Universidade Federal do Rio de Janeiro
Instituto de Biologia

André Queiroz de Padua

Aspectos da individualidade de esponjas calcáreas (Porifera)

Regeneração, alorreconhecimento, fragmentação e fusão

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André Queiroz de Padua

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas (Biodiversidade e Biologia Evolutiva), Instituto de Biologia, Universidade Federal do Rio de Janeiro, como requisito parcial à obtenção do título de Doutor em Ciências Biológicas Biológicas (Biodiversidade e Biologia Evolutiva).

Orientadora: Dr[a]. Michelle Klautau

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Data: 29/02/2016

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Rio de Janeiro
Fevereiro de 2016

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“O começo de todas as ciências é o espanto de as coisas serem o que são”.

(Aristóteles)

“A ciência nunca resolve um problema sem criar pelo menos outros dez”.

(George Bernard Shaw)

RESUMO

PADUA, André Queiroz de. **Aspectos da individualidade de esponjas calcáreas (Porifera)**. Rio de Janeiro, 2016. Tese (Doutorado em Ciências Biológicas - Biodiversidade e Biologia Evolutiva). Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2016.

O Filo Porifera é o grupo animal mais antigo ainda vivo no planeta e seus indivíduos, as esponjas, apresentam hoje uma enorme diversidade de morfologias externas, com grande plasticidade de seus elementos celulares. Nesta tese, dividida em quatro capítulos, abordamos aspectos observados em indivíduos de esponjas da classe Calcarea que nos mostram como eles apresentam e mantêm a sua integridade e individualidade. O primeiro Capítulo trata da regeneração em uma espécie com forma corporal definida e polaridade (*Ernstia* sp.) e da comparação desta com outras espécies da classe Calcarea com morfologias diferentes. Concluímos que a capacidade e velocidade de regeneração estão ligadas à morfologia externa e à presença de uma polarização corporal, principalmente quando o ferimento afeta uma parte importante do corpo do indivíduo. O Capítulo dois estuda a reconstituição de indivíduos funcionais de duas espécies (*Paraleucilla magna* e *Clathrina aurea*) a partir de células dissociadas e formação de primorfos. Observamos que, em *P. magna*, a reconstituição de indivíduos funcionais apresenta diferenças tanto na ordem quanto na cronologia dos eventos quando comparada com o desenvolvimento a partir da larva. No Capítulo três, acompanhamos por 18 meses a dinâmica de fragmentação e fusão de *C. aurea* e a possível existência e formação de quimeras *in situ* que pudesse explicar a alta diversidade genética da espécie. Não observamos fusão entre indivíduos geneticamente diferentes e, consequentemente, aparentemente não há a formação natural de quimeras nessa espécie. No quarto capítulo estudamos o alorreconhecimento e formação de quimeras *in vitro* de *P. magna* e *C. aurea* após a dissociação celular. Observamos que, para as duas espécies, ocorre a fusão de células de indivíduos geneticamente diferentes, sugerindo a perda do alorreconhecimento após a dissociação celular. Em *P. magna*, foi possível observar a reconstituição de primorfos quiméricos em indivíduos funcionais. Ao final, é discutido como as esponjas, especialmente da classe Calcarea, enquadram-se nas características classicamente atribuídas a indivíduos biológicos.

ABSTRACT

PADUA, André Queiroz de. **Aspectos da individualidade de esponjas calcáreas (Porifera)**. Rio de Janeiro, 2016. Tese (Doutorado em Ciências Biológicas - Biodiversidade e Biologia Evolutiva). Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2016.

The Phylum Porifera is the oldest animal group still alive in this planet and its individuals, the sponges, present a great diversity of external morphology and high plasticity of cellular elements. In this thesis, divided in four chapters, we studied some aspects observed in individuals of the class Calcarea that show us how they maintain their integrity and individuality. The first chapter deals with the regeneration in a species with a defined external shape and body polarity (*Ernstia* sp.) and compares it with other species with the opposite characteristics. It was concluded that the capacity and speed of regeneration are linked with the external morphology and the presence of a body polarity, especially when the wound affects an important part of the sponge body. The Chapter two studies the reconstitution of functional individuals of two species (*Paraleucilla magna* e *Clathrina aurea*) from dissociated cells and the formation of primmorphs. It was observed that, in *P. magna*, the reconstitution has differences in the order and timing of the events when compared to the normal development from the larvae. In Chapter three, it was followed for 18 months the dynamics of fragmentation and fusion of *C. aurea* and the possible existence and formation of chimeras in situ that could explain the high genetic diversity previously observed in this species. Fusion between genetically different individuals was not observed and, consequently, apparently there is no natural formation of chimeras in this species *in situ*. In Chapter four, the allorecognition and formation of chimeras in vitro of *P. magna* e *C. aurea* were studied after cellular dissociation. In both species, there was fusion and formation of primmorphs composed of cells from genetically different individuals, suggesting that after cellular differentiation, the alorrecognition capacity is lost. In *P. magna*, it was possible to observe the reconstitution of chimeric functional individuals. At the end, it is discussed how sponges, especially from the class Calcarea, fit on the characteristics classically considered for biological individuals.

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INTRODUÇÃO

A multicelularidade animal

A história da vida na Terra é composta por importantes transições evolutivas que levaram a níveis cada vez mais integrados e complexos de organização biológica e geraram condições para a origem da grande diversidade de formas viventes que dominam o nosso planeta. O estabelecimento de organismos multicelulares só foi possível após a integração de unidades biológicos menores e individuais (células) que resultou na formação de outra unidade mais complexa (organismo), prevalecendo a mediação de conflitos celulares internos, o que promoveu a cooperação entre linhagens de células (Szathmáry & Maynard-Smith, 1995; Michod & Roze, 1997; Szathmáry, 2015). A multicelularidade se originou independentemente diversas vezes ao longo da evolução da vida na Terra, várias vezes (com perdas secundárias) em alguns grupos como plantas, fungos e Eubacteria, mas uma única vez entre os metazoários (Bonner, 1998; Kaizer 2001; King, 2004; Grosberg & Strathmann, 2007; Knoll, 2011).

É consenso, atualmente, que os metazoários (animais) formam um grupo monofilético, tendo evoluído de um ancestral comum semelhante a um protista coanoflagelado (Steenkamp *et al.,* 2006; Carr *et al.,* 2008). Apesar de serem, em sua maioria, unicelulares, os coanoflagelados (e outros protistas) já possuíam parte do repertório genético utilizado no desenvolvimento dos animais atuais como, por exemplo, famílias de proteínas de matriz extracelular, adesão e sinalização celular, fatores de transcrição, além de processos de diferenciação e morte celular programada (Abedin & King, 2008; Ruiz-Trillo *et al.,* 2008; Shalchian-Tabrizi *et al.,* 2008; Degnan *et al.,* 2009; Sebé-Pedrós *et al.,* 2010; Adamska *et al.,* 2011; Knoll, 2011). Tais moléculas, provavelmente, possuíam outras funções antes do surgimento dos animais, tendo sido cooptadas para as funções atuais e permitiram, pela sua diversificação e aumento de complexidade, a origem de novos tipos de organização, planos corporais e morfologias (King *et al.,* 2003; Nielsen, 2008; Knoll, 2011).

Outras características importantes herdadas do ancestral dos animais, como a celularização (ausente na maioria dos fungos) e a ausência de parede celular (presente em fungos e plantas) determinaram os potenciais sinergismos e conflitos nas interações entre células animais (Buss, 1987). Assim, o advento da multicelularidade animal trouxe vantagens, facilitou o surgimento de outras novidades e promoveu desafios a serem ultrapassados. O aumento imediato de tamanho provavelmente conferiu vantagens ecológicas, permitiu a diferenciação celular, especialização e divisão de trabalho, assim

como impôs a adesão, interação e comunicação entre as células, enquanto que a necessidade de reconhecer outros organismos geneticamente diferentes e impedir a proliferação de linhagens traiçoeiras, evitando conflitos celulares internos, mostraram-se como desafios durante a origem e o desenvolvimento dos animais (Abedin & King, 2010; Bonner, 1998, 2003; Knoll, 2011). Entre os animais atuais, as esponjas (Filo Porifera) são o grupo mais antigo, podendo nos indicar como ocorreu o desenvolvimento do sistema de integração que permitiu a vida metazoária.

O Filo Porifera Grant, 1836

Origem

O Filo Porifera abriga os organismos multicelulares popularmente conhecidos como esponjas. Elas são consideradas os animais mais antigos ainda vivos e o ramo mais basal dentre todos os metazoários atuais (Philippe *et al.,* 2009). No entanto, possivelmente devido à dificuldade de fossilização destes organismos e aos poucos estudos focando em esponjas, o grupo apresenta um registro fóssil muito incompleto (Pisera, 2006). Apesar disso, estudos apontam para a presença de esponjas desde o Neoproterozóico (entre 540 milhões e um bilhão de anos atrás), antes mesmo da grande diversificação de formas corporais dos animais evidenciado no Cambriano. Fósseis de organismos com uma organização corporal semelhante às esponjas foram encontrados em estratos datando entre 600 e 635 milhões de anos atrás (Maloof *et al.*, 2010; Yin *et al.,* 2015), mas estudos utilizando relógio molecular e datação de biomarcadores colocam a origem das esponjas há cerca de 240 milhões de anos antes (período Criogeniano, entre 850 e 630 milhões de anos atrás) do seu aparecimento no registro fóssil (Love *et al.,* 2009; Sperling *et al.*, 2010).

Plano corporal e funcionamento

As esponjas são animais exclusivamente aquáticos (marinhos ou dulciaquícolas), sésseis que apresentam uma estrutura corporal simples, sem órgãos, sistema nervoso ou cavidade digestiva (Ereskovsky, 2010; van Soest *et al.,* 2012). Estes organismos são filtradores ativos (exceto pelas esponjas carnívoras; Vacelet & Boury-Esnault, 1995) e utilizam uma única camada de células flageladas, os coanócitos, para promover um fluxo unidirecional de água através de um sistema de canais, o sistema aquífero, até uma abertura, o ósculo (Bergquist, 1978). É por meio dessa corrente de água que as esponjas

obtêm seu alimento, realizam trocas gasosas, liberam excretas e elementos reprodutivos (Ereskovsky, 2010).

As esponjas apresentam uma enorme plasticidade dos seus elementos celulares. Por não possuírem membrana basal (presente apenas em uma classe do filo - Homoscleromorpha), as células das esponjas apresentam grande mobilidade, podendo se deslocar de seu local de origem para qualquer parte do corpo do animal (Bergquist, 1978; Simpson, 1984). Além dos coanócitos, as esponjas possuem tipos celulares especializados em diversas outras funções. Os pinacócitos revestem a superfície interna dos canais e externa da esponja, delimitam o organismo externamente, são responsáveis pela adesão ao substrato e, provavelmente, pelo reconhecimento quando ocorre contato com outros indivíduos da mesma ou de outras espécies (Van de Vyver, 1970; Simpson, 1984). Os esclerócitos são responsáveis pela produção das espículas, os elementos que compõem o esqueleto mineral das esponjas. Este esqueleto pode ou não (dependendo da classe da esponja) ser complementado ou substituído por fibras de colágeno (espongina) produzidas pelos espongócitos. Entre a coanoderme e a pinacoderme está o mesoílo onde se localizam células secretoras e de reserva, além de células altamente móveis, tais como os arqueócitos (Simpson, 1984). O auge da grande plasticidade celular das esponjas, provavelmente está na sua totipotência e capacidade de transdiferenciação, ou seja, a capacidade de se diferenciar e rediferenciar em qualquer outro tipo celular, inclusive gametas (Simpson, 1984; Funayama, 2010, 2012).

Apesar de serem organismos antigos e apresentarem um plano corporal simples comparado a outros animais, as esponjas compartilham com os outros metazoários um extenso repertório de genes responsáveis pelas mais diversas funções. Representantes de famílias de genes responsáveis pela adesão e sinalização celular, desenvolvimento de sistema nervoso em bilatérios, reconhecimento celular e resposta imune, mecanismos de controle do ciclo e morte celular programada, fatores de transcrição, complexos de proteínas responsáveis pela formação de polaridade, além de vias relacionadas à morfogênese e especialização de tipos celulares (Larroux *et al.*, 2006; Nichols *et al.*, 2006; Srivastava *et al.*, 2010; Adamska *et al.*, 2011) são alguns exemplos do grande arcabouço genético que as esponjas compartilham com os outros metazoários. Acredita-se que algumas destas famílias de genes possam ter surgido junto com o advento da multicelularidade e se expandido após a separação entre esponjas e eumetazoários. Portanto, a diversidade de formas e planos corporais que observamos atualmente entre os

diversos grupos de Metazoa seja talvez mais uma questão de diferenças quantitativas do que qualitativas em genes regulatórios (Srivastava *et al.,* 2010; Adamska *et al.*, 2011).

Diversidade e ecologia

Apesar de haver debates acerca da origem única das esponjas (por exemplo, Nielsen, 2008) e das relações entre os grupos mais internos, o filo Porifera é atualmente considerado um grupo monofilético, representado por cerca de 8.700 espécies divididas em quatro classes: Demospongiae, Homoscleromorpha, Hexactinellida e Calcarea (Van Soest *et al.,* 2016). Dentro dessa diversidade de quase nove mil espécies divididas em quatro classes, as esponjas apresentam diferenças na composição do esqueleto, tipos de sistemas aquíferos (Figura 1), além de uma enorme variedade de morfologias externas, estratégias de vida, de ocupação de ambientes e interações com outros organismos (Tabela 1; Figura 2).

