

Université de Montréal

Développement du squelette du crinoïde *Florometra serratissima* et évolution des protéines de la matrice de spicules chez les ambulacraires

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Résumé

Les crinoïdes sont bien connus pour leurs fossiles, mais la biominéralisation de leurs stades larvaires n'est que peu documentée. La première partie du projet présente le développement des ossicules des trois stades larvaires du comatule *Florometra serratissima* : doliolaria, cystidienne et pentacrinoïde. Les ossicules du crinoïde démontraient de la plasticité phénotypique et de la désynchronisation dans leur développement. Les crinoïdes étant la classe basale des échinodermes modernes, ceci porte à croire que ces traits étaient aussi caractéristiques des échinodermes ancestraux et auraient joué un rôle dans la radiation hâtive et la grande disparité des échinodermes. Pour notre deuxième étude, comme les patrons de morphologie des crinoïdes et des autres échinodermes sont nombreux et sont régulés par des protéines spécifiques, nous avons vérifié la présence de quatre familles de protéines de la matrice de spicules (SMAP) connues chez les oursins dans les transcriptomes des autres échinodermes et d'autres deutérostomes. La famille des *spicules matrix* (SM) et l'anhydrase carbonique CARA7LA étaient absentes chez tout autre organisme que les oursins, les protéines spécifiques au mésenchyme (MSP130) étaient présentes en nombres différents chez tous les ambulacraires suggérant de multiples duplications et pertes, et les métalloprotéases étaient nombreuses chez chacun. Le développement des ossicules chez les échinodermes est un sujet qui a gagné en popularité au cours des dernières décennies, spécialement chez les oursins, et inclure les crinoïdes dans ce type d'étude permettra de nous renseigner sur l'origine et l'évolution des échinodermes modernes.

Mots clés : biominéralisation, comatule, crinoïde, échinoderme, ambulacraire, transcriptome, algue coralline

Abstract

While the fossil record of crinoids is widespread and largely known, biomineralization of their larval stages is poorly documented. The first part of the project focuses on the ossicle development of the three larval stages of the feather star *Florometra serratissima*: doliolaria, cystidean and pentacrinoid. The ossicles of the crinoid showed phenotypic plasticity and asynchronous development. Crinoids form the basal class of living echinoderms; this prompts one to believe that these traits were also characteristic of the ancestral echinoderms and would have played a role in the early radiation and large disparity of the modern echinoderms. For the second study, as patterns of morphology of crinoids and of other echinoderms are numerous and are regulated by specific proteins, we verified the presence of four families of spicule matrix associated proteins (SMAPs) known among sea urchins in transcriptomes of the other echinoderms and deuterostomes. The family of spicule matrix (SMs) proteins and the carbonic anhydrase CARA7LA were absent in any other organism aside from sea urchins, mesenchyme specific proteins (MSP130s) were present in varying numbers in all ambulacrarians suggesting multiple duplications and losses and matrix metalloproteases were numerous in every organisms. The development of ossicles in echinoderms is a topic that has gained popularity in the last decades, especially in sea urchins, and including crinoids in this type of study will inform us about the origin and evolution of the modern echinoderms.

Keywords: biomineralization, feather star, crinoid, echinoderm, ambulacrarian, transcriptome, coralline algae

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Chapitre 3

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Chapitre 3

Figure 3.1. Alignment of the MSP130 proteins in the echinoid *S. purpuratus* (Sp), the holothuroid *P. parvimensis* (Pp), the ophiuroid *A. filiformis* (Af), the asteroid *P. miniata* (Pm), the crinoid *F. serratissima* (Fs) and the hemichordates *Schizocardium sp* (Ssp) and *S. kowalevskii* (Sk). If a gap was present in the same position in six or more of the twenty one sequences, it was removed from the alignment.

Figure 3.2. Phylogenetic relationships and the presentation of the MSP130 proteins in the echinoid *S. purpuratus* (Sp), the holothuroid *P. parvimensis* (Pp), the ophiuroid *A. filiformis* (Af), the asteroid *P. miniata* (Pm), the crinoid *F. serratissima* (Fs) and the hemichordates *Schizocardium sp* (Ssp) and *S. kowalevskii* (Sk) shown as phylogram (**A**) and as radial (**B**). The number at each node represents the bootstrap of 1000 replicates as a percentage.

Figure 3.3. Alignment of the MMPs proteins in the echinoid *S. purpuratus* (Sp), the holothuroid *P. parvimensis* (Pp), the ophiuroid *A. filiformis* (Af), the asteroid *P. miniata* (Pm),

the crinoid *F. serratissima* (Fs), the hemichordates *Schizocardium sp* (Ssp) and *S. kowalevskii* (Sk). and the tunicate *O. dioica* (Od). Only the first sequence obtained with each four echinoid MMP proteins in every transcriptome are part of this alignment, which were sometimes the same for different queries. If a gap was present in the same position in six or more of the sixteen sequences, it was removed from the alignment.

Figure 3.4. Phylogenetic relationships and the presentation of the MMPs proteins in the echinoid *S. purpuratus* (Sp), the holothuroid *P. parvimensis* (Pp), the ophiuroid *A. filiformis* (Af), the asteroid *P. miniata* (Pm), the crinoid *F. serratissima* (Fs), the hemichordates *Schizocardium sp* (Ssp) and *S. kowalevskii* (Sk). and the tunicate *O. dioica* (Od). shown as phylogram (A) and as radial (B). Only the first sequence obtained with each four echinoid MMP proteins in every transcriptome are part of these trees. The number at each node represents the bootstrap of 1000 replicates as a percentage.

Liste des sigles et abréviations

a.a. :	amino acid
<i>A. bifida</i> :	<i>Antedon bifida</i>
<i>A. filiformis</i> :	<i>Amphiura filiformis</i>
<i>A. mediterranea</i> :	<i>Antedon mediterranea</i>
<i>A. wilsoni</i> :	<i>Aporometra wilsoni</i>
ab :	apical blastema
ad :	attachment disk
an :	anal plate
ant :	anterior
ax :	axillary ossicle
As :	arsenic
BLAST :	basic local alignment search tool
blastp :	protein databases searched using a protein query
BMSC :	Bamfield Marine Sciences Centre
b :	basal plate
bp :	basal plate
br :	brachial ossicle
c :	columnar ossicle
C :	carbon
Ca :	calcium
CaCO ₃ :	calcium carbonate
ci :	cirri
cm :	centimeter
cos :	costal ossicle
cr :	concave region
CTL :	C-type lectin
CTLLD :	C-type lectin-like domain
d :	day

dor :	dorsal region
EDS :	energy dispersive spectroscopy
EST :	expressed sequence tag
<i>F. serratissima</i> :	<i>Florometra serratissima</i>
g :	gram
GRN :	gene regulatory network
h :	hour
i :	infrabasal plate
kV :	kilovolt
L :	liter
m :	meter
<i>M. rotundus</i> :	<i>Metacrinus rotundus</i>
Ma :	million years ago
Mg :	magnesium
MgCO ₃ :	magnesium carbonate
mm :	millimeter
mmol :	millimole
MMP :	matrix metalloprotease
mol :	mole
mRNA :	messenger ribonucleic acid
MSP130 :	mesenchyme specific protein 130
mW :	milliwatt
n :	number
Na :	nitrogen
NCBI :	National Center for Biotechnology Information
o :	oral plate
O :	oxygen
<i>O. dioica</i> :	<i>Oikopleura dioica</i>
<i>O. japonicus</i> :	<i>Oxycomanthus japonicus</i>
op :	oral plate
<i>P. miniata</i> :	<i>Patiria miniata</i>

<i>P. parvimensis</i> :	<i>Parastichopus parvimensis</i>
pi :	pinnule
pi o :	pinnule ossicle
PMC :	primary mesenchyme cell
post :	posterior
pr :	proximal
psu :	practical salinity unit
r :	radial plate
<i>S. kowalevskii</i> :	<i>Saccoglossus kowalevskii</i>
<i>S. purpuratus</i> :	<i>Strongylocentrotus purpuratus</i>
SCUBA :	self contained underwater breathing apparatus
SEM :	scanning electron microscopy
SM :	spicule matrix
SMAP :	spicule matrix associated protein
<i>Ssp</i> :	<i>Schizocardium sp.</i>
tblastn :	translated nucleotide databases searched using a protein query
tf :	tube feet
tf o :	tube feet ossicle
ven :	ventral region
wt% :	weight percent
% :	percent
°C :	Celsius degree
µm :	micrometer

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Je tiens à remercier plus que tout mon directeur de recherche, le professeur Christopher Cameron, pour m'avoir donné la chance de réaliser ce projet et d'explorer la recherche du monde marin. Merci pour son soutien, pour sa confiance et pour m'avoir généreusement transmis sa passion pour la biologie développementale et l'évolution. Merci au professeur Cory Bishop, St. Francis-Xavier University pour avoir accepté de me diriger pour un stage dans son laboratoire, professeure Paola Oliveri et David Dylus, University College London, pour le travail collaboratif, Professeur Chris Lowe, Judith Levine et Paul Gonzalez, Stanford University pour la réalisation et le partage des transcriptomes, Kevin Learning, Simon Fraser University pour sa contribution aux tests de fixation des larves aux différents substrats, Siobhan Gray, officière de plongée et de la sécurité au Bamfield Marine Sciences Centre (BMSC) et Ross Whippo, son assistant pour les collectes de crinoïdes et pour m'avoir initiée à la plongée scientifique, docteur Eric Clelland, coordinateur de recherche du BMSC et George Robertson, technicien de recherche à St. Francis-Xavier University pour leur assistance en laboratoire, Marie-Line Fiola pour avoir contribué aux manipulations en laboratoire au BMSC, Maureen Vo, Karma Nanglu et Lina Kabbadj pour avoir été de plaisants camarades de laboratoire, ma famille et mes amis pour leur support perpétuel et sans oublier, merci aux merveilleux crinoïdes pour être aussi adorables et remplis de mystères.

Chapitre 1. Introduction

La capacité de biominéralisation d'innombrables organismes distincts leur ont permis d'être fossilisés depuis plus de 800 millions d'années (Cohen et al., 2011; Macdonald et al., 2010). L'étude des fossiles nous permet de retracer l'histoire de l'évolution, entre et parmi les différents taxons des êtres vivants. Les crinoïdes sont célèbres pour leur grande quantité de spécimens fossilisés (Mooi, 2001) et leur phylogénie est principalement construite par rapport à la configuration de leurs plaques thécales (Ausich, 1996; Moore and Teichert, 1978; Simms, 1993; Ubaghs, 1969). Par contre, les stades larvaires des crinoïdes ne sont que très peu documentés comparativement à leur représentants fossilisés et aux autres échinodermes vivants (Clark, 1921; Lahaye and Jangoux, 1987; Mortensen, 1920b; Shibata et al., 2008). Les oursins représentent un modèle important en biologie développementale et moléculaire, essentiellement parce que les gamètes adultes sont matures en tout temps, leur développement est régulier et leur larve planctonique pluteus est transparente, ce qui est commode pour l'observation. L'oursin pourpre *Strongylocentrotus purpuratus* a son génome complet séquencé et il est souvent utilisé pour représenter les échinodermes en général. Les avancées moléculaires sur l'espèce sont rapides. Le réseau de régulation des gènes qui dirige la formation du squelette dans l'embryon est même déchiffré (Oliveri et al., 2008). Par contre, les échinodermes forment un phylum comportant des organismes qui diffèrent grandement les uns des autres, principalement par leur morphologie générale. On connaît très peu la génétique des concombres de mer, des ophiures, des étoiles de mer et des crinoïdes, les quatre autres classes d'échinodermes. Puisqu'ils constituent la classe basale des échinodermes modernes, l'étude du développement des crinoïdes a le potentiel de compléter les données paléontologiques et moléculaires et de mettre en lumière l'évolution du squelette chez les échinodermes. Le développement des crinoïdes modernes est peu étudié parce qu'il pose plusieurs difficultés. Contrairement aux oursins, le cycle de reproduction de la plupart des crinoïdes est mal compris et probablement irrégulier, et leur larve planctonique est opaque, ce qui rend l'observation des structures plus ardue. Dans le bras de mer vis-à-vis le Bamfield Marine Sciences Centre (BMSC), Barkley Sound, CB, une population de crinoïdes de l'espèce *Florometra serratissima* se trouve à 30 mètres de profondeur. Nous avons donc rapporté quelques adultes dans le laboratoire du BMSC afin de cultiver des larves et étudier le développement du squelette aux trois stades larvaires de *F. serratissima*. Des embryons et des larves ont aussi été rapportés précédemment au Hopkins Marine Lab, Université de Stanford,

afin que les membres du laboratoire en extraient les transcriptomes. Ils nous ont partagé les transcriptomes des crinoïdes, des autres échinodermes et d'un hémichordé, ce qui nous a permis de chercher des séquences homologues aux gènes impliquées dans la biominéralisation des oursins, chez les autres ambulacraires.

L'espèce *F. serratissima* (Clark, 1907) est un crinoïde sessile qui ne vit que sur la côte ouest de l'Amérique de Nord, entre l'Alaska et la Californie, à des profondeurs variant entre 11 et 1 252 m (Clark and Clark, 1967). Plusieurs études sur *F. serratissima* ont été entreprises à partir de la population du bras de mer du Bamfield Marine Sciences Centre (BMSC), Barkley Sound, CB, Canada, depuis les années 80 (Byrne and Fontaine, 1981, 1983; Chia et al., 1986; Holland and Grimmer, 1981a; McEdward et al., 1988; Mladenov, 1983, 1986; Mladenov and Chia, 1983; Shaw and Fontaine, 1990; Telford et al., 2014). L'animal a aussi été recueilli à d'autres endroits dans la région de l'île de Vancouver, Canada (Bickell et al., 1980; Byrne and Fontaine, 1981; Mortensen, 1920b; Scouras and Smith, 2001), en Oregon, États-Unis (Puniwai, 2002) et en Californie, États-Unis (Holland and Grimmer, 1981b; Webster, 1975). Tout de même, peu de recherches ont été entreprises sur le crinoïde *F. serratissima* relativement aux autres échinodermes et une variété d'aspects de l'espèce est encore à clarifier. Jusqu'en 2001, 55% des articles scientifiques ayant comme sujet les échinodermes excluant les fossiles portaient sur les oursins et moins de 7% sur les crinoïdes (Mooi, 2001).

1.1 Phylogénie

Les échinodermes et les hémichordés sont des embranchements frères et forment ensemble le taxon des ambulacraires (Swalla and Smith, 2008) (Figure 1.1). Les échinodermes ont divergé des hémichordés durant l'édiacarien, il y a environ 570 millions d'années (Erwin et al., 2011). Les plus vieux ossicules fossiles d'échinodermes dateraient de plus de 520 millions d'années (Mooi, 2001; Sprinkle and Wilbur, 2005) et ceux des hémichordés de 525 millions d'années, au cambrien inférieur (Hou et al., 2011). Les crinoïdes sont apparus lors du début de l'ordovicien il y a environ 485 millions d'années (Guensburg and Sprinkle, 2001; Hess et al., 1999; Pisani et al., 2012; Ubaghs, 1969). Les crinoïdes vivant aujourd'hui sont soit sessiles suite à la perte du pédoncule au stade juvénile (comatules) ou pédonculés à l'âge adulte (lys de

mer) (Cohen et al., 2004) et forment la classe basale des échinodermes modernes (Ruppert et al., 2004; Smith, 1997) (Figure 1.1).

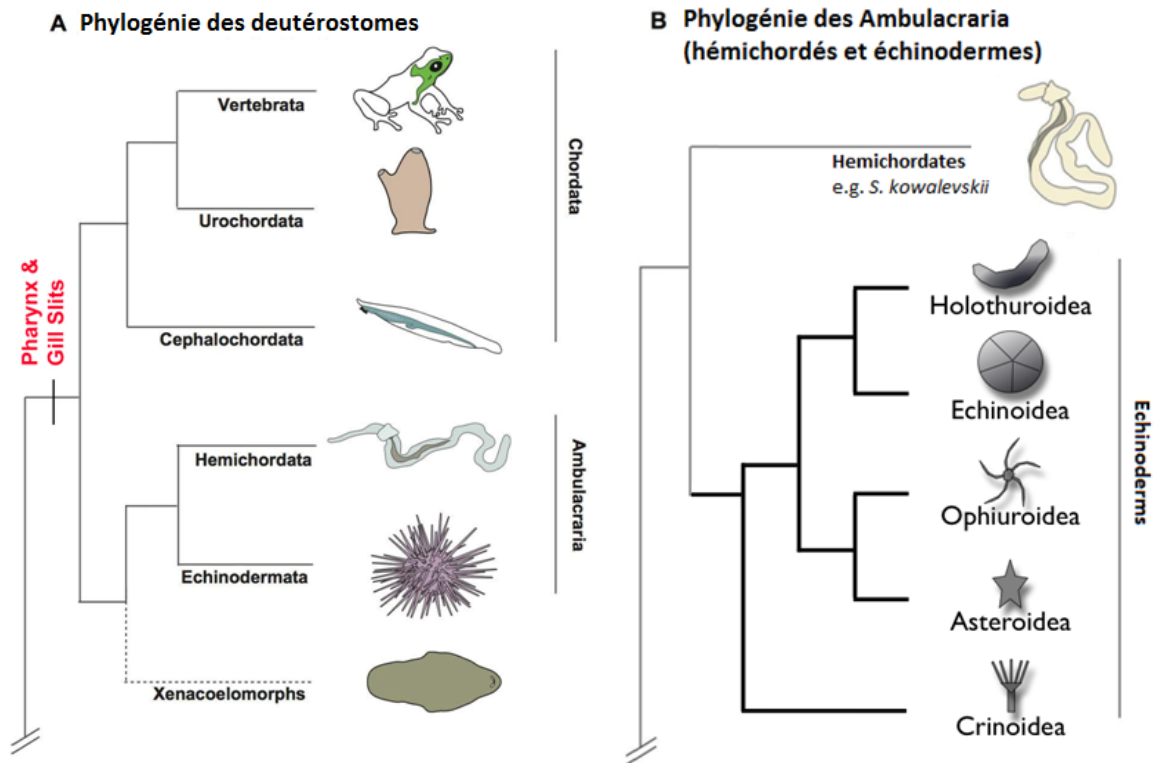


Figure 1.1. Arbres phylogénétiques A : des deutérostomes (modifié de Röttinger and Lowe, 2012) et B : des ambulacraires (modifié de Telford et al., 2014). Les échinodermes et les hémichordés forment le clade des ambulacraires. Les classes d'échinodermes vivant aujourd'hui sont les crinoïdes, les astéroïdes (étoiles de mer), les ophiuroïdes (ophiures), les holothuroïdes (concombres de mer) et les échinoïdes (oursins).

1.2 Reproduction et stades larvaires

Alors que certains crinoïdes vivent 1) en eaux peu profondes, 2) couvent leurs larves et / ou 3) possèdent un cycle de vie relativement court (Haig et al., 2012), *F. serratissima* est un comatule 1) qui est principalement retrouvé en mer profonde, 2) dont les femelles et les mâles relâchent leurs gamètes dans la colonne d'eau pour que la fertilisation y ait lieu et 3) dont le cycle de vie est relativement long et mal compris (Clark and Clark, 1967). De plus, les animaux vivant en eaux profondes sont rarement observés à relâcher leurs gamètes *in situ*, tel

est le cas de *F. serratissima*. Ces aspects rendent le développement de cette espèce une étude à défis. En fait, seulement Mladenov (1986) a publié sur le cycle de reproduction de *F. serratissima* et aucune autre révision n'a été publiée par la suite. Chez d'autres espèces de crinoïdes telles que *Comanthus japonica* et *Lamprometra klunsingeri* (Dan and Dan, 1941; Fishelson, 1968; Mladenov, 1986), les mâles relâchent leurs gamètes en premier. La présence des spermatozoïdes pourrait être nécessaire pour induire la ponte d'œufs des femelles chez *F. serratissima* aussi (Mladenov, 1986). Trois stades larvaires surviennent lors du développement des comatules : doliolaria, cystidienne et pentacrinoïde (Figure 1.2). La doliolaria (Figure 1.2.A) possède des cils et nage afin de trouver un substrat adéquat, s'y attacher et se métamorphoser en larve cystidienne (Figure 1.2.B). Cette dernière développera un pédoncule sur lequel le calice est soutenu. La cystidienne deviendra pentacrinoïde (Figure 1.2.C) lorsque le calice s'ouvrira pour laisser les ambulacres sortir et permettre à la larve de se nourrir comme suspensivore (Mladenov and Chia, 1983). La larve doliolaria de *F. serratissima* peut nager entre 4,6 et 14 jours avant de s'installer au substrat (Mladenov and Chia, 1983). Chez un grand nombre d'invertébrés marins, il y a un stade larvaire planctonique et certains nécessitent un signal spécifique pour s'installer (Bishop et al., 2006). Certaines espèces de crinoïdes s'attachent à une grande variété de substrats tels que des algues et des hydroïdes, alors que d'autres s'installent seulement sur leurs conspécifiques (Clark, 1921). En nature, le peu de pentacrinoïdes de *F. serratissima* qui ont été retrouvés étaient attachés aux

cirres des adultes de la même espèce (Mortensen, 1920b), alors qu'en laboratoire, ils s'attachent à de multiples substrats (Clark, 1921).

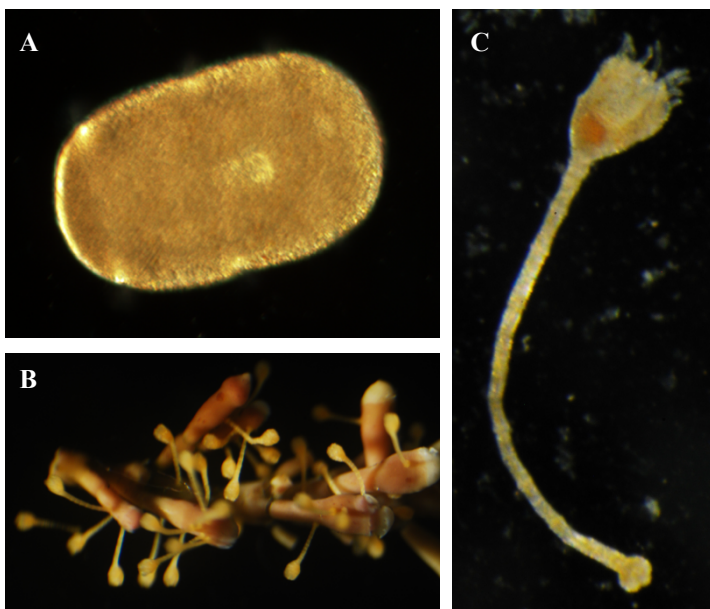


Figure 1.2. Stades larvaires du comatule *F. serratissima* : **A** : Doliolaria, **B** : cystidienne et **C** : pentacrinoïde.

1.3 Biominéralisation

Un squelette de carbonate de calcium (CaCO_3) est sécrété chez de nombreux groupes d'invertébrés à partir des éléments nécessaires qui sont disponibles dans leur environnement (Lowenstam and Weiner, 1989). Le CaCO_3 est retrouvé chez ces animaux sous trois différents polymorphes de cristallisation : aragonite, vaterite et calcite (Lowenstam, 1954). Le mécanisme influençant la production d'un squelette calcaire en aragonite, en vaterite ou en calcite n'est pas tout à fait clarifié. L'aragonite a été récemment découvert dans les biominéraux réduits des hémichordés (Cameron and Bishop, 2012). Les échinodermes forment le seul embranchement possédant un endosquelette complètement cristallisé en calcite (Lowenstam and Weiner, 1989), et ce datant au moins depuis l'hélicoplacoïde, l'un des plus vieux représentants fossiles d'échinodermes (Durham, 1993). Lors du développement larvaire de l'oursin, le CaCO_3 des spicules passe par un stade amorphe avant de devenir calcite, c'est-à-dire qu'il ne possède aucune cristallisation pendant ce stade (Beniash et al., 1997). Les premiers minéraux de la larve bipinnaria des astéroïdes est fait de calcite (Koga et al., 2014). Le polymorphe de cristallisation chez les larves de crinoïdes n'est pas connu et pourrait se modifier lors du développement des ossicules, ou encore ressembler à celui des hémichordés et être en aragonite.

Dans la composition chimique du calcite des échinodermes, le magnésium se trouve en concentration relativement élevée (entre 3 et 43,5 moles % du MgCO_3), et peut varier en fonction de facteurs génétiques, physiologiques et environnementaux, notamment la température, la salinité et le rapport de Mg à Ca dans l'eau de mer (résumé par Gorzelak et al., 2013). Lors des différentes ères géologiques, il y a eu des fluctuations de ratio Mg/Ca des océans. Les périodes avec des océans à ratio élevé en Mg/Ca sont appelées aragonites et les périodes à ratio Mg/Ca bas sont calcites. Ainsi, il est suggéré que les espèces ayant évolué récemment formeraient la plupart du temps un squelette calcaire de même minéralogie que leur ère d'apparition. Toutefois, certains passages de la calcite vers l'aragonite et *vice versa* sont survenues chez certaines espèces lors des changements du ratio Mg/Ca des océans. Jusqu'à maintenant, la manière dont la concentration de Mg est contrôlée par ces facteurs est incertaine (résumé par Gorzelak et al., 2013). Au cours de la formation de la nacre de la coquille des abalones, il y a une transition de la phase calcite à la phase plus stable, aragonite.

Il y a transfert entre des protéines solubles et glycoprotéines spécifiques au calcite à celles de l'aragonite pour que cette transition entre les deux cristaux se produise (Fritz et al., 1994; Michenfelder et al., 2003). Dans ce cas-ci, la transition entre les deux cristaux ne dépend donc pas de son environnement ou du ratio Mg/Ca dans l'eau de mer, mais de changements au niveau génétique.

