Secretion of byssal threads in *Mytilus galloprovincialis*: Quantitative and qualitative values after spawning stress

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Abstract

The effect of spawning events of the mussel *Mytilus galloprovincialis* on both quantitative and qualitative values of byssus secretion and its associated attachment force was investigated. Byssogenesis rates and absorption efficiency values were significantly reduced after spawning of individuals. However, the maintenance of individuals under sub-optimal conditions (lack of microalgae in the diet) for a week caused no effect on thread's number. Surprisingly, attachment force varied within a narrow range of values (1.7-1.9 N) with the exception of a significant drop in the experimental group spawned and kept unfed (1.0 N; $P<0.001$) most likely due to a similar pattern of the thread's thickness variability.

Qualitative analysis concerned to the amino acid composition of the byssus highlighted a higher presence of the basic residues histidine and lysine in threads secreted by spawned individuals. The presence of both histidine and lysine residues in the byssal collagen is associated to the formation of cross-links and specifically histidine has a functionality with a pronounced effect on metal chelation to stabilise the integrity of the byssus. Results reported here evidence the necessity to integrate all components that eventually determine the attachment strength of the mussels to get more insight the plasticity of such secretion. Morphology of the byssus (thickness) secreted under different endogenous conditions of

mussels was the major parameter to explain variability in attachment force. Moreover, aminoacidic composition as quality term of the byssus secreted may also contribute to understand plasticity of this secretion and needs to be extended in further surveys.

Keywords: *Mytilus galloprovincialis*, byssus, attachment, amino acids

Introduction

Mussels have the ability to secrete byssal threads that ensure a secure attachment point of individuals to the substratum in nature (Yonge 1962; Price 1983), as a mode of dispersion of young individuals (Sigurdsson et al. 1976; Lane et al. 1985) and as a predatory escape to immobilize predators (Farell and Crowe 2007). Secretion of byssus represents a dynamic process that has been widely described to occur from the foot and resembling a polymer injection-molding (Gerzeli 1961; Waite 1992). Byssus secreted by the mussel foot is composed by numerous byssal threads, each connecting proximally to a common stem that is rooted within the byssus gland of the foot and ultimately connects to the byssus retractor muscles (Brown 1952; Price 1983; Waite et al. 2002). The byssal thread itself can be divided into distinct sections from the morphological and compositional point of view i.e. proximal, connecting with the soft tissues and distal, which in turn, together with the adhesive disc ensure an anchorage point (Brown 1952; Waite et al. 2002). The strength of this byssal apparatus relative to the forces imposed on it from nature determines whether a mussel will remain attached to the substrate. Considering the plasticity that bivalves may express in terms of byssus secretion under certain stressful conditions, McDowell et al. (1999) have presented the first evidence for the formation of quinone-derived cross-links in mussel byssal plaques with

enhanced levels of 5, 5´-dihydroxyphenyl-alanine cross-links when individuals are exposed to increasing flow regimes. The whole thread structure is mainly collagenous (Pujol et al. 1970; 1976; Sun and Waite 2005) but the distal part has a supplementary composition in alanine and glycine that make it similar to silk fibroin (Qin and Waite 1998) whereas proximal section has additional components similar to those encountered in elastin (Coyne et al. 1997; Waite et al. 2002). Both proximal and distal sections have common histidine-rich residues at their terminal flanking domains with important implications for the intra- and intermolecular stabilization of assembled preCols in the byssus (Qin and Waite 1998). Specifically for the case of the byssal collagens, metal chelate complexes joining Zn^{2+}, Cu^{2+} and Fe^{2+} represent a significant cross-link alternative involving histidine, dopa (3,4-dihydroxyphenylalanine) or even cysteine residues (Lucas et al. 2002; Harrington and Waite 2007) that gives integrity and structural strength to the byssus apparatus. The axial gradient of dopa along the thread has been reported to be similar to that of iron which may suggest that mussels have the ability to exploit the interplay between dopa and metals to tailor the different parts of threads (Sun and Waite 2005). Under specific extreme environments, it has been recently described that metals can be sequestrated and deposited in byssus of the deep sea hydrothermal mussel *Bathymodiolus azoricus* with the participation of bacterial flora associated to the threads (Kadar 2007).

The dynamic process of byssus secretion in mussels is influenced by a number of both exogenous and endogenous factors. Initially, emphasis was focused on the importance of abiotic factors, specifically those related to the hydrodynamic character of the environment as most likely candidates to explain high proportion of the variability encountered in the thread production and attachment strength of individuals (Price 1982; Lee et al. 1990; Bell and Gosline 1997; Hunt and Scheibling 2001). However, other factors may also help to explain such variability by establishing a link with the energetic status of the individuals not only as a function of the available food resources (Clarke 1999) but also with regard to their

reproductive status (Carrington 2002a). Energy requirements for gamete formation are relatively high but this fact does not seem to influence negatively the attachment strength of mussels (Carrington 2002a; Zardi et al. 2007; Lachance et al. 2008). Zardi et al. (2007) have suggested that attachment strength and reproductive status are independently driven by environmental factors i.e. wave action or sea surface temperature and that its correlation could be purely coincidental although they also assumed that both processes are linked as competing energetic demands.