As esponjas ocupam praticamente todos os ambientes aquáticos ao longo de todas as latitudes: desde a zona entre marés, poças de maré, costões rochosos, recifes, cavernas submarinas até profundidades abissais (8840m); e massas de água salobra e doce como manguezais, rios e lagos internos e de altitude (Van Soest *et al.,* 2012). Nesses ambientes, elas desempenham papeis ecológicos importantes para a comunidade bentônica se associando a micro-organismos (Taylor *et al.,* 2007), servindo de proteção e alimento para outros animais (Wulff, 2006), promovendo a ciclagem e disponibilidade de compostos e matéria orgânica dissolvida (Diaz & Rützler, 2001; Maldonado *et al.,* 2012; de Goeij *et al.,* 2013), atuando como agentes de distúrbio através da bioerosão e competição por espaço, servindo de substrato e permitindo o aumento da heterogeneidade e biodiversidade de habitats pouco complexos (Diaz & Rützler, 2001; Buhl-Mortensen *et al.,* 2009; Bo *et al.,* 2012) e na criação, estabilização e consolidação do substrato bentônico (Bell, 2008). A enorme plasticidade das esponjas permitiu a ocupação de diversos ambientes e o desenvolvimento de uma enorme gama de hábitos de vida e morfologias. Em regiões abissais, extensos "jardins de esponjas" formados por esponjas hexactinelidas ou demosponjas são conhecidos e geram substrato para outros organismos, além de promover o aumento da diversidade local e heterogeneidade geográfica (Maldonado *et al.,* 2015). O hábito carnívoro em esponjas que apresentam redução total ou parcial do sistema aquífero é considerado uma adaptação evolutiva a ambientes oligotróficos e é restrito a uma família de demosponjas (Hestetun *et al.,* 2016). As esponjas são os componentes mais diversos e abundantes em ambientes recifais e costões rochosos, onde podemos encontrar desde

espécies de hábito perfurante, medindo milímetros de espessura, até metros de altura, com as mais diversas cores e morfologias externas, inclusive variando dentro da mesma espécie ou gênero (Figura 2; Diaz & Rützler, 2001; Van Soest *et al.*, 2012; Ruiz *et al.*, 2014; Zea *et al.*, 2014).

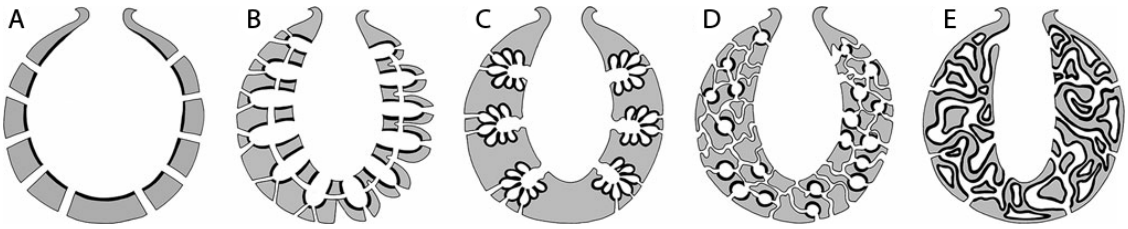

Figura 1: Tipos de sistemas aquíferos encontrados no Filo Porifera. (A) Asconóide, (B) Siconóide, (C) Sileibide, (D) Leuconóide, (E) Solenóide. As linhas pretas grossas representam a coanoderme. (Modificado de Cavalcanti & Klautau, 2011).

Tabela 1: Principais características das quatro classes do filo Porifera. Fontes: (1) Hooper & van Soest, 2002; Ereskovsky, 2010; (2) Gazave *et al.*, 2012; (3) Reiswig, 2002; (4) Manuel *et al.*, 2002. (*) número de espécies e representatividade (%) baseado em Van Soest *et al.*, 2016.

	Demospongiae[1]	Homoscleromorpha[2]	Hexactinellida[3]	Calcarea[4]
N° de espécies (%)*	7.264 (83,5%)	103 (1,2%)	618 (7,1%)	715 (8,2%)
Ambientes	Marinho e água doce	Marinho (águas rasas)	Marinho (águas profundas)	Marinho
Sistema aquífero	Leuconóide e semelhante à sileibide	Leuconóide e semelhante à sileibide	Leuconóide e semelhante à siconóide	Asconóide, siconóide, sileibide, leuconóide e solenoide (Figura 1)
Esqueleto	Quando presente é formado por espículas e/ou fibras de espongina	Espículas ou ausente	Espículas, podendo ser fusionadas ou não	Espículas
Espículas	Sílica	Sílica	Sílica	Carbonato de cálcio
Estratégia reprodutiva	Ovípara e vivípara	Vivípara	Vivípara	Vivípara
Desenvolvimento	Direto e indireto	Indireto	Indireto	Indireto
Tipo(s) larval(is)	Parênquimela, disférula ou coeloblástula	Cinctoblástula	Triquimela	Anfiblástula (Calcaronea) e Calciblástula (Calcinea)
Outras características	Algumas espécies apresentam esqueleto basal hipercalcificado ou de aragonita com espículas fusionadas	Presença de junções celulares do tipo *zonula adherens* e membrana basal sob a pinacoderme e coanoderme. Endo- e exopinacócitos flagelados	Membrana derma e coanoderme sincicial	Algumas espécies apresentam esqueleto basal calcário não espicular onde espículas podem estar aderidas ou embebidas

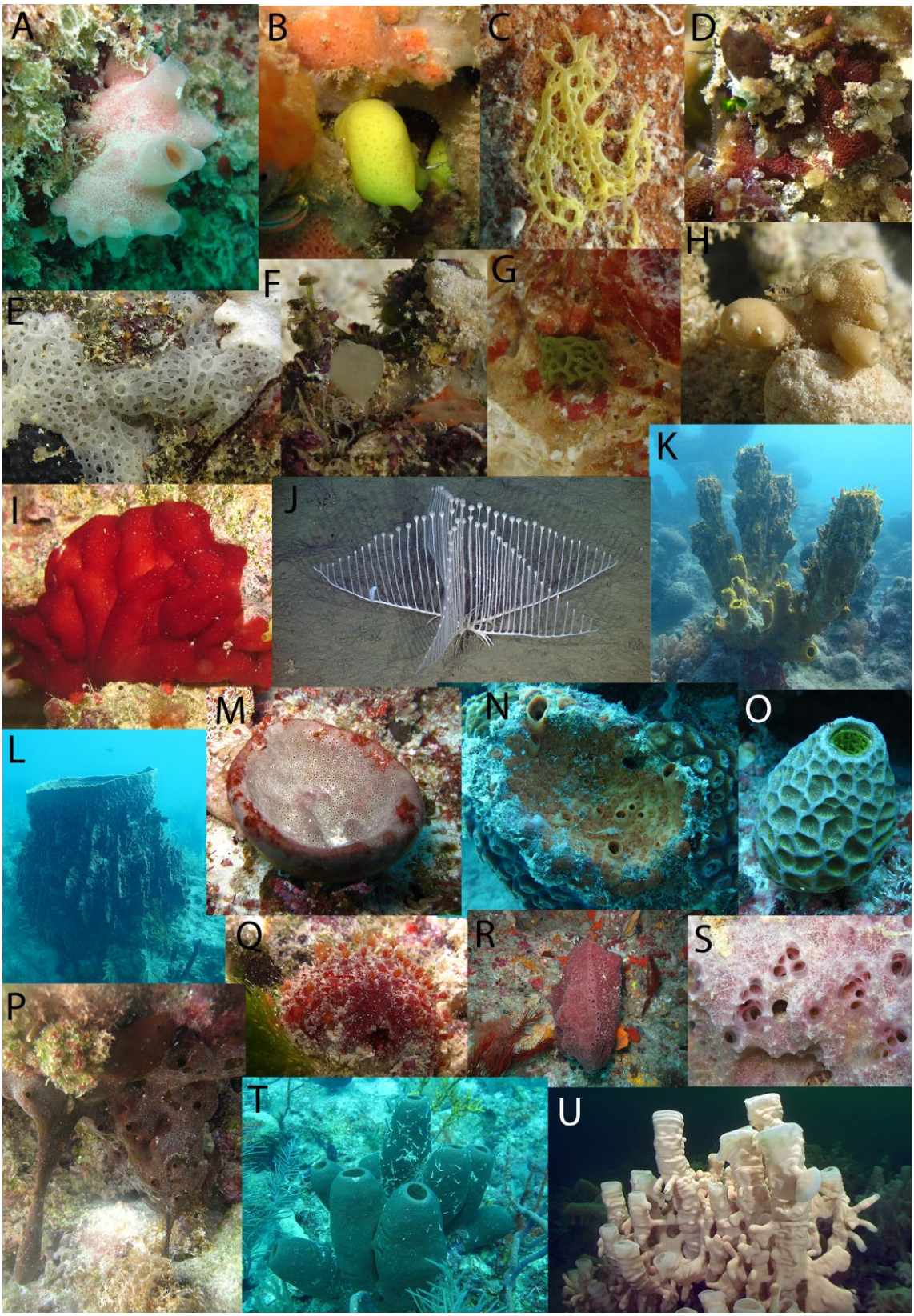

Figura 2: Diversidade de formas encontradas no Filo Porifera. (A-H) Classe Calcarea; (I) Classe Homoscleromorpha; (J-T) Classe Demospongiae; (J) a esponja carnívora *Chondrocladia lyra*; retirado de Lee *et al.*, 2012; (U) Classe Hexactinellida (retirado de www.spawsbc.org); Fotos: (A-I e K-T), autoria de André Padua.

Reprodução e diversidade genética nas populações

Esponjas não possuem gônadas e, portanto, não apresentam a separação de uma linhagem germinativa no início do seu desenvolvimento exclusivamente para produzir os seus elementos reprodutivos. Sendo assim, os gametas se originam a partir das células somáticas de forma difusa no corpo do animal (Ereskovsky, 2010). Além disso, como a maioria dos animais que não apresentam separação de linhagem germinativa no início de seu desenvolvimento, as esponjas são capazes de realizar reprodução assexuada (Buss, 1983, 1987; Blackstone & Jasker, 2003).

Em relação à reprodução sexuada, as espécies podem ser hermafroditas ou gonocóricas, apresentando, em sua grande maioria, desenvolvimento indireto com a produção de uma larva livre-natante. Existem espécies ovíparas, com fertilização e desenvolvimento externos, e espécies vivíparas, com fertilização interna, incubação e posterior liberação da larva (Maldonado & Bergquist, 2002; Maldonado & Riesgo, 2008). No entanto, larvas de esponjas são lecitotróficas, ou seja, se alimentam do próprio vitelo armazenado, além disso, elas costumam ter pouco tempo de duração na coluna d'água e, consequentemente, apresentam uma baixa capacidade de dispersão com consequente comportamento filopátrico e populações com altos índices de endocruzamento (Maldonado & Bergquist, 2002; Maldonado, 2006).

A reprodução assexuada em esponjas se dá principalmente por brotamento, gemulação ou fragmentação (Fell, 1993). No primeiro caso, massas de células se formam no interior da esponja, são revestidas por colágeno e levadas para a superfície da esponja, de onde se destacam, aderem ao substrato e originam um novo indivíduo, enquanto as gêmulas são estruturas de resistência formadas principalmente por arqueócitos e células de reserva que são envoltos em uma estrutura de proteção (geralmente contendo espículas e material colagenoso). As gêmulas são encontradas principalmente em espécies de água doce e são fundamentais para a sobrevivência da espécie quando as condições ambientais são desfavoráveis (Simpson, 1984; Fell, 1993; Maldonado & Riesgo, 2008). Fragmentação pode ser tanto um evento endógeno controlado pela esponja quanto ocasionada acidentalmente. Quando associada à reprodução sexuada, pode auxiliar inclusive na dispersão e colonização de novos ambientes, bem como no aumento da variabilidade genética das populações (Wulff, 1991; Maldonado & Uriz, 1999).

Populações de esponjas costumam apresentar alta estruturação genética (independente do marcador molecular utilizado), provavelmente devido à alegada baixa capacidade de dispersão das larvas (Van Oppen *et al.*, 2003; Boury-Esnault & Solé-Cava,

2004). Nesses casos de populações estruturadas, espera-se observar uma baixa variabilidade genética e altos índices de homozigose. No entanto, não é isso que se tem observado nas populações de esponjas, que sempre apresentam elevada variabilidade genética e excesso de indivíduos heterozigotos (Solé-Cava & Thorpe, 1994; Boury-Esnault & Solé-Cava, 2004). Já foi sugerido, por exemplo, que a fusão durante os estágios larval, juvenil ou adulto de indivíduos geneticamente diferentes (Solé-Cava & Thorpe, 1994; Boury-Esnault & Solé-Cava, 2004; Blanquer & Uriz, 2011), seria uma possível explicação para este padrão de alta estruturação populacional aliado à alta diversidade genética e ao excesso de heterozigotos.

Sobre indivíduos de esponjas

Populações são compostas por indivíduos. Portanto, estudar as particularidades de indivíduos de organismos tão plásticos como as esponjas nos ajuda a compreender como estes interagem, inserem-se no ambiente, relacionam-se com outros de sua mesma espécie e como isso influencia processos em escalas ecológica e evolutiva a curto, médio e longo prazos.

Apesar de parecer uma definição simples e trivial, especialmente para outros grupos animais, durante muito tempo a ausência de uma definição formal e prática do que representa um indivíduo de esponja utilizando critérios claros levou a diversas definições por parte de vários autores. Durante o século XIX, esponjas foram basicamente consideradas organismos coloniais. No entanto, diversas interpretações diferentes foram levantadas acerca do que representaria a unidade (indivíduo ou zoóide) dentro da "colônia": células amebóides, coanócitos, câmaras coanocitárias ou o ósculo (Hartman & Reiswig, 1973; Fry, 1979; Ereskovsky, 2003). A ideia de que esponjas seriam organismos modulares, formadas por uma "população de módulos aquíferos", representados por um volume de esponja composto por canais inalantes e exalantes e câmaras coanocitárias conectadas a um único ósculo, também foi sugerida (Fry, 1970, 1979). O termo "colônia" foi utilizado durante muito tempo, inclusive em meados do século XX, principalmente para as esponjas com mais de um ósculo, enquanto esponjas monosculares eram consideradas indivíduos (Brien, 1967; Korotkova, 1963, 1970; ver também: Simpson, 1973; Hartman & Reiswig, 1973; Fry, 1979; Ereskovsky, 2003). No entanto, tal definição é inadequada, pois as esponjas apresentam enorme plasticidade morfológica, com constante reorganização do sistema aquífero e migração de seus elementos celulares (Simpson, 1963; Bond, 1992), sincronização de eventos da gametogênese (Maldonado &

Riesgo, 2008) e capaz de movimentos coordenados e resposta a estímulos mecânicos e ambientais (Leys *et al.*, 1999; Nickel, 2006) em um mesmo organismo. Além disso, um mesmo indivíduo pode apresentar variação no número de ósculos ao longo do seu ciclo de vida (Hartman & Reiswig, 1973; Fry, 1979; Gaino *et al.*, 1995) e, nos casos de algumas esponjas carnívoras, os elementos do sistema aquífero (coanócitos, câmaras, canais e ósculo) podem estar ausentes (Vacelet & Boury-Esnault, 1995). Portanto, outra definição se fez necessária. Atualmente um indivíduo de esponja é definido por uma pinacoderme contínua, ou seja, é composto por todos os componentes celulares englobados dentro de um revestimento celular (pinacoderme) comum (Borojevic *et al.*, 1968). Assim definidos, os indivíduos de esponjas são reconhecidos como unidades morfológicas e funcionais coesas. Pode-se acrescentar a isso um fator imunológico, uma vez que a capacidade de reconhecer outros indivíduos da mesma espécie (alorreconhecimento) está presente nesses organismos (Curtis, 1979; Gaino *et al*., 1999).