1.4 Protéines impliquées dans la biominéralisation

L'endosquelette des échinodermes est nommé stétérome, il est caractérisé par une structure fenestrale (Lowenstam and Weiner, 1989) et par des cavités appelées stroma (Ruppert et al., 2004). Dans cette structure de carbonate de calcium, des protéines sont présentes afin de permettre la formation et le maintien de la structure (Benson et al., 1986; Wilt, 2005). La minéralisation des oursins est régulée par des protéines associées à la matrice de spicules (SMAP). Quatre familles de protéines sont particulièrement abondantes et se trouvent dans la carapace, la dent, les épines et les spicules de l'oursin (Mann et al., 2010). i) Les protéines *spicule matrix* (SM) et d'autres protéines à domaine lectine de type C (CTLCC) sont de loin les plus abondantes et omniprésentes des SMAP d'oursins et sont principalement impliquées dans la cristallisation. ii) Les protéines spécifiques au mésenchyme (MSP130) sont le deuxième groupe de protéines les plus abondantes et sont nécessaires à la croissance des spicules. iii) Les anhydrases carboniques sont des enzymes qui catalysent la conversion réversible du dioxyde de carbone en protons et en bicarbonate. iv) Les métalloprotéases sont impliquées dans la maturation protéolytique des précurseurs de matrice. Le rôle de chacun des membres de ces familles de gènes pour la biominéralisation a été expérimentalement vérifié chez l'oursin (Mann et al., 2010). Par contre, leur présence dans les autres classes d'échinodermes n'est pas connu, excepté quelques unes chez l'ophiure : Les SM et les MSP130 n'ont pas été identifiés dans le transcriptome du gastrula de l'espèce *Ophiocoma wendtii* (Vaughn, 2012). Chez l'hémichordé *Saccoglossus kowalevskii*, les protéines SM sont absentes, trois MSP130 sont présentes, l'anhydrase carbonique CARA7LA ainsi que des homologues aux métalloprotéases de la matrice sont présents (Cameron and Bishop, 2012).

1.5 Objectifs

L'objectif général du projet est de mettre en lumière deux aspects majeurs reliés aux crinoïdes : le développement des ossicules présents aux stades larvaires et les protéines liées à la biominéralisation des crinoïdes et autres ambulacraires. La biologie développementale et comparative sont les deux principales disciplines qui ont été utilisées lors de ce projet. Nous mettons en parallèle ces deux aspects étant donné que la formation et la morphologie des ossicules dépendent des protéines qui y sont présentes et qui régulent leur formation. Les échinodermes fossiles et modernes diffèrent grandement les uns des autres par la structure de leur squelette. Jusqu'à maintenant, les protéines qui synthétisent cette structure sont inconnues chez presque tous les échinodermes sauf chez les oursins modernes. La combinaison de différentes disciplines telles que l'ontogénie, la paléontologie et la biologie moléculaire est nécessaire afin de construire des arbres phylogénétiques robustes et comprendre l'évolution rapide au sein du phylum (Roux et al., 2013).

Le deuxième chapitre présente le premier article portant sur le développement du squelette aux stades larvaires du crinoïde *F. serratissima*. Le but est de caractériser les premières formes d'ossicules dans les larves, de déterminer leur composition chimique et leur cristallisation à des fins de comparaison avec d'autres espèces de crinoïdes et de compréhension du cycle de vie du comatule.

Le troisième chapitre présente le deuxième article et porte sur les protéines associées à la matrice de spicules (SMAP) chez les ambulacraires. L'objectif de cette étude est d'identifier des familles de protéines du SMAP de l'oursin chez les autres ambulacraires et qui par conséquent pourrait aussi avoir dans ces organismes un rôle pour la biominéralisation.

1.6 Méthodologie

Afin d'étudier le développement des ossicules aux stades larvaires de *F. serratissima*, pendant l'été 2013, nous avons recueilli des adultes crinoïdes de la population du bras de mer du Bamfield Marine Sciences Centre, Île de Vancouver, Colombie-Britannique. Cette population se trouvant à environ 30 mètres de profondeur, nous avons eu recours à la plongée sous-

marine pour recueillir les crinoïdes adultes. Les mâles et les femelles récoltés étaient gardés dans des réservoirs d'eau salée courante. Leurs gamètes étaient utilisés pour fertilisation en laboratoire afin d'élever des larves et en observer les ossicules à différents stades de développement, sous microscope optique équipé de filtres polarisées. Des larves ont été fixées afin de conserver leur squelette à un stade précis pour observation ultérieure. Le squelette de certaines larves fixées a été observé et analysé sous spectroscopie à l'Université St. Francis-Xavier, Antigonish, Nouvelle-Écosse, afin de déterminer le polymorphe de cristallisation et la composition élémentaire constituant les ossicules. Pour ce faire, l'analyse dispersive en énergie (EDS) et un microscope électronique à balayage (SEM) ont été utilisés.

La formation de ces ossicules larvaires dépend de protéines spécifiques qui jusqu'à maintenant sont inconnues chez les crinoïdes et chez la plupart des autres échinodermes. Ces protéines sont connues chez l'oursin *S. purpuratus* et font partie du squelette de l'animal (Mann et al., 2010). Elles s'appellent les protéines associées à la matrice de spicules (SMAP). Quatre familles de protéines se retrouvent à l'intérieur de toutes les parties du squelette de l'oursin (carapace, dent, épines et spicules) et elles ont une fonction dans la régulation du squelette qui a été expérimentalement vérifiée (Mann et al., 2010). Nous avons cherché ces quatre familles de protéines dans le transcriptome de chacune des autres classes d'échinodermes (crinoïde, astéroïde, ophiuroïde et holothuroïde), d'un hémichordé et dans le génome d'un tunicier avec l'outil *tblastn*. Les séquences de protéines de l'oursin ont été récupérées sur le SpBASE portal (<http://www.spbase.org/SpBase/>). Pour être considérées homologues, les séquences retrouvées dans les transcriptomes devaient atteindre le seuil de ressemblance établi, qui était de minimalement 25% identique sur la longueur d'au moins 100 acides aminés, avec un e-value inférieur à 10^{-10} , et ce en dehors des domaines conservés (Cameron and Bishop, 2012). Les alignements de séquences et les arbres phylogénétiques étaient construits à l'aide des logiciels CLC Main Workbench 7 (<http://www.clcbio.com/products/clc-main-workbench/>), Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) et Jalview 2.8 (<http://www.jalview.org/>).

1.7 Contribution des auteurs

L'article présenté au Chapitre 2 s'intitule *Skeletal development in larval stages of the feather star* *Florometra serratissima*. Les coauteurs sont Cory Bishop et Christopher Cameron. J'ai contribué à cet article en le rédigeant et en exécutant la plupart des manipulations. L'expérimentation comparant l'installation sur algue, coquille de moule, biofilm, tube de ver serpulide et rouille ont été effectuées par l'étudiant Kevin Learning de l'Université Simon Fraser pendant l'été 2011 et j'ai réalisé les manipulations avec l'algue coralline ramifiée. Christopher Cameron m'a dirigé tout au long de mes expérimentations au Bamfield Marine Sciences Centre et à l'Université de Montréal. Cory Bishop m'a dirigé lors des manipulations à l'Université de St. Francis-Xavier.

Le deuxième article est présenté au Chapitre 3 et s'intitule *Among Ambulacrarians, sea urchins have a complex, derived protein skeletal matrix*. Les coauteurs sont David Dylus, Paola Oliveri et Christopher Cameron. J'ai contribué à cet article en le rédigeant et en effectuant la partie bioinformatique, c'est-à-dire ce qui fait suite à l'obtention des transcriptomes. J'ai effectué la recherche de protéines dans les transcriptomes de chacun des organismes, à l'exception de celui de l'ophiuroïde, effectuée par David Dylus. La préparation des transcriptomes a été performée par Chris J. Lowe, Judith Levine, Paul Gonzalez (Hopkins Marine Lab, Université de Stanford), Paola Oliveri, David Dylus (University College London) et Christopher Cameron (Université de Montréal). Christopher Cameron m'a dirigé lors de mes manipulations et Paola Oliveri a dirigé David Dylus.

Chapitre 2. Skeletal development in larval stages of the feather star *Florometra serratissima*

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2.1 Abstract

Crinoids constitute the basal group of living echinoderms. They are well known for their fossils, and their adult stage skeleton has been much studied, whereas biomineralization in early larval stages is not well documented. In this work we describe ossicle development of the feather star *Florometra serratissima* at its three larval stages doliolaria, cystidean and early pentacrinoid. The earliest form of plate ossicles were tri-radiate spicules and appeared at the doliolaria stage. By the late doliolaria, three types of ossicles developed: calyx plates (5 basals and 5 orals), columnar ossicles and an attachment disk. The columnar ossicles began development as ellipsoidal spicules. We document the elaboration of these plates through the cystidean to the 56 day old early pentacrinoid stage. The oldest pentacrinoids had no radial plates. The crystal polymorph of the pentacrinoid ossicles is calcite, as found in adult crinoids and all other echinoderms. Doliolaria larvae grown in the presence of coralline algae settled in greater numbers and the development of the ossicles was accelerated. Contrary to what is reported for sea urchins, crinoid larval development is asynchronous, and highly phenotypically plastic, two evolvable traits that may have contributed to the early radiation and phenotypic disparity of echinoderms.

Keywords: feather star, biomineralization, calcite, ossicle, larva, doliolaria, cystidean, pentacrinoid.

2.2 Introduction

Florometra serratissima (Clark 1907) is an unstalked crinoid with a range from Alaska to South California, occurring at depths of 11 to 1 252 m (Clark and Clark, 1967). Among extant echinoderms, crinoids are the sister group to the remaining echinoderm classes, the Eleutherozoa (Smith, 1997) (Figure 2.1). The subclass Articulata are the only living crinoids and include the stalked sea lily forms and the comatulids (Cohen et al., 2004). The pentacrinoid stage of comatulids is stalked, reminiscent of the sea lilies, but the stalk is abandoned in the transition to the mobile juvenile stage. Morphological phylogenies have placed feather stars and sea lilies as monophyletic sister taxa. Molecular studies on the other

hand suggest that some sea lilies have evolved from a paedomorphic pentacrinoid stage of a feather star, which makes the sea lilies a polyphyletic group (Rouse et al., 2012). Relationships among the comatulids are also in contest as members of the family Antedonidae, including *Florometra* and *Antedon*, may be para- or polyphyletic (Hemery et al., 2013). Ontogenetic investigations on crinoids are rare but they certainly should provide useful information about phylogeny. Moreover, the fossils of adult crinoids are numerous but their early stages are rarely found. Of what is observed, Paleozoic crinoids seem to follow similar developmental trajectories than to the modern crinoids (Brower, 1974). Thereby, the study of the skeleton in early stages of modern crinoids is required for a better understanding of the fossil crinoid ontogeny.

During post-embryonic development, feather stars pass through three larval stages: doliolaria, cystidean and pentacrinoid. Doliolaria larvae swim with their cilia to find a substrate on which to settle and attach with an adhesive pit. *F. serratissima* doliolariae begin to settle at around 4.6 days after fertilization and settlement can be delayed up to 14 days after fertilization. During the first hour after settlement, attachment to the substrate is temporary and the larva can resettle if removed. This capacity to resettle is lost after about one hour post settlement. At this point the larva metamorphoses into the sessile non-feeding cystidean stage, it loses its cilia, the calyx rotates to an upward position and the stalk begins to elongate. About one month after settlement, the sessile cystidean becomes a pentacrinoid when it extends its tube feet from the calyx and begins to feed. Pentacrinoids of *F. serratissima* abandon the stalk to become a free moving juvenile months later (Mladenov and Chia, 1983).

2.2.1 Larval ossicle development

The endoskeletal material of adult echinoderms is called stereom. It is characterized by a fenestrated structure (Lowenstam and Weiner, 1989) and by labyrinthine cavities called stroma (Ruppert et al., 2004). Contrary to feather stars, the auricularia and doliolaria stages of sea lilies and sea cucumbers lack ossicles (Massin et al., 2000; Nakano et al., 2003). Instead, sea cucumbers possess one or more hyaline spheres also referred to as elastic balls (Hamel et al., 2001; Ito and Kitamura, 1998; Massin et al., 2000; Mortensen, 1937). The only stalked crinoid larvae observed so far are *Metacrinus rotundus* auriculariae and doliolariae and they both lack ossicles (Nakano et al., 2003). The auricularia larval stage is not present in feather

stars, only being seen in stalked crinoids, asteroids and holothuroids. The earliest spicules in larval sea urchins (echinopluteus) and brittle stars (ophiopluteus and vitellaria) are tri-radiate, with no stereom (Hendler and Meyer, 1982; Mortensen, 1921, cited by Yamashita, 1985). In bipinnaria and brachiolaria larvae of asteroids, larger spicules are shaped as plates in a mesh-like arrangement (Hamanaka et al., 2011; Koga et al., 2014) like the plates of the feather star doliolaria (Mladenov and Chia, 1983; Shibata et al., 2008). The development of ossicles in larval stages in some species of feather stars has been described and illustrated in the twenties (Clark, 1921; Mortensen, 1920b) and has been investigated more recently (Haig and Rouse, 2008; Lahaye and Jangoux, 1987; Mladenov and Chia, 1983; Shibata et al., 2008), but the shapes of plates and columnar ossicles in their earliest forms remained unknown for *F. serratissima*.

Developed ossicles are present in advanced doliolaria of *F. serratissima* (Mladenov and Chia, 1983). About 4.5 days after fertilization when the water temperature is between 9.5° to 11.5°C, ten skeletal plates are located in the posterior region, which will become the oral and basal plates in the cystidean calyx. The primary stalk projects along the dorsal side of the doliolaria, terminating at the anterior end of the larva. The attachment disk is the last ossicle of the stalk, located just under the adhesive pit, where the larva attaches to the substrate. In cystidean and pentacrinoid stages, the stalk is xenomorphic and the individual ossicles have different lengths. The stalk is differentiated into the apical, median and basal regions (Lahaye and Jangoux, 1987). The apical and basal ossicles are shorter. The larval skeleton of the feather star *Aporometra wilsoni* develops relatively early. This species is a small ovoviviparous comatulid with a life cycle of less than a year (Haig et al., 2012). Unlike *F. serratissima*, the attachment disk is already well developed in late doliolaria, before the contact with the substrate and the development of the radial plates between the basal and oral calyx plates in recently settled larvae (Haig and Rouse, 2008). In *Oxycomanthus japonicus*, infrabasal plates appear below the calyx basal plates at the cystidean stage and pentacrinoids develop five radials and an anal plate two weeks after metamorphosis, around 40 days post-fertilization (Shibata et al., 2008). In the feather star *Antedon bifida*, radial plates appear ten days after the pentacrinoid stage has begun, between 20 and 22 days post-fertilization (Lahaye and Jangoux, 1987). Cystideans of *A. bifida* and *A. wilsoni* possess homeomorphic stalk

ossicles (Haig and Rouse, 2008; Lahaye and Jangoux, 1987). Pentacrinoid larvae of all four species possess xenomorphic stalk ossicles. In *F. serratissima*, the radials and anal plate appear during pentacrinoid stage but the infrabasal never been observed in this species (Mladenov and Chia, 1983; Mortensen, 1920b).

Larval and adult skeletal ossicles develop in echinoid and ophiuroid pluteus larvae. The first set of ossicles is uniquely embryonic and larval and does not persist after metamorphosis. The adult ossicles, first appearing in the juvenile rudiment during the planktonic larval stage, do persist through metamorphosis into juvenile and adult stages (Heyland and Hodin, 2014; Yajima, 2007). In contrast, feather stars develop ossicles in the larval stages that are maintained into the adult stage. The only exceptions are the stalk and attachment disk ossicles, which are abandoned when the pentacrinoid detaches from the stalk to become a free living adult. In this study we use the term larval ossicles for all biominerals present in the larval stages. We also use spicule for the early ontogenetic stage of ossicle development, and ossicle as a later developmental stage when a stereom, with at least one complete hole has developed. As the larval skeleton of feather stars remain after metamorphosis into juveniles, their larval skeleton development might have interesting implications for the phylogeny of the class, especially because the adult thecal plates are highly used for the construction of the crinoid phylogeny (Ausich, 1996; Moore and Teichert, 1978; Simms, 1993; Ubaghs, 1969).

The terminology of thecal plate homologies of crinoids differs in the literature. Among crinoids, there are the monocyclic forms and the dicyclic forms. They differ by the arrangement of their thecal plates. In the classic terminology, the plates forming the first circlet from the top of the theca are the orals, where the arms of the crinoid are attach to. The next circlet contains the radials, followed by the basals, in both monocyclic and dicyclic forms. The dicyclic possess an additional row of plates just above the stem, the infrabasals (Moore and Teichert, 1978). Though, three other schemes for crinoid plate homologies have been used in literature. Simms (1993) argued that radial and basal plates were not homologous between monocyclic and dicyclic crinoids. He proposed an approach to name the plate according to their position relatively to the stem instead of relatively to the arms. Therefore, the dicyclic plate terminology stays the same, but in monocyclic, the plates called basals in the classic scheme are here called the infrabasals, and the radials are called basals. Disagreeing

with the approach of Simms, Ausich (1996) proposed a four plate circlet model, based on the morphology of the ancestral *Aethocrinus*, a tricyclic crinoid. Guensburg and Sprinkle (2003) proposed that the plates just above the stem are always the infrabasals, in monocyclic and in dicyclic crinoids. In dicyclic forms, the infrabasals are followed by the basals and then the radials, like in the classic scheme. In the monocyclic forms though, the infrabasals are followed by the radials. In our work, we follow the classic scheme to identify plates in larvae of *F. serratissima*, a monocyclic species (no infrabasals).

2.2.2 Crystal polymorphs and composition of calcium carbonate in invertebrates

With the exception of teeth of echinoids and the ring and anal teeth of holothurians (Donnay and Pawson, 1969), all skeletal elements of adult echinoderms are composed of single crystals of magnesium-rich calcite. However, in hemichordates, the sister group to echinoderms, ossicles are composed of aragonite (Cameron and Bishop, 2012). Magnesium is found in relatively high concentration in echinoderm calcite. It varies greatly between 3 to 43.5 mole % MgCO_3 depending on genetic, physiological and environmental factors including salinity, temperature and the ratio of Mg to Ca in seawater (summarized by Gorzelak et al., 2013). For example, in the starfish *Asterias rubens*, salinity increases linearly the Mg/Ca ratio in its skeleton, from 0.94 to 1.69 (mmol/mol)/psu (Borremans et al., 2009). In the sea urchin *Paracentrotus lividus*, the Mg/Ca ratio is positively related to temperature (from 13 °C to 24 °C, the Mg/Ca ratio increases from 0.093 to 0.119 Mg/Ca mol/mol). Contrary to starfish, the salinity does not influence the Mg/Ca ratio in sea urchins (Hermans et al., 2010). In the sea urchin *S. purpuratus*, the Mg content is lower in the tip of spines soon after the initial regeneration and it increases while the regenerating tip matures (Davies et al., 1972). The magnesium content in adult stalked crinoid ossicles varies from 1.83 to 3.55 % weight in MgCO_3 and significant variation may occur within a single ossicle (Gorzelak et al., 2013). Here we determine that the larval ossicles of *F. serratissima* are also calcite, and report on their Mg concentration.

2.2.3 Larval settlement

In most marine invertebrates the transition from a planktonic larval life to a benthic juvenile involves settlement and developmental metamorphosis. Many species settle and

metamorphose without any external signal while others require a specific cue (Bishop et al., 2006). For example, the juveniles of the sea urchin *Holopneustes purpurascens* are primarily found on a specific algal host, *Delisea pulchra*. The larvae of the species settle in response of floridoside, a soluble sugar by-product that is only produced by red algae (Williamson et al., 2000). The green sea urchin *Strongylocentrotus droebachiensis* displays no cue specificity to settle, but larger percents of larvae settle and metamorphose on coralline and non-coralline red algae (Pearce and Scheibling, 1991). While some species of comatulids attach themselves to various kind of substrate, some will only settle on cirri or pinnules of larger individuals of their respective species (Clark, 1921). The feather star *F. serratissima* has a long competent period before settlement and metamorphosis from 4.6 to 14 days after fertilization (Mladenov and Chia, 1983), where the doliolaria locates a preferential benthic substrate (Toonen and Tyre, 2007). In nature, the very few pentacrinoids of *F. serratissima* found were attached to cirri of the adults (Mortensen, 1920b).

The goals of this study are threefold; i) to characterize ossicle development in the doliolaria, cystidean and early pentacrinoid stages of the feather star *F. serratissima*, ii) to determine the calcium carbonate crystal polymorph of the ossicles, and determine its elemental composition and iii) to quantify the effect of coralline algae on larval settlement and the timing of ossicle growth.

2.3. Materials & Methods

Manipulations on living *F. serratissima* took place at the Bamfield Marine Sciences Centre (BMSC), Vancouver Island, B.C. from mid April to early July 2013. Individual *F. serratissima* were collected in 30 meters of water in Bamfield Inlet (Latitude: 48.83454395 Longitude: -125.13720095) by SCUBA and transferred in plastic bags to BMSC where they were kept in the dark in flow through seawater tables (Figure 2.2A-B). Crinoids are diecious. To determined individual's sex, a pinnule, which houses a tubular gonad, was removed from an arm and dissected out to find oocytes or sperm. To reduce the risk of natural spawning, males and females were kept in separate tables. Within an individual gonad gametogenesis is non-synchronous. Mature oocytes, which are round and non-sticky were separated from the

immature oocytes which adhere into clusters (Figure 2.2C-D) (Mladenov and Chia, 1983). Pinnules from each of the females were removed daily and checked for mature oocytes (Figure 2.2E-F). Sperm, also taken from pinnules by dissection, was in most cases mature and ready for fertilization. When mature oocytes were obtained, fertilization was conducted in small glass bowls with a minimal quantity of sperm, followed by several rinses with 50 μ m filtered seawater. Individual cultures were kept at approximately 8 °C and placed on a shallow sea table, and washed every other day. A developmental timetable of the species is provided in Table 2.1. Doliolaria larvae were reared in clean glass bowls and bowls containing glass covered with a biofilm, coralline algae, rust, mussel shells and serpulid worm tubes. After several days of growth, feeding pentacrinoids were fed a mixture of *Rhodomonas*, *Isocrysis* and *Chlorella* every other day. The oldest larvae cultured were pentacrinoids of 56 day-old after fertilization (or 30 days old as pentacrinoids, for the quickest of the culture to metamorphose). This culture contained about 200 fertilized eggs. The majority survived and reached the pentacrinoid stage.

2.3.1 Ossicle isolation and observation

Larvae were collected and fixed in cold 2 % paraformaldehyde for one hour and then washed with Millipore filtered sea water. For ossicle observation, larvae were fixed in sodium borate solution between pH 8 and pH 10 and kept in a storage buffer (5.0 g/L sodium glycerophosphate powder in 70 % ethanol). Ossicles were isolated from living or fixed doliolaria, cystidean and pentacrinoid larvae by dissolving the tissues with one drop of 6 % bleach added to the drop of water pipetted with the larva directly on slides for microscopic observation, then rinsed with distilled water. Observations were made with an Olympus FluoView™ 300 compound microscope equipped with polarizing filters and photographs were taken with a Coolsnap ProCF camera made by Roper Scientific Photometrics.

Table 2.1. Timetable of developmental events of *F. serratissima*, from fertilization to pentacrinoid, between 9.5 and 11.5 °C. All descriptions are taken from Mladenov and Chia (1983). *F. serratissima* development begins by free spawning of gametes into the water where fertilization takes place.

Age since fertilization	Stage	Characteristics
before fertilization	Ovulated egg (Unfertilized)	Pale pink, perfectly spherical, diameter of 207 ± 6 µm
0 - 3 min	Fertilization	Ridged fertilization membrane, perivitelline space
Embryonic development		
2 h - 2.25 h	First cleavage	Meridional, unilateral
2.25 h - 3.5 h	Second cleavage	Meridional, unilateral, right angle with first
3.5 h - 4.75 h	Third cleavage	Equatorial, radial, slightly unequal, 8-cell stage
4.75 h - 6 h	Fourth cleavage	Meridional, 16-cell stage, three pores
9 h	Spherical embryo	Only vegetal pore remains
12.5 h	Coeloblastula	About 200 cells, disappearance of vegetal pore
19 h		Vegetal pole of blastula flattens into vegetal plate, 1000 cells
21 h	Gastrula	Gastrulation
27 h		Cilia
Larval development		
35 h to 47 h	Pre-doliolaria	Hatch from fertilization membrane
4 d	Doliolaria	Ciliated bands, vestibular invagination, antero-ventral adhesive pit, delicate glycocalyx. Sinusoidal swim path near the water surface
4.5 d		Developed skeleton: 10 skeletal plates, primordia of the oral and basal plates of the future cystidean. Exploration of substratum
Metamorphosis		
4.6 d (possible delay of 9 d)	Cystidean	Attachment disk, loss of cilia, cuticle replaces glycocalyx, covering over the vestibular invagination, 90 degree rotation of the vestibule, stalk. Gregarious settlement, non-feeding
16 d (possible delay of 14 d)	Pentacrinoid	Extension of the 15 papillate tube feet, further stalk elongation. Feed on small food particles
4 months		10 adult arms. New ossicles: axillary, brachial, costal, radial
6 months		Arm span of 6.5 mm, cirri and pinnules not yet present

2.3.2 Settlement observations

In an effort to increase the settlement success of our larvae, so that a sufficient number of later developmental stages could be studied, five substrates were collected from the site of the adult population for settlement preference trials: i) shards of glass slides that had rested in sea water for four weeks (glass biofilm), ii) crustose coralline algae, iii) rust, iv) fragments of mussel shells and v) pieces of serpulid worm casings. Rust was taken from the wood pinches used to identify the bowls of culture. It was included in the analysis to see if fallen rust from pinches would affect larvae survival and settlement. For the rest of the study, pinches to identify cultures were new and lacked rust. One of each substrate was added to three replicate bowls, and an additional three control bowls, without substrate were prepared. At 5 days post-fertilization, 10 to 13 doliolaria larvae were transferred in each of the eighteen culture bowls. The number of settled larvae in each culture was counted at both 7 and 9 days post-fertilization. They were considered settled when they remained attached to the substrate following a light blast of water from a pipette. Settling proportions were compared using two one-way analysis of variance (ANOVA) tests, one test for day 7 and one test for day 9 after arcsine transformations of the data. The significance of the differences between treatments was determined using a Tukey post-hoc test.