Spawning events in mussels may cause a number of perturbations in several physiological rates of the organisms as consequence of such abrupt change in the soft tissues state by gamete release. Spawning represents a very stressful event that may weaken individuals and even cause massive mortalities (Myrand et al. 2000). Under these circumstances of stress, mussels still need to renovate the byssal apparatus permanently as consequence of the thread's ageing in order to keep optimal attachment strength values. Lucas et al. (2002) have suggested that inconsistencies found in the bibliography with regard to the mechanical aspects of the byssal threads in the *Mytilus* complex are speculative but factors like sample size, mussel health, reproductive stage and thread age among others could help to understand such variability. Indeed, during gonadal development mussels are subjected to highly variable energetic demands and may invest up to 90% of their energy in gamete production (Seed and Suchanek 1992). The replacement of decayed byssal threads, however, can take up to 8-15% of total energy expenditure (Griffiths and King 1979; Hawkins and Bayne 1985). Nevertheless, the latter energetic component towards byssus secretion might represent a limiting action under certain stressful circumstances like post-spawning period with a corresponding weaker energetic status. Recently, Lachance et al. (2008) have highlighted that spawning of *Mytilus edulis* seemed to be correlated with significant decreases in attachment strength. A hypothetical lower potential to secrete byssus after spawning of mussels might

cause a negative impact in the viability of individuals when facing additional stressors in nature i.e. food scarcity, adverse meteorological processes etc.

Considering these aspects of byssus secretion research, we have tested here the hypothesis that byssus secretion of *M. galloprovincialis* and its attachment force associated are negatively affected by the spawning events under laboratory conditions. Accordingly, we have followed both quantitative and qualitative aspects of byssus secretion as the number of threads secreted and its amino acid composition, respectively that in turn might be related to the potential of establishing optimal attachment strength. With the aim to test the incidence of an additional stressor to the spawning event on both quantitative and qualitative aspects of byssus secretion, we have exposed part of the experimental mussel population (spawned and unspawned individuals) to non-feeding conditions for a week in the laboratory.

Material and Methods

Maintenance of individuals in the laboratory and byssal thread secretion

Individuals of *Mytilus galloprovincialis* were collected in the Ría de Arousa (NW Spain) from adjacent ropes of the same raft used for mussel culture in Galicia. Mussels were isolated from the clumps by cutting carefully and individually their byssal threads. Mean size of animals sampled was 72.3 ±1.3 mm (shell length) and 1.6 ±0.4 g (dry soft tissues). Gonadal index (see below) values of the individuals were 44% ±2.5. A total number of forty-eight animals were placed individually on glass Petri dishes (one animal per dish) on the bottom of a series of four 19-litre experimental tanks (45 x 40 x 14 cm, length x width x height; twelve animals per tank) and maintained for a week under controlled laboratory conditions in an open flow system (see

below). Glass Petri dish was selected as substrate based on the capacity to isolate individuals for specific measurements i.e. faeces collection for absorption efficiency as well as the fact that represents the second only to slate surface in mussel's choice of substratum (Young 1983). An input flow was distributed into the series of four 19-litre experimental tanks with values of approx. 3 l min^{-1} each tank, which in turn represented a relatively calm flow regime of 0.10 cm s^{-1} in our experimental system. The tanks were of open flow design using filtered (10 μm) seawater (Cartridge CUNO Super Micro-Wynd 10 μm) with controlled salinity and temperature values of 35.5‰ and 13ºC, respectively. The filtered seawater was supplemented with a mixture of microalgae (Tahitian *Isochrysis* aff. *galbana*, T-ISO) and sediment from the seafloor below the rafts (40:60 microalgae:sediment, by weight) supplied with a peristaltic pump at constant flow, so that particulate material load was maintained at 1.0 mg l^{-1} with an organic content percentage of 50%, simulating the mean values of food availability for the animals in their natural environment of Galician Rías (Babarro et al. 2000).

After one week of acclimation period, spawning was provoked in half of the mussel population (2 experimental tanks) during two consecutive days by temperature and air exposure shocks alternatively until spawning ceased. Byssal threads secreted during acclimation period were then removed carefully by severing them at the byssal gape with a razor blade and the experimental time began. Both spawned (two tanks) and unspawned (two tanks) mussels were maintained with the open flow system described before and two different feeding regimes were established: i) half population of both recently spawned (1 tank; n=12) and unspawned (1 tank; n=12) individuals was normally fed in a similar way than that of acclimation conditions (see before; 1.0 mg l^{-1} of total particulate matter with an organic content percentage of 50%), ii) the other half population of both spawned (1 tank; n=12) and unspawned (1 tank; n=12) individuals was maintained only with filtered seawater in an open flow but without any supplementation of the microalgae:sediment basis.

During both acclimation and experimental periods, orientation of the individuals within open flow system was considered to be at random in the Petri dishes although if any position is more repetitive than others that was the dorsal upcurrent to the input flow according to classification made by Dolmer and Svane (1994). Neither the latter authors that studied the effect of flow regime between 0 and 7.7 cm s^{-1} nor ourselves with much lower flow regime have observed a significant effect on number of threads secreted relative to the orientation of individuals unless high current values are considered ≈19.4 cm s^{-1} (Dolmer and Svane 1994).

Number of threads secreted by the individuals was counted daily in all Petri dishes with a binocular (Nikon SMZ-10 at 4x) until asymptotic values were obtained. New byssal threads were counted by viewing both upside and underside of the mussel through the transparent glass Petri dishes that were clearly visible and new plaques were marked each day on that underside of the dish with permanent ink marker.