No entanto, devido à sua enorme plasticidade morfológica e dos elementos celulares, a individualidade nas esponjas se manifesta de forma diferente dos outros animais. Por exemplo, sabe-se que grande parte das esponjas realizam fragmentação e fusão dos seus corpos e são capazes de reconhecer outros indivíduos geneticamente diferentes da mesma ou de outras espécies quando ocorre contato. Além disso, esponjas também possuem uma enorme capacidade regenerativa, reconstituindo partes perdidas do corpo ou até mesmo reconstruindo um novo (ou vários) indivíduo(s) a partir de células dissociadas. No entanto, essas características variam muito entre as diferentes espécies de esponjas e mesmo dentro da mesma espécie, em alguns casos estando relacionadas à forma do corpo do indivíduo. Nessa tese abordaremos alguns eventos observados em indivíduos de esponjas da classe Calcarea e que colocam o entendimento da individualidade destes organismos à prova: regeneração de partes do corpo, reagregação celular e formação de novos indivíduos, fragmentação, fusão e reconhecimento de indivíduos geneticamente diferentes.

Regeneração e reagregação celular

A regeneração de tecidos ou partes do corpo é observada em todos os filos animais e é considerada uma característica intrínseca e fundamental à vida (Sanchez-Alvarado, 2000; Carlson, 2007). No entanto, apesar de amplamente presente entre os metazoários, é um processo bastante heterogêneo, com muitas diferenças entre os meios pelos quais a regeneração é atingida (Carlson, 2007; Brockes & Kumar, 2008; Kürn *et al.*, 2011;

Ereskovsky *et al.,* 2015). A regeneração de tecidos, partes do corpo e até organismos completos é especialmente marcante entre os invertebrados mais basais. Nestes organismos, regeneração e reprodução assexuada (tais como brotamento e fragmentação) são eventos muitas vezes semelhantes, complementares e sobrepostos, provavelmente utilizando cascatas de mecanismos moleculares semelhantes (Sanchez-Alvarado, 2000; Carlson, 2007).

Dentre os invertebrados marinhos, as esponjas provavelmente figuram entre os animais com maior capacidade e potencial de regeneração (Simpson, 1984). Esta alta capacidade regenerativa das esponjas talvez esteja relacionada ao fato de estes serem organismos sésseis e não possuírem, em sua maioria, estruturas externas rígidas (como conchas e escamas) para proteção contra ferimentos, tornando a regeneração um evento permanente ao longo de sua vida. A regeneração é também muito importante para aquelas espécies que vivem em ambientes altamente dinâmicos e onde o risco de danos devido à predação e movimentação da água é intenso, podendo comprometer atividades vitais do organismo e sua sobrevivência (Jackson & Palumbi, 1979; Ayling, 1983; Hoppe, 1988; Korotkova, 1997; Borisenko *et al.,* 2015).

A capacidade regenerativa das esponjas é conhecida há muito tempo. Há cerca de 2400 anos, o filósofo grego Aristóteles já havia notado a capacidade que as esponjas possuem de reconstituir partes perdidas do seu corpo (Aristóteles, *Historia Animalium* 548b18; Voultisiadou, 2007). É neste conhecimento e grande potencial que a indústria de pesca e fazendas de esponjas se baseia. Esta indústria iniciou na Grécia antiga e, posteriormente, se difundiu para todo o Mediterrâneo, Polinésia e Caribe (Pronzato, 1999). No entanto, foi somente nos últimos 60 anos que a capacidade e o processo de regeneração de partes do corpo de indivíduos adultos de esponjas de mais de 30 espécies com as mais diferentes formas de crescimento e linhagens evolutivas foram estudados.

O processo de regeneração em esponjas é influenciado por fatores intrínsecos e extrínsecos ao animal, tais como a morfologia externa e características do ferimento e do ambiente, por exemplo (Henry & Hart, 2005). Ele é também independente do crescimento normal de uma dada espécie, resultando em taxas de regeneração muito maiores do que as de crescimento, além disso, não parece ter qualquer relação filogenética, ou seja, espécies próximas (de um mesmo gênero, por exemplo) podem apresentar taxas de regeneração muito diferentes (Ayling, 1983; Wulff, 2010). A existência de uma morfologia externa bem definida parece ser um fator crucial para a regeneração de esponjas, especialmente quando partes essenciais ao seu funcionamento e sobrevivência são afetados (Jackson,

1979; Bell, 2002). A questão da forma externa já foi também relacionada ao "nível de integração e de individualidade" da esponja, influenciando na velocidade, capacidade de reconstituir partes perdidas do corpo e na variabilidade do resultado final do processo (Korotkova, 1963, 1970). No entanto, em última instância, o processo de regeneração é operado a nível celular, onde as esponjas apresentam uma enorme plasticidade. A ausência de tecidos verdadeiros (exceto em Homoscleromorpha), permitindo que células de diferentes partes do corpo migrem para o local afetado, e a capacidade de transdiferenciação em outros tipos celulares, mostram-se essenciais para a regeneração e tornam este processo altamente rápido e eficiente nas esponjas (Borisenko *et al.*, 2015; Ereskovsky *et al.,* 2015).

No entanto, a enorme capacidade de regeneração de esponjas adultas não se restringe a partes perdidas do corpo. Talvez a maior manifestação da grande plasticidade das esponjas seja sua capacidade de reagregação celular e formação de um novo indivíduo após dissociação. Esta capacidade foi demonstrada pela primeira vez *in vitro* por Wilson (1907) que foi seguido por diversos autores estudando o processo de reagregação celular e reconstrução de um indivíduo funcional em diversas espécies. O processo de reconstituição de esponjas funcionais a partir de células dissociadas passa por alguns estágios morfogenéticos que são essenciais. O primeiro estágio é o de reagregação celular, seguido do estágio de revestimento desses agregados por uma camada de pinacócitos separando os meios interno e externo. Tais agregados revestidos por pinacoderme foram chamados primeiramente por termos genéricos como "esférula" ou "agregados" e depois receberam o nome de diamorfos (Borojevic *et al*., 1968). Esses diamorfos representam o estágio entre o final da agregação celular e o início do seu processo de reorganização em uma esponja funcional (Borojevic *et al*., 1968; Borojevic, 1971). Posteriormente, os diamorfos passaram a ser chamados de primorfos (Custódio *et al.,* 1998) e têm sido utilizados como alvo de estudos de espiculogênese e morfogênese (Le Pennec *et al.,* 2003; Zhang *et al.,* 2003b; Krasko *et al.,* 2004; Valisano *et al.,* 2012), reconhecimento (Van de Vyver, 1975; Johnston, 1990; Yin & Humphreys, 1996; Custódio *et al.,* 2004), diferenciação celular (Borojevic & Levi, 1964; Ereskovsky *et al*., 2015) e obtenção de produtos naturais (Müller *et al.,* 2000; Thakur *et al.,* 2003).

Os estágios seguintes à formação do primorfo (diamorfo) são a adesão ao substrato e reorganização celular para a reconstituição de um indivíduo com sistema aquífero funcional (Eerkes-Medrano *et al.,* 2014; Lavrov & Kosevich, 2014). No entanto, a agregação celular, formação de primorfos, adesão ao substrato e posterior organização do

indivíduo não parecem ser etapas simples de serem alcançadas, uma vez que poucos trabalhos observaram a reconstituição completa *in vitro* de uma esponja (Lavrov & Kosevich, 2014). Vários fatores como a saúde e o estágio do ciclo de vida da esponja (Valisano *et al.,* 2006), a composição e concentração de células em suspensão (De Sutter & van de Vyver, 1977; Buscema *et al.,* 1980; Sipkema *et al.,* 2003; Zhang *et al.,* 2003a, 2003b), temperatura (Zhang *et al.,* 2003a; Valisano *et al.,* 2007; Chergonor *et al.,* 2001), presença de micro-organismos e composição do meio de cultura (Pomponi & Willoughby, 1994; Osinga *et al.,* 1999; Le Pennec *et al.,* 2003; Sipkema *et al.,* 2003; Zhang *et al.,* 2004; Pomponi, 2006; De Caralt *et al.,* 2007; Valisano *et al.,* 2007) podem contribuir, comprometer ou afetar o alcance de cada uma das etapas da reconstituição da esponja a partir de células dissociadas (Lavrov & Kosevich, 2014).

No entanto, os processos que ocorrem durante a regeneração de partes perdidas do corpo e a reconstituição de indivíduos a partir de tecidos ou células dissociadas apresentam diferenças, mas parecem ocorrer por processos semelhantes. Em ambos os casos, uma intensa movimentação de células com eventos de transdiferenciação em outros tipos celulares é observada (Carlson, 2007; Tanaka & Reddien, 2011; Chergonor *et al.,* 2011; Ereskovsky *et al.,* 2015). Entre os animais, enquanto a regeneração é um evento observado em todos os filos animais e pode ocorrer por diversos processos diferentes, geralmente com a ação de tipos celulares específicos (Carlson, 2007; Tanaka & Reddien, 2011), a reconstituição de indivíduos é observada principalmente em animais capazes de reprodução assexuada e que não apresentam separação precoce da linhagem germinativa (Buss, 1983, 1987) e é caracterizada por uma intensa desintegração e desorganização das relações estabelecidas entre as células, tecidos e do próprio organismo como um todo, tendo sido chamada de embriogênese somática no caso de esponjas (Tokin, 1963).

Fragmentação, fusão, alorreconhecimento e quimerismo

A fragmentação e fusão de indivíduos são eventos considerados presentes e comuns no ciclo de vida de todas ou, pelo menos, da maioria das espécies de esponjas, independente da classe a qual pertence (Johnson, 1979; Fell, 1993; Wulff, 1991; Gaino *et al.,* 1991, 1996; Tsurumi & Reiswig, 1997). Fragmentação pode ser tanto um evento controlado pela esponja, e atuar como uma estratégia de dispersão, ocupação do espaço ou controle de patógenos, como pode ocorrer acidentalmente por ação mecânica externa (Reiswig, 1973; Wulff, 1991, 1995; Zilberberg *et al.*, 2006a; 2006b; Blanquer *et al.*, 2008). Já a fusão pode oferecer vantagens ecológicas e evolutivas aos indivíduos

(Grosberg, 1988) e ocorre pelo contato entre as partes, o que pode ser muito frequente em ambientes dinâmicos e com grande competição por espaço, como costões rochosos.

Em ambos os casos, geralmente assume-se que tanto os produtos da fragmentação quanto os indivíduos que fusionam sejam geneticamente idênticos. No entanto, casos de heterogeneidade genética intraorganísmica (IGH, do inglês *intraorganismal genetic heterogeneity*) são reportados frequentemente em todos os Reinos e podem ser gerados por fusão de indivíduos, mutações ao longo da vida ou pela replicação de linhagens clonais (Buss, 1982; Lushai & Loxdale, 2002; Loxdale & Lushai, 2003; Pineda-Krch & Lehtilä, 2004; Santelices, 2004; Rinkevich, 2011). Os dois principais tipos de IGH são o mosaicismo e o quimerismo, diferindo principalmente na origem da heterogeneidade genética. Enquanto quimeras são geradas a partir da fusão de duas ou mais linhagens celulares geneticamente diferentes, mosaicos se originam a partir de mudanças genéticas, tais como mutações, conversões gênicas, duplicações de genes e alterações no número de cromossomos. A ocorrência de IGH pode trazer vantagens como maior variabilidade genética para os indivíduos e para a população, maior resistência frente a alterações no ambiente e a patógenos ou problemas como parasitismo e conflitos entre linhagens celulares (Pineda-Krch & Lehtilä, 2004; Rinkevich, 2011).

Mecanismos de reconhecimento tanto de linhagens co-específicas geneticamente diferentes (alorreconhecimento) quanto de espécies diferentes (xenorreconhecimento), são comumente observados entre invertebrados marinhos sésseis, como esponjas, cnidários e ascídias. Tais mecanismos são ativados pelo contato e geram uma resposta imune a fim de manter a integridade dos indivíduos e evitar a fusão entre genótipos diferentes e os possíveis danos do quimerismo (Grosberg, 1988; Feldgarden & YUND, 1992; Brusini *et al.*, 2014). Apesar de a resposta alo-imune ser, geralmente, muito precisa, a ocorrência de quimeras é comumente reportada entre organismos marinhos sésseis, sugerindo que apesar de apresentar grandes custos com consequências evolutivas (Buss, 1982; Rinkevich & Weissman, 1987), existam formas de compensá-los (Foster *et al.*, 2002). No filo Porifera, já foi sugerida a existência de quimeras formadas por duas espécies (Little, 1966) e recentemente foi reportada pela primeira vez a ocorrência de IGH (quimerismo ou mosaicismo) em uma espécie de demosponja (*Scopalina lophyropoda* Schmidt, 1862) que apresenta frequentes eventos de fragmentação e fusão (Blanquer & Uriz, 2011). Neste caso, a presença de IGH em vários indivíduos de uma população foi considerada a principal causa da grande variabilidade genética observada nessa espécie (Blanquer & Uriz, 2010, 2011). Tanto em esponjas como em outros invertebrados marinhos sésseis, a

formação natural de quimeras é atribuída principalmente a dois fatores: fusão nos estágios larval ou juvenil (Ilan & Loya, 1990; Maldonado, 1998; Bishop & Sommerfeldt, 1999; Barki *et al.,* 2002; McGhee, 2006; Gauthier & Degnan, 2008) e alto polimorfismo de alelos em genes de histocompatibilidade, permitindo a fusão apenas entre indivíduos que possuam alelos específicos, sejam cepas ou parentes próximos (Van de Vyver, 1970; Kaye & Ortiz, 1981; Neigel & Avise, 1985; Grosberg & Quinn, 1986, 1988; Rinkevich *et al.*, 1995; Fernandez-Busquets & Burger, 1999).

