis* in the presence of multivalent metal ions or salt-bridges acting as sacrificial bonds (Byette et al., 2016). The absence of a DOPA-rich cuticle could explain the low mechanical performances of *I. bicolor* threads as the hardness and rigidity of the coating are ensured by the DOPA–metal ion bonds, and are one order of magnitude greater than the thread core (Messersmith, 2010). Thus as for *P. nobilis* and *X. securis* threads, the low DOPA content could have an important contribution to weakening mechanical performance. Other post-translationally modified amino acids could play a role in metal ion binding, as reported for the glycosylated hydroxyl-tryptophan in the cuticle protein pvfp-1 in *Perna viridis* byssal threads (Zhao et al., 2009); however, the amino acid analysis method used in our study could not detect other post-translational modifications.

Contrary to mytilids, *I. bicolor* produces threads with no evident proximal portion, which is known to provide extensibility to the threads. The important post-yield extensibility observed in the case of *I. bicolor* threads (with viscoelastic behaviour up to a maximum strain of 75±20%) suggest that broken bonds are not the only mechanism involved. Considering that the extensibility of preCol does not exceed 2% (Harrington et al., 2009), disruption of the metal–histidine bridges linking adjacent preCols could also occur.

Byssal threads from *M. edulis*, *M. trossulus* and *M. galloprovincialis* obtained in this study could be grouped together as they are generally considered to be close congeners (McDonald et al., 1991). The similarity was striking not only according to amino acid composition but also to mechanical features, in agreement with previous reviews (Bell and Gosline, 1996; Carrington and Gosline, 2004; Harrington and Waite, 2007; Lucas et al., 2002). However, despite its lower thread diameter, *M. galloprovincialis* excelled at the beginning of stress–strain curve with a modulus similar to

M. californianus as well as with a similar curve shape characterized by a short plateau before undergoing strain stiffening. This could be explained by a comparable cuticle thickness (Holten-Andersen et al., 2009b). However, *M. californianus* threads remained stronger and more extensible. This mechanical difference could be related in part to the difference in the degree of protection coating against abrasion and degradation. Holten-Andersen et al. (2009b) reported that cuticle granular size gives threads of *M. californianus* more surface area contact and interaction with the matrix than those of *M. galloprovincialis*.

The behaviour of the threads of Mytilidae (*Mytilus* and *Perna* genus) under tension was highly conserved, but the intensities of their properties were much different. For instance, the mechanical properties of *M. californianus* byssal threads were superior to those obtained from other bivalves and particularly from *Mytilus* species as previously reported (Bell and Gosline, 1996; Harrington and Waite, 2007; Lachance et al., 2008; Lucas et al., 2002). This could be explained not only by its adaptation to highly exposed shores, but also by its taxonomic affiliation. According to the genetic tree proposed by Santaclara et al. (2007), *M. californianus* is phylogenetically distant from the other three *Mytilus* species, and even more distant from *P. perna*. Therefore the mechanical properties of byssal threads probably reflect a combination of habitat and phylogenetic relationships between species.

The threads of *P. perna* shared several properties with those of other mytilids, i.e. common diameters and morphology as well as similar proportions of amino acid residues, although it showed a higher content in inorganic ions, in particular divalent ones. Mechanically, *P. perna* threads were as extensible as those of *M. californianus* and they were as stiff as those of *M. edulis*, but their strength was much lower compared with the rest of the Mytilidae. The coexisting species *P. perna* and *I. bicolor* had byssal threads with similar strength and extensibility. These results could explain much about their ability to coexist and to compete for space in the same intertidal zone, from where they had been collected. Further structural analyses would be necessary to better understand the differences at a molecular level.

Biochemical profiles of other species

Although the mechanical properties of *X. securis* and *B. solisianus* threads could not be evaluated, their amino acid and metal ion profiles allowed interesting comparisons with other bivalves. *Brachidontes solisianus* is part of the Mytilidae family and shared similar amino acid content – with a slightly higher proportion in DOPA – suggesting a common strategy for byssal thread formation. Its inorganic ion content resembled that of the other species sampled in Brazil, indicating again that the selection of metals would depend both on the valence and occurrence in the local environment.