Absorption efficiency (AE), gonadal and condition indexes

Volumes of the experimental diet used to feed the mussels (seawater + T-ISO + sediment; see before) and the faeces produced by fed individuals of both spawned and unspawned groups were collected at different sampling times (2^{nd} and 4^{th} experimental days). Faeces were collected from the glass Petri dishes where mussels are located on the bottom of the experimental tanks whereas the experimental diet was collected from the input flow of the system. The calm water treatment used in the experimental design allowed us to ensure that faeces collected at each Petri dish corresponded to those produced by the corresponding animal and not others. Both experimental diet and faeces samples were filtered on Whatman GF/C filters and processed for total particulate matter (TPM) and particulate organic matter (POM). Absorption efficiency (AE) was then quantified according to Conover (1966) as follows: AE=

(F-E) / [(1-E).F], where F and E are the organic content (by weight) of food and faeces, respectively.

Gonadal index was obtained as a simply proportion of mussel biomass composed of mantle tissue (site of gametogenesis in *Mytilus*) after weighting the gonad mass before and after spawning has been provoked i.e. as the weight lost in the spawning. Wet mantle was dissected from the wet body and together with the rest of organs lyophilised for 48 hours. Samples of the mantle and the rest of tissues were weighted to the nearest 0.001 g and gonadal index was calculated as the dry weight of the mantle divided by the whole soft body (sum of the dry weight of the mantle and remaining tissues). A high correlation between this simply parameter to obtain the gonadal index and that more precise value provided by image analyses of mantle lobe sections embedded in paraffin was confirmed by Carrington (2002a) (r^2=0.92; P<0.001). Condition index was obtained according to the formula: $CI=(DW_{tissue}/DW_{shell})x100$, where DW_{tissue} corresponds to dry weight of soft tissues and DW_{shell} to dry weight of the shell (Freeman 1974). A similar temporal variation between both gonadal and condition indexes was also observed by Moeser et al. (2006) for *M. edulis* confirming gonadal index used here as good factor for establishing the condition of experimental individuals. In the present study, we have obtained a high correlation coefficient between both condition and gonadal indexes (r^2=0.82) (Figure 1).

Attachment force

Attachment force of the mussels was measured as Newtons (1 kg=9.81 N) by connecting the individual to a spring scale (Kern MH, resolution of 0.01N) through a thin multifilament fishing line and then, quantifying the force needed to dislodge mussels from the substrate (after 1 to 3 s). The spring scale was pulled perpendicular (normal) to the substrate (Petri dish) once all byssal threads were observed to be at full extension until dislodgement occurred.

Dislodgment force needed to detached mussels was measured when asymptotic values of byssal threads secreted were obtained at the end of the experimental period.

Image Analysis

After measuring the attachment force of individuals, thread thickness was obtained by image analysis (IA) performed in a number of five byssal threads per individual and six different animals in each experimental treatment. IA measurements were performed using the software QWin (© Leica Imaging Systems) on a PC (AMD Athlon XP 3000+) connected to a video camera (Leica IC A) on a stereo microscope (Leica MZ6). Camera and light settings were established at the beginning of the analysis and kept constant throughout the whole analysis. Thread's thickness values refereed approximately to the 2/3 thread's length that corresponded mainly to the distal region of the thread that remained attached to the Petri dish after dislodgement of individuals.

Amino acid composition of byssal threads

Approximately 2/3 of the byssal thread's length used to measure thickness i.e. distal section was also considered for amino acid analysis. Hydrolysis of the byssal proteins were performed following Lucas et al. (2002). Briefly, distal segments of the threads were hydrolysed in 6 mol^{-1} HCl with 0.01 ml of redistilled phenol. A number of three replicates of each experimental treatment were considered for HPLC analysis, each being integrated by 3 animals (3-5 distal segments from each animal). Threads were hydrolysed *in vacuo* for 24h at 110ºC and samples were then flash-evaporated at 60ºC. A volume of PCA (perchloric acid) was added to the dry hydrolysed thread material and amino acids were quantified following Babarro et al. (2006).

Determination of amino acids was performed by reverse-phase high-performance liquid chromatography of the dabsyl derivatives. All amino acids standards and dabsyl chloride were purchased from Sigma. Amino acid separation method consisted in a slight modification of that reported by Krause et al. (1995). The chromatograph was a Waters Alliance HPLC System with a 2690 separations module and a Waters 996 photodiode array detector (440-480 nm). The stationary phase was a C_{18} column (Waters Symmetry, 150 x 4.6 mm, 3.5 µm particle size, 100 Å pore size) thermostated at 50ºC either by an Alliance System column oven. Twenty µL of the derivatized samples were injected. Dabsylated amino acids were eluted at a flow-rate of 1 mL/min using a gradient made with phase A (9 mM sodium dihydrogenphosphate, 4% dimethylformamide and 0.1-0.2% triethylamine titrated to pH 6.55 with phosphoric acid) and B (80% aqueous acetonitrile) with a gradient profile that corresponds to that used by Pinho et al. (2001). For quantification, nor-leucine was used as internal standard.

Statistical analysis

Number of byssal threads produced by the mussels were compared by means of ANOVA. Cumulative values of threads are presented as the mean ± standard errors of twelve individuals in each experimental group**.** One-way ANOVA was also used to compare absorption efficiency as well as gonadal and condition indexes. Two-way ANOVA was used to estimate the effects of both gonadal index and feeding regime on the attachment force and thread's thickness values (log transformed data). Homogeneous groups among experimental mussels could be established *a posterior*i by using Tukey´s test. When variances were not homogeneous (Levene´s test), non-parametric test Kolmogorov-Smirnov and Mann-Whitney were used. Correlation analyses were performed following Pearson's correlation coefficients. For all analyses performed a statistical computer package STATISTICA 6.0 was used.