Entre as esponjas, apesar de haver variabilidade intraespecífica na intensidade e tempo de resposta das reações, tanto o alorreconhecimento quanto o xenorreconhecimento são largamente observados entre indivíduos adultos (Hildemann *et al.,* 1980; Buscema & van de Vyver, 1983; Fernandez-Busquets & Burger, 1999; Gaino *et al.,* 1999). Diversos estudos utilizando enxertos de indivíduos mostraram a fusão de tecidos provenientes da mesma esponja e rejeição entre espécies e indivíduos diferentes (Van de Vyver, 1970; Van de Vyver & De Vos, 1979; Hildemann *et al.,* 1980; Kaye & Ortiz, 1981; Jokiel *et al.*, 1982; Bigger *et al.,* 1983; Buscema & van de Vyver, 1983, 1984a, 1984b, 1984c, 1985; Johnston & Hildemann, 1983; Van de Vyver, 1983; Van de Vyver & Barbieux, 1983; Van de Vyver *et al.*, 1985; Amano, 1990; Ilan & Loya, 1990; Van de Vyver & Buscema, 1990; Mukai, 1992; Gaino *et al.,* 1999; Gauthier & Degnan, 2008; Saito, 2013). Geralmente, o contato de tecidos de esponjas diferentes gera reações de rejeição, ativadas por genes específicos (Müller *et al.,* 2002) e que se caracterizam principalmente por uma reação citotóxica ou pela formação de uma barreira de colágeno entre os enxertos, separando os dois indivíduos (Gaino *et al*., 1999). Acredita-se que a reação se inicie com o reconhecimento por parte dos pinacócitos, que formam a camada mais externa e delimitam o corpo do indivíduo, seguido da migração até o local de contato de outras células, tais como arqueócitos, para produzir uma barreira de material colagenoso (van de Vyver, 1970; van de Vyver & de Vos, 1979; Johnston & Hildemann, 1983; Buscema & van de Vyver, 1984a; Gaino *et al.,* 1999). No entanto, alguns estudos mostraram casos de fusão entre indivíduos diferentes, sugerindo que, assim como em outros invertebrados marinhos, a fusão ou rejeição estejam associadas a diferenças em *loci* específicos de histocompatibilidade, permitindo a fusão entre os indivíduos que possuam os mesmos alelos, ou a existência de cepas nas espécies (Van de Vyver, 1970; Kaye & Ortiz, 1981; Jokiel *et al.*, 1982; Neigel & Schmahl, 1984; Neigel & Avise, 1985; Amano, 1990; Fernandez-Busquets & Burger, 1999).

O reconhecimento e resposta ao contato entre indivíduos diferentes também podem ser observados quando ocorre a dissociação celular. É comum se observar inicialmente a mistura de células de indivíduos ou até mesmo de espécies diferentes. Nesses casos, ou o alorreconhecimento é readquirido ao longo do tempo, observando-se a separação das células e formação de agregados específicos ou reações citotóxicas acabam por matar as células de ambos os indivíduos ou espécies (Wilson, 1907; Galtsoff, 1923; de Laubenfels, 1927; Curtis, 1962; Humphreys, 1963, 1970; Johnston, 1990; Humphreys, 1994; Yin & Humphreys, 1996; Custódio *et al.*, 2004). No entanto, casos de não reação e permanência de agregados formados por duas espécies também já foram reportados (de Laubenfels, 1928; Curtis, 1962; Sarà, 1956; Sarà *et al.*, 1966; Curtis, 1970a, 1970b).

Espécies estudadas e organização da tese

Apesar de serem os animais mais basais ainda vivos, as esponjas possuem mecanismos que as permitem recuperar e manter a integridade do indivíduo (como a regeneração de partes perdidas do corpo e o reconhecimento de indivíduos diferentes), apesar de esta integridade poder ser totalmente desfeita (a partir da dissociação celular) e ser altamente plástica e passível de perturbações (por fusão e fragmentação de indivíduos). No entanto, muitos desses aspectos da individualidade das esponjas são amplamente estudados e discutidos em espécies da classe Demospongiae, havendo poucos estudos com esponjas das demais classes, incluindo a classe Calcarea.

Portanto, esta tese está dividida em quatro capítulos, escritos em forma de artigos científicos, e abordará a regeneração de partes perdidas do corpo, a capacidade de reagregação e formação de novos indivíduos a partir da dissociação celular, eventos de fragmentação e fusão de indivíduos adultos e o alorreconhecimento de células dissociadas em três espécies da classe Calcarea: *Ernstia* sp., *Clathrina aurea* Solé-Cava *et al.*, 1991 e *Paraleucilla magna* Klautau *et al.*, 2004.

Ernstia sp. (Clathrinidae, Clathrinida, Calcinea) é uma espécie provisoriamente endêmica das ilhas oceânicas brasileiras (Atol das Rocas (RN) e Abrolhos (BA); Azevedo *et al., in prep.*). Ela apresenta cor amarela e possui sistema aquífero asconóide formado por tubos densamente anastomosados e apresenta uma morfologia polarizada e bem definida, caracterizada por uma forma semi-globular com um único ósculo apical (Figura 3A).

Clathrina aurea (Clathrinidae, Clathrinida, Calcinea) é uma espécie amplamente distribuída na costa do Brasil, sendo encontrada desde o Rio Grande do Norte até Santa

Catarina (Muricy *et al.*, 2011). Ela apresenta cor amarela, possui sistema aquífero asconóide com tubos frouxamente anastomosados e é comumente encontrada em ambientes protegidos e com pouca incidência de luz, tais como paredes e tetos de pequenas tocas (Klautau & Borojevic, 2001; Monteiro & Muricy, 2004; Figura 3B). Suas populações no Sul e Sudeste do Brasil se mostram altamente estruturadas, mas com alta diversidade genética e excesso de indivíduos heterozigotos (Padua, 2012). Apesar da abundância e ampla distribuição observada, elementos reprodutivos foram raramente observados nessa espécie (Lanna & Klautau, 2015). Como eventos de fragmentação e fusão são conhecidos e frequentes em outras espécies do mesmo gênero ou de gêneros próximos (Johnson, 1979; Gaino *et al.*, 1991, 1996), existe a possibilidade da alta variabilidade genética nessa espécie estar relacionada à formação de quimeras, por exemplo.

Paraleucilla magna Klautau et al., 2004 (Amphoriscidae, Leucosolenida, Calcaronea) é amplamente distribuída nas costas Sul e Sudeste do Brasil e também no Mar Mediterrâneo, especialmente próximo a regiões portuárias e fazendas de mexilhões, o que a leva a ser considerada uma espécie introduzida nesses locais (Longo *et al.*, 2007; Cavalcanti, 2013). Tanto no Brasil quanto no Mar Mediterrâneo, as populações de *P. magna* são altamente estruturadas e apresentam alta diversidade genética (Cavalcanti, 2013; Guardiola *et al.*, 2013). Ela possui sistema aquífero leuconóide e não apresenta forma externa definida, podendo apresentar um único ou diversos ósculos no mesmo indivíduo (Figura 3C). Nessa espécie nunca foram observados eventos de fragmentação e fusão de indivíduos (Cavalcanti *et al.*, 2013; Guardiola *et al.*, 2013).

Figura 3: Espécies estudadas. (A) *Ernstia* sp. (B) *Clathrina aurea* (C) *Paraleucilla magna*; no detalhe, indivíduo com vários ósculos.

OBJETIVOS

O objetivo geral desta tese é:

Avaliar como indivíduos de esponjas da classe Calcarea apresentam e mantêm sua integridade e individualidade.

Os objetivos específicos (de cada capítulo) são:

CAPÍTULO 1: Estudar a capacidade regenerativa de uma espécie de esponja calcárea (*Ernstia* sp.) com sistema aquífero asconóide e forma do corpo polarizada em relação a duas partes diferentes do indivíduo: ósculo e corpo.

CAPÍTULO 2: Estudar a formação de primorfos em duas espécies de esponjas calcáreas (*Paraleucilla magna* e *Clathrina aurea*) e o posterior desenvolvimento em indivíduos funcionais.

CAPÍTULO 3: Investigar a possível presença e formação de IGH em indivíduos adultos de *Clathrina aurea*, acompanhando sua dinâmica de fragmentação e fusão, além de avaliar aspectos da sua história de vida, tais como longevidade e tamanho.

CAPÍTULO 4: Investigar a ocorrência de IGH *in situ* em *P. magna* e se existe alorreconhecimento após a dissociação celular de *P. magna* e *C. aurea*.

CAPÍTULO 1

TÍTULO: Regeneration in calcareous sponges (Porifera)

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Regeneration in calcareous sponges (Porifera)

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Wounds caused by predation and/or physical disturbances to sessile marine animals are common. Consequently, these organisms had to develop strategies to endure such injuries and survive in such a dynamic environment. Sponges are known to possess one of the greatest capacities of regeneration among living metazoans, but this feature has been largely studied only in Demospongiae. In Calcarea, very few species have been investigated. Hence, we analysed the regeneration and speed rates from two regions (osculum and choanosome) of the body of a calcareous sponge: Ernstia *sp. Only the osculum regenerated until the end of the experiment, while the choanosome simply cicatrized. Calcareous sponges seem to have a polarized regeneration closely related to their external morphology and level of individuality and integration. A brief review of the regeneration capacity in Calcarea is presented.*

Keywords: body symmetry, Calcarea, choanosome regeneration, *Ernstia*, osculum regeneration, polarization

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INTRODUCTION

The marine benthic environment is highly dynamic and its sessile fauna is constantly susceptible to physical injuries. These injuries, caused by predation or physical disturbances, may result in a decrease in the animal's feeding capacity and, if extensive, constitute a potential threat to survival (Jackson & Palumbi, 1979). Reconstitution and/or replacement of lost structures and tissues through regenerative processes are the way to recover from such harmful events. Regeneration can overlap and be complementary to asexual reproduction, but the first is involved in the development of a lost structure in a non-embryonic scenario, while the latter is involved in the modification of a part of the parent into a new and independent individual (Carlson, 2007; Kürn *et al.*, 2011). Among sessile marine invertebrates, sponges are known to possess great capacity for regeneration, maybe higher than any other living metazoan (Simpson, 1984; Duckworth, 2003). Regeneration is probably a permanent event in the life history of adult sponges. In highly dynamic habitats, for example, where the presence of grazers is ecologically significant, and in tropical waters, where fishes and turtles are specialist sponge-predators, this regeneration capacity seems to be vital to guarantee the survival of sponge species (Ayling, 1983; Hoppe, 1988).

There are many factors (intrinsic or extrinsic) that could influence positively or otherwise the regeneration rates in sponges, such as external morphology and place and size of the injury (for a review see Henry & Hart, 2005). For example, when a wound occurs in an individual with a well-defined morphology (e.g. tubular) or when it affects a vital body region/structure (e.g. osculum), the individual should act rapidly to restore its optimal shape, otherwise it could cause structural failure and death (Jackson, 1979; Bell, 2002). As it is a vital process for survival, regeneration rate is usually much faster than growth rate in sponges, reaching 2900 times growth velocity in some species (Ayling, 1983; Leys & Lauzon, 1998; Turon *et al.*, 1998; Wulff, 2010). It is also interesting to note that regeneration capacity is species specific and does not seem to follow any phylogenetic sense, meaning that even species with shared evolutionary history may present different regeneration rates (Wulff, 2010).

Although works concerning sponge regeneration have been published mainly in the last 30 years, the regenerative capacity of these animals has been known since the ancient Greek civilization, when the fishing tradition of bath sponges started and developed through the Mediterranean and later the Caribbean and Polynesia. Today, sponge-farming techniques continue to exploit the great capacity of these animals of regenerating a whole individual from a small fragment. This is even considered one of the solutions for the biotechnology industry seeking new metabolites and for the reconstitution of declining natural stocks due to disease and overfishing (Verdenal & Vacelet, 1990; Osinga *et al.*, 1999; Pronzato, 1999).

Since the pioneering work of Wilson (1907), where he observed and discussed the cell aggregation capacity of sponges, many experiments exploring this issue and the regeneration of lost parts were undertaken in the class Demospongiae. Discussions about the independence between regeneration and growth processes (Reiswig, 1973; Hoppe, 1988) and regeneration speed related to sponge morphology (Hoppe, 1988; Rohde & Schupp, 2012) took place. In this context, more than 30 demosponge species with the most diverse growth forms and from different evolutionary lineages have been studied since then (Reiswig, 1973; Ayling, 1983; Hoppe, 1988; Bell, 2002; Duckworth, 2003; Gilliam *et al.*, 2008; Wulff, 2010; Rohde & Schupp, 2012). Regeneration in

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hexactinellid sponges was studied and discussed in recent works (Leys & Lauzon, 1998; Leys *et al.*, 2007), while the class Calcarea was the focus of such interest mainly in works published in the 1960s and 1970s (Korotkova, 1961a, b, 1963a, b, 1969, 1970, 1972; Tuzet & Paris, 1963). Since then, to our knowledge, the calcareous sponges were neglected as the subject of regeneration studies. In contrast to the number of demosponges studied, only five species of calcareous sponges were investigated concerning their regenerative capacity: *Sycon lingua* (Haeckel, 1870), *S. raphanus* Schmidt, 1862, *S. ciliatum* (Fabricius, 1780), *Leucosolenia variabilis* (Haeckel, 1870) and *L. complicata* (Montagu, 1814). All of them are syconoid and polarized (*S. raphanus* – solitary; *S. lingua* and *S. ciliatum* – several tubes) or asconoid and amorphous (*L. variabilis* and *L. complicata* – several tubes emerging from stolons).

In the present work, we investigated the regenerative capacity of an asconoid and polarized species concerning two body parts: osculum and choanosome. We compared our results with previous data and suggest whether the regenerative capacity is influenced by the polarity or body organization. Besides bringing new information about the regeneration in calcareous sponges, we also collected and reviewed the current knowledge about the regeneration process in the class Calcarea.

MATERIALS AND METHODS

We investigated the regeneration capacity of *Ernstia* sp. (Clathrinida, Calcinea) concerning two regions of its body: (1) osculum and (2) choanosome. This yellow sponge has a well-defined globular shape, formed by regularly anastomosed tubes, asconoid aquiferous system and a clear polarized basal-apical symmetry with, in general, one apical osculum. It was referred by Klautau *et al.* (2013) as *Ernstia* sp. nov. 2. The experiment was carried out in a tide pool (Falsa Barreta) in Rocas Atoll (Rio Grande do Norte state, north-eastern Brazil; 3°51′S 33°49′W). This species is considered endemic from the Rocas Atoll, where it is very abundant, being found in all tide pools, hanging from the ceiling of small caves or living in small crevices. Eight individuals were selected for each of the two regeneration experiments. (1) In the first experiment, only the osculum of each individual was totally removed with a razor blade. (2) For the second experiment, a longitudinal section of the choanosome was made, removing apical, central and basal regions. The 16 individuals were checked every other day for 8 days. Photographs were taken with a rule as a reference of size of the individuals. The measurements of growth were acquired with the software AxioVision 4.6 (Carl Zeiss Imaging Solutions). A one-way ANOVA was undertaken to compare regeneration and speed rates.