The invasive species *X. securis* was collected in Spain, in the same environment as *M. galloprovincialis*. They have similar concentrations of divalent (e.g. Ca, Mg, Cu, Zn) and multivalent inorganic ions (e.g. B, Al, Fe, Ti, V, Mo), although the proportion of iron in *X. securis* threads is twice that of the mytilid. This is consistent with a previous study (Jaworski et al., 2015) that related this to the different microstructures of the threads. The Gly:Ala:Pro proportion (30:10:7) compared well with the Mytilidae, suggesting the presence of collagen in the threads. Also, a high content (~26%) in charged or potentially charged amino acids (Arg, Asx, Glx, Lys) was measured. These data suggest that *X. securis* would rely on collagen, DOPA–metal and His–metal cross-links as well as electrostatic interactions in its byssal thread formation. However, the extreme thinness of the threads prevented the structural studies that are necessary to better understand the macromolecular organization of threads.

Conclusions

Similarities in mechanical features provide crucial information on the evolution and distribution of these bivalve species. Given the large size differences of animals and thread materials collected in diverse coastal areas around the world, interspecific variations in mechanical features are expected to be observed. Of the nine species we examined, *I. bicolor* and *P. nobilis* threads showed the most distinct response to tensile tests. Taxonomically close species such as *P. perna*, *M. galloprovincialis*, *M. trossulus* and *M. edulis* demonstrated similar morphological and biochemical characteristics while *M. californianus* – adapted to environments exposed to high wave action – produced the most robust fibres, in agreement with previous studies.

The comparison of byssus threads from a variety of species collected in different parts of the world showed that mechanical performance depends on the interrelation between morphometric characteristics as well as the amino acid and metal ion content. The fibre organization, such as the presence of a cuticle, of distal and proximal parts, and oriented fibrils in the core, is an important parameter. The analysis of the amino acid and metal ion content – which is related to the molecular structure of the fibres – indicated that the mechanical properties would depend on the strategy used by the bivalves in their byssal thread biosynthesis. Except for *P. nobilis*, which had a particular content of Gly, Ala and Pro, all species seemed to rely on the use of collagen as well as amino acid cross-linking with metals, but to different extents. *Pinna nobilis* and *X. securis* would, however, at least partially rely on a byssal protein assembly via electrostatic interactions. The data showed that the metal profile was mostly conserved, and that the metal selection would depend on its ability to cross-link with histidine and DOPA. Variations would depend on the surrounding environment, and other factors such as the molecular organization, not characterized in this study, could play a prominent role.

Acknowledgements

We thank Molly Roberts (Friday Harbor Laboratories) and Elsi Silva (Instituto de Investigaciones Marinas) for field assistance, Mathieu Babin (Institut des Sciences de la Ser de Rimouski) and Marc-Olivier Séguin-Heine (Université du Québec à Montréal) for their help in biochemical data collection, and Alexandre Arnold (Université du Québec à Montréal) and Frédéric Byette (Université de Montreal) for stimulating discussions.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Z.B. conducted sampling, data collection and analysis, and wrote the manuscript. B.G. assisted statistical analysis. N.I. contributed to the measurements of the mechanical properties. R.T., I.M. and C.P. contributed to the conception and the design of the technical and analytical protocols, to the data analysis, and to writing the manuscript. J.M.F.B, A.A.V.F., E.C. and A.L. supervised byssus sampling in Spain, Brazil, the USA and Turkey, respectively, and contributed to editing the manuscript and data analysis.

Funding

This work was supported by the Fonds de Recherche du Québec sur la Nature et les Technologies (FRQNT, team project 2012-PR-145239) and the Natural Sciences and Engineering Research Council of Canada (NSERC, discovery grant 299100 to R.T.). Z.B. would like to thank the Tunisian government and Ressources Aquatiques Québec (Ressources Aquatiques Québec, Rimouski, Canada) for the award of scholarships. R.T. and I.M. are members of the RAQ.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.141440.supplemental

Journal of Experimental Biology

References

Adams, C. B. (1845). Specierum novarum conchyliorum, in Jamaica repertorum synopsis. *Proc. Boston Soc. Nat. Hist.* **2**, 1-17.