Results

Spawning effects: corporal parameters and byssogenesis

The effect of mussel spawning on endogenous indexes is illustrated in Table 1. As expected, values of dry soft tissues and gonadal/condition indexes of individuals dropped significantly after spawning of fed animals with values that represented a decrease of 22%, 42% and 24% in the latter corporal parameters, respectively and compared to unspawned fed mussels ($0.05>P<0.001$; Table 1). The latter decreases as consequence of spawning were even more abrupt when individuals were maintained under non-feeding conditions for a week (38%, 51% and 40% decrease in soft tissues, gonadal and condition indexes, respectively; $0.05>P<0.001$; Table 1).

The number of threads secreted by the mussels subjected to the experimental conditions is illustrated in Figure 2 (A-D). Byssogenesis of individuals after spawning was significantly affected ($P<0.05$), spawned mussels secreted significantly lower amount of threads as compared to unspawned individuals and this result was observed regardless feeding regimens (Figure 2A-B). Then, considering both subgroups spawned and unspawned mussels separately, the non-feeding exposure caused no effect on thread's numbers (Figure 2 C-D) although soft tissues (and gonadal/condition indexes) dropped significantly in the recently spawned and maintained unfed mussels ($P<0.01$; Table 1).

Absorption efficiency (AE)

AE of mussels subjected to the experimental diet was significantly lower in recently spawned mussels as compared to unspawned individuals ($P<0.01$) two days after the beginning of the experimental time (Figure 3). Both groups of experimental mussels, however, showed values of AE above 80%. At day 4^{th} of the experiment, AE differences were observed to be statistically not significant between both groups of mussels as consequence of a slight decrease in AE of unspawned mussels (Figure 3).

Attachment force and thread's thickness

A comparison of the results obtained for the quantitative values of byssus secreted, its attachment force associated and the byssal thread's thickness values are presented in Figure 4. Despite the fact that quantitative values of threads secreted were significantly affected by the spawning of the mussels (Figure 4 A), two-way ANOVA performed on attachment force values (log transformed) showed no effect of both gonadal index and feeding regime as independent factors but a significant interaction term gonadal index * feeding regime ($P<0.05$) which meant that the significant effect of spawning events depended on the non-feeding maintenance of individuals (Figure 4 B). Force values to dislodge animals from the experimental substratum varied within a narrow range of 1.7-1.9 N for all experimental groups with the only exception of the spawned mussels maintained unfed that showed a significant drop in attachment force to values of 1.0 N ($P<0.001$) (Figure 4 B).

After attachment force measurements, values of thread's thickness were recorded in the distal regions of the threads that remained attached to the substratum (see Material and Methods; Figure 4 C). Two-way ANOVA performed on byssal thickness values (data not shown) showed the same pattern than the previous one for the attachment force in which only the interaction term gonadal index * feeding regime was presented as significant factor

(P<0.05). Accordingly, it can be observed that distal thread's diameter values varied within a range of 80-83 µm for all experimental groups although recently spawned mussels maintained unfed for a week produced threads significantly thinner (72 µm) (P<0.05; Figure 4 C).

Amino acid composition of byssal threads

Amino acid compositional analyses of the acid-hydrolysed distal regions of the threads secreted by the experimental mussels are listed in Table 2. In all cases, glycine represents approx. 1/3 of the thread's amino acidic composition, alanine 12-13% and proline 5-6% of the total residues analysed (Table 2). The sum of these three amino acids corresponded approximately to half amount of residues (Table 2). Amino acid residues are rather constant in all comparisons, nevertheless, significant differences referred mainly to the basic amino acids (histidine and lysine) and, in lower magnitude, phenylalanine and threonine (Table 2). Mussels that were forced to spawn secreted byssal threads with significantly higher presence of histidine and lysine residues (3.5% ±0.2 and 5.8% ±0.2, respectively) as compared to unspawned individuals (2% ±0.4 and 4.4 ±0.5, respectively) (P<0.001; Table 2). For both amino acids, the lowest values were observed in mussels maintained unfed for a week which means that such a drop was of higher magnitude in spawned mussels (1.4-1.6% and 3.2-3.4% for histidine and lysine, respectively, for both spawned and unspawned mussels; Table 2).

Discussion

Internal processes in bivalve molluscs concerning to the reproductive cycle are subjected to relatively large energetic demand (Seed and Suchanek 1992). Therefore, the influence of this energy expenditure associated to the sexual activity on the byssus secretion may be a limiting

factor for the individuals with important consequences for their performance and survival in nature considering that both processes may be linked as competing energetic demands (Zardi et al. 2007). Earlier studies had pointed out that prediction of byssus strength of mussels in different months could be lower than expected according to the variability of the most common analysed factors i.e. wind and temperature and that in such gape, the effect of spawning of individuals might play an important role (Price 1982). More recently, Carrington (2002a) has highlighted the importance of the reproductive internal status of the mussels, apart from the classical hydrodynamic view, as new insight to explain the seasonal variation in the byssus secretion and its associated attachment strength of the individuals.