RESULTS

Experiment 1

All the eight individuals studied fully regenerated their oscula after 8 days. Two days after the removal, a small growing osculum could be observed in all individuals (Figure 1). During this period (first 2 days), the osculum reached a regeneration rate that varied from 26.6 to 100% (48.3% in average $\pm$8.0 of standard error (SE)) of its original size, with a regeneration speed varying from 0.02 to 0.12 cm day^{-1}

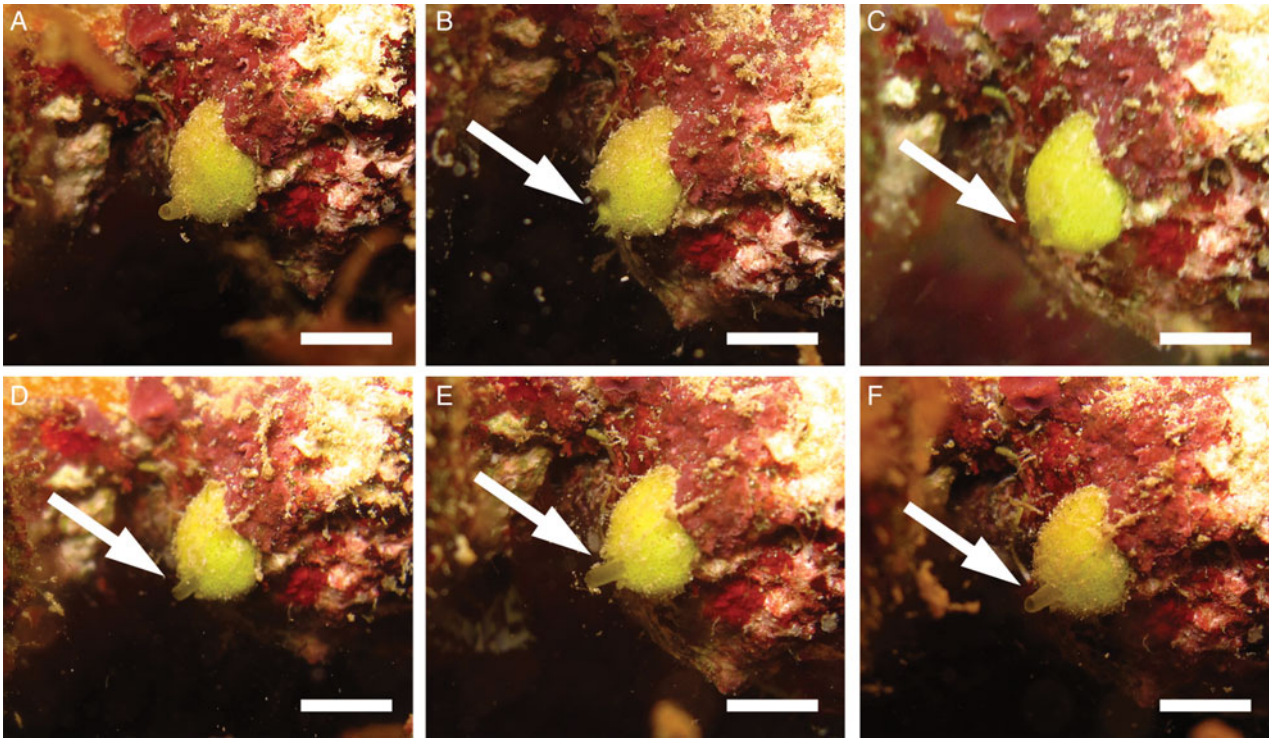

Fig. 1. Sequence of photographs following the osculum regeneration of one individual of *Ernstia* sp. (A) Before; (B) just after injury; (C) 2 days; (D) 4 days; (E) 6 days and; (F) 8 days after the induced injury. The white arrow points to the location and growth of the osculum. Scale bar: 0.5 cm.

(0.05 cm day^{-1} in average ±0.01 SE) (Figure 2). Between subsequent intervals (4, 6 and 8 days after being wounded), the regeneration rates slightly declined, reaching 44.2% (±14.3), 23.7% (±11.2) and 24% (±9.6) of average, respectively (Figure 2A). Nonetheless, differences in the regeneration rates among all intervals were not significant (ANOVA, $P = 0.271$). After the 8th day of the injury (and the end of the experiment), all individuals had regenerated their oscula on average 93.25% (±8.5 SE) compared with the original size (before the injury). The regeneration speed also declined after the first 2 days, reaching 0.01 (±0.007), 0.01 (±0.003) and 0.02 (±0.007) cm day^{-1} in the subsequent intervals respectively, and 0.02 cm day^{-1} (±0.002 SE) on average during the whole period. The average regeneration speed in the first 2 days after the wounding (0.05 cm day^{-1}) was significantly higher when compared with the regeneration speed in the other intervals (ANOVA, $P = 0.004$; Tukey test, $P < 0.05$).

Experiment 2

In contrast to the osculum experiment, all the sponges that had a fragment of their choanosome removed did not fully regenerate the lost part after the 8th day of the study. However, 2 days after the injury, the sponges managed to heal it completely, closing the opened wound. As they only cicatrized the wound, no statistical analysis was done concerning choanosome regeneration.

DISCUSSION

Since the beginning of the last century it was known that calcareous sponges are capable of regeneration after a wound and reaggregation after cellular dissociation (Maas, 1910; Huxley, 1912), but it was especially between the 1950s

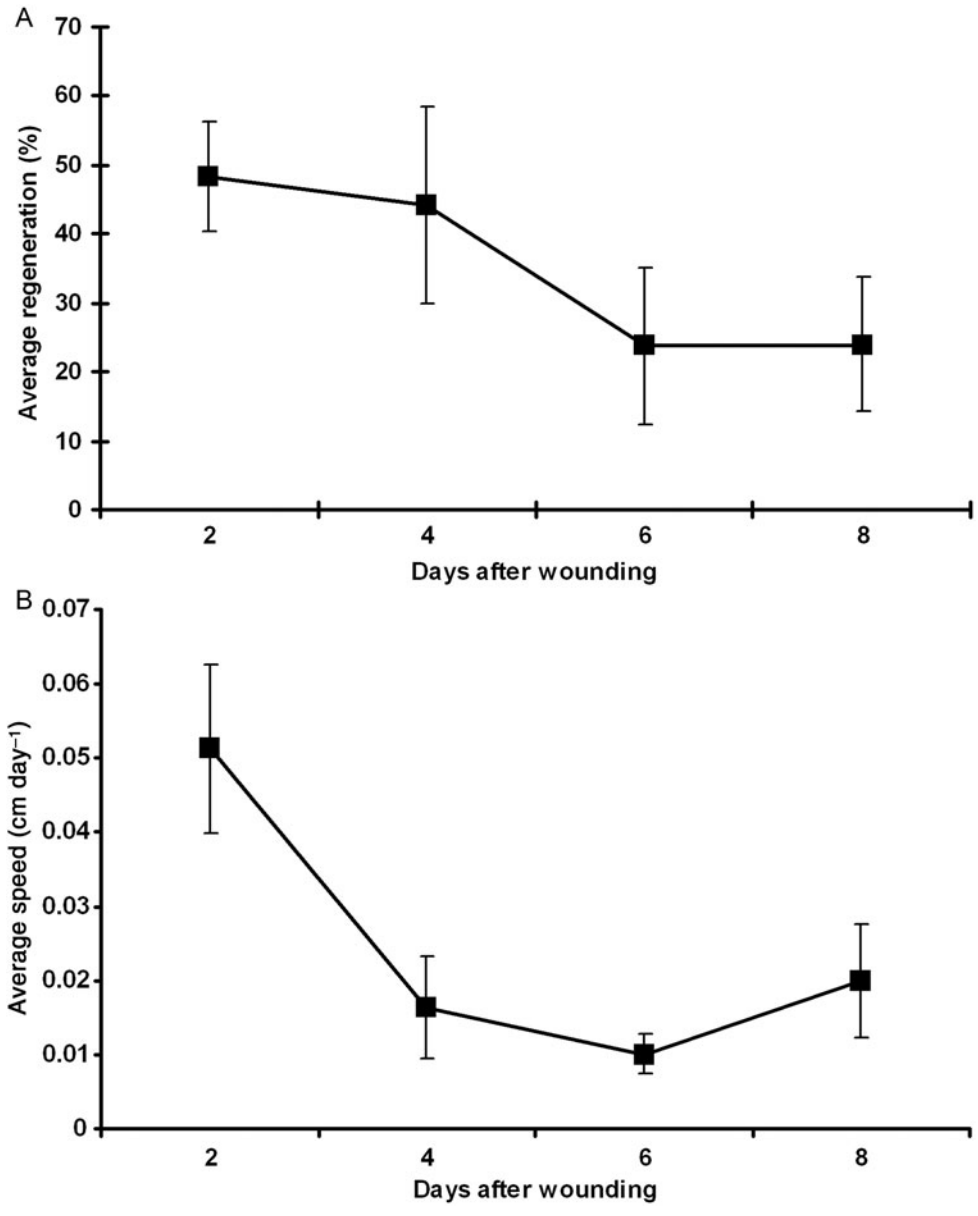

Fig. 2. (A) Average regeneration of the osculum (in %; with standard error) and (B) average speed of regeneration (in cm day^{-1}; with standard error) along the days after the wounding of the eight individuals of *Ernstia* sp. analysed.

and the 1970s that studies with the class Calcarea were published (Jones, 1957, 1958; Korotkova, 1961a, b, 1963a, b, 1969, 1970, 1972; Tuzet & Connes, 1962; Korotkova & Gelihovskaia, 1963; Tuzet & Paris, 1963). Since then little attention was given to this topic.

Some works that preceded the ones cited above focused on the 'level of integration' of different sponge species and the differences between regeneration and somatic embryogenesis (Korotkova, 1963a). Tokin (1963) dedicated one of his works to establish both terms among sponges and other organisms due to misuse. Concerning Porifera, he concluded that the wound healing process should be called regeneration, while the formation of new individuals after cell aggregation or deep injury is a product of somatic embryogenesis because it consists of a radical cellular reorganization, which alters the existing correlations among cells in a process similar to asexual reproduction. In this work he also stated that 'weakly integrated' (usually amorphous, multioscular) sponges often show somatic embryogenesis. Instead, 'highly integrated' (polarized, unioscular) sponges would restore their tissue *via* regeneration, recovering their original shape and functioning. The 'integration level' in sponges was sometimes discussed together with its concept of individuality. In those multioscular sponges (that sometimes were called colonies) composed of several tubes (units) connected by stolons at the base (e.g. *Leucosolenia*), the level of individuality and integration of each 'unit' and the whole sponge was considered very low. On the other hand, in single, polarized (unioscular) species the level of integration and individuality was considered higher. In those single forms the regeneration power should be higher and the restoration faster than in multioscular forms due to a higher individuality, integration and polarized body (Korotkova, 1963a, 1970).

The regeneration differences depending on the morphology can be demonstrated even in species of the same genus, suggesting that (as in Demospongiae; Wulff, 2010) this event does not obey a phylogenetic sense. When *Sycon lingua* and *S. raphanus* were cut longitudinally in two or four parts, individuals of *S. lingua* bent inwards, closing into a bean-shape, and a new osculum was formed in a position different from the original, while in *S. raphanus* the opposite margins joined, closing the individuals again and maintaining the original shape with the original osculum (figure 2 in Korotkova, 1963b). In these experiments, the majority of specimens of *S. lingua* changed polarity, while in *S. raphanus* it was maintained. When individuals of *S. lingua, S. raphanus* and *S. ciliatum* were cut transversally in two or three parts, the fragments regenerated the lost parts (osculum and base), maintaining the original polarity. Only in a few individuals of *S. lingua* (15%) did a distortion of polarity occur (Figure 6 in Korotkova, 1963b). These differences may be due to the level of 'integration and individuality' observed in each species. Tuzet & Paris (1963) found the same results cutting *S. raphanus* longitudinally and transversally, but when the cut passed over two-thirds of the sponge body, two tubes with one osculum each were originated.

Korotkova (1961a) carried out several regeneration experiments using *Leucosolenia complicata*, obtaining different results. When doing partial longitudinal cuts in the osculum, most specimens regenerated the original shape, but some doubled oscula and deformed regeneration were also observed. When the osculum was totally removed, only 15% regenerated, while up to 60% closed the opening and new lateral buds appeared. Korotkova & Gelihovskaia (1963) also did experiments with *L. complicata* and *L. variabilis* and observed 100 and 87% of regeneration respectively when the oscula were cut. An inversion of polarity was observed in 12 and 50% of the cases when the apical and basal parts of the tubes were cut. The orientation of spicules and, sometimes, the change of the oscular rim position were also observed in *L. complicata* and *L. variabilis* (Jones, 1958).

The level of integration and the polarity of sponges related to their external morphologies are the main issues discussed by many authors (Korotkova & Gelihovskaia, 1963; Tokin, 1963; Tuzet & Paris, 1963; Korotkova, 1970; Tuzet, 1973). Many of the examples suggest that the mechanisms of integration in calcareous sponges are very unstable and the changes in polarity after an injury may depend on the degree of disintegration of the system caused by the trauma (Tuzet, 1973). The influence of the morphology on the capacity of regeneration in calcareous sponges seems very clear. *Sycon raphanus* usually is found in a solitary form, with a single tube and well-defined body symmetry, while *S. lingua* and *S. ciliatum*, when achieving a certain size, are found with more than one tube arising with indistinct boundaries at the same base (Korotkova, 1970). *Leucosolenia complicata* and *L. variabilis* are formed by several branching tubes arising from a common base spreading on the substrate with several stolons (Korotkova, 1961a). Unioscular and polarized species (as *S. raphanus*) are more capable of restoring their original shape, while in multioscular species (*S. lingua, S. ciliatum, L. complicata* and *L. variabilis*) it seems to be a random process.