Arnold, A. A., Byette, F., Séguin-Heine, M.-O., LeBlanc, A., Sleno, L., Tremblay, R., Pellerin, C. and Marcotte, I. (2013). Solid-state NMR structure determination of whole anchoring threads from the blue mussel *Mytilus edulis*. *Biomacromolecules* **14**, 132-141.

Babarro, J. M. F. and Carrington, E. (2013). Attachment strength of the mussel *Mytilus galloprovincialis*: effect of habitat and body size. *J. Exp. Mar. Biol. Ecol.* **443**, 188-196.

Babarro, J. M. F. and Lassudrie, M. (2011). Ecophysiological responses of invasive and indigenous Mytilids in the Ría de Vigo (NW Spain). *Aquat. Living Resour.* **24**, 303-315.

Babarro, J. M. F. and Reiriz, M. J. F. (2010). Secretion of byssal threads in *Mytilus galloprovincialis*: quantitative and qualitative values after spawning stress. *J. Comp. Physiol.* **180**, 95-104.

Bell, E. C. and Gosline, J. M. (1996). Mechanical design of mussel byssus: material yield enhances attachment strength. *J. Exp. Biol.* **199**, 1005-1017.

Bell, E. C. and Gosline, J. M. (1997). Strategies for life in flow: tenacity, morphometry, and probability of dislodgment of two *Mytilus* species. *Mar. Ecol. Prog. Ser.* **159**, 197-208.

Benedict, C. V. and Waite, J. H. (1986). Composition and ultrastructure of the byssus of *Mytilus edulis*. *J. Morphol.* **189**, 261-270.

Brazee, S. L. and Carrington, E. (2006). Interspecific comparison of the mechanical properties of mussel byssus. *Biol. Bull.* **211**, 263-274.

Byette, F., Pellerin, C. and Marcotte, I. (2014). Self-assembled pH-responsive films prepared from mussel anchoring threads. *J. Mater. Chem. B* **2**, 6378-6386.

Byette, F., Laventure, A., Marcotte, I. and Pellerin, C. (2016). Metal-ligand interactions and salt bridges as sacrificial bonds in mussel byssus-derived materials. *Biomacromolecules* **17**, 3277-3286.

Carrington, E. and Gosline, J. M. (2004). Mechanical design of mussel byssus: load cycle and strain rate dependence. *Am. Malacol. Bull.* **18**, 135-142.

Conrad, T. A. (1837). Descriptions of new marine shells from Upper California, collected by Thomas Nuttall, Esq. *J. Acad. Nat. Sci. PA.* **7**, 227-268.

Coombs, T. L. and Keller, P. J. (1981). *Mytilus* byssal threads as an environmental marker for metals. *Aquati. Toxicol.* **1**, 291-300.

Coyne, K. J., Qin, X.-X. and Waite, J. H. (1997). Extensible collagen in mussel byssus: a natural block copolymer. *Science* **277**, 1830-1832.

d'Orbigny, A. (1846). Mollusques lamellibranches. *Voyage dans l'Amérique Méridionale*. **5**, 489-758.

García-March, J. R., Pérez-Rojas, L. and García-Carrascosa, A. M. (2007). Influence of hydrodynamic forces on population structure of *Pinna nobilis* L., 1758 (Mollusca: Bivalvia): the critical combination of drag force, water depth, shell size and orientation. *J. Exp. Mar. Biol. Ecol.* **342**, 202-212.

George, S. G., Pirie, B. J. S. and Coombs, T. L. (1976). The kinetics of accumulation and excretion of ferric hydroxide in *Mytilus edulis* (L.) and its distribution in tissues. *J. Exp. Mar. Biol. Ecol.* **23**, 71-84.

Gould, A. A. (1850). Shells from the United States exploring expedition. *Proc. Boston Soc. Nat. Hist.* **19**, 292-296.

Guerra, A., Pascual, S., Garcil, M. E., Roura, A., Mucientes, G. and Gonzalez, A. F. (2013). The black-pygmy mussel *Limnoperna securis* in Galician Rias (north-eastern Atlantic): new records and first evidence of larval stages predation by copepods. *Mar. Biodivers. Rec.* **6**, e15.