In the present survey, spawning events of the mussels maintained under laboratory conditions had adverse effects on different ecophysiological parameters of *M. galloprovincialis*. Number of threads secreted and absorption efficiency of food were significantly lower in recently spawned mussels as compared to unspawned individuals under optimal feeding conditions in the laboratory (Figure 2 and 3). For the specific case of AE, nevertheless, values remained relatively high in both experimental populations (above 80%), and were similar 4 days after the beginning of the experiment. Contrarily, maintenance of individuals in filtered seawater without any food addition for a week did not cause significant changes in the amount of byssal threads secreted either by spawned or unspawned mussels (Figure 2 C-D) whereas soft tissues (and gonadal/condition indexes) dropped significantly in the spawned population maintained unfed ($P<0.01$; Table 1). Individuals kept unfed might have continued to derive energy towards byssal threads production most likely at the expense of the transfer of organic tissue reserves to byssogenesis. This fact is suggested from the drop in soft tissues (and gonadal index) of individuals within the worst experimental condition (spawning plus non-feeding conditions) (Table 1). Clarke (1999) had showed that starved zebra mussels (*Dreissena polymorpha*) also continued to partition energy to byssal threads formation

1 although total mass was compromised with lower amount of threads formed. The non-feeding
2 time tested, however, seemed to be no longer enough to observe a significant decrease in soft
3 tissues of mature animals (see also gonadal index), the byssus formation rates being not
4 significantly affected in case the energetic reserves in soft tissues of unspawned animals are
5 high (Table 1).

6 In agreement with the present results for *M. galloprovincialis*, lower number of threads
7 secreted by mussels with lower gonadal index values was also observed for *M. edulis* by
8 Moeser et al. (2006) that highlighted the importance of such endogenous factor in the
9 variability of byssogenesis. In a more complete analysis than that reported initially by Price
10 (1982**)**, up to 90% of the variability in thread production was, therefore, explained by the latter
11 authors (Moeser et al. 2006) according to changes in temperature, wave height and
12 reproductive condition.

13 Once a negative effect of mussel spawning on byssogenesis rates was observed, one
14 might have expected a similar significant incidence on attachment force values since the
15 number of threads has been refereed as one of the most important factors influencing
16 attachment force of mussels (Bell and Gosline 1997; Zardi et al. 2007). Our own studies have
17 reported a significant relationship between attachment force and number of byssal threads for
18 *Mytilus galloprovincialis* of different size maintained in the laboratory (Babarro et al. 2008).
19 The latter relationship attachment force *vs*. number of byssus was not clear here as
20 consequence of the similar attachment force values reported (Figure 4 B). The only exception
21 is represented by the most stressful experimental condition (spawned plus maintained unfed
22 animals) that caused a drop in the attachment force up to 1.0 N (Figure 4 B) which in turn can
23 be clearly linked to the lowest thread's thickness value reported for the byssus secreted by this
24 experimental group ($P<0.05$; Figure 4 C). Indeed, apart from the number of threads secreted,
25 the way by which individuals might vary its attachment force values can be related to

differences in thread's diameter and/or material properties of the byssus (Bell and Gosline 1997; Brazee and Carrington 2006).

Attachment force profiles reported here followed a similar pattern than that of the byssal thread's thickness for each experimental group of mussels (Figure 4 B-C). However, we were also interested in hypothetical modifications that animals may carry out at qualitative level of the byssus to cope with endogenous stress. In the present study, quality was considered as biochemical composition of threads secreted by individuals with different gonadal index values. Surprisingly, lower number of threads secreted by spawned mussels was counteracted by changes in the biochemical composition of the threads that can be linked to processes to get optimal structural integrity of the byssus. Specifically, significant changes came from variability of basic amino acids histidine and lysine that were present in higher number of residues in threads secreted by recently spawned mussels (74% and 32%, respectively) as compared to unspawned individuals (Table 2). Considering the total number of residues, such increase of the basic amino acids in the distal collagen of threads secreted by spawned individuals are counterbalanced by slight decreases in a number of amino acids (Table 2) but both threonine and phenylalanine represented up to 30-50% of such histidine/lysine increases. No information is available to us for the importance of threonine/phenylalanine in the byssal collagen. However, it is well-known that residues of both lysine and histidine produce cross-links, joining two or more molecules by a covalent bond. Specifically for the case of histidine, it has been reported a functionality with a pronounced effect on metal chelation and/or cross-link ability (Waite et al. 1998) as well as the capacity to form a significant part (up to 22 mol% in protein mcfp-4) of the junction between collagen fibres and foam-like adhesive plaques in the mussel *Mytilus californianus* (Zhao and Waite 2006). Whenever histidine-rich domains occur in proteins, they usually bind with metal and, byssal collagen of *Mytilus galloprovincialis* has been reported to contain additional histidine residues in their flanking

domains that can help to utilise more metal chelate cross-link for byssal stability and integrity (Lucas et al. 2002). According to its functionality in the cross-link potential, *M. galloprovincialis* would be expected to produce stronger and stiffer threads by virtue of having more histidine in the flanking domains of all its precols that might help to counteract lower byssus secreted in those mussels recently spawned. At first view, this result might be considered as an example of qualitative modification of the byssus properties that would derive eventually in a better performance of the byssal apparatus to cope with specific stress i.e. post-spawning performance of individuals. Plasticity patterns in the mussel byssus were also reported by McDowell et al. (1999) with an increased formation of quinone-derived cross-links in mussel byssal plaques when individuals are exposed to higher flow regimes which might cause better attachment of the individuals to the substratum. Nevertheless, it can be also observed that histidine and lysine residues in byssal threads of both spawned and unspawned individuals dropped significantly in the absence of food resources (Table 2) although attachment force values of the whole individual for the unspawned experimental group were similar than that of fed individuals (Figure 4 B). This inconsistency between compositional analysis of the threads and the actual attachment force values is solved with the inclusion of the thread's thickness values of the latter experimental group (unspawned kept unfed) similar to the fed experimental groups (Figure 4 C).