Like *S. raphanus*, the species studied here (*Ernstia* sp.) clearly shows a well-defined polarized body but with an asconoid aquiferous system and a single, apical osculum, which certainly exerts a crucial role in the functioning of the aquiferous system of the individuals. In the present work, we observed a fast regeneration oriented to the apical region (osculum) in order to restore the shape of the individuals, while the choanosome did not reconstitute the lost part but, at least in the short term, regenerated the injured tissue (Carlson, 2007). Osculum regeneration in demosponges also tends to be faster than choanosome regeneration (Hoppe, 1988; Walters & Pawlik, 2005; Rohde & Schupp, 2012). As a 'solitary' and unioscular species with a polarized external morphology, the osculum regeneration in *Ernstia* sp. was complete (not random) and directed to restore the optimal shape of the individuals. It can then be considered the true regeneration process cited by Tokin (1963) driven by the clear polarization of the body and by the importance of the structure. The same was observed by Korotkova in her works with *S. raphanus*.

Differently from the osculum, *Ernstia* sp. did not regenerate the entire lost choanosome, but healed the opened wound in less than 2 days. It is known that some calcareous species may take longer periods to heal lost pieces of the choanosome after an induced injury: *Leucosolenia complicata* took up to 10 days (Korotkova, 1961a), while *S. raphanus* did not regenerate nor cicatrized but, after up to 6 days, the sponge retreated at the level of the cut and separated in two (Tuzet & Paris, 1963). It is possible that, if followed for a longer period, the individuals studied here would regenerate the lost part to regain the lost substrate in a regular process of growth. The non-immediate recovering of the lost part of the choanosome in *Ernstia* sp. could be explained by its asconoid aquiferous system.

Possibly the lost part did not compromise the functioning and survival of the individuals, and the fast healing was necessary only to avoid, for example, the settlement of particles or organisms in the exposed parts that could preclude the normal function of the aquiferous system (Hoppe, 1988; Leys & Lauzon, 1998).

As the mesohyl of calcareous sponges is almost acellular (except for sclerocytes), the totipotency of choanocytes and their possible role in the regeneration process of sponges were already proposed (Funayama, 2010, 2012). Research shows that cells that will work in the regenerative process probably are derived from the neighbouring tissue around the wounded area (Tuzet & Paris, 1963; Boury-Esnault, 1976) and the role of choanocytes and sclerocytes are evident in Calcarea, while in Demospongiae the archaeocytes are more important (Korotkova, 1963a, 1972). Choanocytes originate oocytes and spermatozoa in some calcareous sponges (Gaino *et al.*, 1987; Lanna & Klautau, 2010) and may also be the main source of stem cells in the class (Funayama, 2012). In a histological study of the regeneration process in *Sycon ciliatum*, a decrease in the number of choanocyte chambers and an increase of amoebocytes around the regenerative area were observed (Tuzet & Paris, 1963). These amoebocytes may be derived from mobile cells of the mesohyl or from the differentiation of nearby choanocytes (Tuzet & Paris, 1963) and could be acting in the regenerative process. It has already been observed that choanocytes may detach from choanocyte chambers, lose their flagella, differentiate into amoeboid cells, and move into the mesohyl to act as stem cells in calcareous sponges (Korotkova, 1972; Gaino *et al.*, 1987; Funayama, 2012). Nevertheless, Korotkova (1970) also proposed that, in asconoid species, the regenerative process takes place with the pinacocytes and the posterior spread of underlying choanocytes to the recovered area.

Another issue of sponge regeneration that is not commonly investigated are the biochemical mechanisms involved in the process. Basile *et al.* (2009) studied the activity of ADPRC (ADP-ribosyl cyclase) in sponges during 2 years and observed that it can increase up to 10 times in regenerating specimens of the calcareous sponge *Clathrina clathrus* (Schmidt, 1864). It is known that the signal transduction pathway that involves ADPRC is related to physiological activities in sponges, such as stem cell duplication, respiration, water filtration and protein synthesis (Zocchi *et al.*, 2001, 2003).

In conclusion, the simple morphology of calcareous sponges together with the totipotency of their choanocytes may be mainly responsible for the efficiency of the regenerative process in these animals (Jackson & Palumbi, 1979). Besides, the body's polarity probably conducts and streamlines the regenerative process in order to restore the species' optimal shape (Jackson, 1979; Hoppe, 1988; Bell, 2002; Walters & Pawlik, 2005; Rohde & Schupp, 2012). Although more studies are needed using species with different external morphologies and a broader phylogenetic range, the studies undertaken with calcareous sponges (including this one) suggest that regeneration in this class is strongly dependent on morphology and body polarity.

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CAPÍTULO 2

TÍTULO: “There and back again”: reconstitution of calcareous sponge (Porifera, Calcarea) from dissociated cells

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"There and back again": reconstitution of calcareous sponges (Porifera, Calcarea) from dissociated cells.

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Abstract

Sponges (phylum Porifera) are considered the oldest animals still alive and they retained a great plasticity of its morphological characteristics and cellular elements. One of the most striking features of sponge individuals is their capacity of total reconstruction after cellular dissociation. Isolated sponge cells are able to reaggregate, form spherical structures (termed primmorphs) and develop again into fully functional individuals *in vitro*. Works describing such reconstitution of sponges from dissociated cells focused mainly on the class Demospongiae. Here, we describe the development of primmorphs and functional individuals from dissociated cells of two calcareous sponge species: *Paraleucilla magna* (Calcaronea) and *Clathrina aurea* (Calcinea). Both species developed primmorphs from dissociated cells within 24 hours. All *Clathrina aurea* primmorphs remained unchanged until the sixth day, when they died trying to adhere to the substrate, while in 22% of *P. magna* experiments, primmorphs resulted in functional sponges. The development of *P. magna* individuals from primmorphs is similar as that from the larvae; however, it bypasses some steps and differs in the timing of events.

Keywords: Cell aggregation, Primmorphs, *Paraleucilla magna*, *Clathrina aurea*.

Introduction

Sponges (phylum Porifera) are considered the most ancient metazoan group and have the simplest body organization among extant animals. They are multicellular organisms lacking distinct nervous, digestive, muscular and/or reproductive systems and rely their vital activities on a filter feeding system (aquiferous system) composed of flagellated collar cells (choanocytes) inside canals and chambers (Ereskovsky 2010). Sponges are animals with high morphological plasticity and mobility of cellular elements, being capable of constant body reorganization in response to diverse conditions (Bond 1992; Nickel 2006; Ereskovsky 2010). Perhaps the most extreme example of such plasticity is the capacity of reaggregation and formation of a new functional individual from dissociated cells.

Wilson (1907) described the phenomenon of sponge cell reaggregation for the first time and, since then, it has been a target for studies on spiculogenesis, cell-cell interactions, adhesion and behaviour, molecular mechanisms, and bioactive compounds (*e.g.* Fernández-Busquets and Burger 1999; Gaino and Magnino 1999; Müller et al. 2000; Zhang et al. 2003; Valisano et al. 2012). Sponge dissociated cells aggregate and form spherical structures delimited by a cell layer of pinacocytes separating an inner cell mass from the external environment. These structures were described and first termed as diamorphs in order to replace vague names as “spheres” or “balls” (as in Huxley 1912), and represents the last stage before the reorganization into functional individuals (Borojevic et al. 1968; Borojevic 1971). Later on diamorph received the name primmorph (Custódio et al. 1998; Müller et al. 1999). The success of the reaggregation, however, depends on several factors such as culture conditions, the achievement of important morphogenetic stages and species-specific traits (Lavrov and Kosevich 2015).

Despite the large number of studies describing the formation of primmorphs and their development into functional individuals, few works were performed with sponges of the class Calcarea. Since Huxley (1912) work on *Sycon raphanus* Schmidt, 1862, only Korotkova (1972) - with *Leucosolenia complicata* (Montagu, 1818) and *S. lingua* (Haeckel, 1870) - and Eerkes-Medrano et al. (2014) - with *S. coactum* (Urban, 1906) - used calcareous sponges as models for cell reaggreggation and morphogenetic studies. Therefore, the aim of this work was to study the formation of primmorphs and their development into functional individuals of calcareous sponges.

Materials and Methods

Sample collection

Two species, one of each subclass of Calcarea, were studied in the present work. Specimens of *Paraleucilla magna* Klautau et al. 2004 (n=18), subclass Calcaronea, and *Clathrina aurea* Solé-Cava et al. 1991 (n=12), subclass Calcinea, were collected at Praia Vermelha, Rio de Janeiro (22°57'18"S, 43°09'42"W) and Cabo Frio, Southeastern Brazil (22°53'50"S, 41°58'56"W), respectively. Specimens were brought to the laboratoty in vials with a pumping air device and water temperature similar to that of the collecting site to minimize stress to the sponges.

Cell dissociation protocol

At the laboratory, the sponges were washed and kept in 0.22 μm filtered seawater (FSW) and their surface was gently cleaned with a fine brush and a forceps to remove epibionts. The sponges were cut into small fragments around 2 mm^2 and placed in falcon tubes with sterile calcium and magnesium-free artificial seawater (CMFSW) containing a chelating component (EDTA). The composition of the CMFSW-EDTA

used was: NaCl: 0.4585 mol/L; $NaSO_4$: 0.0082 mol/L; KCl: 0.0099 mol/L; Hepes buffer: 0.0099 mol/L; EDTA: 0.0032 mol/L; pH 8.0-8.2. The ratio of fragments to CMFSW-EDTA was 1:10 and incubation time was one hour. During this time, tubes were gently shaken every 5 minutes to facilitate cell dissociation. After this step, the tubes were centrifuged for 10 seconds at 1200 rpm and the supernatant was transferred to a new tube, which was centrifuged for 5 minutes at the same speed. The supernatant was discarded and the cells were gently resuspended using a Pasteur pipette and placed in a sterile 6-well plate containing FSW. One-third of the FSW content in each well was changed every day. The development of the primmorphs was followed at a Nikon TS100 inverted microscope.

Results

In both species, cell suspension sank to the bottom of the well and, at this moment, many dissociated cells and amorphous aggregates were observed (Figure 1). Isolated cells actively explored the surroundings with pseudopods or assumed a rounded shape, being less active (probably choanocytes). Both types of cells started to group soon, forming primary amorphous aggregates, which progressively increased in size, detached from the bottom and took a spherical shape (Figures 1, 2). Approximately four hours after dissociation, most primary aggregates were floating. At this point, it was possible to observe a slight movement around their surface, probably caused by the flagella of choanocytes. It is important noticing that, when cell aggregates failed to detach from the plate bottom, the culture did not go ahead and died within a maximum of two days (Figure 2c). In *P. magna* and *C. aurea*, the formation of the first primmorphs, *i.e.*, cell aggregates with pinacoderm, started 4 hours after dissociation. *Paraleucilla magna* primmorphs were characterized by a translucent space between the

inner mass of cells (IMC) and the pinacoderm, while in *C. aurea*, the IMC occupied all the inner space of the primmorph (Figure 1, 2). In both species, the first 24-48 hours were marked by the completion of primmorphs formation and their increase in size, either by fusion with others or perhaps by cell divisions. After 48 hours, differences between both species were observed. The primmorphs of *C. aurea* remained unchanged until the sixth day, when they tried to adhere to the substrate and died, while in some *P. magna* experiments, primmorphs adhered to the substrate and developed into functional sponges.

In 39% of *P. magna* cultures, successful primmorph adhesion occurred between the third and sixth days (Figure 2e), but unattached primmorphs were observed for at least eight days and 33% of our cultures ended due to massive primmorph death upon adhesion on substrate. Twenty-eight percent of *P. magna* cultures presented aggregates adhered to the bottom since the beginning and never formed primmorphs. In these cultures all cells died. In the fourth day after dissociation, the secretion of the first spicules started in both adhered and no-adhered primmorphs. The first spicule secreted was always a regular triactine. In the fifth day, the skeleton organization started in the adhered primmorphs with the synthesis of cortical tetractines and sub-atrial triactines (Figure 2d). At the beginning of the primmorph fixation at the substrate, the position of the primordial oscula and the inner cell mass could be observed, but on the fifth it became a single central choanocyte chamber (Figure 2e, 2f).

Fusions also occurred among adhered sponges and, by the seventh day after dissociation, many settlers presented multiple choanocyte chambers (Figure 2g), but settlers with a single choanocyte chamber were still present and remained alive for at least 30 days. Oscula were observed by the seventh day, in both settlers with one or multiple choanocyte chambers (Figure 2g), completing the morphogenetic process of

reconstruction of a functional individual from dissociated cells. From the 39% of cultures where we observed successful settlement, 67% (22% from the total number of cultures) reached this final stage, while 33% (17% from the total number of cultures) developed choanocyte chambers but not an osculum. Eleven days after the beginning of the experiment, the newly formed sponges started to die. Deaths of sponges was characterized mainly by loss of spicules and decrease in size and started with the larger ones. *Paraleucilla magna* cultures were maintained for seven days in average, with functional sponges living a maximum of 30 days.

Discussion

The morphogenetic process of development of functional sponges from dissociated cells probably does not occurs in nature and its complete development is a difficult task *in vitro,* as it depends on the achievement of some stages, such as the formation of primary aggregates, epithelization into primmorphs, adhesion to the substrate, cell differentiation and organization into a new sponge (Korotkova, 1979; Eerkes-Medrano et al. 2014; Lavrov and Kosevich 2014; Ereskovsky et al. 2015). The success of these stages may also depend on the health of the sponge, cellular composition, cell concentration, microorganism contamination, presence/absence of antibiotics, ions concentration, temperature, physiological state and life cycle stage of the sponge (Pomponi and Willoughby 1994; Le Pennec et al. 2003; Sipkema et al. 2003, Pomponi 2006; Valisano et al. 2006; 2007; Chergonor et al. 2011; Lavrov and Kosevich 2015). The process of cell reaggregation and primmorph formation was described for more than 40 species, with many differences among them. Despite this, few works were able to observe the complete reconstruction of functional individuals and this data is even scarcer for calcareous sponges, with only four species studied to date:

Leucosolenia complicata, *Sycon raphanus*, *S. lingua* and *S. coactum* (Huxley 1912; Korotkova 1972; Eerkes-Medrano et al. 2014).