Hagenau, A., Scheidt, H. A., Serpell, L., Huster, D. and Scheibel, T. (2009). Structural analysis of proteinaceous components in byssal threads of the mussel *Mytilus galloprovincialis*. *Macromol. Biosci.* **9**, 162-168.

Hagenau, A., Papadopoulos, P., Kremer, F. and Scheibel, T. (2011). Mussel collagen molecules with silk-like domains as load-bearing elements in distal byssal threads. *J. Struct. Biol.* **175**, 339-347.

Harrington, M. J. and Waite, J. H. (2007). Holdfast heroics: comparing the molecular and mechanical properties of *Mytilus californianus* byssal threads. *J. Exp. Biol.* **210**, 4307-4318.

Harrington, M. J., Gupta, H. S., Fratzl, P. and Waite, J. H. (2009). Collagen insulated from tensile damage by domains that unfold reversibly: *in situ* X-ray investigation of mechanical yield and damage repair in the mussel byssus. *J. Struct. Biol* **167**, 47-54.

Harrington, M. J., Masic, A., Holten-Andersen, N., Waite, J. H. and Fratzl, P. (2010). Iron-clad fibers: a metal-based biological strategy for hard flexible coatings. *Science* **328**, 216-220.

Holm, R. H., Kennepohl, P. and Solomon, E. I. (1996). Structural and functional aspects of metal sites in biology. *Chem. Rev.* **96**, 2239-2314.

Holten-Andersen, N. and Waite, J. H. (2008). Mussel-designed protective coatings for compliant substrates. *J. Dent. Res.* **87**, 701-709.

Holten-Andersen, N., Slack, N., Zok, F. and Waite, J. H. (2005). Nano-mechanical investigation of the byssal cuticle, a protective coating of a bio-elastomer. *Mater. Res. Soc. Symp. Proc.* **841**, 111-116.

Holten-Andersen, N., Mates, T. E., Toprak, M. S., Stucky, G. D., Zok, F. W. and Waite, J. H. (2009a). Metals and the integrity of a biological coating: the cuticle of mussel byssus. *Langmuir* **25**, 3323-3326.

Holten-Andersen, N., Zhao, H. and Waite, J. H. (2009b). Stiff coatings on compliant biofibers: the cuticle of *Mytilus californianus* byssal threads. *Biochemistry* **48**, 2752-2759.

Holten-Andersen, N., Harrington, M. J., Birkedal, H., Lee, B. P., Messersmith, P. B., Lee, K. Y. C. and Waite, J. H. (2011). pH-induced metal-ligand cross-links inspired by mussel yield self-healing polymer networks with near-covalent elastic moduli. *Proc. Natl. Acad. Sci.* **108**, 2651-2655.

Jaworski, J. S., Karasiński, J., Bulska, E. and Babarro, J. M. F. (2015). Effects of species and sites on metal concentrations in byssal threads of two mytilids. *Int. J. Environ. Anal. Chem.* **95**, 657-664.

Krauss, S., Metzger, T. H., Fratzl, P. and Harrington, M. J. (2013). Self-repair of a biological fiber guided by an ordered elastic framework. *Biomacromolecules* **14**, 1520-1528.

Lachance, A. A., Myrand, B., Tremblay, R., Koutitonsky, V. and Carrington, E. (2008). Biotic and abiotic factors influencing attachment strength of blue mussels *Mytilus edulis* in suspended culture. *Aquat. Biol.* **2**, 119-129.

Lamarck, J. B. M. (1819). *Histoire naturelle des animaux sans vertèbres*. *Les Mollusques* **7**, 1-343.

Legendre, P. and Legendre, L. (2012). *Developments in Environmental Modelling, Vol. 24. Numerical Ecology. 3rd edn*. Amsterdam: Elsevier Science.

Lin, Q., Gourdon, D., Sun, C., Holten-Andersen, N., Anderson, T. H., Waite, J. H. and Israelachvili, J. N. (2007). Adhesion mechanisms of the mussel foot proteins mfp-1 and mfp-3. *Proc. Natl. Acad. Sci.* **104**, 3782-3786.

Linnaeus, C. (1758). *Systema Naturae*. Editio decima. 1. Regnum Animale Holmiae, *Laurentii Salvii*. 824 pp.