After finding the greatest settlement success in the presence of coralline algae, we then compared the timing of settlement in a culture with branched coralline algae versus a culture without any substrate aside from a glass bowl. We divided a culture of embryos in two glass bowls just after fertilization. Branched coralline algae were added three days after fertilization, when the doliolariae were ciliated and swimming. Twenty-two larvae were present in the culture with algae and twenty-five larvae were in the culture without algae. Eleven days after fertilization, some larvae of the culture without algae were transferred to a bowl with algae to determine if they would settle. In other cultures, broken sea urchin tests and crustose coralline algae were added.

The diameters of doliolaria and cystidean ossicles at different ages were measured with a $\pm 5 \mu\text{m}$ precision. An ANCOVA test was conducted to see if the presence of branched coralline algae significantly affected the average ossicle diameter per larva depending on their age.

Also, five newly settled larvae were purposefully dislodged from the substrate with a sharp glass tool to see if they would grow normally without being attached.

2.3.3 Scanning electron microscopy (SEM) and electron-dispersive spectroscopy (EDS)

SEM and EDS analyses were performed on pentacrinoid ossicles at St. Francis-Xavier University. Ossicles were cleaned in 6 % bleach for two minutes on a slide until the soft tissues were dissolved. Ossicles were washed several times with distilled water, dried in 95 % ethanol, and transferred to an aluminium SEM stub. Ossicles were carbon coated using a Devon D205A sputter coater, analyzed and imaged using a JEOL JSM-6010LA SEM. EDS allowed the identification of elements present in the pentacrinoid ossicles and provided an estimate of their abundance. Twenty-six EDS analyses were performed on ten different plates, columnar ossicles and attachment disk of five pentacrinoids of 22 d old with an accelerate voltage of 10 to 20 kV, spot size of 50 to 60 and working distance of 8 to 11 mm.

2.3.4 Confocal Raman spectroscopy

Confocal Raman spectroscopy was used at St. Francis-Xavier University to identify the crystallization of CaCO_3 on different ossicles of the pentacrinoids (see Methods in Cameron and Bishop, 2012). A Renishaw InVia Raman microspectrometer attached to a Leica microscope with a 50X objective equipped with a deep depletion CCD detector, 1800 mm^{-1} , holographic notch filter, argon ion 514.5 nm laser, 28 mW output and 5 mW on sample allowed us to acquire spectra of the samples.

2.4. Results

2.4.1 Ossicles in larvae of *F. serratissima*

The ossicles of approximately twenty larvae for each three larval stages were isolated for microscopy observations. The first spicules appeared in doliolariae at an age of four days after fertilization. There were normally ten columnar ossicles in doliolariae and the number increased during the later stages. Rare cases of 9 or 11 columnar ossicles in doliolaria were

observed. After settlement, from zero to three columnar ossicles were added in cystideans and from zero to five were added in pentacrinoids. From the doliolaria to the early pentacrinoid stage, contrary to the number of columnar ossicles, the number of calyx plates did not increase: there were five basal plates and five oral plates and their appearance in an individual was synchronized with the ten first columnar ossicles. Again, some larvae had an eleventh plate ossicle as detailed below.

Plate ossicles of doliolariae and early cystideans are shown in Figure 2.3 to illustrate the steps of their development. The earliest plates observed were tri-radiate (Figure 2.3A) but some seemed to have developed in a quadra-radiate or penta-radiate way (Figure 2.3B). In doliolaria and early cystidean stages, the basal and oral plates were not distinguishable by their shapes (Figure 2.3C-D-E). Their shapes began to differentiate in advanced cystideans. Plates were located in the posterior half of the doliolaria (Figure 2.3F) and dictated the form of the cystidean calyx (Figures 2.3G & 2.4). No ossicle was identical to another and they all grew through different shapes (Figures 2.3H to Q & 2.11). In plate ossicles, the spicules elongated and eventually closed off the circular holes of the stereom (Figure 2.5A-B). They seemed to begin to grow in width at the advanced cystidean stage.

The earliest columnar stalk spicules observed were ellipsoidal (Figure 2.6A); subsequently they form a semicircle (Figure 2.6A-B) before closing into an O-shape (Figure 2.6C-E). From doliolariae to early cystideans, the columnar ossicles were wider than long. Subsequently, their lengths grew while the size of their widths stopped growing (Figure 2.6G-J). The adhesive pit, located just anterior to the apical tuft of the doliolaria was the larva attachment site, and the site of the aboral end of the cystidean stalk (Figure 2.6F), which began development in the dorsal part of the larva (Figure 2.6H). The posterior region of the doliolaria contained the plate ossicles that became the oral region of the newly settled cystidean larva. The cystidean calyx, recognized by the oral and basal plates, developed in the posterior ventral region of the doliolaria. After settlement, the calyx rotated from its position against the stalk, to an upright position on the stalk (Figure 2.6I-J). The stalk ossicles were xenomorphic at the cystidean stage as the ossicles of the median region of the stalk were longer and older than the ones of the apical region (Figure 2.4). In advanced cystideans, new ossicles were added at the base of the calyx in the apical region of the stalk (Figure 2.7).

Between ten to thirteen stalk ossicles including the attachment disk were observed in advanced cystideans.

The attachment disk was sometimes differentiated in doliolariae (Figures 2.8A & 2.12E) but it greatly developed after the larva settled (Figure 2.8B-C). It was flat on a glass bowl substrate but developed into many shapes to accommodate uneven substrates (Figure 2.8D).

Pentacrinoids appeared between 22 and 29 days post-fertilization. This feeding stage is recognized by the tube feet that project outwardly, from inside the oral plates of the calyx (Figure 2.14A). The calyx (Figure 2.9A) was composed of five oral plates (Figure 2.9B) and five basal plates (Figure 2.9C). Each oral and basal plate was imbricated in pairs towards their larger part. The oral end of the oral plate was narrowest and inwardly concave to permit closure of the oral cavity. The narrowest aspect of the basal plate was the aboral end, which interlocked in the first columnar ossicle (Figure 2.9D). Like in the cystidean stage, columnar ossicles at the terminal ends (apical and basal regions) of the stalk were shorter than those in the middle (median region) and the new ossicles developed under the calyx, at the apical region (Figure 2.9E). Interestingly, the columnar ossicles of the median region resembled adult pinnules in regeneration (Figure 2.10). The oldest pentacrinoids cultured were 56 days. At that age, no new type of ossicles appeared since doliolaria stage yet, none had developed radial plates. In pentacrinoids, between ten (22 days old) and fifteen (56 days old) stalk ossicles (including the attachment disk in the counting) were observed.

The shapes of plates and stalk ossicles inside a single larva varied greatly (Figure 2.11) as well as between two larvae of the same age and same culture (Figure 2.12). The differences in diameter of oral and basal calyx plate ossicles between doliolariae, cystideans and early pentacrinoids are shown in Figure 2.13. Average sizes of plate ossicles of cystideans tended to be wider than those of the same age in unsettled doliolariae. In a few doliolariae and cystideans, an additional small plate ossicle that seemed to be attached between the basal plate and the stalk was perceived and could be homologous to the infrabasals (Figure 2.14 B-C). Also, one pentacrinoid exceptionally possessed a sixth narrow basal plate (Figure 2.14 D-E).

2.4.2 Elemental composition and crystallization of pentacrinoid ossicles

Twenty-six EDS scans (example shown in Figure 2.14F) were performed on the 22 day old pentacrinoid ossicles (plates, columnar ossicles and attachment disk). They were composed of oxygen, carbon, calcium, magnesium and sometimes sodium and arsenic (Table 2.2). The average mass of magnesium in the ossicles was 1.55 % (SD of 0.9 %), but it ranged from 0 % to 3.05 %.

Spectra of crystallization in pentacrinoid ossicles were generated with Raman confocal spectroscopy and four major peaks appeared at 166 cm^{-1} , 289 cm^{-1} , 720 cm^{-1} and 1095 cm^{-1} (Figure 2.15). These are characteristic of the calcite polymorph of calcium carbonate (Dandeu et al., 2006).

Table 2.2. EDS analysis of 22 days old pentacrinoid ossicles (plates, columnar ossicles and attachment disk). $n_{\text{scans}} = 26$, on 10 ossicles. Quantities are reported as mass percentage.

	C	O	Na	Mg	Ca	As
average (mass %)	25.35	50.35	0.44	1.55	22.29	0.03
median (mass %)	24.29	54.71	0.00	1.83	18.06	0.00
standard deviation	9.52	11.74	0.65	0.90	17.75	0.09
number of scans with no detection of the element	1 / 26	0 / 26	16 / 26	5 / 26	3 / 26	24 / 26

2.4.3 Settlement

Doliolariae explored and sometime settled onto the substrate before any appreciable skeletal development occurred (i.e. before any stereom holes formed in calyx plates or columnar ossicles). On days 7 and 9 after fertilization, the substrate preference for settlement from high to low was algae, mussel shell, glass biofilm, serpulid worm tubes, rust and the control glass bowls (Figure 2.16). The settling proportions on day 7 and day 9 in the algae and mussel shell treatment groups were significantly higher than the control ($p=0.003$ and $p=0.004$ for algae and $p=0.010$ and $p=0.007$ for mussel shell on day 7 and day 9 respectively). The trends seen on day 7 and day 9 of the proportion of settled larvae increased for every group between the two days (Figure 2.16). Separately from these experiments, it was observed that branched

coralline algae were preferred over crustose coralline as substrates. In addition, five newly settled larvae (less than a day settled) were removed from their substrate and grew normally, despite never reattaching to the substrate. This was in sharp contrast to the control larvae that never attached. In these larvae, doliolariae lost their cilia and stopped swimming, and their later development was abnormal; the stalk elongated but the calyx did not rotate into an erect orally projecting position. These results suggest that attachment is required for normal development.

Competent doliolariae selected coralline algae as a settlement site and attached to it in aggregates (Figure 2.17). The preference for coralline algae was determined in paired cultures (Figure 2.18). On day 3 after fertilization, algae were added to one culture. On day 5, 82 % of larvae in presence of algae were settled, while only 4 % were settled in a clean glass bowl. From day 8 to day 10, 95 % larvae in the presence of algae were settled while 28 % of larvae were settled in the control. On day 10, seven larvae without algae were transferred to a bowl with algae to see if they would settle. Forty eight hours later, only one of them was not settled.

In addition to greater settlement and metamorphosis in the presence of coralline algae, ossicle development was accelerated in the presence of algae. Cystideans with algae had statistically significant larger average plate ossicle diameter than doliolariae without algae ($f = 8.240$, $p = 0.011$) (Figure 2.19). Not enough ossicles of doliolariae with algae were measured because larvae settled and metamorphosed quickly in the presence of algae. Only a small number of ossicles of cystideans without algae were measured, so it is uncertain if the larger average of plate ossicle diameter in cystidean with algae was due to the metamorphosis of the larvae or to the presence of algae.

2.5. Discussion

2.5.1 Comparison of *F. serratissima* ossicles with those of other crinoids and echinoderm larvae

In the twenties, A.H. Clark (1921) and T.H. Mortensen (1920b) described and compared some aspects of skeleton development in larvae of various species. Afterwards, only little

documentation on the subject has been undertaken. Here we compare what have been documented since their comparison on the subject. It includes the comatulids *Aporometra wilsoni* (Haig and Rouse, 2008), *Antedon bifida* (Lahaye and Jangoux, 1987), *Oxycomanthus japonicus* (Shibata et al., 2008) and *Florometra serratissima* (Mladenov and Chia, 1983). The earliest pentacrinoids we observed were 22 days old (in about 8 °C water temperature), in contrast to those of Mladenov and Chia (1983), that were 16 days old (in 9.5 to 11.5 °C water temperature). The oldest pentacrinoids observed here were 56 days post-fertilization (30 days post-metamorphose into pentacrinoid). Therefore this study complements and reproduces some of Mladenov and Chia's work, but also extends it to earlier and later ontogenetic stages (except for the 4 months old pentacrinoids studied by them). The timeline in Table 2.3 encompass what is known about the appearance of the skeletal elements in larval stages of the four comatulids mentioned above. The stalked crinoid *M. rotundus* (Nakano et al., 2003) is also included in the timeline but no biominerals were observed in its larval stages.

In *F. serratissima*, the earliest and most common shape of oral and basal plate spicules was tri-radiate. This is similar to early stages of sea urchin and brittle star larval skeletal development (Mortensen, 1921, cited by Yamashita, 1985), although some quadra-radiate and penta-radiate plates were also found. Larval stages from the doliolaria to young pentacrinoids have five basal and five oral plates. At the doliolaria stage, the shape and size of the ossicles varies within and between larvae. Similar developmental plasticity was observed between larvae of the same age. Plate ossicles were positioned in the posterior half of the doliolaria that became the anterior calyx of the cystidean. In advanced cystideans and pentacrinoids, the basal plates were attached to the first columnar ossicle on their narrow aboral side and each was attached to an oral plate at the larger oral side end, reminiscent of the pentacrinoid of *A. bifida* (Lahaye and Jangoux, 1987). The top of the oral plates touched and formed a concave shape to close the oral end of the calyx. In pentacrinoids, the oral plates opened when the podia are extended for feeding. At the 56 day old pentacrinoid stage, no radial ossicles were seen in any individual, so the timing of their appearance is unknown. However, the radials are well developed in pentacrinoids after four months, as well as the axillary, brachial and costal ossicles (Mladenov and Chia, 1983). This differs from other comatulids in which the radial

Table 2.3. Timeline of skeletal elements appearance from fertilization to pre-juvenile stage in four feather stars and one sea lily. Information is taken from the works listed under the respective species. The color code indicates the larval stage at which the first skeletal element was observed (purple for auricularia (sea lily only), blue for doliolaria, red for cystidean and green for pentacrinoid). The geometric symbols indicate the beginning of the larval stages if known (blue circle for doliolaria, red triangle for cystidean and green diamond for pentacrinoid). Two of the same larvae symbols on one line indicate its possible delay before metamorphosis into the larvae symbolized. The circle with a bar (\emptyset) indicates that no biominerals were observed. A question mark followed by an ellipsis (?...) indicates that the skeletal formation events up to this time are unknown and an ellipsis followed by a question mark (...?) indicates that later skeletal formation events are unknown. an: 1 anal (radial) plate; ad: 1 attachment disk; ax: axillary ossicles; b: 5 basal plates; br: brachial ossicles; c: columnar ossicles; ci: cirri; i: infrabasal plates; cos: costal ossicles; o: 5 oral plates; p: 1 proximal; r: 5 radial plates.

days old post-fertilization	<i>F. serratissima</i> (Current work; Mladenov and Chia, 1983)	<i>A. wilsoni</i> * (Haig and Rouse, 2008)	<i>A. bifida</i> (Clark, 1921; Lahaye and Jangoux, 1987)	<i>O. japonicus</i> (shibata et al., 2008)	<i>M. rotundus</i> (Nakano et al., 2003)
0		?...			
2		o, b, c, r, ad... only 2 to 3 days after cystidean, becomes pentacrinoid.	•o, b, c, ad Δ	o, b, c, Δ, i, ad	
4	•	br	Δ		\emptyset
6	Δ				
8		...?			
10			◇**		\emptyset
12					...?
14	Δ				
16	◇			r, an	
18					

Table 2.3 (continued). Timeline of skeletal elements appearance from fertilization to pre-juvenile stage in four feather stars and one sea lily.

days old post-fertilization	<i>F. serratissima</i> (Current work; Mladenov and Chia, 1983)	<i>A. wilsoni</i> * (Haig and Rouse, 2008)	<i>A. bifida</i> (Clark, 1921; Lahaye and Jangoux, 1987)	<i>O. japonicus</i> (shibata et al., 2008)	<i>M. rotundus</i> (Nakano et al., 2003)
20			an		
22			r		
...					
30	◇				
35			br		
40					
50			anal cone		
55					
60	...?			loss of stalk	
65					
70					
75			p		
80			ci		
...					
120	?... r, br, ax, cos		loss of stalk		

* Time is not given for *A. wilsoni*

** In Mortensen studies, pentacrinoid individuals of *A. bifida* possessed infrabasals

ossicles appear in pentacrinoids of *A. bifida* at 20 to 22 days after fertilization (Lahaye and Jangoux, 1987) and in doliolariae of the small ovoviviparous comatulid *A. wilsoni*, which has

a short life cycle (Haig and Rouse, 2008). The number of infrabasals varies between species from zero to five (Clark, 1921). In the species *Antedon mediterranea*, between three to five infrabasals were observed in doliolariae (Clark, 1921). In *O. japonicus*, infrabasal plates appear at the cystidean stage and radials and an anal plate appears at the pentacrinoid stage (Shibata et al., 2008) (Table 2.3). The presence of the infrabasal plates in *A. bifida* is controversial. While Lahaye and Jangoux (1987) claimed that they did not find infrabasal plates in any pentacrinoid of the species, Mortensen (1920a) identified three infrabasal plates forming a small ring hidden inside the basal plates of all *A. bifida* pentacrinoids he observed. This leads one to think that this variance could depend on a genetic factor where sister larvae or even inside a population of *A. bifida* must have either all the three infrabasal plates or either none. Similarly in *F. serratissima*, in addition to the five oral and five basal plates, a small plate was found in only some doliolariae and cystideans. When found, it seemed to be positioned at the base of the basal plates, like the infrabasal plates. *A. bifida* and *F. serratissima* would be two monocyclic species that pass through a facultative dicyclic stage. As it is the row of thecal plates closest to the stalk that is normally missing in pentacrinoids of *F. serratissima*, this leads one to believe that the classic scheme of crinoid plate homologies by Moore and Teichert (1978) would be the one to follow. Some fossil specimens of the genus *Bactrocrinites*, dating from the Middle Devonian, exhibit anomalies by losing a complete basal circlet and this misled taxonomy in the past (McIntosh, 1979).

The earliest columnar spicules began development as ellipsoids. They then formed an O-shape that was wider than long in doliolariae and early cystideans. Columnar ossicles were xenomorphic at the cystidean stage, regardless of when the individuals metamorphosed from 4.6 to 14 days after fertilization. In *A. bifida*, and *A. wilsoni*, columnar ossicles are homeomorphic in cystideans and become xenomorphic in pentacrinoids (Haig and Rouse, 2008; Lahaye and Jangoux, 1987). The morphology of the cystidean stalk of *O. japonicus* has not been documented but it is xenomorphic at the pentacrinoid stage, like the three other species (Shibata et al., 2008). Doliolaria of *F. serratissima* possessed ten columnar ossicles, and more were added following metamorphosis. Including the attachment disk, from ten to thirteen stalk ossicles were present in cystidean stalks and from ten to fifteen ossicles in pentacrinoid stalks depending on individuals. In *A. bifida*, the addition of columnar ossicles

ceases at around 65 day old pentacrinoids (75 days after fertilization) and the number is limited to 17 to 24 ossicles in various individuals (Lahaye and Jangoux, 1987). Twenty one columnar ossicles form the stalk of *A. wilsoni* pentacrinoids (Haig and Rouse, 2008). In every species, the newly added columnar ossicles in cystideans and pentacrinoids likely form at the base of the calyx because these are much shorter than those in the middle section of the stalk. Likewise, sea lilies generate and regenerate the stalk under the basal plate of the calyx, and after awhile these intercalate new columnals between older ones (Breimer, 1978; Nakano et al., 2004; Simms, 1989; Webster, 1974). The intercalation procedure is commonly seen in Paleozoic crinoids (Brower, 1974; Haude, 1980; Moore et al., 1968). Some small grains between the doliolaria columnal ossicles have been observed in the comatulid *Tropiometra carinata* but it is not certain if they are part of the main columnals or if they are newly form intercalated ossicles (Mortensen, 1920b). The attachment disk in *F. serratissima* was sometimes differentiated in doliolariae and invariably well developed after the larva settled. In *A. wilsoni*, the attachment disk is well developed at doliolaria stage (Haig and Rouse, 2008).

In contrast to stalked crinoid and sea cucumber doliolariae (Massin et al., 2000; Nakano et al., 2003), ossicles are present in the doliolaria stage of feather stars. Plate ossicles of larvae in crinoids resemble the ossicles of other echinoderm larvae, as in the spicules of bipinnaria and brachiolaria larvae of the asteroid *Patiria (Asterina) pectinifera* (Hamanaka et al., 2011; Koga et al., 2014) and in the terminal arm plate of the vitellaria larva of *Ophionereis annulata*, a brittle star that does not pass through an ophiopluteus stage (Hendler, 1982). These plate ossicles also resemble the flat plates of the adult sea cucumber *Psolus chitonoides* (Emlet, 1982) and the tube foot end plates of juvenile *Holothuria scabra* (Massin et al., 2000).

Some irregularities were observed from the doliolaria to pentacrinoid stages: 1) shapes and sizes of doliolaria plates differ from each other and 2) particular types of plates do not appear at the same age among larvae, 3) the number of columnar ossicles in larvae of the same age varies, 4) the attachment disk shape conforms to the shape of the substrate, 5) some small plate ossicles were found in some larvae and 6) one pentacrinoid possessed a sixth basal plate. Other feather star species also exhibit variation in their ossicle development, like the infrabasal plates in *A. bifida* mentioned above. Also, while most late doliolariae of *A. wilsoni* possess five radial plates, some early cystideans lack these ossicles (Haig and Rouse, 2008). These

variations indicate a plasticity of ossicle development in crinoid larvae that has not been reported in sea urchins when exposed to identical environments.

The distribution of the various types of larvae in echinoderms obscures the relationships among the classes because the larval morphology patterns display high homoplasy compared to the adult morphology. Clearly, the larval morphology evolved independently of the adult morphology (Smith, 1997). However, some larval morphologies are specific to clades (Byrne, 2006) Further studies and comparisons of skeletal development are needed in crinoid larvae to see if this feature is correlated with phylogeny. Some recently discovered extinct Cambrian blastozoan echinoderms exhibited high phenotypic variability in the skeleton of their feeding appendages, in which the fundamental organization is the same as crinoid arms (Zamora and Smith, 2012). The plasticity and asynchrony exhibited in crinoid development were arguably significant during the early evolution and radiation of echinoderms and may explain the early disparity of the group.

2.5.2 Elemental composition and crystallization of pentacrinoid ossicles

Although crinoids constitute the basal group of living echinoderms, the composition of the larval ossicles has, heretofore, not been verified. Given that aragonite and vaterite can be transformed into calcite (Manoli and Dalas, 2000), that sea urchin larvae pass through an amorphous calcium carbonate stage (Beniash et al., 1997), and that hemichordates have ossicles composed of aragonite (Cameron and Bishop, 2012), we sought to determine the crystal polymorph of the crinoid larvae. Oxygen, carbon and calcium were the most abundant elements in pentacrinoid ossicles, indicating a CaCO_3 composition. The peaks of the spectra of pentacrinoid ossicles are characteristic of calcite (Dandeu et al., 2006) as in adult echinoderms. The mass percentage of magnesium varied from 0 % (5 of 26 scans) to 3.05 % with an average of 1.55 ± 0.90 %. In adult crinoids, Mg is always present and in a range from 1.83 to 3.55 % mass (Gorzalak et al., 2013). Gorzelak et al. (2013) found that magnesium content varies between individuals and within a single animal and that the Mg/Ca ratio was highest in the inner stereom. Our findings show that Mg concentration may vary within a single larval ossicle and may increase in concentration with ossicle development and skeletal growth. These variations support some authors claim that using fossils of crinoid to

reconstruct Mg/Ca paleo-variations of the seas must be done carefully (Borremans et al., 2009; Gorzelak et al., 2013).

2.5.3 Settlement

Newly settled larvae that were dislodged within one hour after settlement were unable to settle again, perhaps due to the loss of cilia (Mladenov and Chia, 1983) or the loss of ability to produce more adhesive. These larvae, if left on their sides, continued to grow and form normal cystideans. This is in sharp contrast to the larvae grown in glass bowls that never settled: these animals eventually rested on the bottom, abandoned their cilia and began stalk elongation, but the calyx did not undergo rotation. Settlement, but not an extended attachment, seems to be a requisite for normal cystidean development.

Larvae of *F. serratissima* preferentially settled on coralline algae, though larvae occasionally aggregated on glass. Larval settlement may have been in response to a chemical cue from coralline algae, or an algal-specific biofilm. The presence of coralline algae is known to be a settlement cue for larvae of various invertebrate species (summarized by Bishop et al., 2006) and in some cases may be due to bacteria living on the surface of the algae (Johnson et al., 1991). Aggregation occurs often in larval settlement and adult populations of *F. serratissima* alike (Hyman, 1955; Mladenov and Chia, 1983). The aggregation behaviour suggests that the presence of conspecifics may also play a role in site choice.