Previous authors observed the amoeboid aspect and high mobility of calcareous sponges isolated cells after dissociation (Huxley 1912; Korotkova 1972). The cell movements on the substrate are possible through expansions of the cellular membrane due to actin polymerization (Gaino and Magnino 1999). While the precise determination of most cell types is difficult and these are generally termed amoebocytes, the abundance and behaviour of choanocytes (which loses the collar, retain the flagellum and assume a rounded shape with an inactive condition) is clear in calcareous cells suspensions (Gaino and Magnino 1999). Choanocytes are considered the most abundant cell type in clathrinid cell suspensions and one of the most important cell types involved in the morphogenesis of calcareous sponges from dissociated cells (Huxley 1912; Korotkova 1972; Gaino and Magnino 1999). Apparently, the choanocyte's morphological changes results in a dedifferentiation process (Gaino and Magnino 1999) allowing differentiation in all other cell types (Huxley 1912; Korotkova 1972). These facts put them as putative stem cells in the class Calcarea (Funayama 2012), although they alone are unable to rebuild a new functional individual by themselves, as the presence of other cell types are required (Huxley, 1912). In demosponges, archaeocytes seem to be the stem cells (Borojevic 1966; Ereskovsky 2010; Funayama 2012), as they can differentiate into all other cell types and alone form a new individual (van de Vyver and Buscema 1981). Previous studies observed that pure fractions of archaeocytes (or at least with their presence) are able to differentiate into other cell types, form primmorphs and new functional individuals, while suspensions containing other cell types (except archaeocytes) were not able to reach all

stages into the reconstruction of a sponge (de Sutter and van de Vyver 1977; Korotkova 1979; Buscema et al. 1980; Zhang et al. 2003; Lavrov and Kosevich 2014).

In the present work, the reconstruction of a functional individual *in vitro* was not achieved without passing through the stage of primmorph. When the cells aggregated and adhered at the bottom of the well after dissociation, the culture died in at least two days (Figure 2c). Complete formation of primmorphs was finished by the end of the second day in both species (*C. aurea* and *P. magna*) in the present study. The same period was observed for *S. coactum* (Eerkes-Medrano et al. 2014), while primmorphs were observed only after the fourth and sixth days for *S. raphanus* and *S. lingua*, respectively (Huxley 1912; Korotkova 1972).

In the present study, we were not able to confirm the origin of the primmorph's pinacocytes, but we can suggest that they originate from the most external cells in the aggregate, the choanocytes. In calcareous sponges, the primmorph's pinacoderm was suggested to originate from pinacocytes present among the cells of the primary aggregates or by the differentiation of choanocytes (Huxley 1912; Korotkova 1972). In demosponges, ultrastructural studies of marine and freshwater species suggest that there are three main sources of primmorph's pinacocytes: choanocytes, archaeocytes and amoebocytes (Borojevic and Levi 1964; Ereskovsky et al. 2015). Independently of the pinacocytes origin, the epithelization process and consequent isolation of the developing individual seem to be crucial steps both in the development from larvae and from dissociated cells in calcareous sponges. The differentiation of the baso- and exopinacoderm are the very first steps of the larval metamorphosis of *P. magna* (Lanna 2012) and are important steps for the development from dissociated cells into functional individuals.

Most studies with primmorph cultures were not able to observe the reconstitution of functional individuals from dissociated sponge cells *in vitro* (see table in Lavrov and Kosevich 2015). The complete process of the individual reconstruction from dissociated cells of calcareous sponges was observed in *L. complicata*, *S. lingua* and *S. raphanus*; although in the last two species the sponges did not achieve the syconoid aquiferous system organization of the adult remaining as "*olynthus*-like" individuals (Huxley 1912; Korotkova 1972). Dissociated cell of *S. coactum* (Eerkes-Medrano et al. 2014) and *C. aurea* did not develop into functional sponges, remaining at the primmorph stage until death, while in 22% of the *P. magna* experiments, we observed the complete development of fully functional sponges with oscular openings and several choanocyte chambers, typical of leuconoid aquiferous system (Figure 2g, 2h). The difficulty of syconoid species in achieving its adult organization may evidence the developmental complexity of their aquiferous system, which is characterized by radially disposed elongated chambers extending from the external surface to the atrium, in contrast to the discrete and scattered disposition of choanocyte chambers of the leuconoid system (Manuel et al. 2002a; Manuel 2009).

Every developmental pathway must follow precise and well stablished steps to generate viable organisms. In sponges, the natural development occurs *via* larval metamorphosis, but formation of individuals can also be achieved by cellular dissociation of adult sponges. In the case of the calcareous sponge *Paraleucilla magna*, there are interesting differences between the development from cell aggregates (primmorphs) and from the larvae. The most striking difference concerns the timing of the events, which was also noticed by Huxley (1912) comparing the development from larvae and the reorganization from cell aggregates of *Sycon raphanus*. In *P. magna*, the differentiation and organization of a pinacoderm points out the first steps in both types

of development (Figure 3). Larval metamorphosis starts prior to the settlement when the first steps of cellular organization and differentiation occurs. Pinacocyte differentiation is observed as the larvae settles and spreads in the substrate (Lanna 2012). Dissociated cells from *P. magna* must pass through the stage of primmorphs to develop into functional individuals and the first evidences of cellular organization and differentiation on primary aggregates start around four hours and finishes between 24-48 hours after dissociation with the complete primmorph formation (Figure 2, 3).

One interesting point is the fact that the first spicule secreted by primmorphs appears only on the fourth day and it is a triactine (Figure 3), while in the larval metamorphosis the secretion of the first spicule starts around eight hours after settlement, and it is a diactine, a spicule that is lost during ontogenesis and is not found in the adult of *P. magna* (Lanna 2012). The production of a diactine as the first spicule in Calcaronea is a synapomorphy of this subclass (Manuel et al. 2002b), but this phylogenetic memory seems to be lost during the development from primmorphs. The cortical skeleton of the juvenile was complete by the fifth day after settlement, while at this same moment the cortical skeleton of the sponge developed from the primmorph was beginning to be secreted, with the production of other spicule types, such as the cortical tetractines (Figure 2).

Differences were also observed in the development and organization of the primordial aquiferous system. In the larval development, six hours after settlement the first choanocytes appear and the primordial osculum becomes evident two hours later (Lanna 2012). The settled larvae will develop into an *olynthus* (with a sac-like choanoderm), with its osculum-base axis defined, which will be completed by the fifth day after settlement (Lanna 2012). From primmorphs, evident choanocytes were observed as the primmorph adhered and stablished at the substrate, between the third

and sixth days, although it did not show any evident sign of axial symmetry. It did not develop into the juvenile form of the *olynthus*, developing initially a single rounded choanocyte chamber, typical of the leuconoid aquiferous system of the adult (Figure 2f), while a functional osculum was observed only between the seventh and eighth days (Figure 2g, 2h). Young sponges originated from cellular aggregation were fully functional, presenting an osculum with several choanocyte chambers at the ninth day after dissociation, while sponges originated from larval development only started the organization of the leuconoid aquiferous system around 16 days after settlement. These comparisons evidence the timing differences and sequence of events between larval and primmorph development in the calcareous sponge *P. magna* and suggest that adult cells follow a different morphogenetic pathway into a functional individual, bypassing stages present on the larval development. The timing differences and sequence of events observed between the development from dissociated cells and larvae in the calcareous sponge *Paraleucilla magna* are an interesting issue for further studies on cellular differentiation and comparison of the molecular mechanisms underlying both types of developments.

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Figures

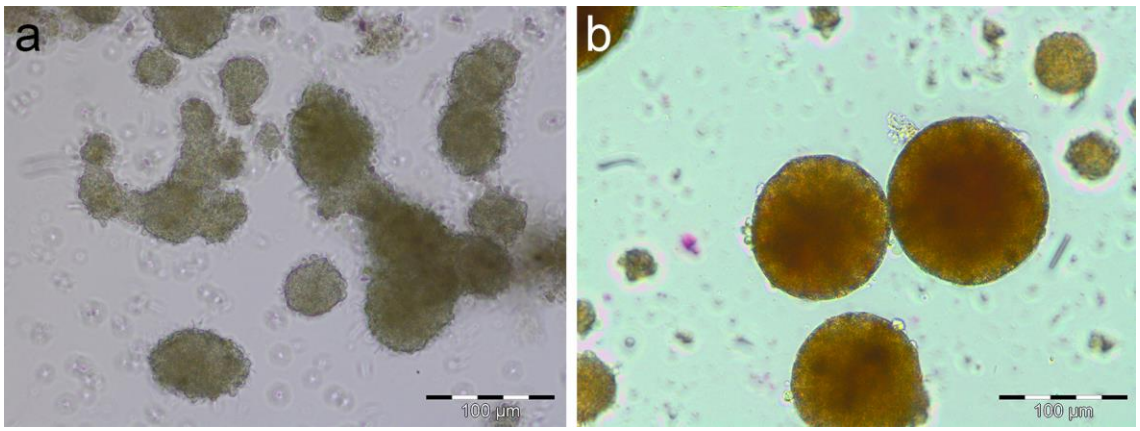

Fig 1 *Clathrina aurea* primmorphs (a) primary aggregates, after beginning of the experiment; and (b) primmorphs, 48 hours after dissociation

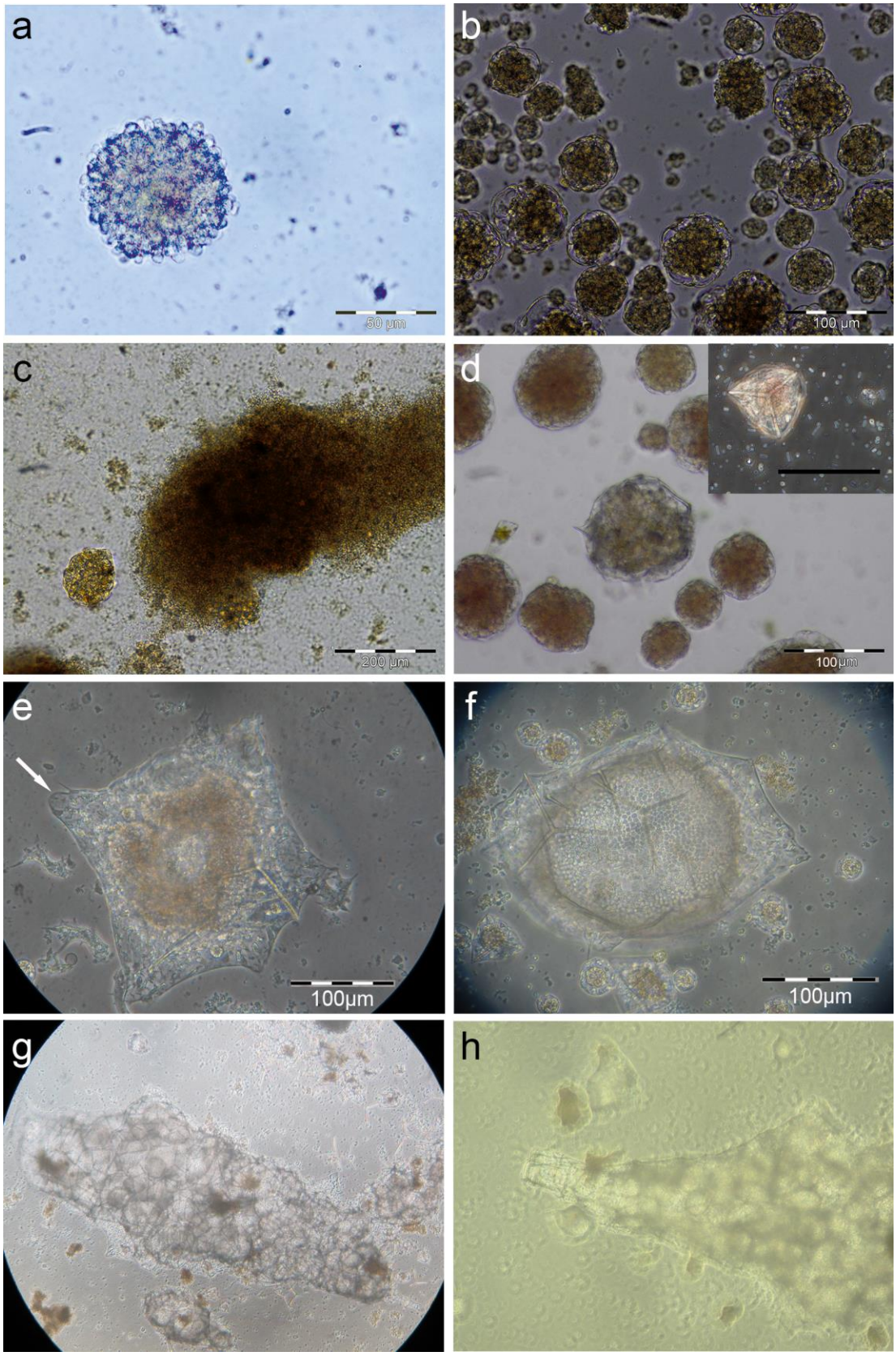

Fig 2 *Paraleucilla magna* primmorphs. (a) primary aggregate, four hours after dissociation; (b) primmorpgs and primary aggregates, 24 hours after dissociation; (c) adhered cell mass on failed experiment; (d) primmorph with spicules, four days after dissociation and, in detail, a primmorph with one spicule scale=100µm; (e) adhered

primmorph with inner cell mass, four days after dissociation, arrowhead shows the position of the primordial oscula; (f) adhered primmorph with a single choanocyte chamber and spicules, five days after dissociation; (g) functional sponge, seven days after dissociation; (h) detail of the oscula of a functional sponge, eight days after dissociation.

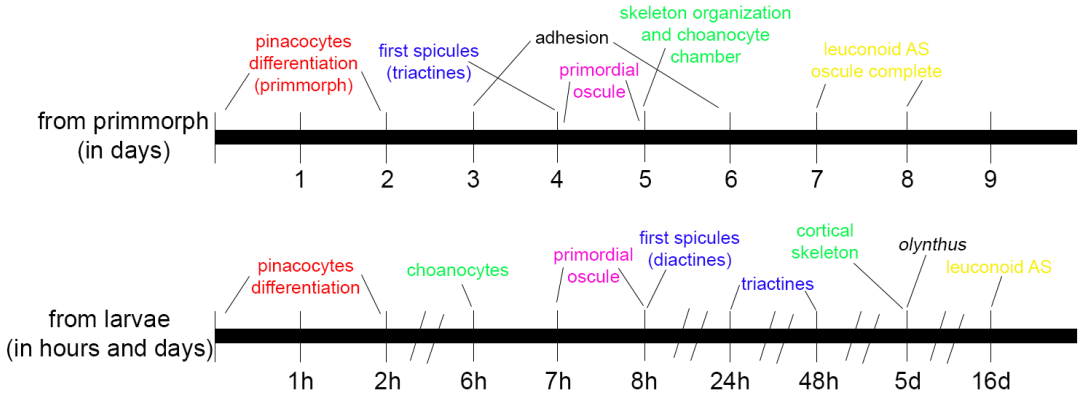

Fig 3 Scheme comparing the reconstitution from dissociated cells (upper line; scale in days) and development from larvae (lower line; scale in hours and days). Colours represents the same events in both time lines. Events in black are observed in the respective type of development. Consecutive bars (//) represents time advances.