Lucas, J. M., Vaccaro, E. and Waite, J. H. (2002). A molecular, morphometric and mechanical comparison of the structural elements of byssus from *Mytilus edulis* and *Mytilus galloprovincialis*. *J. Exp. Biol.* **205**, 1807-1817.

Marigómez, I., Soto, M., Cajaraville, M. P., Angulo, E. and Giamberini, L. (2002). Cellular and subcellular distribution of metals in molluscs. *Microsc. Res. Technol.* **56**, 358-392.

Marshall, B., Bouchet, P. and Gofas, S. (2015). *Xenostrobus securis* (Lamarck, 1819). In: *MolluscaBase* (2016). Accessed through: World Register of Marine Species at http://www.marinespecies.org/aphia.php?p=taxdetails&id=140485 on 28 July 2016.

McDonald, J. H., Seed, R. and Koehn, R. K. (1991). Allozymes and morphometric characters of three species of *Mytilus* in the Northern and Southern Hemispheres. *Mar. Biol.* **111**, 323-333.

Messersmith, P. B. (2010). Holding on by a hard-shell thread. *Science* **328**, 180-181.

Moeser, G. M. and Carrington, E. (2006). Seasonal variation in mussel byssal thread mechanics. *J. Exp. Biol.* **209**, 1996-2003.

Pearce, T. and LaBarbera, M. (2009). A comparative study of the mechanical properties of *Mytilid* byssal threads. *J. Exp. Biol.* **212**, 1442-1448.

Price, H. A. (1981). Byssus thread strength in the mussel, *Mytilus edulis*. *J. Zool.* **194**, 245-255.

Qin, X.-X. and Waite, J. H. (1995). Exotic collagen gradients in the byssus of the mussel *Mytilus edulis*. *J. Exp. Biol.* **198**, 633-644.

Qin, X.-X., Coyne, K. J. and Waite, J. H. (1997). Tough tendons: mussel byssus has collagen with silk-like domains. *J. Biol. Chem.* **272**, 32623-32627.

Sagert, J. and Waite, J. H. (2009). Hyperunstable matrix proteins in the byssus of *Mytilus galloprovincialis*. *J. Exp. Biol.* **212**, 2224-2236.

Santaclara, F. J., Espiñeira, M. and Vieites, J. M. (2007). Molecular detection of *Xenostrobus securis* and *Mytillus galloprovincialis* larvae in Galician coast (Spain). *Mar. Biotechnol.* **9**, 722-732.

Scarabino, F. (2003). Lista sistemática de los Bivalvia marinos y estuarinos vivientes de Uruguay. *Comun. Soc. Malacol. Uruguay*. **8**, 229-259.

Schmitt, C. N. Z., Winter, A., Bertinetti, L., Masic, A., Strauch, P. and Harrington, M. J. (2015). Mechanical homeostasis of a DOPA-enriched biological coating from mussels in response to metal variation. *J. Roy. Soc. Interface* **12**, 0466.

Séguin-Heine, M.-O., Lachance, A.-A., Genard, B., Myrand, B., Pellerin, C., Marcotte, I. and Tremblay, R. (2014). Impact of open sea habitat on byssus attachment of suspension-cultured blue mussels (*Mytilus edulis*). *Aquaculture* **426**, 189-196.

Sun, C. and Waite, J. H. (2005). Mapping chemical gradients within and along a fibrous structural tissue, mussel byssal threads. *J. Biol. Chem.* **280**, 39332-39336.

Sun, C., Lucas, J. M. and Waite, J. H. (2002). Collagen-binding matrix proteins from elastomeric extraorganismic byssal fibers. *Biomacromolecules* **3** 1240-1248.

Tanaka, M. O. (2005). Recolonization of experimental gaps by the mussels *Brachidontes darwinianus* and *B. solisianus* in a subtropical rocky shore. *Braz. Arch. of Biol. Tech.* **48**, 115-119.

Tanaka, M. O. and Magalhães, C. A. (2002). Edge effects and succession dynamics in *Brachidontes* mussel beds. *Mar. Ecol. Prog. Ser.* **237**, 151-158.