The size of ossicle plates varied in *F. serratissima* sibling larvae within the same developmental time period. Cystideans had slightly larger plates than doliolariae of the same age and growth slowed between the late cystidean and early pentacrinoid stages. Phenotypic plasticity has also been reported in echinoids when larvae are exposed to different environments. In period of high food concentration, *Paracentrotus lividus* larvae reduce the growth of the feeding apparatus and juvenile structures appear later than in larvae provided with little food (Strathmann et al., 1992). Similarly, the feeding larva of the sand dollar *Dendraster excentricus* reduce the arm spicule length and accelerate the development of the juvenile rudiment in the presence of a thyroid hormone (Heyland and Hodin, 2004). These examples contrast greatly with the non-feeding larvae of *F. serratissima* that exhibited asynchronous development and metamorphosis along with high variation of ossicle sizes

within a sibling culture in a controlled environment. This plasticity in ossicle development among a single culture of larvae has also been observed in the feather star *A. mediterranea* (formerly called *Antedon adriatica*). Additionally, larvae whose mothers are kept in aquarium exhibit smaller, thinner and less developed ossicles than the larvae fertilized by mothers living free in the sea (Clark, 1921).

The timing of ossicle development varied tremendously within a culture of sibling embryos and doliolaria ossicle development was accelerated in the presence of coralline algae. The stage of ossicle development was variable at settlement, and accelerated following settlement. Plate ossicle development proceeded in a manner that results in phenotypic variation; no plate ossicle appeared to be a precise copy of another. Most plate ossicles began development as tri-radiate spicules, but quadra- and penta-radiate spicules were also found. This suggests that the physical process of crystallization of plate formation varies, as do late stage ossicle phenotypes. This variation in ossicle shapes was particularly pronounced in the attachment disk. This disk may appear in the doliolaria, and was necessary for attachment of the cystidean. It conformed to the surface of its settlement site. Assuming the gene regulatory pathway that underlies attachment plate development is the same as that for other plates, its malleability may account for the maintenance of skeletal plasticity among ossicles in general. Asynchronous development and phenotypic plasticity are evolvable traits that likely characterized the echinoderms since their early evolution and may have accounted for the extraordinary disparity of the group.

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2.9 Figures

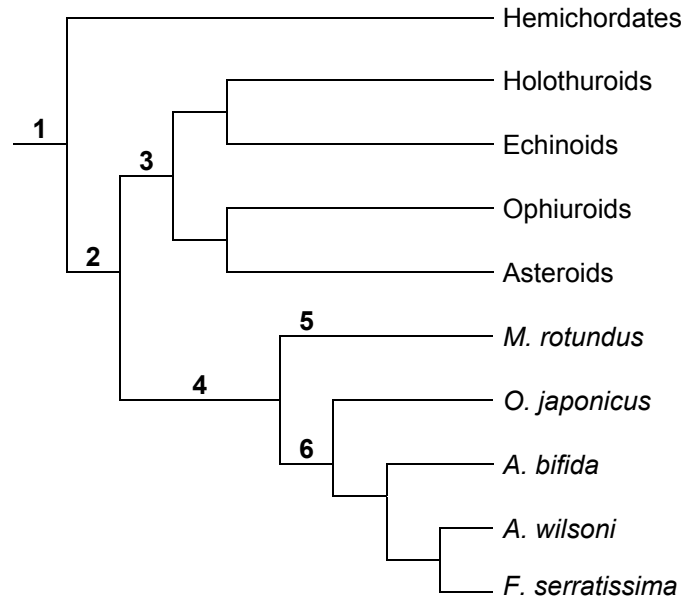


Figure 2.1. Cladogram of ambulacrarians phylogeny including example species of crinoids. **1:** Ambulacrarians; **2:** Echinoderms; **3:** Eleutherozoa (sister group of crinoids); **4:** Crinoids (Articulata are the extant crinoids); **5:** Sea lilies; **6:** Comatulids (feather stars). Phylogeny of Eleutherozoa and of crinoids is in debate. This cladogram was constructed following the works of Telford et al. (2014) and Hemery et al. (2013), two recent studies using molecular data to construct trees.

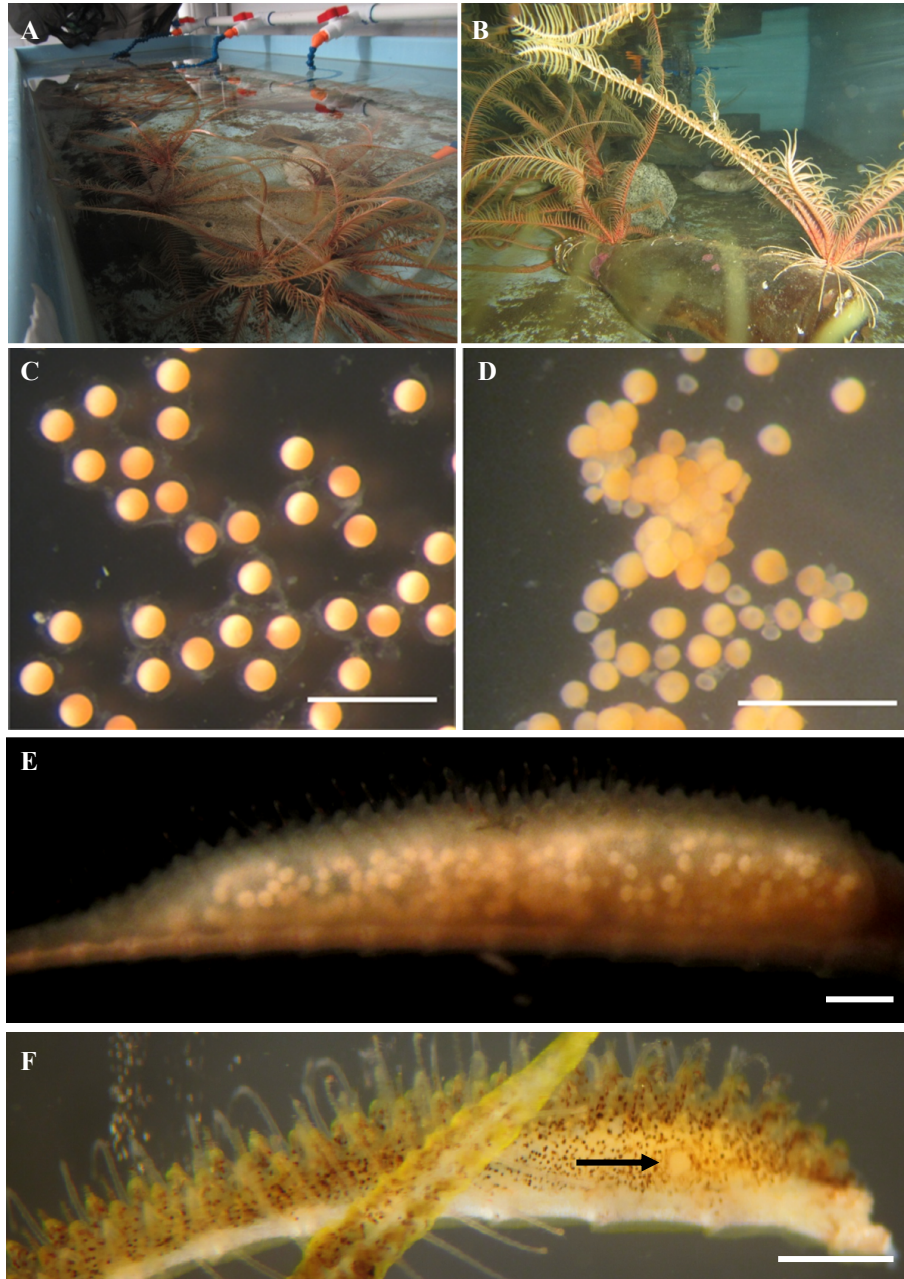


Figure 2.2. **A:** Crinoids were kept in the dark in tables with flow through seawater. **B:** Rocks and bottles taken from their habitat were added to the tables because the animals like to settle on raised objects from the bottom. **C:** Mature eggs are perfectly spherical. **D:** Immature eggs are irregularly shaped and aggregated into masses. **E:** An individual pinnule that has been dissected from an arm is showing immature eggs. **F:** *In vivo*, eggs and sperm are released by the gonopore (shown by the arrow). Scales = 800 μ m

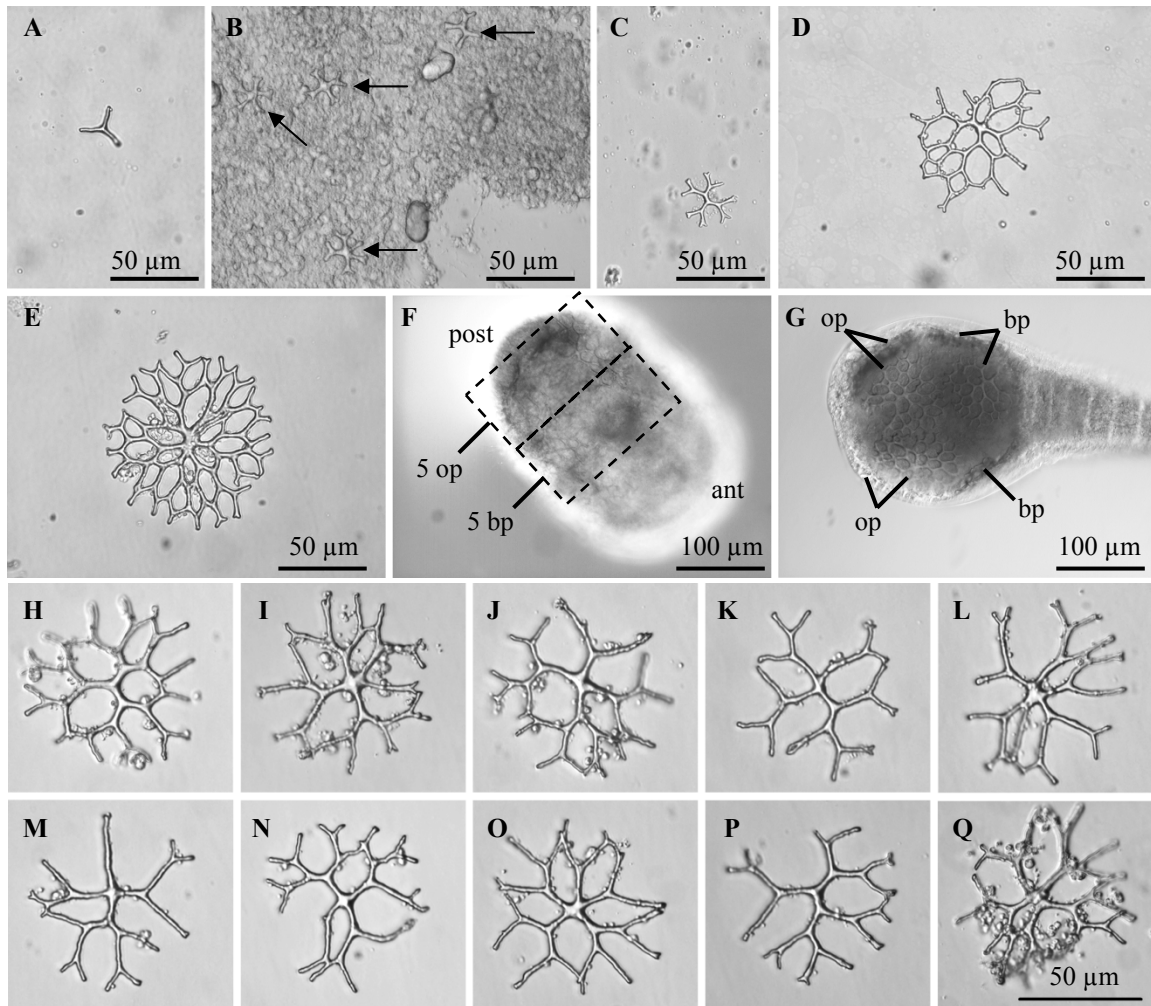


Figure 2.3. Development of plate ossicles from doliolaria to cystidean. **A:** 5d21h old doliolaria tri-radiate early plate; **B:** 4d5h old doliolaria plate; arrows point early spicules becoming plates that did not have a tri-radiate form; **C:** 7d old doliolaria plate; **D:** 10d19h old doliolaria plate; **E:** 7d7h old cystidean plate; **F:** 11d old doliolaria, the square encompasses the area where the five oral and five basal plates in the larvae are located; **G:** Photo montage of 11d2h old cystidean calyx with some visible plates. **H to Q:** Set of the ten plate ossicles from an individual 8d7h doliolaria. No plate is identical to another; basal and oral plates are not distinguished here. ant: anterior; bp: basal plate(s); op: oral plate(s); post: posterior

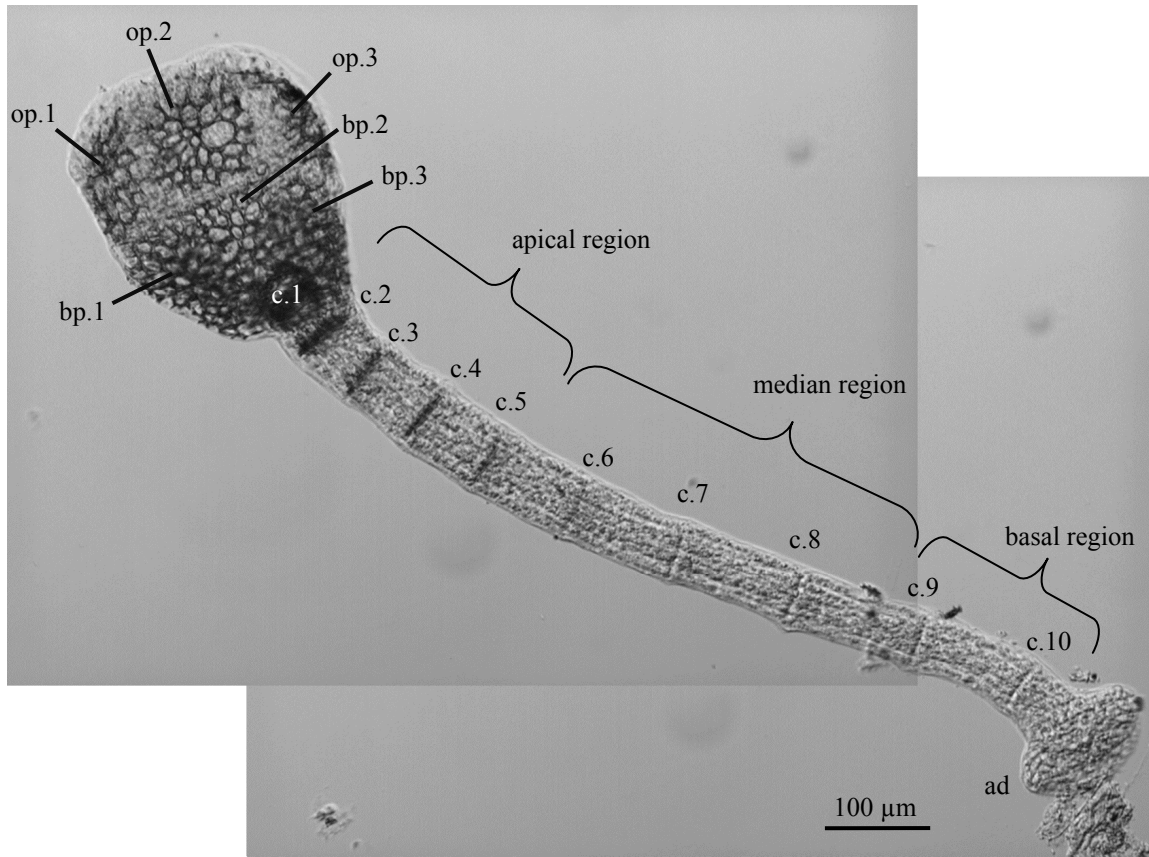


Figure 2.4. Skeletal parts of a cystidean. Some of the 5 oral and 5 basal plates are visible. The stalk is divided into three regions, apical, median and basal regions. Around 10 columnar ossicles and the attachment disk form the xenomorphic stalk of this cystidean. The attachments between them are not visible here. The line marks are bulge of the middle of each ossicle. ad: attachment disk; bp.1 to 3: basal plates; c.1 to 10; columnar ossicles of the stalk starting at the calyx; op.1 to 3: oral plates

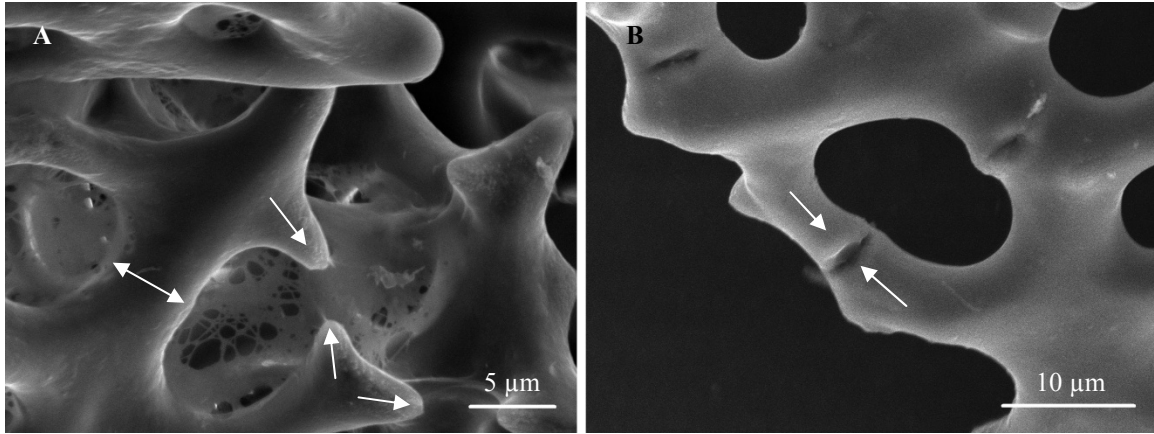


Figure 2.5. A-B: Spicule growth in two oral plate ossicles of 22 day old pentacrinoids. Arrows show the direction of the growth of ossicles when the spicules elongated to form stroma of the stereom. They also grew in width (illustrated by double arrow in **A**).

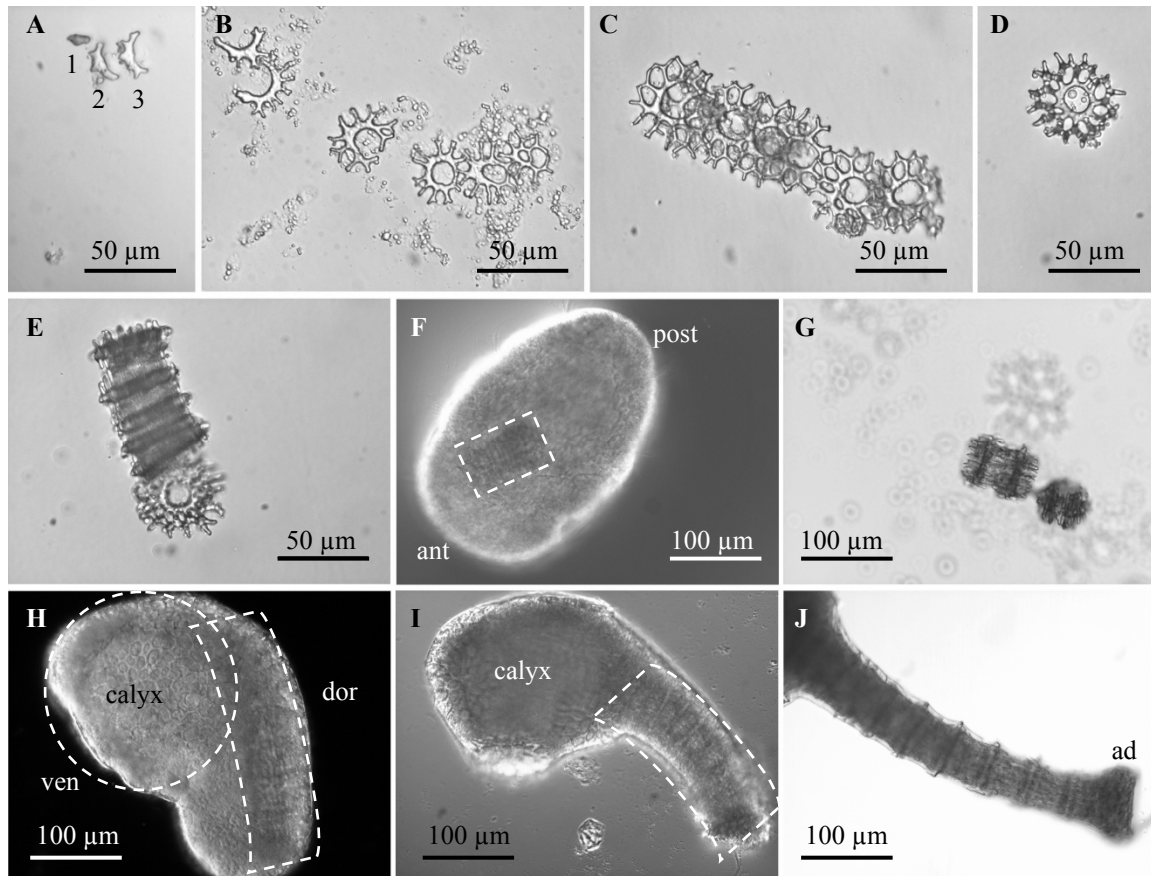


Figure 2.6. The columnar ossicles from ten larvae from different developmental time periods. **A** : 5d5h old doliolaria columnar spicules, at earliest ellipsoidal shape (**1**), semicircle shapes (**2-3**) ; **B**, **C**, **D**: 6d1h, 8d7h and 9d17h old doliolaria columnar ossicle, respectively; **E** : 9d17h old portion of a doliolaria stalk; **F** : 11d3h old doliolaria; **G**: 9d old cystidean columnar ossicles; **H**: Photo montage of 10d2h old recently settled cystidean; **I**: The calyx has rotated into a nearly upright position in this 7d7h old cystidean; **J**: 13d old cystidean stalk with xenomorphic columnar ossicles. Rectangular dash areas demarcate the stalk position in **F**, **H** and **I** and the circle dash area demarcates calyx position in **H**. ad: attachment disk; ant: anterior; dor: dorsal region; post: posterior; ven: ventral region

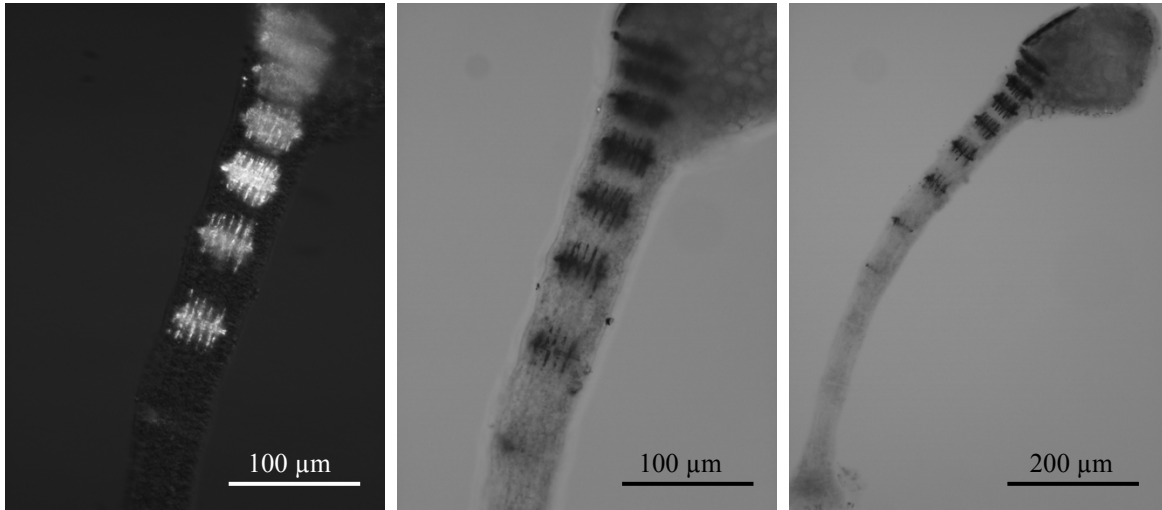


Figure 2.7. New columnar ossicles of cystidean seem to appear under the calyx and they are shorter than the ossicles in the middle of the stalk.