Here we present evidences to suggest certain plasticity with regard to compositional values of the mussel's byssus facing endogenous stress i.e. after spawning, in order to get eventually optimal attachment force. However, it is important to highlight that, on one hand, biochemical analyses carried out in the present study refer only to the distal sections of the byssus and that is necessary to extent such knowledge to other sections i.e. proximal and adhesive plaque in order to obtain a more significant view. Our own experience suggests that proximal region of the byssus is much less variable than distal sections (and a number of

mechanical properties) when mussels are transplanted between very different environments within the same estuary (not published results). On the other hand, despite differences encountered in biochemical analysis of the threads, actual attachment force values were significantly linked to the differences encountered in thread's diameter as crucial factor. Complete analyses including quantitative and qualitative values of the byssus might help to understand ecophysiological plasticity of individuals facing stress in order to establish an eventual optimal attachment force in the substratum.

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Table 1. *Mytilus galloprovincialis*. Mean values (± SE) of shell length (mm), soft tissues dry weight (g), and condition and gonadal indexes of all experimental groups of mussels under study. Statistical comparisons between experimental groups are also presented.

	Shell Length (mm)	Tissue DW (g)	Gonadal index (GI)	Condition index (CI Freeman)
Fed Spawned (FS)	72.08 ±0.43	1.21 ±0.05	25.09 ±1.42	12.46 ±0.62
Fed Unspawned (FU)	72.63 ±0.32	1.55 ±0.11	43.50 ±3.50	16.36 ±0.95
Unfed Spawned (US)	72.04 ±0.53	0.96 ±0.07	20.62 ±1.16	9.77 ±0.53
Unfed Unspawned (UU)	72.46 ±0.35	1.55 ±0.08	42.31 ±1.85	16.20 ±0.72
	FS-FU ns US-UU ns FS-US ns FU-UU ns	FS-FU p<0.05 US-UU p<0.001 FS-US p<0.01 FU-UU ns	FS-FU p<0.001 US-UU p<0.001 FS-US p<0.05 FU-UU ns	FS-FU p<0.01 US-UU p<0.001 FS-US p<0.01 FU-UU ns

Table 2. *Mytilus galloprovincialis*. Representative amino acid composition from hydrolysed thread portions (distal regions) of mussels subjected to different experimental conditions.

Amino Acid	Fed Spawned (FS) mean	SE	Fed Mature (FM) mean	SE	Unfed Spawned (US) mean	SE	Unfed Mature (UM) mean	SE
Hyp (Hydroxyproline)	6.697	0.090	6.665	0.293	6.808	0.256	6.592	0.255
Asx	2.299	0.118	2.288	0.122	2.223	0.098	2.312	0.138
Thr	6.581	0.089	7.260	0.153	7.534	0.133	8.160	0.220
Ser	5.557	0.147	5.766	0.391	5.212	0.455	5.416	0.312
Glx	2.177	0.055	2.206	0.084	2.261	0.104	2.198	0.062
Pro	5.817	0.120	6.088	0.289	5.915	0.153	5.640	0.233
Gly	29.159	0.414	29.705	0.849	30.115	0.697	30.397	0.509
Ala	12.813	0.349	13.040	0.667	13.719	0.501	13.609	0.406
Cys/2	0.049	0.014	0.091	0.060	0.016	0.008	0.015	0.004
Val	3.020	0.101	2.924	0.374	2.628	0.353	2.336	0.234
Met	0.127	0.018	0.156	0.018	0.117	0.064	0.038	0.021
Ile	1.485	0.093	1.568	0.273	1.299	0.231	1.256	0.208
Leu	3.005	0.075	3.355	0.110	3.441	0.144	3.296	0.195
Dopa	0.072	0.018	0.067	0.017	0.092	0.034	0.106	0.017
Tyr	1.208	0.111	0.991	0.088	0.971	0.067	1.219	0.095
Phe	3.050	0.086	3.531	0.460	4.684	0.674	5.002	1.222
His	3.535	0.204	2.030	0.391	1.629	0.246	1.386	0.317
Hlys (Hydroxylysine)	0.033	0.008	0.060	0.006	0.021	0.009	0.089	0.017
Lys	5.824	0.217	4.395	0.548	3.398	0.629	3.236	0.454
Arg	7.492	0.126	7.814	0.169	7.917	0.103	7.697	0.139
Gly-Ala-Pro	47.789	0.705	48.833	1.367	49.749	1.080	49.646	0.819
Acid amino acid	4.555	0.126	4.494	0.179	4.484	0.187	4.510	0.168
Basic amino acid	16.851	0.384	14.239	1.077	12.944	0.771	12.319	0.466

Legend of Figures

Figure 1. Linear correlation between gonadal index and condition index values obtained for the experimental groups of mussels under study.

Figure 2. Quantitative values of byssal threads secreted by the experimental unspawned and spawned mussels (mean values ± SE). The effect of spawning is reported in both fed (A) and unfed (B) mussels. The effect of feeding is reported in both spawned (C) and unspawned (D) mussels.

Figure 3. Absorption efficiency (mean values ±SE) of both spawned and unspawned mussels maintained fed at different days of the experimental period.

Figure 4. Comparison of the gonadal index values and the cumulative number of threads secreted at the end of the experimental period (A) and the attachment force of the whole individual (B). Values of the byssal thread diameter for the experimental individuals considering distal sections (C).