Taylor, S. W., Chase, D. B., Emptage, M. H., Nelson, M. J. and Waite, J. H. (1996). Ferric ion complexes of a DOPA-containing adhesive protein from *Mytilus edulis*. *Inorg. Chem.* **35**, 7572-7577.

Trovant, B., Ruzzante, D. E., Basso, N. G. and Orensanz, J. M. L. (2013). Distinctness, phylogenetic relations and biogeography of intertidal mussels (*Brachidontes*, Mytilidae) from the south-western Atlantic. *J. Mar. Biol. Assoc. UK* **93**, 1843-1855.

Tsukada, M., Gotoh, Y. and Yasui, H. (1995). Comparison of chemical and physical properties of the byssus of *Mytilis edulis* with those of silk fibroin fibers. *J. Seric. Sci. Jpn.* **64**, 435-445.
Vaccaro, E. and Waite, J. H. (2001). Yield and post-yield behavior of mussel byssal thread: a self-healing biomolecular material. *Biomacromolecules* **2**, 906-911.
Waite, J. H. (1985). Catechol oxidase in the byssus of the common mussel, *Mytilus edulis* L. *J. Mar. Biol. Assoc. UK* **65**, 359-371.
Waite, J. H. (2002). Adhesion a la moule. *Integr. Comp. Biol.* **42**, 1172-1180.
Waite, J. H., Qin, X.-X. and Coyne, K. J. (1998). The peculiar collagens of mussel byssus. *Matrix Biol.* **17**, 93-106.
Waite, J. H., Vaccaro, E., Sun, C. and Lucas, J. M. (2002). Elastomeric gradients: a hedge against stress concentration in marine holdfasts? *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **357**, 143-153.
Waite, J. H., Lichtenegger, H. C., Stucky, G. D. and Hansma, P. (2004). Exploring molecular and mechanical gradients in structural bioscaffolds. *Biochemistry* **43**, 7653-7662.
Waite, J. H., Weaver, J. C. and Vaccaro, E. (2006). Mechanical consequences of biomolecular gradients in byssal threads. In *Bionanotechnology: Proteins to Nanodevices* (ed. V. Renugopalakrishnan and R. V. Lewis), pp. 25-37. Netherlands: Springer.
Xu, Z. (2013). Mechanics of metal-catecholate complexes: the roles of coordination state and metal types. *Sci. Rep.* **3**, 2914.
Yap, C. (2012). Byssus as a means of metal excretion route and high metal levels in fecal materials as metal retention: an experimental laboratory study using *Perna viridis*. *Int. J. Adv. Appl. Sci.* **1**, 191-196.
Zeng, H., Hwang, D. S., Israelachvili, J. N. and Waite, J. H. (2010). Strong reversible Fe^{3+} mediated bridging between DOPA-containing protein films in water. *Proc. Natl. Acad. Sci.* **107**, 12850-12853.
Zhao, Y.-H. and Chi, Y.-J. (2009). Characterization of collagen from eggshell membrane. *Biotechnology* **8**, 254-258.
Zhao, H. and Waite, J. H. (2006). Linking adhesive and structural proteins in the attachment plaque of *Mytilus californianus*. *J. Biol. Chem.* **281**, 26150-26158.
Zhao, Y. H., Sagert, J., Soo Hwang, D. and Waite, J. H. (2009). Glycosylated hydroxytryptophan in a mussel adhesive protein from *Perna viridis*. *J. Biol. Chem.* **284**, 23344-23352.

Table S1: Total inorganic elements composition of byssal threads for each species expressed in µmol·g^{-1}. *Values correspond to means and respective standard deviations*