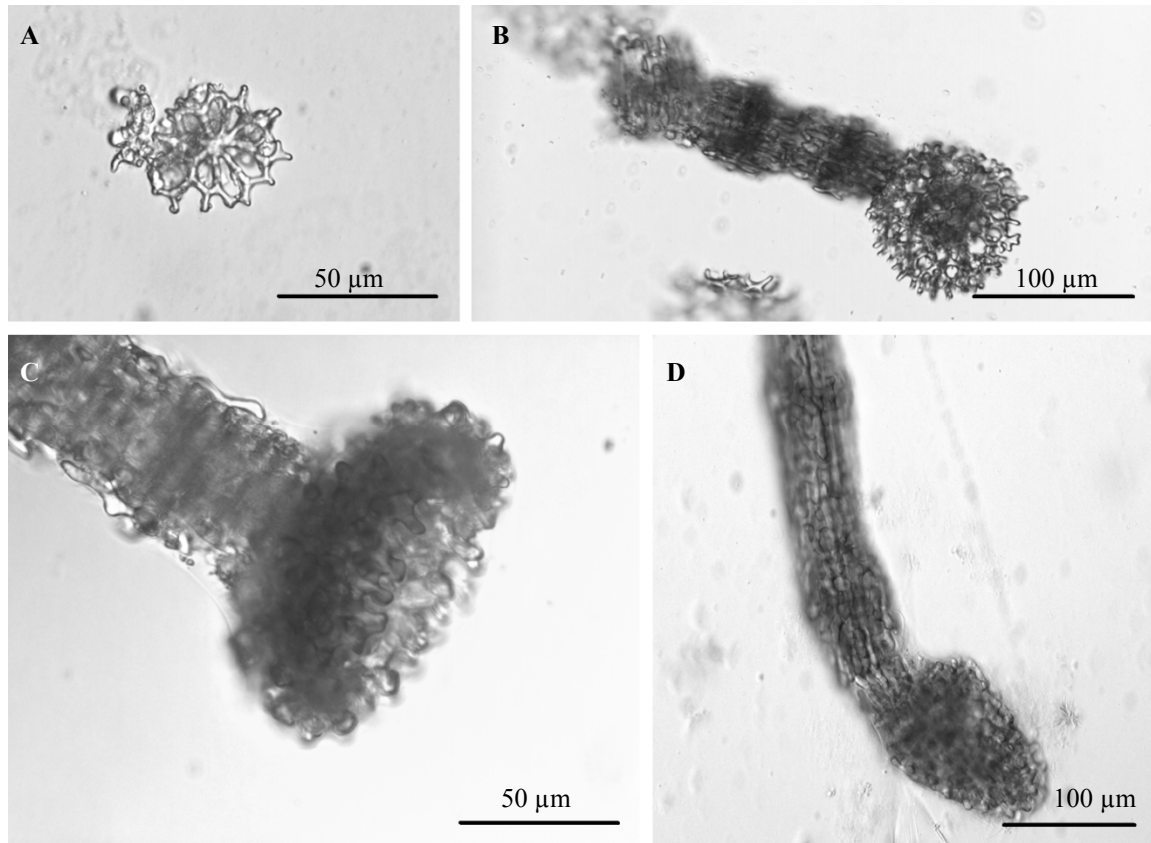


Figure 2.8. The attachment disks from four larvae from at developmental time periods. **A:** Ossicle of an 8d1h old doliolaria is an early form of the attachment disk; **B:** Attachment disk and a part of the stalk of an 11d4h old cystidean that had settled five days earlier; **C:** Photo montage of a 15d old cystidean that had settled on a flattened substrate with its attachment disk having taken a flattened enlarged disk-shape; **D:** The attachment disk of this 45d old pentacrinoid has an irregular cylindrical shape compared to the flattened shape illustrated in **B** and **C** and it is a consequence of attaching on a non-flat substrate.

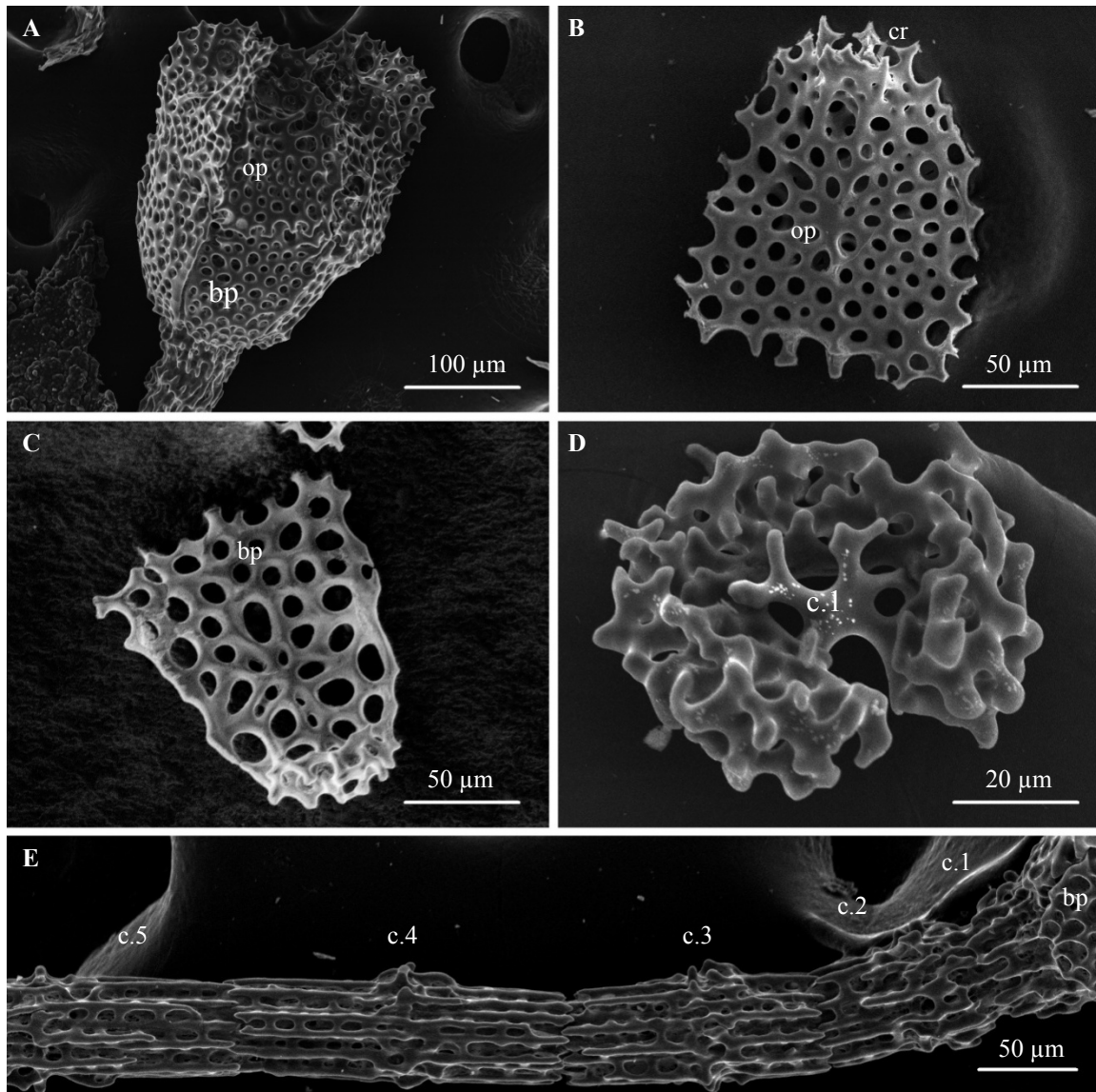


Figure 2.9. Ossicles of pentacrinoid of 22 days old. **A:** Calyx; **B:** Oral plate; **C:** Basal plate; **D:** Immediately under the calyx with newer columnar ossicle. bp: basal plate; c.1 to c.5: first five columnar ossicles of the stalk starting at the calyx; cr : concave region; op: oral plate

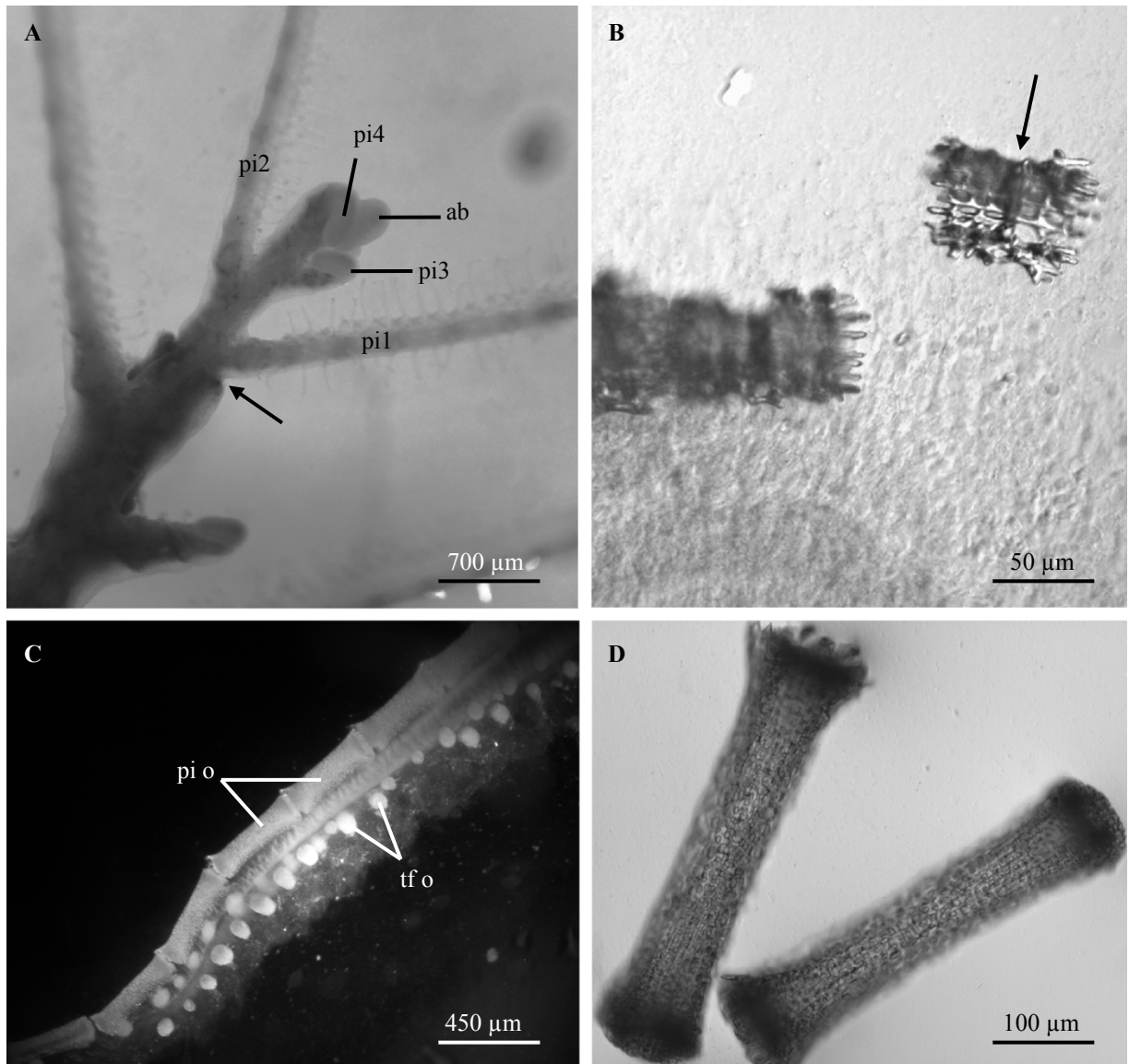


Figure 2.10. Pinnules at the tip of an arm in regeneration of an adult crinoid *F. serratissima* possessed ossicles that are reminiscent to cystideans and pentacrinoids columnar ossicles. **A:** The tip of an adult arm in regeneration possessed two new pinnules in formation (pi3 and pi4). The arrow shows the beginning of the part in regeneration. **B:** Ossicles were isolated from a pinnule in formation and like the cystideans and pentacrinoids, the middle of the columnar ossicles are marked by a bulge (indicated by the arrow). **C:** The ossicles of a newly regenerated pinnule (see **A:** pi1 and pi2) were aligned like the stalk of the pentacrinoids. **D:** Two isolated ossicles of the newly regenerated pinnule. ab: apical blastema; pi: pinnule; pi o: pinnule ossicles; tf o: tube feet ossicles

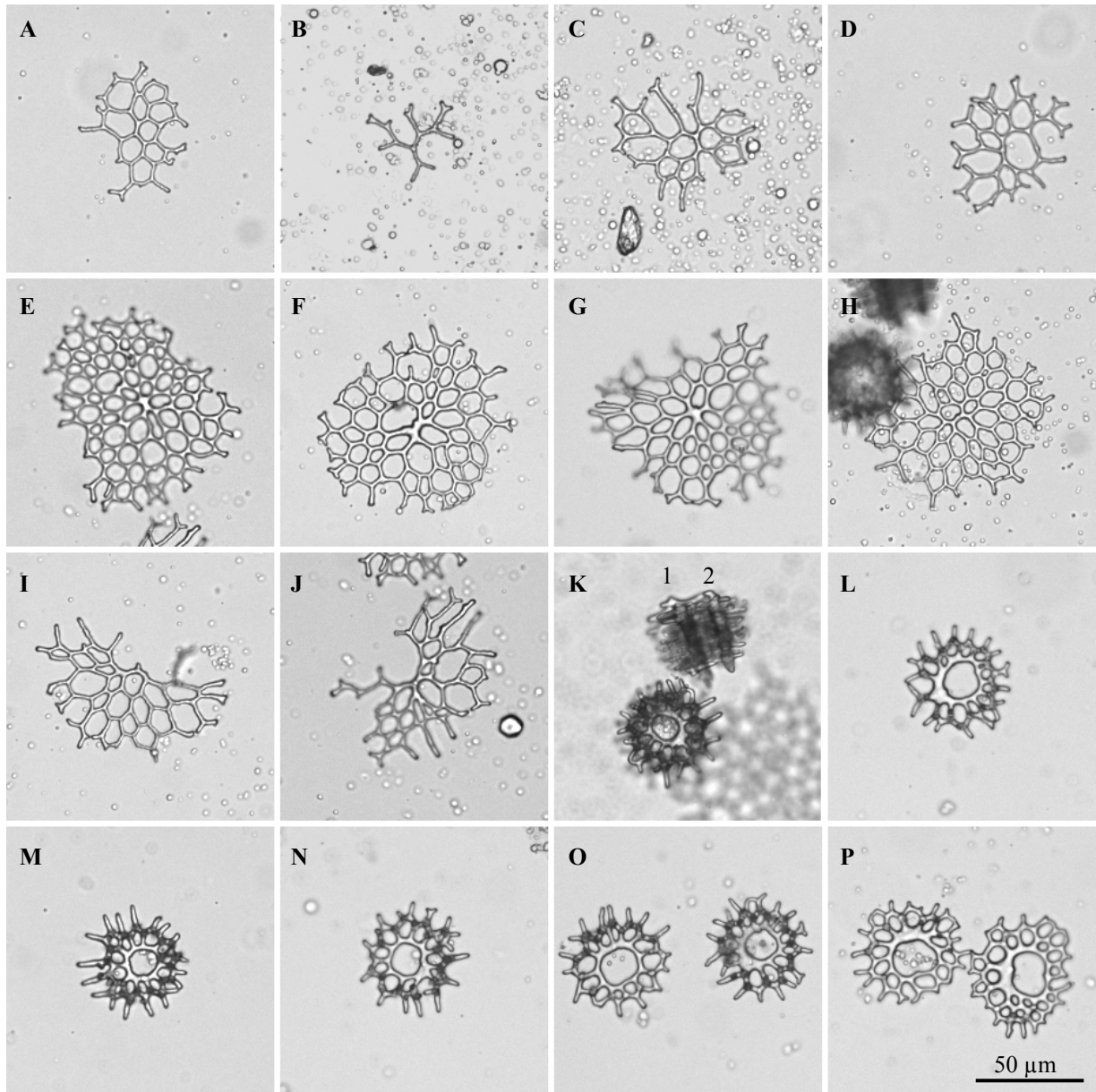


Figure 2.11. Complete skeleton of a 9d7h old doliolaria consisting of 10 plates (**A** to **J**) and 10 columnar ossicles (**K** to **P**) shows the disparity of their shapes and sizes within a single larva. The basal and oral plates were not identified here. At that stage, some plates were composed of numerous stereom holes (**E** to **H**) while other lacked of them (**B**). **K**: Two columnar ossicles were still imbricated together (**1-2**). At that stage, most of the columnar ossicles had also spicules developed perpendicularly on both sides of the circle to assure the imbrications of the stalk in addition to the basic circular shape (**K** to **O**). Other columnar ossicles lacked of the perpendicularly spicules and were probably positioned in the apical region of the stalk under the pre-calyx (**P**). This individual did not possess a recognizable attachment disk.

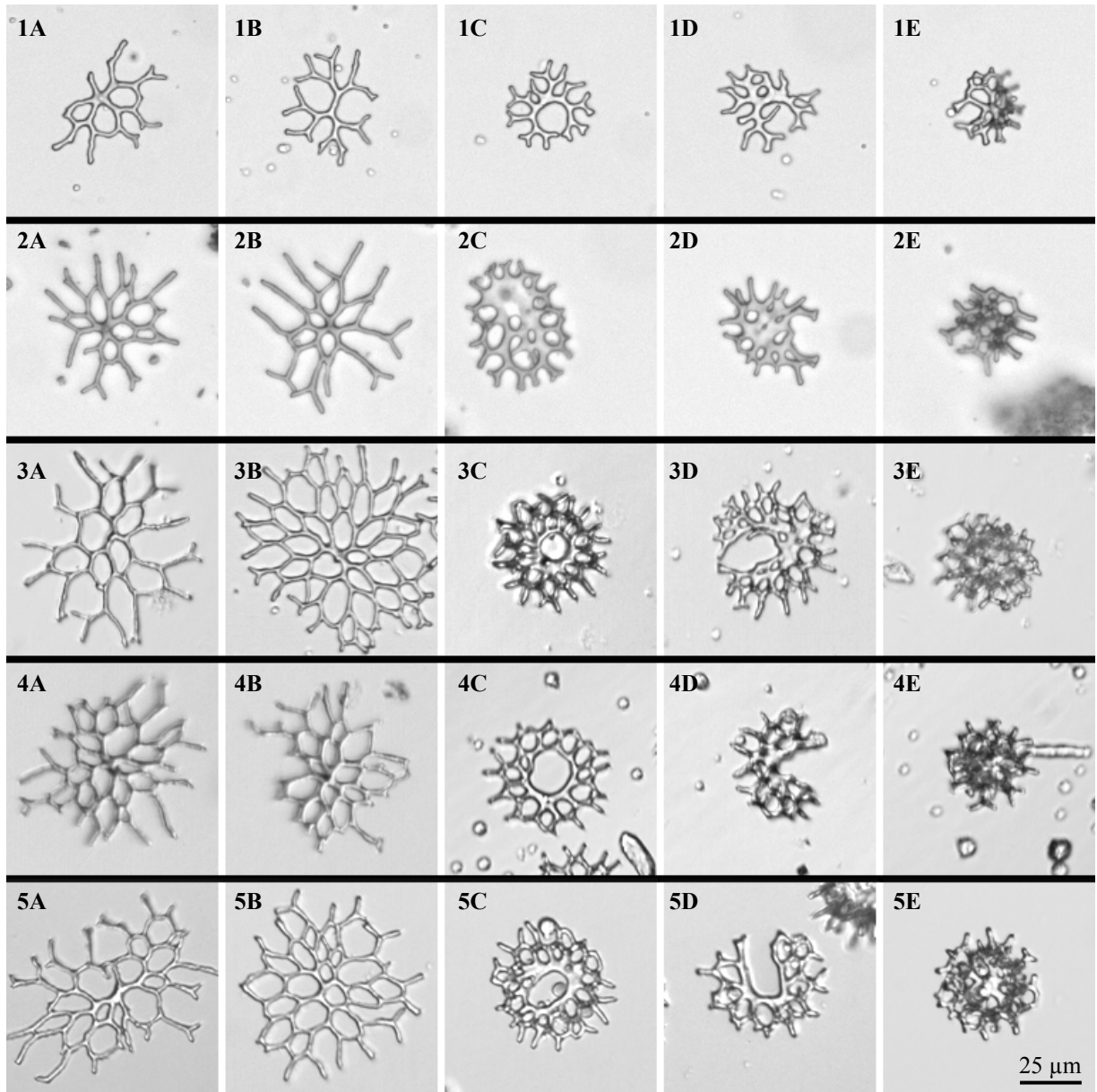


Figure 2.12. Selected ossicles of five sister doliolariae of 9d7h old show the shape disparity between and among larvae. Each row (larvae **1** to **5**) represents two plates (columns **A** and **B**), two columnar ossicles (columns **C** and **D**) and the attachment disk (column **E**) of one larva. At that stage, two patterns were retrieved several times among columnar ossicles. In a same larva, some columnar ossicles fully formed the middle stereom (**C**) while others were not completely closed (**D**). These latter were found in the apical region of the stalk under the pre-calyx. A rare pattern of columnar ossicles consists of a more oval shape with smaller stereom holes (**2C-2D**). The attachment disk (**E**) was also circular but was recognized by the absence of the middle stereom and by the dense perpendicular spicules on the top of one side. The attachment disk was not retrieved in all doliolariae of the study. The plate shapes, the developmental rates, and the diameter of the ossicles differed between larvae. The development of larva **1** seems the

slowest compared to the other larvae as its ossicles are smaller. The individual shown in Figure 2.11 was from the same culture of these larvae.

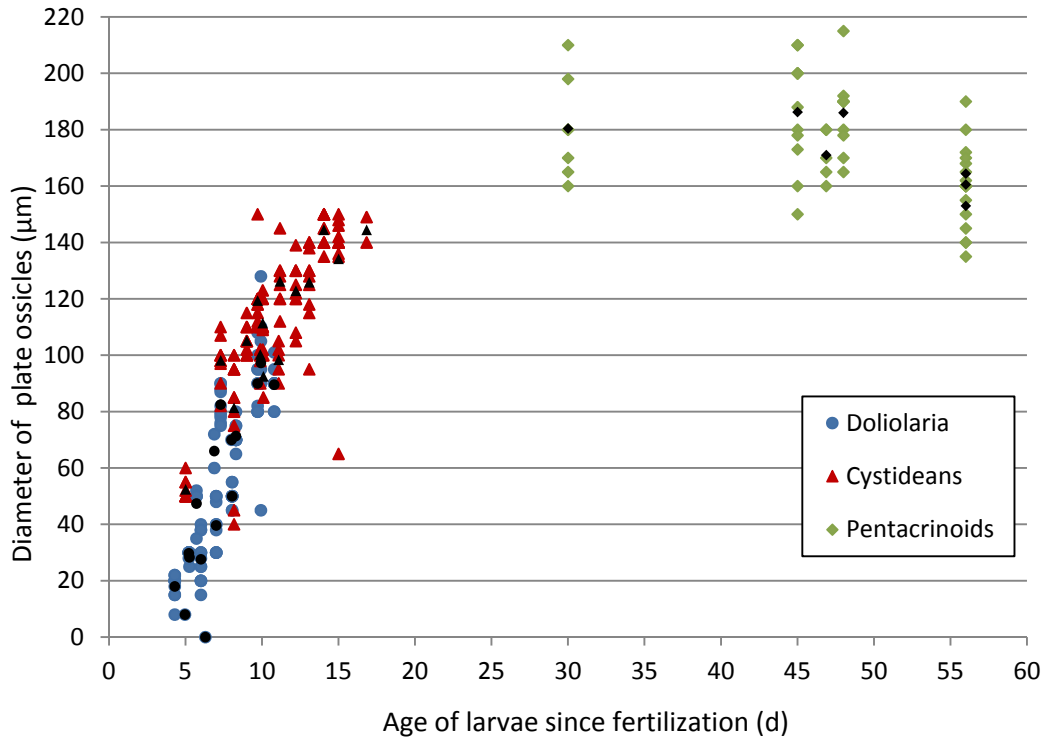


Figure 2.13. Maximum dimension of oral and basal calyx plate ossicles according to the age of doliolaria ($n_{\text{larvae}} = 16$; $n_{\text{ossicles}} = 115$), cystideans ($n_{\text{larvae}} = 15$; $n_{\text{ossicles}} = 124$) and pentacrinoids ($n_{\text{larvae}} = 7$; $n_{\text{ossicles}} = 52$) defined as the time after fertilization. Average sizes of ossicles in each larva are shown in black. Some were only spicules (biominerals without stereom holes). Some larvae grew in the presence of algae which may affect the speed of ossicle growth.