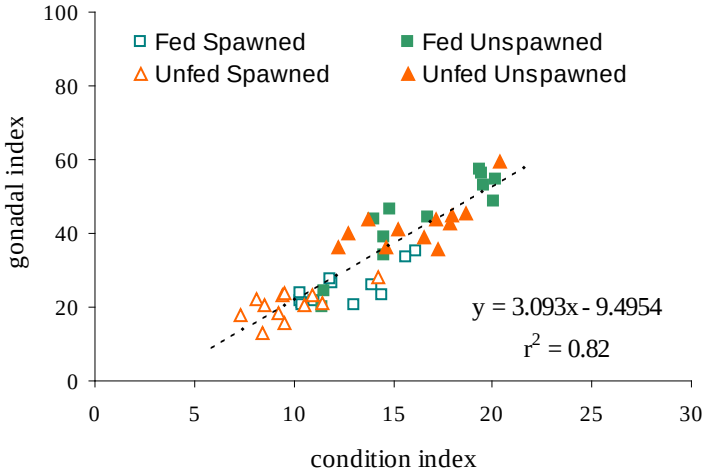

Fed Spawned
Fed Unspawned
Unfed Spawned
Unfed Unspawned
gonadal index
condition index
y = 3.093x - 9.4954
r2 = 0.82
0
20
40
60
80
100
0
5
10
15
20
25
30

Figure 1

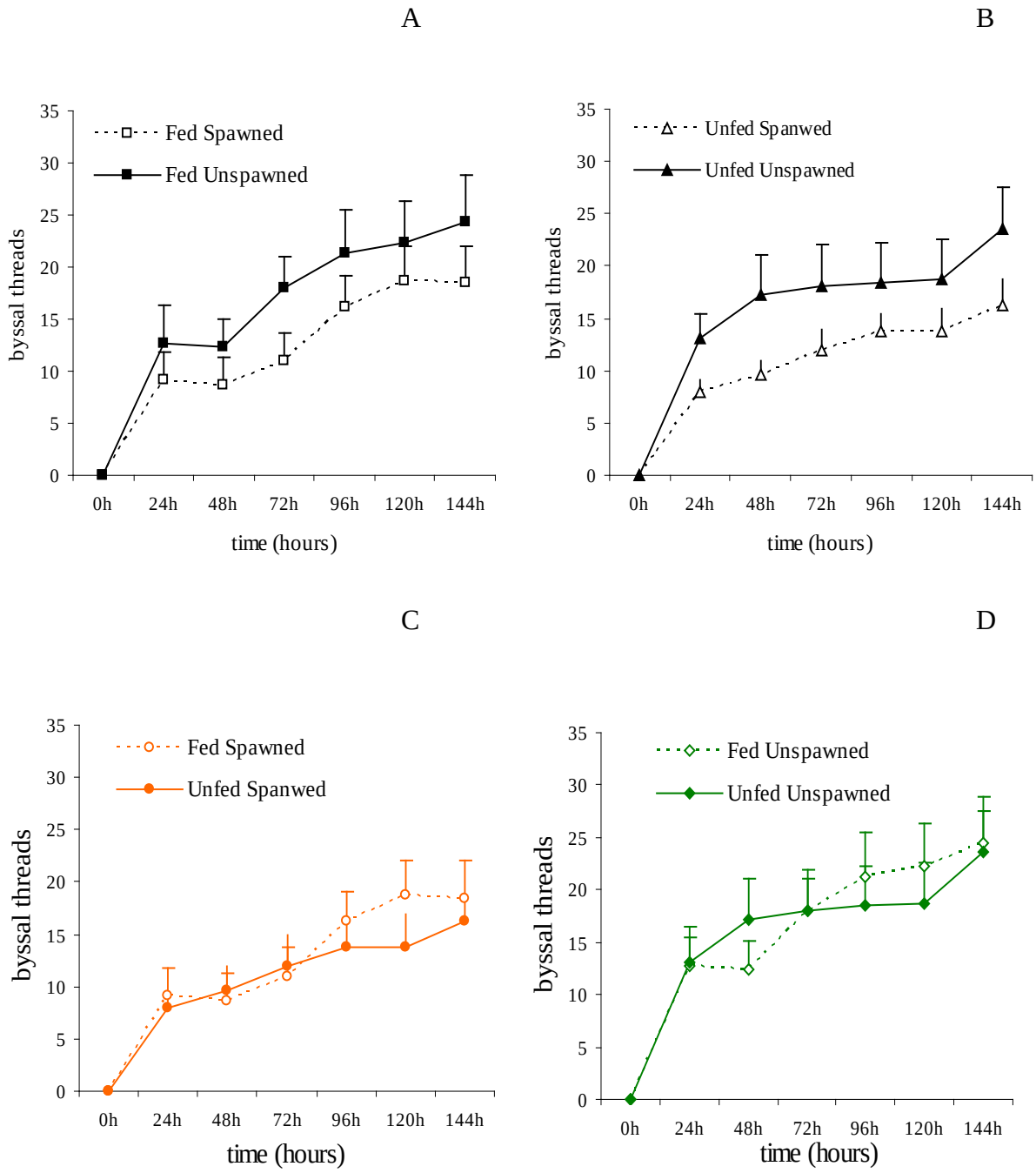

A
B
C
D
Fed Spawned
Fed Unspawned
Unfed Spanwed
Unfed Unspawned
Fed Spawned
Unfed Spanwed
Fed Unspawned
Unfed Unspawned
byssal threads
time (hours)
0h
24h
48h
72h
96h
120h
144h
0
5
10
15
20
25
30
35