Group		*M. edulis*	*M. trossulus*	*M. galloprovincialis*	*M. californianus*	*P. nobilis*	*P. perna*	*I. bicolor*	*X. securis*	*B. solisianus*
	B	2.7 ± 0.2	2.0 ± 0.4	7.4 ± 0.5	10.7 ± 0.8	2.0 ± 0.2	7.2 ± 0.7	2.4 ± 0.8	1.0 ± 0.3	0.5 ± 0.09
	Fe	22.5 ± 0.7	23 ± 4	17 ± 2	7.0 ± 0.7	43 ± 5	14 ± 3	50 ± 10	80 ± 10	23.6 ± 0.9
	Al	10 ± 1	29 ± 2	22 ± 2	5.6 ± 0.8	24 ± 5	20 ± 3	53 ± 10	33 ± 8	35 ± 3
I	**Mg**	11 ± 2	6.6 ± 0.5	6 ± 1	15 ± 2	36 ± 6	126 ± 25	184 ± 52	13 ± 6	62 ± 1
	Ca	24 ± 4	11 ± 2	15.4 ± 0.6	16 ± 6	70 ± 10	25 ± 6	64 ± 21	28 ± 5	31.1 ± 0.8
	Mn	46 ± 6	15 ± 2	2.0 ± 0.3	2.4 ± 0.3	0.16 ± 0.03	0.53 ± 0.05	7 ± 1	0.4 ± 0.2	1.73 ± 0.09
	K	2.2 ± 0.2	2.6 ± 0.2	5 ± 3	1.5 ± 0.2	1.6 ± 0.2	10 ± 3	13 ± 4	4 ± 2	21 ± 10
	Ti	0.31 ± 0.05	0.84 ± 0.08	0.4 ± 0.1	0.29 ± 0.06	0.5 ± 0.1	0.65 ± 0.07	1.3 ± 0.1	1.4 ± 0.1	1.1 ± 0.2
	V	1.42 ± 0.05	0.82 ± 0.06	1.47 ± 0.08	1.9 ± 0.1	1.2 ± 0.2	0.42 ±0.02	0.42 ± 0.02	0.8 ± 0.2	0.18 ± 0.02
	Cr	0.176 ± 0.006	0.21 ± 0.02	0.08 ± 0.01	0.052 ± 0.004	0.08 ± 0.02	0.07 ±0.01	0.20 ± 0.05	0.11 ± 0.01	$(9.93 \pm 0.01)10^{-2}$
	Ni	1.38 ± 0.03	1.61 ± 0.06	0.19 ± 0.01	0.27 ± 0.03	0.45 ± 0.06	0.073 ± 0.005	0.48 ± 0.05	0.43 ± 0.06	0.70 ± 0.01
II	**Cu**	0.25 ± 0.02	3.7 ± 0.3	1.0 ± 0.5	0.12 ± 0.06	2.6 ± 0.3	0.4 ± 0.2	2.2 ± 0.5	2.1 ± 0.4	1.48 ± 0.05
	Zn	0.52 ± 0.03	1.4 ± 0.1	2.0 ± 0.2	0.27 ± 0.04	1.0 ± 0.1	0.28 ± 0.04	2.9 ± 0.5	2.6 ± 0.6	1.55 ± 0.09
	Sn	0.010 ± 0.008	0.024 ± 0.002	0.05 ± 0.04	0.01 ± 0.01	0.020 ± 0.007	0.15 ± 0.04	0.2 ± 0.1	0.09 ± 0.03	0.3 ± 0.2
	Sr	0.12 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.09 ± 0.03	0.37 ± 0.04	0.6 ± 0.1	1.2 ± 0.4	0.13 ± 0.03	0.62 ± 0.06
	Mo	0.128 ± 0.002	0.04 ± 0.02	0.17 ± 0.07	0.49 ± 0.05	0.16 ±0.02	0.017 ± 0.001	$(4.1 \pm 0.1)10^{-3}$	0.021 ± 0.004	$(3.15 \pm 0.02)10^{-3}$
	Ba	0.064 ± 0.004	0.053 ± 0.006	0.033 ± 0.008	0.017 ± 0.003	0.013 ± 0.002	$(3.0 \pm 0.3)10^{-3}$	0.008 ± 0.001	0.04 ± 0.02	$(6.9 \pm 0.2)10^{-3}$
	Be	$(1.33 \pm 0.04)10^{-3}$	$(7 \pm 2)10^{-4}$	$(9 \pm 1)10^{-3}$	$(8 \pm 1)10^{-4}$	$(7 \pm 2)10^{-3}$	$(2.0 \pm 0.3)\ 10^{-3}$	$(8 \pm 2)10^{-3}$	$(1.4 \pm 0.3)10^{-2}$	$(3.2 \pm 0.2)10^{-3}$