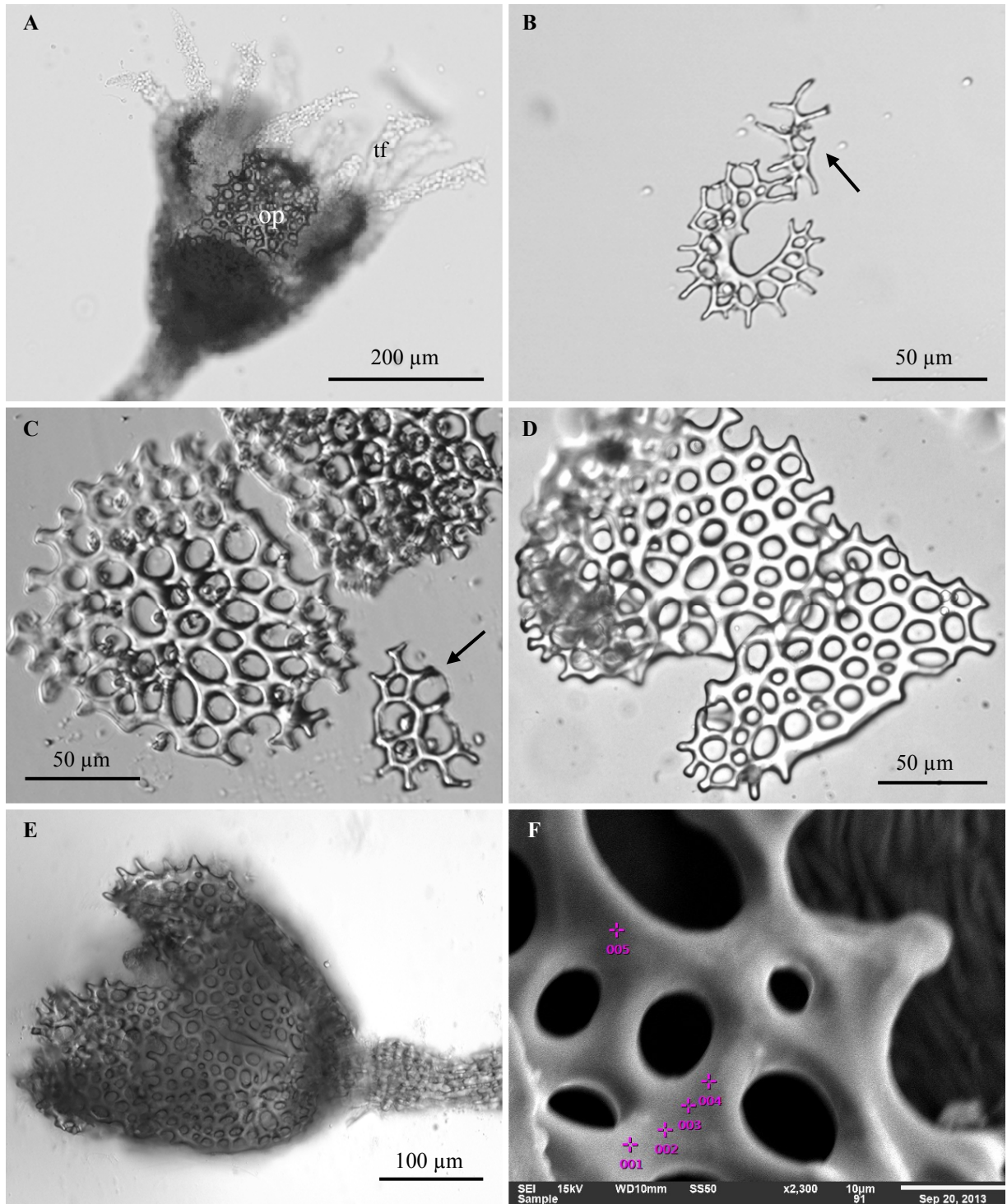


Figure 2.14. **A:** Oral plates are mobile in pentacrinoids to allow the tube feet to get out and become a suspension feeder. **B:** Additional small plate (pointed by the arrow) was found in a 9d7h old doliolaria close to a columnar ossicle. **C:** Additional small plate (pointed by the arrow) was found in a 15d old cystidean close to the basal plates. The plates shown in B and C might be homologous to the infrabasals seen in other species. **D:** A sixth thinner basal plate

was occasionally found in a 45 days old pentacrinoid. **E**: The plate was aligned with the normal basal plates (photo montage). The abnormal plate is shown within the dashed area. **F**: EDS scan with five points on a plate ossicle of a 22 days old pentacrinoid. op : oral plate; tf : tube feet

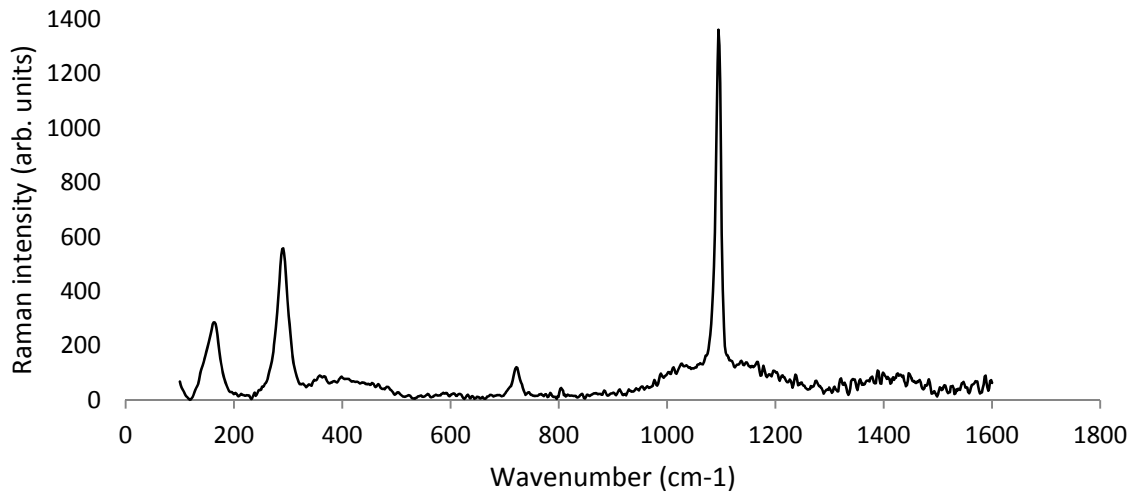


Figure 2.15. Crystal spectrum of ossicles in pentacrinoids using confocal Raman spectroscopy is characteristic of calcite.

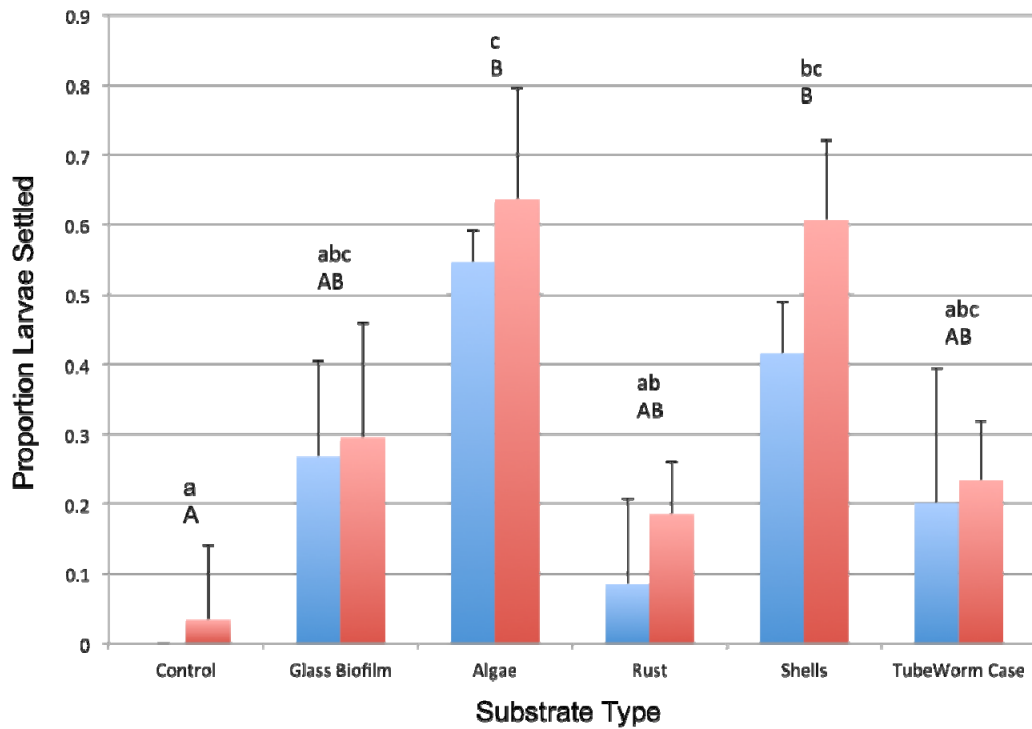


Figure 2.16. Proportion of larvae settled in the different substrate treatment groups. Blue bars illustrate proportions at day 7 post fertilization while red bars show proportions at day 9 post fertilization. Groups with corresponding letters (lower case letters correspond to day 7 and upper case letters correspond to day 9) are not significantly different from each other according to a Tukey post-hoc test.

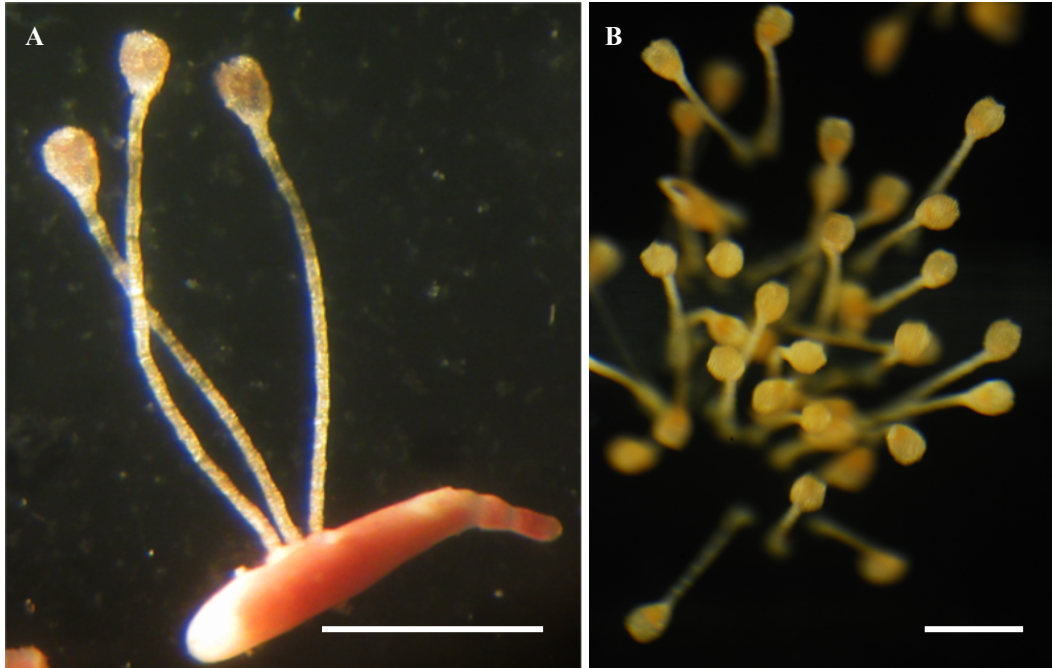


Figure 2.17. Aggregation of advanced cystideans. **A:** Three cystideans aggregated on a piece of a branched coralline alga; **B:** Many aggregated cystideans of a culture settled on the glass bowl, which was found more rarely than on algae. Scales = 1 mm

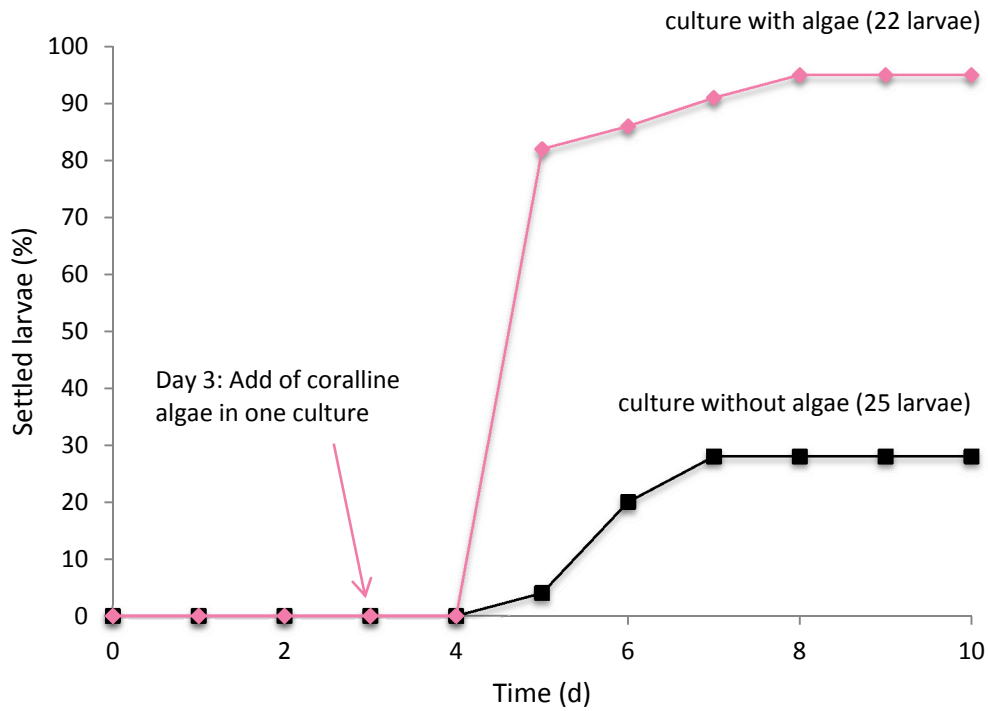


Figure 2.18. Effect of coralline algae addition on the abundance of settled larvae. A culture of 47 sister larvae was made and separated in two bowls after fertilization, 25 larvae were transferred to a first bowl and 22 larvae were transferred to a second bowl. On day 3 post-fertilization, coralline algae was added to the bowl containing the 22 larvae. On day 5 post-fertilization, only 4% of the larvae without algae was settled (one larva) while 82% of the larvae with algae was settled.

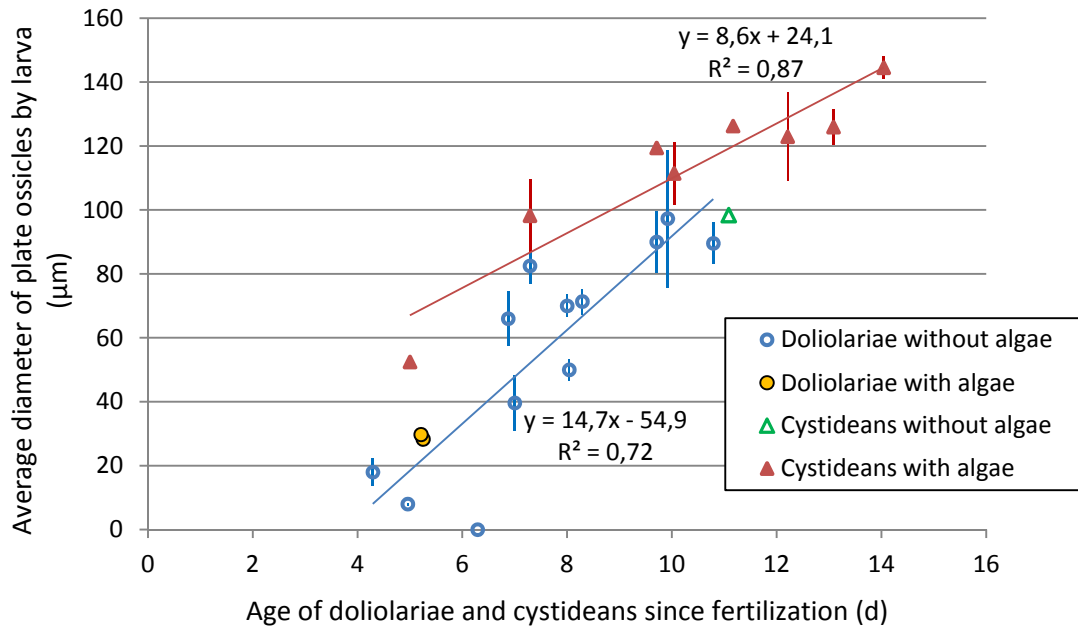


Figure 2.19. Effect of coralline algae on ossicle growth of doliolariae and cystideans. Some were only spicules (biominerals without a stereom hole). Error bars show the standard deviation. The average ossicle diameter per larva was significantly larger in cystideans with algae (red linear regression line) than the doliolariae without algae (blue linear regression line) ($F = 8.540$, $p = 0.011$).

Chapitre 3. Among Ambulacrarians, sea urchins have a complex, derived protein skeletal matrix

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3.1 Abstract

Echinoderms have a skeleton composed of calcium carbonate and spicule matrix associated proteins (SMAPs). SMAPs have been well characterized in the developmental model sea urchin *Strongylocentrotus purpuratus*, and in the transcriptome of the hemichordate *Saccoglossus kowalevskii*. The objective of this study was to search for SMAPs of *S. purpuratus* in the transcriptomes of representatives from the remaining echinoderm classes (i.e., a holothuroid, an ophiuroid, an asteroid and a crinoid), *Schizocardium* sp. (an acorn worm with a tornaria larva) and a tunicate. The spicule matrix family of proteins (SMs) was unique to the sea urchin. Whereas six copies of mesenchyme specific proteins (MSP130s) were found in the sea urchin, five homologous transcripts were present in the ophiuroid, three in *Saccoglossus*, two in the holothuroid, crinoid and *Schizocardium*, one in the asteroid, and none were recovered from the tunicate genome. Matrix metalloproteases (MMPs) were found in all groups. Carbonic anhydrases are well represented in all groups, but the specific Cara7LA protein that is involved in sea urchin biomineralization was unique to the sea urchin. These results show that SMAPs were present in the ambulacrarian ancestor, but the SM proteins, the Cara7LA protein, and a rapid proliferation of MSP130 proteins are unique to sea urchin.

Keywords: Biomineralization, skeleton, SMAPs, Echinodermata, deuterostomes, transcriptome

3.2 Introduction

Among the invertebrate deuterostomes, biominerals occur in tunicates (Lambert, 1998), hemichordates (Cameron and Bishop, 2012) and echinoderms. We know most about echinoderm skeletons because they have an extensive fossil record dating back 520 million years (Mooi, 2001; Sprinkle and Wilbur, 2005; Zamora et al., 2013). Echinoderm skeletal elements provide detailed taxonomic information, and the larval skeleton of the sea urchin *Strongylocentrotus purpuratus* is one of the best understood structures in molecular developmental biology (Oliveri et al., 2008). The skeletal elements, or ossicles, of echinoderms are composed of numerous single calcite crystals, with 2-3% magnesium

(Lowenstam and Weiner, 1989), that lies on the same crystal axis and originates either extracellularly or sometimes intracellularly (Bottjer et al., 2006; Wilt, 2005). The ossicles are positioned just under the ectoderm forming an endoskeleton. They are usually perforated by a network of sponge-like cavities forming a complex stereom, making the endoskeleton a key synapomorphy of the phylum (Bottjer et al., 2006).

The cells that form the pluteus larval skeleton of *S. purpuratus* are generated at the fifth cleavage stage of embryonic development. A quartet of vegetal pole located large micromere cells divides into a lineage that forms the primary mesenchyme cells (PMCs) (Oliveri et al., 2008; Wilt et al., 2003) and that during gastrulation give rise the larval skeleton. The micromere to PMC gene regulatory network (GRN) is one of the most complete developmental GNRs in an experimental model (Ettensohn, 2009; Ettensohn, 2013; Oliveri et al., 2008; Rafiq et al., 2012; Rafiq et al., 2014). The various cellular activities that underlie skeletogenesis in other echinoderms, in contrast, are not well understood.

Mann et al. (2010) identified 231 SMAPs present in the sea urchin spicule matrix. Four protein families are most abundant in the sea urchin test, tooth, spine, and spicule ossicles (Mann et al., 2010) and their functions have been experimentally verified. Spicule Matrix (SM) proteins and other C-type lectin domain proteins are the most abundant in sea urchin SMAPs and are mostly involved in crystallization (Drickamer and Taylor, 1993; Illies et al., 2002; Killian and Wilt, 1996; Livingston et al., 2006; Wilt et al., 2003). Mesenchyme Specific Proteins (MSP130) and related proteins are the second most abundant proteins among the SMAPs and function in spicule growth; they inhibit calcium accumulation and skeleton formation (Carson et al., 1985). Matrix Metalloprotease (MMP) proteins are involved in the proteolytic processing of matrix precursors (Ortega et al., 2003). Carbonic anhydrases are enzymes that catalyze the reversible conversion of carbon dioxide to protons and bicarbonate (Lindskog, 1997).

Recently, biomineralized ossicles composed of aragonite were discovered in Hemichordata (Cameron and Bishop, 2012), the sister phylum to echinoderms. The presence of several of these sea urchin SMAPs in the translated genome and expressed sequence tag (EST) libraries of the hemichordate *Saccoglossus kowalevskii* (Cameron and Bishop, 2012) suggest that some

aspects of echinoderm skeletogenesis were present already in the ancestor of hemichordates and echinoderms, together called the Ambulacraria. Three members of the sea urchin MSP130 family, a matrix metalloprotease and a carbonic anhydrase are present and transcribed during the development of *S. kowalevskii*, whereas the SM family of proteins is absent from the hemichordate genome (Cameron and Bishop, 2012).

The objective of the study was to identify SMAP protein families involved in sea urchin biomineralization in the other living echinoderm belonging to different classes. We queried the transcriptomes of a holothuroid, an ophiuroid, an asteroid a crinoid and the acorn worm *Schizocardium* to compare with the purple sea urchin and the direct developing acorn worm *Saccoglossus kowalevskii*. A tunicate is included as an outgroup taxon. Based on these data we discuss the evolution of SMAPs in the Ambulacraria.

3.3 Materials & Methods

Sea urchin SMAPs were searched in other ambulacrarians' transcriptome using the same criteria as in Cameron and Bishop (2012); i) they are present in sea urchin tests, tooth, spines and spicules and ii) they have been experimentally verified to be involved in skeletogenesis. These criteria narrowed the list of SMAPs amino acids sequenced (see Mann et al 2010, Supplementary File #5) to 22 proteins belonging to four different protein families (Table 3.1). Transcriptomes were developed from the crinoid *Florometra serratissima*, the asteroid *Patiria miniata*, the ophiuroid *Amphiura filiformis* and the holothuroid *Parastichopus parvimensis*. The genes of the hemichordate *S. kowalevskii* (Cameron and Bishop, 2012) were included for a comprehensive analysis, and the transcriptome SMAPs of the hemichordate *Schizocardium sp.* were added. The larvacean tunicate *Oikopleura dioica* was queried on Oikobase (<http://oikoarrays.biology.uiowa.edu/Oiko/index.html>) and was as an outgroup taxon. Larvaceans do not produce biominerals and should not have SMAPs. The presence of SMAPs in a larvacean questions the fact that SMAPs in the transcriptomes of our ingroup ambulacrarians are involved in biomineralization.

For each of the five representative taxa (i.e., the crinoid, asteroid, ophiuroid, holothuroid and the acorn worm *Schizocardium*), the RNA samples were pooled (excepted ophiuroid) and prepared for sequencing using the Illumina TruSeq stranded mRNA sample prep with oligo-dT selection, and then sequenced using HiSeq 100bp paired end reads. Samples were sequenced in a single lane of Illumina HiSeq2000 and reads were trimmed using Trimmomatic and *de novo* transcriptome assembly was done with Trinity (Grabherr et al., 2011).

F. serratissima gastrulas and late doliolariae were cultured at the Bamfield Marine Sciences Centre (BMSC), BC, Canada, following the methods of Chapter 2. Developmental stages were frozen in liquid nitrogen. *P. miniata* and *P. parvimensis* embryos were cultured at the Hopkins Marine Laboratory (Stanford University) and libraries of transcriptomes were made from pooled RNA of blastulas through to juveniles, including larvae with rudiments. *Schizocardium sp.* blastula, early and late gastrula, late larva (about a month old) and newly metamorphosed juveniles (48h after metamorphosis) were cultured at the University of Texas Marine Science Institute, Port Aransas, Texas. *A. filiformis* embryos were cultured at the Sven Loven Marine Station in Kristineberg, Sweden and the sample included stages from embryo cleavages to blastula. Total RNA was prepared using the Ambion RNA Aqueous mini kit.

The SpBASE portal (<http://www.spbase.org/SpBase/>) was used to retrieve protein sequences from the purple sea urchin *S. purpuratus*. These sequences were searched using BLAST (tblastn) against the transcriptome of each of the holothuroid, ophiuroid, asteroid, crinoid and hemichordate databanks and against the genome of the tunicate. Library sequences that resemble the query sequence above the threshold of 25% identity over more than 100 amino acids in length with the e-value less than 10^{-10} were kept. These sequences were then reverse BLASTed (blastp) in NCBI protein database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>), against all organisms in the non-redundant protein database to ensure that the *S. purpuratus* sequences appeared in the top hits. This ensured that sequence similarity was due to homology and not conserved, shared domains.

The software CLC Main Workbench 7 (<http://www.clcbio.com/products/clc-main-workbench/>) was used to align, and edit sequences. The alignments were made using the

“Very accurate (slow)” setting and the default settings. When two or more query sequences matching sea urchin proteins from a same protein family were recovered from a transcriptome, an unrooted phylogenetic phylogram and a radial phylogenetic tree were constructed to visualize the similarity between the library sequences and the sea urchin query sequences. Default values were used for tree construction and the estimations for the maximum-likelihood phylogenies were based on protein distance measures with a Jukes-Cantor distance correction. The multiple sequence trimmed alignments (some gaps were kept), consisting of 402 and 438 amino acids for the MSP130 family and for the MMPs family of proteins respectively, were used to construct two neighbour joining amino acid trees with 1000 bootstrap replicates. Nearly identical trees were constructed using two additional software packages, Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) and Jalview 2.8 (<http://www.jalview.org/>). In Jalview, trees were calculated with Neighbour joining using BLOSUM62. Carbonic anhydrases are ubiquitous and abundant in animals. In our transcriptomes, most are more likely to be involved in cellular processes than skeletogenesis. For this reason, only one sea urchin carbonic anhydrase sequence, CARA7LA, known to be involved in sea urchin skeletal development, was queried against our taxa.

3.4 Results

Homologous amino acid sequences of 22 SMAPs of the sea urchin *S. purpuratus* were queried against the transcriptome libraries of five ambulacrarians; the holothuroid *P. parvimensis*, the ophiuroid *A. filiformis*, the asteroid *P. miniata*, the crinoid *F. serratissima*, the hemichordate *Schizocardium sp.* and the genome of the larvacean *O. dioica*. Table 3.1 shows the presence and absence of sequences from the spicule matrix (SM) family (including the C-type lectin-like domain (CTLLD) proteins), mesenchyme specific protein (MSP130) family, matrix metalloprotease (MMP) family and carbonic anhydrase family of proteins respectively. The hemichordate *S. kowalevskii* data are from Cameron and Bishop (2012).

Mann et al. (2010) recovered six spicule matrix proteins from test, tooth, spine and spicule ossicles of the purple sea urchin: PM27, SM29, SM30, SM32, SM37, SM50. The SM protein family and C-type lectin-like domain (CTLLD) proteins were not retrieved from the

transcriptomes of any other ambulacrarian or the genome of the tunicate. C-type lectin (CTL) shared domains are present in all organisms, but outside the domain no homologous sequence was found.