Figure 2

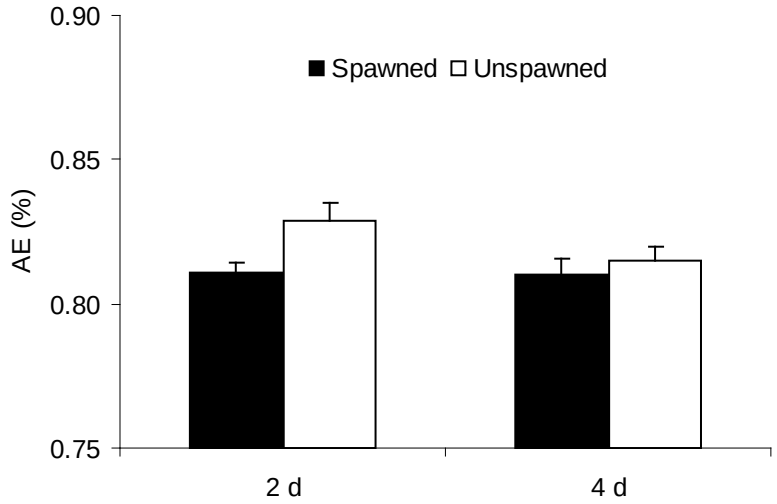
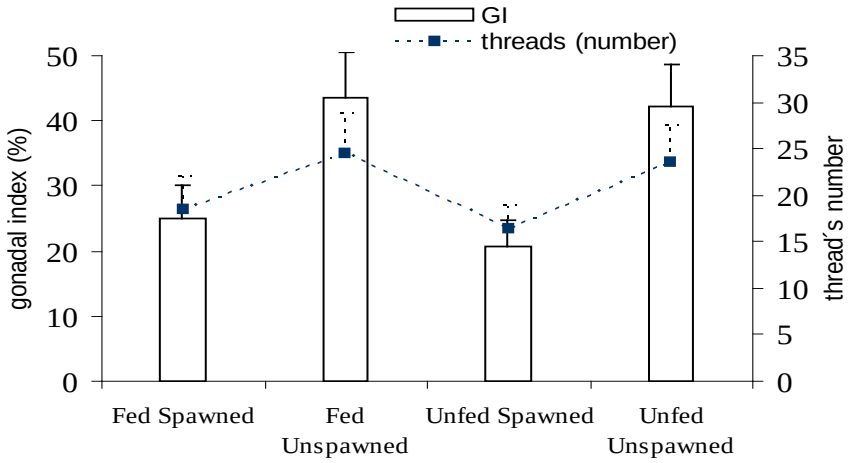
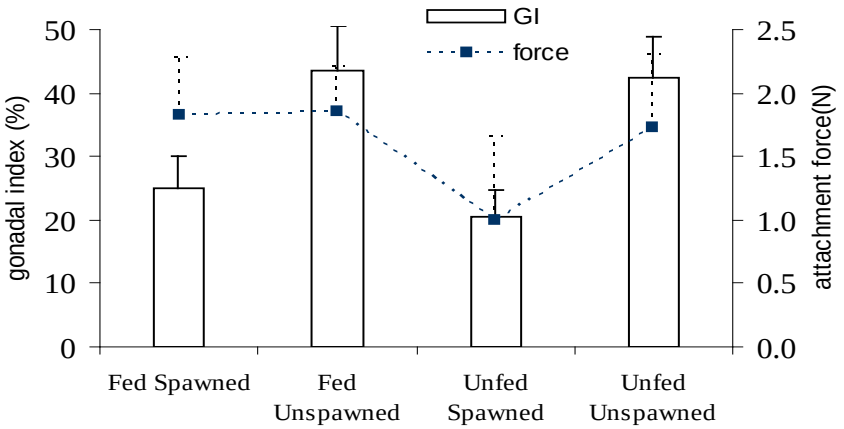


Figure 3

A


GI
threads (number)
gonadal index (%)
thread´s number
50
40
30
20
10
0
35
30
25
20
15
10
5
0
Fed Spawned
Fed Unspawned
Unfed Spawned
Unfed Unspawned

B


GI
force
gonadal index (%)
attachment force(N)
50
40
30
20
10
0
2.5
2.0
1.5
1.0
0.5
0.0
Fed Spawned
Fed Unspawned
Unfed Spawned
Unfed Unspawned

C

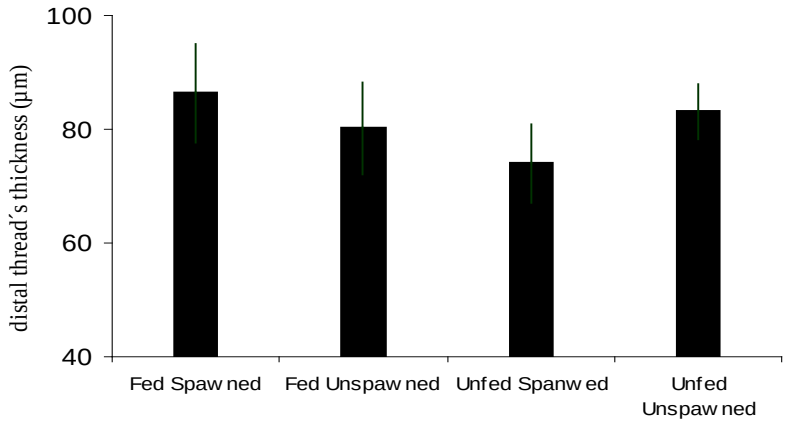

distal thread´s thickness (µm)
100
80
60
40
Fed Spaw ned
Fed Unspaw ned
Unfed Spanw ed
Unfed Unspaw ned

Figure 4