	Co	$(33.4 \pm 0.8)10^{-3}$	0.062 ± 0.007	0.06 ± 0.03	0.016 ± 0.004	0.026 ± 0.006	$(8.8 \pm 0.4)10^{-3}$	0.051 ± 0.005	0.064 ± 0.009	0.134 ± 0.002
	As	0.014 ± 0.001	0.023 ± 0.002	0.026 ± 0.005	$(5.6 \pm 0.6)10^{-3}$	0.07 ± 0.02	$(13.8 \pm 0.9)\ 10^{-3}$	0.034 ± 0.004	0.05 ± 0.01	0.012 ± 0.002
	Se	$(9.3 \pm 0.9)10^{-3}$	0.032 ± 0.006	0.18 ± 0.05	0.06 ± 0.01	n.e	0.057 ± 0.002	0.10 ± 0.06	0.12 ± 0.02	0.038 ± 0.004
	Ag	0.017 ± 0.008	$(3.6 \pm 0.6)10^{-3}$	0.004 ± 0.002	0.026 ± 0.003	0.011 ± 0.001	0.008 ± 0.001	0.031 ± 0.005	0.011 ± 0.002	0.013 ± 0.002
	Cd	$(1.7 \pm 0.1)10^{-3}$	$(2.4 \pm 0.4)10^{-3}$	$(3.9 \pm 0.3)10^{-3}$	0.024 ± 0.003	0.001 ± 0.001	$(2.84 \pm 0.09)10^{-3}$	$(3.0 \pm 0.7)10^{-3}$	$(2.8 \pm 0.5)10^{-3}$	0.022 ± 0.001
III	**Sb**	$(4.0 \pm 0.2)10^{-3}$	$(3.3 \pm 0.2)10^{-3}$	$(2.7 \pm 0.2)10^{-3}$	$(2.5 \pm 0.3)10^{-3}$	0.010 ± 0.002	$(1.3 \pm 0.3)10^{-3}$	$(6.0 \pm 0.7)10^{-3}$	0.007 ± 0.001	$(3.37 \pm 0.06)10^{-3}$
	Pb	$(1.29 \pm 0.08)10^{-3}$	$(13 \pm 1)10^{-3}$	$(60 \pm 7)10^{-3}$	$(1 \pm 1)10^{-3}$	0.14 ± 0.04	0.022 ± 0.007	0.038 ± 0.006	0.080 ± 0.009	0.023 ± 0.007
	U	0.041 ± 0.001	0.028 ± 0.003	0.036 ± 0.002	0.064 ± 0.005	0.05 ± 0.02	0.018 ± 0.001	0.028 ±0.002	0.08 ± 0.03	0.033 ± 0.003
	Rb	$(5.4 \pm 0.6)10^{-3}$	0.002 ± 0.001	ne	$(1.9 \pm 0.3)10^{-3}$	0.008 ± 0.002	0.008 ± 0.001	0.033 ± 0.007	0.003 ± 0.002	0.019 ± 0.003
	Cs	n.e.	n.e.	n.e.	n.e.	$(1.2 \pm 0.3)10^{-3}$	$(5 \pm 4)10^{-4}$	$(1.8 \pm 0.4)10^{-3}$	n.e.	$(7.29 \pm 0.03)10^{-4}$
	Tl	$(5 \pm 2)10^{-5}$	ne	n.e.	$(8 \pm 2)10^{-5}$	$(4.3 \pm 0.9)10^{-5}$	n.e	$(1.2 \pm 0.4\ )10^{-4}$	ne	ne
	Hg	$(2 \pm 1)10^{-4}$	$(1.7 \pm 0.1)10^{-4}$	$(2.1 \pm 0.2)10^{-4}$	$(4 \pm 1)10^{-5}$	$(1.1 \pm 0.2)10^{-3}$	$(3.5 \pm 0.3)10^{-4}$	$(1.08 \pm 0.08)10^{-3}$	$(5.7 \pm 0.8)10^{-4}$	$(6.0 \pm 0.4)10^{-4}$
	Total	123 ± 7	98 ± 4	81 ± 4	62 ± 9	184 ± 20	205 ± 35	384 ± 97	167 ± 23	182 ± 8

n.e. notation indicates that these measurements could not be estimated.