The sea urchin *S. purpuratus* has six copies of the MSP130 proteins between 475 and 956 amino acids in length. Two MSP130s sequences were recovered from the holothuroid (477 and 266 a.a. long), five from the ophiuroid (438, two identical of 420 and two identical of 416 a.a. long), one from the asteroid (432 a.a. long), two from the crinoid (424 and 101 a.a. long), two from the hemichordate *Schizocardium* (465 and 174 a.a. long), three from the hemichordate *Saccoglossus* (566, 558 and 522 a.a. long) and no MSP130 protein sequences were recovered from the tunicate genome. In the sea urchin, glycine-rich domains are present in the middle of four of the MSP130 sequences, Sp-Msp130 (Glean3_02088), Sp-Msp130_1 (Glean3_13821), Sp-Msp130L (Glean3_06387), Sp-Msp130r3 (Glean3_13823) that cluster together on the phylogenetic tree. These glycine-rich domains have not been found in any of the MSP130 sequences retrieved in the other organisms. Sister to these genes are the Sp-Msp130r1 (Glean3_13822) and Sp-Msp130r2 (Glean3_16506), which lack glycine-rich domains. A conserved domain was detected only in Sp-MSP130 (Glean3_02088) of sea urchin.

Three transcriptomes from different developmental time periods were queried for the crinoid. None of the MSP130 proteins were expressed at gastrulation (24 hours) but all four copies were recovered at 5d6h, through the late doliolaria stage. At that age, first rudiment of ossicles are present (Mladenov and Chia, 1983). This is in contrast to what observed in sea urchin, ophiuroid and hemichordate. In *S. purpuratus*, MSP130s are expressed in skeletogenic cells in early development as well as during the juvenile skeleton formation (Gao and Davidson, 2008). In the hemichordate *S. kowalevskii*, three MSP130s were recovered in the gastrula and early juvenile stages (Cameron and Bishop, 2012) and five copies were recovered from the early development of the ophiuroid *A. filiformis*. This is in sharp contrast to the ophiuroid *Ophiocoma wendtii*, which no MSP130 has been identified in the reported gastrula transcriptome (Vaughn, 2012).

Our phylogram of the ambulacrarian MSP130s proteins suggests that one copy was present in acorn worms that underwent duplications to three in *Saccoglossus* and two in *Schizocardium*. The two distantly related copies in crinoids suggest that two copies existed at the base of the echinoderms. Fs-Msp130_1 is most similar to the acorn worm clade, as well as to the single copy present in the seastar (Pm_MSP130). Its paralog, Fs-Msp130_2, is most closely related to the two sea cucumber copies. The five copies in the ophiuroid transcriptome came from two distinct ancestral copies by one and two duplication events, respectively. This is in contrast to the six paralogs in the sea urchin genome that share a common ancestor (Figure 3.2). MSP130 is absent from the tunicate *O. dioica*.

Four members of the MMP gene family are expressed in all four sea urchin skeletal matrices and have a homologue in all EST libraries. One of these copies, Sp-Mt1-4/MmpL7 is verifiably involved in biomineralization in the sea urchin (Ingersoll and Wilt, 1998). Inasmuch as matrix metalloproteases are numerous in animal genomes and are not all involved in biomineralization, we kept only the first most similar sequence found in each transcriptome and genome to each of the four sea urchin MMPs to find the most probable homologous sequence (see Figure 3.3 for alignment and Figure 3.4 for trees). We add to the four known copies in the sea urchin, two from the crinoid, two from the holothuroid, one from the seastar, three from the brittle star, one from each of the acorn worms and two from the tunicate. The sea urchin gene Sp-Mt14/MmpL7 (Glean3_28748) is most similar to the acorn worms, the holothuroid (Pp-Mmp_2), the crinoid (Fs-Mmp_2) and two of the ophiuroid sequences (Figure 3.3 and 3.4). The remaining three sea urchin MMPs cluster closely with an ophiuroid (Af-Mmp_2), a holothuroid (Pp-Mmp_1), the solitary asteroid sequence (Pm-Mmp_1) and the two tunicate sequences.

Carbonic anhydrases are upregulated in the sea urchin postgastrulation stages and localized to the leading edge of the growing larval arms of the sea urchin *Heliocidaris tuberculata* (Love et al., 2007). One, Sp-Clara7LA (Glean3_12518), is the most abundant of all four skeletal matrix proteins identified in the proteome, suggesting a role in biomineralization in sea urchins (Mann et al., 2010). Beyond the conserved domain of this protein, there is no homologous sequence among our study taxa beyond what has been reported for *S. kowalevskii* (Cameron and Bishop, 2012).

Table 3.1 Homologous sequences of 22 SMAPs of echinoid *S. purpuratus* found (ID or Accession numbers) or not found (None) in transcriptome and genome of seven invertebrate deuterostomes. Echinoid and hemichordate *S. kowalevskii* data are taken from two other works, Mann et al. (2010) and Cameron and Bishop (2012) respectively and are shown here in italics. E-values are shown following each sequence between brackets. E-values for the MSP130 sequences are based on comparison with Sp-Msp130r2 except for Ssp-Msp130_2 that is based on comparison with Sp-Msp130r3 as it was not retrieved with Sp-Msp130r2. Selected sequences of MMPs and their e-values are the most similar sequence retrieved in transcriptomes and genome for each four sea urchin MMPs. There are less than four sequences in each organism because some retrieved sequences were identical for different sea urchin MMP proteins.

Protein families	Echinoid <i>S. purpuratus</i> (Mann et al., 2010)	Holothuroid <i>P. parvimensis</i>	Asteroid <i>P. miniata</i>	Ophiuroid <i>A. filiformis</i>	Crinoid <i>F. serratissima</i>	Hemichordate <i>Schizocardium sp.</i>	Hemichordate <i>S. kowalevskii</i> (Cameron and Bishop, 2012)	Tunicate <i>O. dioica</i> (do not possess biominerals)
SMs	<i>Sp-Sm29</i> <i>Sp-Sm30E</i> <i>Sp-Sm32</i> <i>Sp-Sm37</i> <i>Sp-Sm50</i> <i>Sp-Pm27</i>	None	None	None	None	None	None	None
CTLLDs	<i>Sp-Clect_13</i> <i>Sp-Clect_14</i> <i>Sp-Clect_25</i> <i>Sp-Clect_76</i> <i>Sp-Clect/PMC1</i>	None	None	None	None	None	None	None
MSP130s	<i>Sp-Msp130</i> <i>Sp-Msp130L</i> <i>Sp-Msp130_1</i> <i>Sp-Msp130r1</i> <i>Sp-Msp130r2</i> <i>Sp-Msp130r3</i>	Pp-Msp130_1 (5e-63) Pp-Msp130_2 (3e-28)	Pm-Msp130 (2e-53)	Af-Msp130_1 (7e-58) Af-Msp130_2 (7e-58) Af-Msp130_3 (4e-56) Af-Msp130_4 (4e-56) Af-Msp130_5 (5e-55)	Fs-Msp130_1 (6e-49) Fs-Msp130_2 (9e-12)	Ssp_Msp130_1 (2e-45) Ssp_Msp130_2 (2e-19)	<i>XP_002739468.1</i> (3e-54) <i>XP_002733636.1</i> (6e-37) <i>XP_002739469.1</i> (3e-47)	None
MMPs	<i>Sp-Mt1-4/MmpL5</i> <i>Sp-Mt1-4/MmpL6</i> <i>Sp-Mt1-4/MmpL7</i> <i>Sp-Mt5/MmpL2</i>	Pp-Mmp_1 (2e-79) Pp-Mmp_2 (1e-77)	Pm-Mmp_1 (1e-72)	Af-Mmp_1 (2e-92) Af-Mmp_2 (1e-76) Af-Mmp_3 (1e-127)	Fs-Mmp_1 (2e-76) Fs-Mmp_2 (8e-150)	Ssp-Mmp_1 (4e-106)	<i>XP_002738586</i> (3e-93)	Od-Mmp_1 (2e-31) Od-Mmp_2 (2e-33)
Carbonic anhydrases	<i>Sp-Cara7LA</i>	None	None	None	None	None	<i>XP_002735813</i> (4e-39)	None

3.5 Discussion

In the purple sea urchin, biomineralization is regulated by secreted proteins that are closely associated with the mineral element component of the biomineral. The proteins make only a minor contribution to its biomineral mass (Wilt et al., 2003), but probably play an important role in regulating the growth and physical characteristics of the biomineral. The sea urchin larval skeleton is produced by the PMCs—a specialized population of embryonic mesodermal cells derived from the large micromere lineage. PMCs express a family of SM proteins, and a family of cell surface proteins consisting of at least five MSP130-related proteins and a matrix metalloproteinase (Ingersoll and Wilt, 1998) that may function in biomineralization (Farach-Carson et al., 1989; Illies et al., 2002; Leaf et al., 1987).

Spicule matrix (SM) genes are clustered together in the sea urchin genome indicating a recent duplication (Livingston et al., 2006; Rafiq et al., 2012). This family of proteins has an important function in the regulation of the conversion of amorphous calcium carbonate to a crystal calcite state (Gong et al., 2012). SM proteins are also expressed in the juvenile skeletogenic centers (Gao and Davidson, 2008) and are abundant in all parts of the adult endoskeleton of sea urchins (Mann et al., 2010). Our analysis did not recover SM proteins from the transcriptomes of any ambulacrarian taxon besides the sea urchin. Another key gene in the embryonic skeletogenic GRN of the sea urchin, *Pmar1* is involved in the establishment of the double negative gate that de-represses skeletogenesis (Oliveri et al., 2008), and is expressed in the quartet of skeletal micromeres, is also unique to echinoids. Telford et al. (2014) did not find evidence of a *Pmar1* ortholog in the crinoid, asteroid, holothuroid or ophiuroid EST databases except for *S. purpuratus*. Whereas SM proteins and *Pmar1* are central to skeletal development in the echinopluteus, they are absent from the ophiopluteus. The ophiopluteus skeleton does not develop from a quartet of embryonic micromeres (summarized by Ettensohn, 2009) suggesting that the sea urchin SM proteins and *Pmar1* may have originated in concert with the quartet of skeletal micromeres. The absence of SM proteins and the *Pmar1* protein from the ophiuroid supports Gao and Davidson's (2008) hypothesis that the GRN specifying embryonic skeleton formation in plutei is likely inherited from the adult skeletal formation pathway.

Our findings support the hypothesis that a single MSP130 gene was present in the ambulacraria ancestor (Cameron and Bishop, (2012). The two distantly related copies in the crinoid suggest that two copies existed at the base of the echinoderms (Figure 3.2). One copy, Fs-Msp130_1 is clustered with the hemichordates and the single asteroid sequence, whereas the Fs_Msp130-2 copy clusters with the two sea cucumber sequences (Figure 3.2). The six purple sea urchin sequences form a monophyletic group. Several duplications of the gene family occurred before the divergence of cidaroids and euechinoids (Ettensohn, 2014), dated around 250 Ma (Smith et al., 2006). Ophiuroids possess five copies but rather than being monophyletic, they seem to arise from two different ancestral copies.

In a comprehensive search for MSP130 proteins in eukaryotes, Ettensohn (2014) recovered sequences from deuterostomes, molluscs, algae and several strains of bacteria. Notably, they are absent from the genomes of several non-bilaterian metazoans, including two cnidarians, a sponge, a placozoan, and a ctenophore (Ettensohn, 2014). From these findings he concluded that the MSP130 is a bacterial specific gene family that was acquired in animals by horizontal gene transfer on at least one occasion. This is a compelling interpretation, but our results hint at an alternate explanation. We see that two copies are in the ancestral crinoid but only one in the asteroid (Figure 3.2). The sea cucumber also does not have an ortholog of the crinoid Fs_MSP130 transcript. The ophiuroid family of MSPs has a dual origin, but the echinoids do not. In other words, there have been repeated losses of MSP130 genes over the course of echinoderm evolution. The absence of MSP130 genes from the genomes of non-bilaterian metazoans may be due to a repeated loss of MSP130 genes from several different animal and non animal lineages.

Carbonic anhydrases and matrix metalloproteases (MMPs) are widespread in animal genomes and are not necessarily involved in biomineralization. No carbonic anhydrases that were clear echinoderm homologues were recovered from our study taxa, but MMPs were retrieved from each of the EST libraries, and the genome of *O. dioica*, a tunicate that does not form skeletal tissue. Until developmental or proteomic studies verify that these MMPs are involved in ambulacrarian skeletogenesis it is premature to infer anything from these data with confidence.

The absence of the SM protein family from the acorn worm genome (Cameron and Bishop, 2012) prompted Etensohn (2013) to suggest that hemichordates biomineralization appeared independently from than of echinoderms. Here we show that SM proteins are absent from a second acorn worm, *Schizocardium* sp., transcriptome as well as from that of a crinoid, asteroid, ophiuroid and holothuroid. Sea urchins moreover are the only class that possess a *Pmar1* ortholog (Telford et al., 2014), six MSP130 paralogs and the Cara7LA protein. The absence of these genes from other classes does not mean that the sea urchin skeleton arose *de novo* from other ambulacraria. Instead, these findings suggest that biomineralization in the ambulacrarian, at least with respect to part of the SMAP GNR appears homologous. The wealth of knowledge available from the *S. purpuratus* model system has provided a platform of tools to address skeletal evolution in ambulacrarians. We have arrived at a point where parallel comparative molecular, genetic and cellular data is beginning to unravel the mystery of ambulacrarian skeletal evolution. The picture that is emerging from our analysis is that the SMAP GNR of echinoids may be highly derived. This conclusion is not surprising - echinoids are late arrivals in echinoderms evolution (Mooi, 2001) and their skeleton is uniquely complex in possessing an adult test, spines, teeth, pedicellaria and larval feeding arms. The latent origin of echinoids coupled with their skeletal complexity may have permitted and required an equally elaborated skeletal gene regulatory network.

3.7 Acknowledgements

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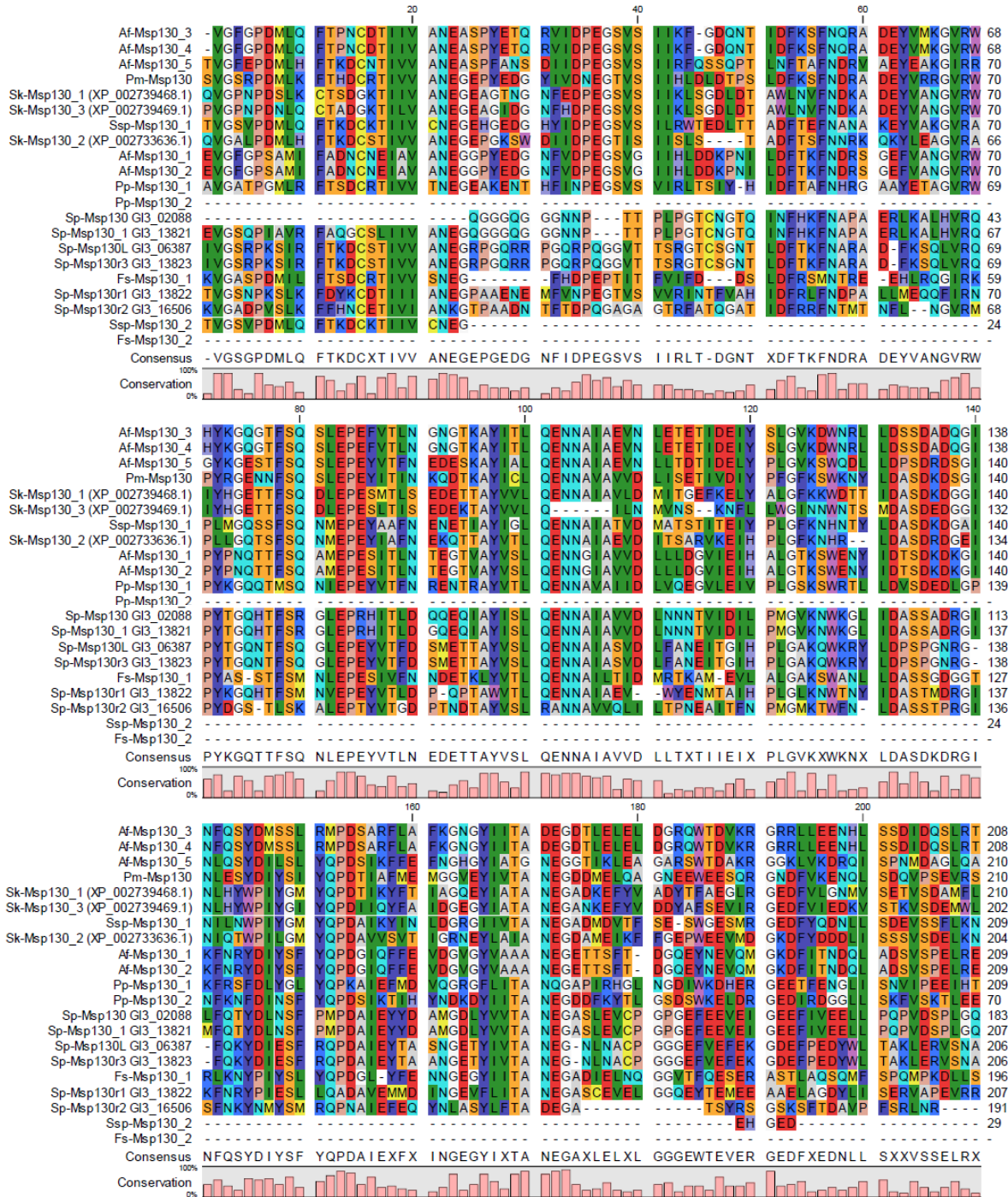
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3.9 Figures



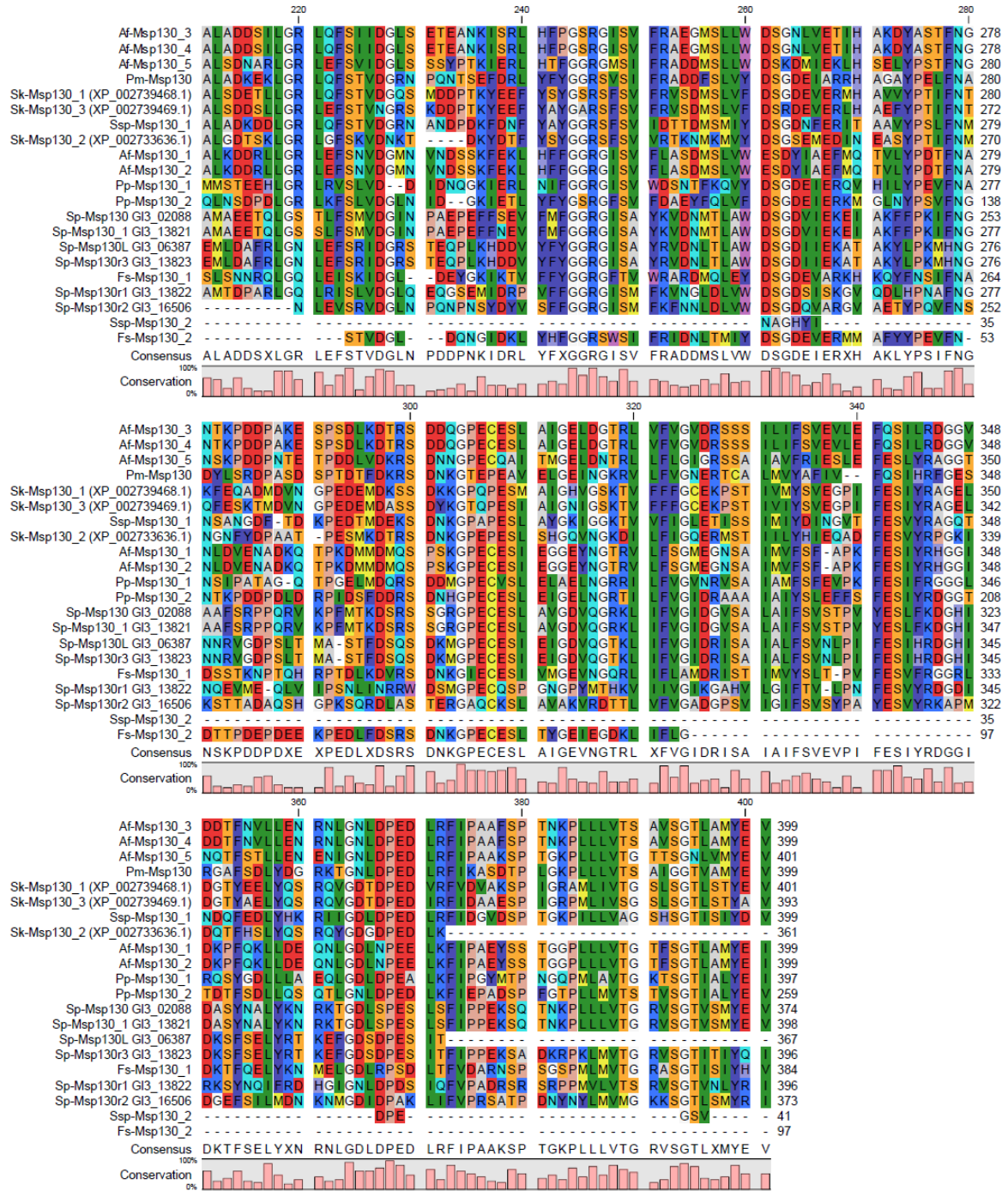


Figure 3.1. Alignment of the MSP130 proteins in the echinoid *S. purpuratus* (Sp), the holothuroid *P. parvimensis* (Pp), the ophiuroid *A. filiformis* (Af), the asteroid *P. miniata* (Pm), the crinoid *F. serratissima* (Fs) and the hemichordates *Schizocardium sp* (Ssp) and *S. kowalevskii* (Sk). If a gap was present in the same position in six or more of the twenty one sequences, it was removed from the alignment.

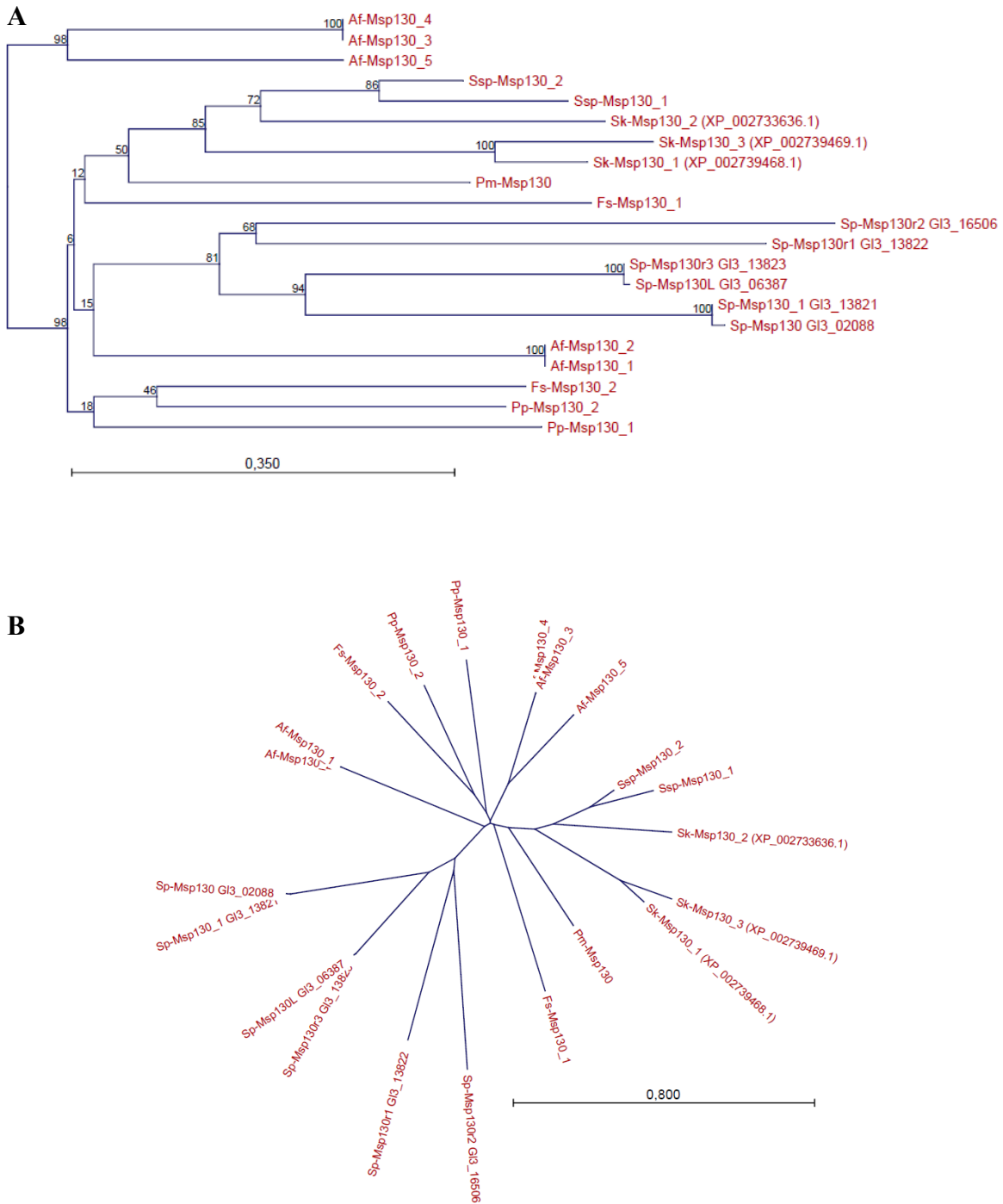
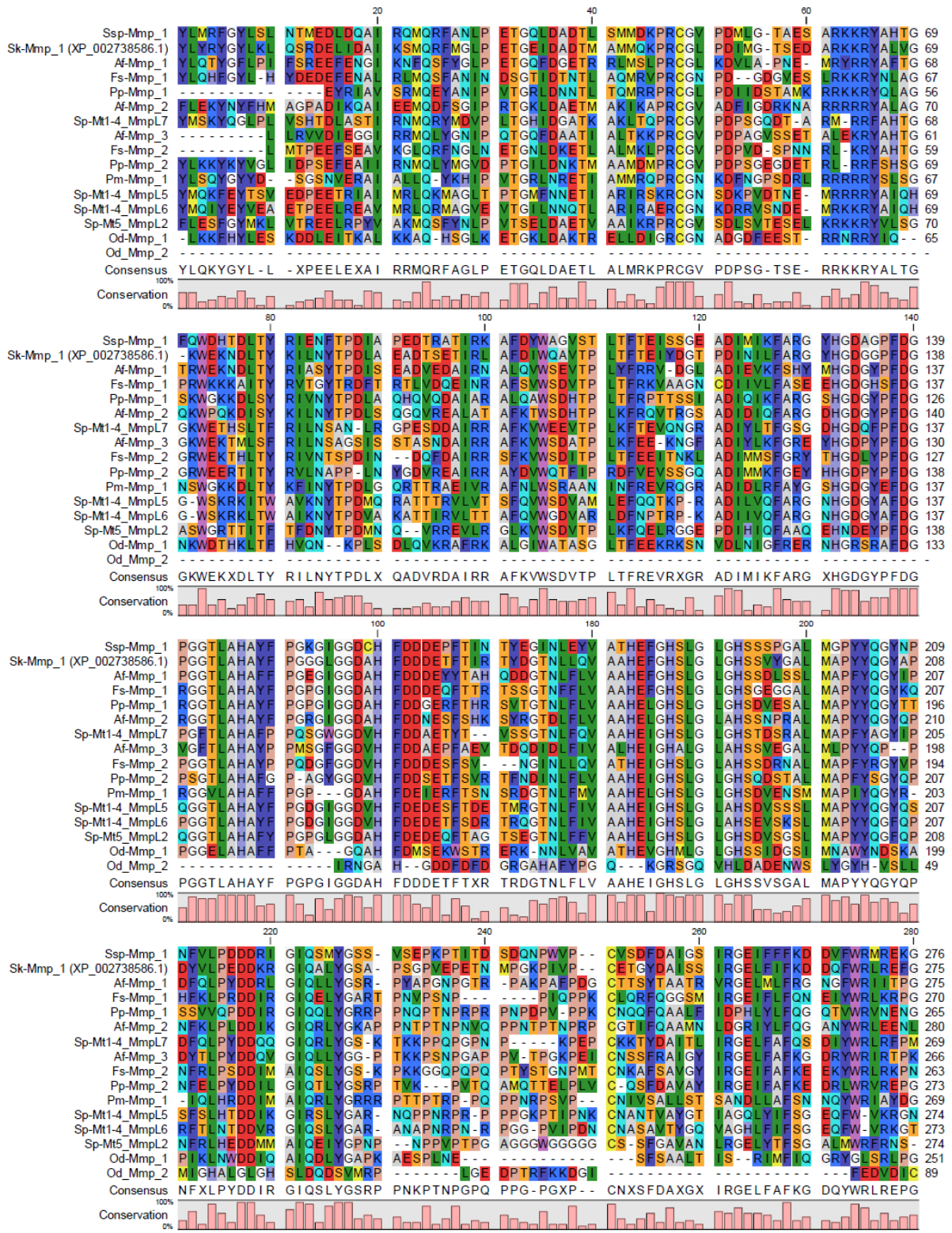


Figure 3.2. Phylogenetic relationships and the presentation of the MSP130 proteins in the echinoid *S. purpuratus* (Sp), the holothuroid *P. parvimensis* (Pp), the ophiuroid *A. filiformis* (Af), the asteroid *P. miniata* (Pm), the crinoid *F. serratissima* (Fs) and the hemichordates *Schizocardium sp* (Ssp) and *S. kowalevskii* (Sk) shown as phylogram (A) and as radial (B). The number at each node represents the bootstrap of 1000 replicates as a percentage.



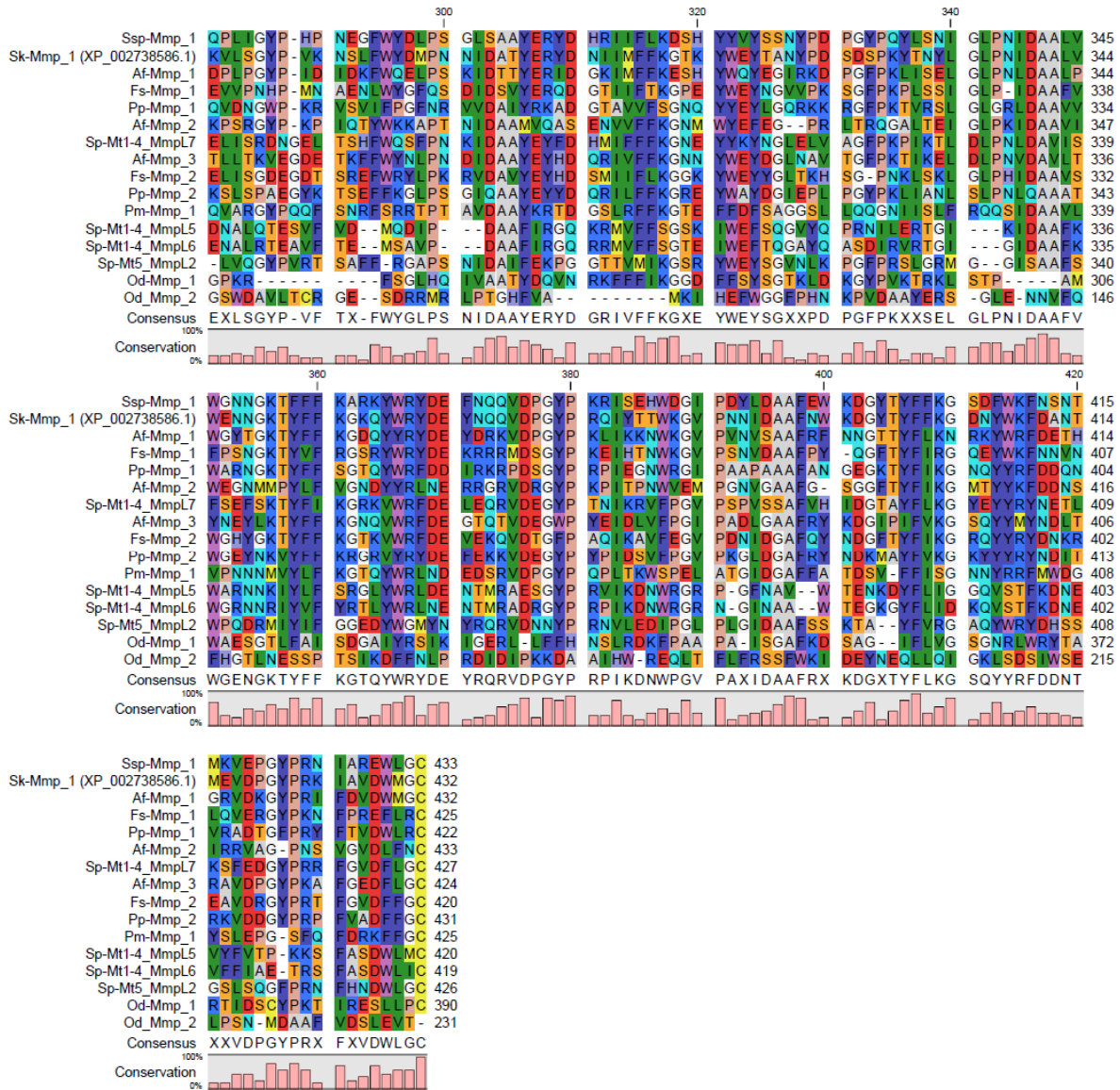


Figure 3.3. Alignment of the MMPs proteins in the echinoid *S. purpuratus* (Sp), the holothuroid *P. parvimensis* (Pp), the ophiuroid *A. filiformis* (Af), the asteroid *P. miniata* (Pm), the crinoid *F. serratissima* (Fs), the hemichordates *Schizocardium sp* (Ssp) and *S. kowalevskii* (Sk) and the tunicate *O. dioica* (Od). Only the first sequence obtained with each four echinoid MMP proteins in every transcriptome are part of this alignment, which were sometimes the same for different queries. If a gap was present in the same position in six or more of the sixteen sequences, it was removed from the alignment.

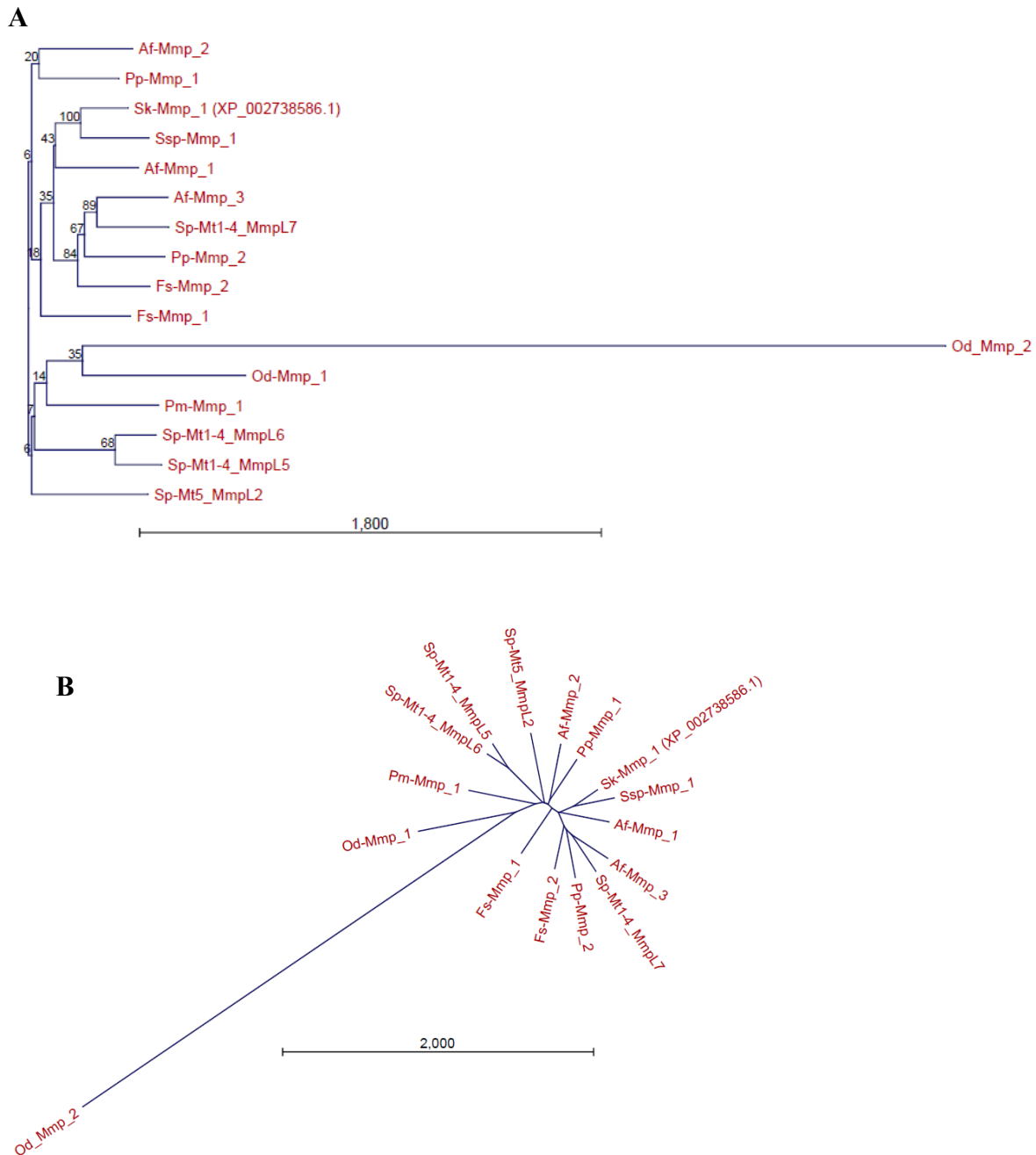


Figure 3.4. Phylogenetic relationships and the presentation of the MMPs proteins in the echinoid *S. purpuratus* (Sp), the holothuroid *P. parvimensis* (Pp), the ophiuroid *A. filiformis* (Af), the asteroid *P. miniata* (Pm), the crinoid *F. serratissima* (Fs), the hemichordates *Schizocardium sp* (Ssp) and *S. kowalevskii* (Sk) and the tunicate *O. dioica* (Od), shown as phylogram (A) and as radial (B). Only the first sequence obtained with each four echinoid MMP proteins in every transcriptome are part of these trees. The number at each node represents the bootstrap of 1000 replicates as a percentage.

Chapitre 4. Discussion générale

Le cycle de reproduction des crinoïdes est complexe. Par conséquent, peu d'études sont entreprises sur leur développement larvaire. La monographie colossale de A.H. Clark combine les travaux de plusieurs auteurs portant sur la description et la comparaison des espèces de comatules (Clark, 1915, 1921, 1931, 1941, 1947, 1950; Clark and Clark, 1967). Par contre, le développement des ossicules des stades larvaires de seulement quelques unes de ces espèces a été observé. Mortensen (1920b) n'a pas réussi à cultiver des embryons de *F. serratissima*. Il a pu illustrer seulement les quelques pentacrinoïdes qu'il avait retrouvés en nature. Les premiers à étudier le développement squelettique des trois stades larvaires de l'espèce sont Mladenov et Chia (1983). Dans la première partie de notre étude, nous complétons leurs travaux. Dans la doliolaria, dix plaques orales et basales débutent leur développement par une forme de spicules tri-radiés. Les premiers rudiments des ossicules du pédoncule apparaissent sous une forme ellipsoïdale. Le nombre d'ossicules du pédoncule augmente lors des stades de larves cystidienne et pentacrinoïde et le dernier ossicule du pédoncule est modifié en disque d'attachement. Le nombre d'ossicule du pédoncule varie entre les individus entre 10 et 11 chez les doliolaria, 10 et 13 chez les cystidiennes et 10 et 15 chez les pentacrinoïdes en bas de 56 jours. Après la formation des 10 premiers ossicules, les nouveaux ossicules se développent sous le calice de la larve.

Le développement squelettique aux stades larvaires entre les différentes espèces de comatules suit un patron similaire. Chez chacune, les plaques orales, basales et les ossicules du pédoncule apparaissent pendant le stade larvaire doliolaria. Seulement l'espèce *A. mediterranea* développe les premiers rudiments de squelette avant de se libérer de sa membrane de fertilisation (Clark, 1921). La larve y est déjà bien développée, mais se libère de la membrane tardivement. Chez certaines espèces, le disque d'attachement est bien développé avant l'installation au substrat et la métamorphose en cystidienne, alors que pour *F. serratissima*, le disque d'attachement est parfois distingué dans la doliolaria, mais se développe essentiellement une fois attaché au sol. De plus, la forme du disque d'attachement se moule dépendant de l'état du substrat auquel la larve s'est attachée. Les larves les plus avancées que nous avons cultivées étaient pentacrinoïdes et avaient 56 jours. À cet âge, elles ne possédaient toujours pas les plaques radiales. Comparativement à d'autres espèces, l'arrivée de ces plaques est tardive pour *F. serratissima*. *A. wilsoni* a un développement rapide

et possède les plaques radiales dès le stade doliolaria (Haig and Rouse, 2008). Les plaques infrabasales n'ont jamais été observées chez *F. serratissima*, et leur présence chez *A. bifida* est controversée. (Lahaye and Jangoux, 1987; Mortensen, 1920b). Lors de nos expérimentations, nous avons observé chez quelques individus un petit ossicule sous les plaques basales, à la position des infrabasales chez d'autres espèces. Ceci pourrait suggérer que de manière similaire à *A. bifida*, *F. serratissima* expose une plasticité au niveau de la présence des infrabasales. Le nombre de rangées de plaques dans la thèque des crinoïdes adultes dicte si l'espèce fait partie des monocycliques (une rangée de plaques sous les radiales) ou dicycliques (deux rangées de plaques sous les radiales) (Moore and Teichert, 1978). Par contre, l'identification classique des plaques radiales, basales et infrabasales est controversée, étant donné que d'une espèce à l'autre, la terminologie n'indiquerait pas des plaques homologues (Ausich, 1996; Guensburg and Sprinkle, 2003; Simms, 1993). Peu est connu sur la présence et la résorption des plaques infrabasales pendant les stades larvaires et leur étude à ces stades indiquerait l'homologie réelle des plaques. La plasticité phénotypique retrouvée au niveau du squelette des crinoïdes est aussi observée chez les échinodermes ancestraux (McIntosh, 1979; Zamora and Smith, 2012). Par rapport aux larves d'oursins, les larves de crinoïdes ont une grande désynchronisation au niveau de leur développement.

Les crinoïdes et les concombres de mer sont les deux seules classes à posséder le stade larvaire doliolaria. Par contre, contrairement aux lys de mer et les concombres de mer, seuls les comatules développent un squelette au stade doliolaria (Massin 2000, Nakano 2003).

La concentration en magnésium des os des crinoïdes adultes varie entre 1,83 et 3,55 % (Gorzalak et al., 2013). Chez les adultes crinoïdes, le ratio Mg/Ca n'est pas distribué de manière uniforme à l'intérieur des os. Il se trouve en plus grande concentration dans la partie intérieure du stéréome (Gorzalak et al., 2013). Les ossicules des pentacrinoïdes étaient composés entre 0 et 3.05 % de magnésium. Comme chez les adultes, la distribution de magnésium varie à l'intérieur d'un ossicule de pentacrinoïde et sa concentration pourrait augmenter au cours de la croissance du squelette. Comme chez les adultes de toutes les classes d'échinodermes, les pentacrinoïdes possèdent un squelette de cristallisation calcique. La cristallisation et la concentration en magnésium chez les doliolaria et les cystidiennes n'ont pu être identifiées lors de cette étude. Des larves doliolaria, cystidiennes et pentacrinoïdes ont été

fixées puis conservées dans une solution tampon à des fins d'analyse. Des larves ont été transportées du BMSC, Île de Vancouver, C.-B. à l'Université de Montréal, Montréal, Québec, et ce dans des glacières afin de garder les solutions à la température et au pH idéals. Rendus à destination, étrangement, seulement les ossicules des pentacrinoïdes étaient intacts, ceux des larves doliolaria et cystidiennes s'étaient dissous. Pourtant, pendant plusieurs semaines au BMSC, les larves étaient fixées et conservés au frais. La cause de cet événement est incomprise. Il se pourrait que des changements involontaires de température et de pH aient eu lieu lors du transport et que les ossicules des doliolaria et cystidiennes soient moins stables que ceux des pentacrinoïdes. Il serait intéressant de vérifier si cette instabilité est due à un changement de cristallisation et de composition des ossicules.

Pour obtenir un développement normal, les larves planctoniques de *F. serratissima* doivent s'installer après un certain délai, qui est de 14 jours maximum après fertilisation (Mladenov and Chia, 1983). Une larve qui continuait de nager après ce délai perdait ses cils qui lui permettent de nager, commençait l'élongation de son pédoncule, mais le calice ne subissait pas la rotation et la larve mourrait. Par contre, si la larve s'attachait au substrat et était par après expérimentalement détachée, la larve se métamorphosait normalement. Ceci indique que le développement normal n'est pas nécessairement dû à l'attachement constant au substrat, mais à l'action de s'y attacher.

En laboratoire, l'algue coralline ramifiée était le substrat préféré des larves de *F. serratissima*. Aussi, de larges agrégations sont survenues directement sur les bols en verre des cultures. Pour plusieurs espèces d'invertébrés marins, l'algue coralline est un signal d'installation et métamorphose (résumé par Bishop et al., 2006). Des pentacrinoïdes du comatule *Antedon serrata* ont été retrouvés en nature installés sur l'algue coralline (Mortensen, 1920b). L'agrégation larvaire lors de l'attachement au substrat ainsi qu'au sein d'une population d'adultes crinoïdes a souvent lieu (Hyman, 1955; Mladenov and Chia, 1983). Les quelques pentacrinoïdes de *F. serratissima* retrouvés en nature étaient sur les cirres des adultes de la même espèce (Mortensen, 1920b). Ce comportement assure la présence de conspécifiques et pourrait être dû à une amélioration dans la possibilité de reproduction.

Plusieurs aspects du squelette des crinoïdes restent à être découverts. Pour mieux comprendre la formation des ossicules, la biologie moléculaire est un outil puissant. Les connaissances sur la biominéralisation au niveau des gènes des oursins sont avancées, mais sont peu étendues au niveau des autres échinodermes. Pour la deuxième partie de notre étude, comme les protéines impliquées dans la biominéralisation des oursins sont connues (Mann et al., 2010), nous avons cherché des séquences homologues à celles-ci dans les transcriptomes des autres échinodermes (crinoïde, étoile de mer, concombre de mer et ophiure), d'un hémichordé et dans le génome d'un tunicier. Les quatre familles de protéines les plus abondantes dans la matrice de spicules de l'oursin *S. purpuratus* sont les SM, MSP130, MMP et anhydrase carbonique (Mann et al., 2010).

Il a été documenté que la famille de SM était absente du génome de l'hémichordé *S. kowalevskii* (Cameron and Bishop, 2012), ce qui a porté Etensohn (2014) à suggérer que l'origine de la biominéralisation des hémichordés n'était pas homologue à celle des échinodermes. Par contre, notre étude démontre que la famille de SM serait une innovation des oursins puisqu'elle est absente chez tout autre échinoderme. Dans le génome de *S. purpuratus*, les gènes SM sont regroupés, ce qui indique des duplications récentes (Livingston et al., 2006; Rafiq et al., 2012).

L'origine des MSP130s chez les métazoaires est mystérieuse (Etensohn, 2014). Ces protéines sont retrouvées chez des deutérostomes, des mollusques, des algues et de nombreuses souches de bactéries. Elles sont par contre absente dans le génome complet de nombreux métazoaires non-bilatéraux tels que deux cnidaires, une éponge, un placozoaire et un cténophore (Etensohn, 2014). Etensohn suggère qu'il y aurait eu au moins un transfert horizontal de la famille de gènes des MSP130 d'une bactérie aux animaux. Dans notre étude, le nombre de séquences homologues aux MSP130 est changeant d'une classe à l'autre. Elles sont présentes en nombre différent chez tous les échinodermes (une chez l'étoile de mer, deux chez le crinoïde et le concombre de mer, cinq chez l'ophiure, six chez l'oursin), deux chez les hémichordés, mais aucun dans le génome du tunicier. Ceci suggère que des pertes répétées ont eu lieu parmi les gènes MSP130 au cours de l'évolution des échinodermes. Nous pensons plutôt que l'absence des gènes MSP130 chez de nombreux animaux et algues serait dû à des pertes répétées. Comme groupe externe, nous avons inclus le tunicier *O. dioica*, mais celui-ci

est une espèce de tunicier qui ne produit pas de biominéraux. Comme les céphalocordés (Ettensohn, 2014), les hémichordés (Cameron and Bishop, 2012) et les échinodermes sont des deutérostomes possédant des séquences homologues aux MSP130 de l'oursin, et il serait intéressant de vérifier si des espèces de tuniciers qui produisent des biominéraux posséderaient des gènes MSP130.

Des métalloprotéases sont présentes chez tous les organismes de cette étude alors que l'anhydrase carbonique Cara7LA est unique à l'oursin. Les métalloprotéases et les anhydrases carboniques sont nombreuses et répandues dans les génomes des animaux et n'ont pas nécessairement un rôle dans la biominéralisation. Des séquences homologues aux protéines cherchées des oursins ont été retrouvées chez les autres échinodermes, mais des études ultérieures sont nécessaires pour vérifier si ces transcrits sont bel et bien des protéines et s'ils ont un rôle dans la biominéralisation de ces organismes.

Chapitre 5. Conclusion

Les échinodermes ne cessent de gagner en popularité auprès des chercheurs en biologie développementale et moléculaire qui tentent de déchiffrer les processus de biominéralisation des stades larvaires. Les crinoïdes sont peu étudiés par rapport aux oursins, notamment à cause de leur cycle de reproduction et leur développement plus difficiles à contrôler. Les oursins représentent un modèle important en biologie du développement. Le génome complet de l'oursin pourpre *S. purpuratus* est séquencé (Consortium, 2006) et il est souvent utilisé pour représenter les échinodermes en général. Cependant, ils ont divergé des autres échinodermes, spécialement par la complexité de la morphologie et de la régulation génétique de leur squelette. Étudier le développement du squelette des crinoïdes aux stades larvaires offrira de nouvelles informations sur leur cycle de vie, permettra de retrouver certains traits ancestraux des échinodermes et mettra en lumière l'origine et l'évolution de ces groupes. Le développement larvaire des ossicules dépend de leur réseau de régulation des gènes et explorer ces deux volets en parallèle permet d'enrichir les connaissances et renforcer la compréhension de la biominéralisation des animaux.

5.1 Perspectives pour des études futures

- 1- Quels sont les facteurs affectant la variation de l'âge auquel les ossicules apparaissent chez les larves de crinoïdes et la vitesse à laquelle ils se développent?
- 2- Comment les ossicules larvaires des autres espèces de crinoïdes sont-ils caractérisés et comment varient-ils d'une espèce à l'autre?
- 3- Comment est-ce que le magnésium est distribué à travers un ossicule larvaire de crinoïde?
- 4- Y a-t-il des changements de cristallisation dans les ossicules des stades doliolaria et cystidienne des crinoïdes?
- 5- Quelle est la solution idéale pour préserver les ossicules des larves doliolaria et cystidiennes des crinoïdes?
- 6- Les espèces de tuniciers qui élaborent un squelette expriment-ils des gènes MSP130s?
- 7- Les gènes MSP130s ont-ils un rôle dans la biominéralisation des ambulacraires autres que les oursins?

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