CAPÍTULO 3

TÍTULO: Fragmentation, fusion, and genetic homogeneity in a calcareous sponge (Porifera, Calcarea)

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Fragmentation, fusion and genetic homogeneity in a calcareous sponge (Porifera, Calcarea)

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Total number of figures: 2

Total number of tables: 1

Running headline: Genetic homogeneity in Calcarea

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Abstract

Sponges can explore their morphological plasticity to obtain ecological advantages in marine hard substrates. Size variations, longer life spans, fragmentation and fusion events are examples of these strategies inherent to the life history of some species. Calcareous sponges are usually small and short-lived and some species of the genus *Clathrina* are known to have frequent events of fragmentation and fusion. However, it is not known yet if fusion occurs only between genetically identical individuals or not. If adult allogeneic fusion is allowed, we would observe intraorganismal genetic heterogeneity (IGH) in nature. This phenomenon is known in almost all animal phyla and was recently described in a demosponge. We investigated the existence of IGH in the calcareous sponge *Clathrina aurea* by following 69 individuals as far as 18 months. We also investigated size variations and longevity of each individual. To verify if fusion events may occur among genetically different individuals, microsatellites were used. Events of growth and shrinkage of individuals were frequently observed, showing that size cannot be associated with age in *Clathrina aurea*. The life span of this species varied from one to 16 months, with a mean of 4.5 months. Such a short life span and variable growth rates have already been observed in other species of Calcarea. Fragmentations and fusions were observed and fusions happened always between genetically identical individuals, as already suggested by graft experiments for adult Demospongiae and other Calcarea. These results suggest that at least adults of *C. aurea* have a mechanism to avoid chimerism.

Keywords: Allorecognition, *Clathrina aurea*, IGH, Life span, Microsatellites

Introduction

Sessile marine invertebrates living on hard substrata usually develop strategies to occupy and compete for space. Sponges (phylum Porifera) are among the most abundant organisms living on hard substrata and due to their morphological plasticity, simple body organization, and totipotency of cellular elements, they are capable of exploring many strategies in response to environmental conditions and interactions with other benthic organisms (van Soest et al., 2012).

One strategy used by some sponges is to fragment their bodies or make fusions among individuals to endure environmental changes or compete for substrate (Jackson, ´86; Zilberberg et al., 2006; Blanquer et al., 2008). Besides, life span, body size and growth rates are also characteristics that can help in ecological disputes and that are usually correlated for a broad range of living organisms (Hernaman and Munday, 2005), although the latter can be highly variable among individuals of the same species or associated with environmental conditions (Sebens, ´87; Duckworth et al., 2004; Blanquer et al., 2008).

Fragmentation and fusion are considered common events in the life cycle of several marine sponge species and they seem to be species-specific events facilitated by the external morphology of the sponge (Wulff, ´91; Tsurumi and Reiswig, ´97; Blanquer et al., 2008). Fusion provides evolutionary and ecological benefits, but usually requires a recognition system to discriminate between self and non-self (Grosberg, ´88; Brusini et al., 2013). The lack of an allorecognition response upon contact, resulting in fusion of genetically different individuals, yields a chimaera, *i.e.*, individuals with two or more cell lineages originated from different zygotes (Pineda-Krch and Lehtila, 2004). Graft experiments have suggested that fusion is restricted to genetically identical individuals in adult sponges (Hildemann et al., ´78; Amano, ´90; van de Vyver and

Buscema, ´90; Gaino et al., ´99), maintaining the individual's genetic homogeneity. However, although avoided in adult sponges, fusion between larvae and juveniles from different individuals is possible (Ilan and Loya, ´90; Maldonado, ´98; McGhee, 2006; Gauthier and Degnan, 2008) and the natural occurrence of intraorganismal genetic heterogeneity (IGH) was already observed in the demosponge *Scopalina lophyropoda*, a Mediterranean species with high genetic diversity and frequent fragmentation and fusion events (Blanquer and Uriz, 2011). Besides fusion, IGH can also arise through somatic mutations and other genetic changes (Pineda-Krch and Lehtilä, 2004), which are not so uncommon as previously thought, especially among clonal organisms (Lushai and Loxdale, 2002; Loxdale and Lushai, 2003).

The calcareous sponge *Clathrina aurea* Solé-Cava et al., ´91 (Clathrinidae, Calcinea) is a common species in Brazilian rocky shores. The species has a great genetic diversity (Padua et al., 2013a) and a body formed by anastomosed tubes and a skeleton composed of triactines disposed without organization. It is commonly observed in a patchy distribution in sciaphilic environments, such as caves, crevices, and overhangs (Monteiro and Muricy, 2004); with individuals living in very close proximity to one another. A similar pattern of distribution is observed in other Clathrinidae species, which also present frequent events of fragmentation and fusion (Johnson, ´79; Gaino et al., ´91).

With its reproduction still unknown (Lanna and Klautau, 2015), the origin of the high genetic diversity observed in *C. aurea* is not yet explained and one possible hypothesis is the occurrence of IGH. In the demosponge *S. lophyropoda*, fusion between genetically different adult individuals or somatic mutations were considered putative explanations to the high levels of genetic diversity observed. Hence, the main objective of the present work was to investigate the possible presence of IGH in a

calcareous sponge (*C. aurea*). This study was performed with adult individuals of *C. aurea*, following their natural dynamics of fragmentations and fusions and using microsatellite *loci* to follow and recognize each individual's genotype. With this study, we were able to suggest the possible presence/absence of an allorecognition system in adults of this sponge and analyse if the high genetic diversity observed in *C. aurea* can be attributed to the occurrence of IGH as it was suggested for *S. lophyropoda*. Additionally, we also assessed some life history aspects of *C. aurea*, such as longevity and size.

Materials and methods

To assess the natural occurrence of IGH in adult individuals of *Clathrina aurea*, we used the same approach as Blanquer and Uriz (2011) for *S. lophyropoda*. In the present work, 10 individuals of *C. aurea* were collected at Papagaios' Island in Cabo Frio, Rio de Janeiro, Southeastern Brazil (22°53'50"S, 41°58'56"W; Fig. 1). To investigate the possible existence of multiple multilocus genotypes (MLG) in the same individual, three fragments (one from two borders and another from the central region) were removed from each analysed specimen. The fragments were fixed in 93% ethanol and DNA was extracted with a phenol-chloroform protocol (Hillis et al., ´96). Seven microsatellite markers previously developed for this species were analysed (Cau_A7; Cau_B2; Cau_C2; Cau_D1; Cau_D8; Cau_E6; Cau_G3) to assess the individual's genotype and followed amplification and genotyping procedures described in Padua et al., (2013a).

Additionally, 69 individuals of *C. aurea* were monitored as far as 18 months (May 2012 to October 2013) in a four-meter deep crevice at the same locality. A fragment of each individual was taken for microsatellite genotyping to access the MLG

of each individual (as explained previously) before and after events of fragmentation and fusion. Underwater photographs of each individual were taken to follow the individual's life span, size variation, and the occurrence of fragmentation and fusion events. Size of individuals was measured as area coverage with the software Axio Vision 4.6.3 (Carl Zeiss Imaging Solutions).

Results

Natural IGH

Intraorganismal genetic heterogeneity (IGH) was not found in *C. aurea*. The three fragments analysed from each individual always presented the same MLG (Table 1).

Fragmentation and fusion

Among the 69 individuals monitored as far as 18 months, there were 58 different MLGs (genets), represented by 48 individuals with exclusive MLGs each and 21 individuals sharing nine identical MLGs.

Eight fragmentation events were confirmed among the 69 studied individuals, usually generating one more individual (there was one case only where one individual generated other two), always with the same MLG. Individuals possessing identical MLGs were found at a mean distance of 0.74cm (±0.32cm SE), ranging from 0.15cm to 6.55cm. Six events of fusion, all between two individuals with the same MLG, were confirmed with microsatellite *loci*. Although many individuals with different MLGs were physically close, fusion events or contacts among them were never observed.

Size and longevity

The individuals studied presented a great variation in size, increasing and decreasing their occupation in the substrate during the study (Fig. 2). Due to the great individual size variation, it is not possible to correlate size with age in this species. In 62.5% of the fragmentation events noticed, we observed a decrease in the size of individuals, while in 100% of fusions the size of the individual increased. However, fragmentation and fusion were not the only responsible for size variations, as many individuals that did not fuse or fragment, presented great increases or decreases in size (Fig. 2). The sponges lived from one (seven individuals: 10%) to 16 months (one individual: 23%). Considering the 69 individuals studied the mean life span was of 4.5 months (±0.3 SE). It was possible to observe two events where an individual disappeared and another one with the same MLG appeared in the same place some months later, suggesting that a microscopic fragment of the sponge had been left in the substrate and regenerated the sponge.

Discussion

Fragmentation and fusion

Fragmentation and fusion events are common among the marine sessile fauna and are recognized as frequent events and part of the life history of some demosponges (Wulff, ´91; Tsurumi and Reiswig, ´97; Garrabou and Zabala, 2001; Tanaka, 2002; Zilberberg et al., 2006; Blanquer et al., 2008, 2009) and Calcarea (Johnson, ´79; Gaino et al., ´91, ´96) species. It has already been suggested that fragmentation could be a strategy to prevent the spread of pathogens, increase chances of dispersal, deal with energetic constraints on growth or as a physiological response to increase the chances of genotype survival after changing environmental conditions, events of partial mortality and population decrease in sponges (Reiswig, ´73; Sebens, ´87; Gaino et al., ´91; Wulff,

´91; Tanaka, 2002), corals and other marine invertebrates (Hughes and Jackson, ´80 and references therein). Somatic fusion could provide immediate ecological benefits related to size and reproduction. In Calcarea, both events have already been observed in *C. clathrus*, *C. coriacea*, *C. blanca* and *Borojevia cerebrum* (Johnson, ´78, ´79; Gaino et al., ´91, ´96), but not in *Paraleucilla magna* (Guardiola et al., 2011; Cavalcanti et al., 2013). Species of these three genera have very different external body morphologies, aquiferous system and skeletal organizations, suggesting that the organization and shape of calcareous sponges may influence their capacity of fragmentation and fusion.

Although widely known in sponges, few works followed fragmentation and fusion events using molecular markers. In the present work, we followed the genotype of each individual before and after events of fragmentation and fusion of the calcareous sponge *C. aurea*. As commonly assumed, fragmentation events always generate individuals with identical MLGs, *i.e.*: clones. This was observed in two species of *Chondrilla* (Zilberberg et al., 2006) and confirmed in our study, at least for the calcareous sponge *C. aurea*. Although this assumption may seem obvious, genetic changes, such as somatic mutations, gene duplications and changes in ploidy level have already been reported during asexual reproduction in invertebrates and plants, evidencing that asexually produced lineages can display genetic variability (Buss, ´85; Santelices, ´99; Lushai and Loxdale, 2002; Loxdale and Lushai, 2003). Such high variability may be beneficial for short-lived organisms occupying niches in dynamic habitats where environmental factors change rapidly and fast adaptations are crucial for survival (Vrijenhoek, ´98; Loxdale and Lushai, 2003).

Fusions in *C. aurea* occurred only between genetically identical individuals, precluding the formation of chimaeras in nature. Although many genetically different individuals were found living very close to each other, different MLGs were not

observed fusing. This may suggest the existence of an allorecognition system at least in adults of *C. aurea* to avoid chimerism. Graft experiments in adult demosponges and Calcarea, showed a high variability in allorecognition responses, probably due to plasticity in histocompatibility genes (Fernandez-Busquets and Burger, ´99). For example, in the demosponge *Aplysina fistularis*, fusion between conspecifics with different morphological phenotypes were attributed to somatic mutations or due to kinship among sponges (Neigel and Schmahl, ´84). On the other hand, Amano (´90) did not find any fusion among individuals of the calcareous sponge *Leucandra abratsbo*, and suggested that the protruding spicules of this species might form a physical barrier that would hamper fusion between individuals. The same explanation cannot be applied to *C. aurea*, since this species does not have a hispid external surface. Therefore, allorecognition in this species may be controlled by the most external cell layer, the pinacoderm. It has already been suggested for demosponges that the pinacocytes initiate the recognition response with further participation of other cell types (Buscema and van de Vyver, ´84).

Although fusion is precluded between allogeneic adults, or possible only among kin (van de Vyver, ´75; Amano, ´90; Gaino et al., ´99), it is possible among larvae and early juveniles from genetically different individuals (Ilan and Loya, ´90; Maldonado, ´98; McGhee, 2006; Gauthier and Degnan, 2008). This indicates the existence of an ontogenetic window when allogeneic fusion is allowed, similarly to what is observed in different classes of cnidarians (Frank et al., ´97; Barki et al., 2002; Fuchs et al., 2002). If such an ontogenetic window exists in *C. aurea*, allowing allogeneic fusion among larvae or juveniles, we would expect to observe IGH in adults and we did not observe that in the present work. Although the larval phase is still unknown in *C. aurea* (Lanna and Klautau, 2015), we may suggest that, if fusion of genetically different larvae

happens, they die due to internal cellular conflicts or one of the cellular lineages is eliminated, such as in colonial tunicates (Rinkevich and Weissman, ´92), precluding IGH in adult individuals.

The occurrence of IGH, either by fusion between genetically different entities (chimerism) or somatic mutations (mosaicism) is described among several metazoan phyla (Buss, ´82; Pineda-Krch and Lehtilä, 2004). In sessile marine invertebrates, it may confer ecological advantages related to size and reproduction and increase the genetic diversity. Chimerism and mosaicism were suggested as putative causes for the IGH identified in one sponge species. Multiple genotypes were found compartmentalized in single individuals of *Scopalina lophyropoda*, a species with frequent fragmentations and fusion events and high genetic variability. In this demosponge, the occurrence of IGH in adults was suggested as the cause for the high genetic diversity of its populations (Blanquer and Uriz, 2011). Although *C. aurea* also shows a high genetic diversity, IGH was not found in this species, suggesting that it is not a consequence of adult allogeneic fusion or somatic mutations and its origins should be investigated.

As the individuals with identical multilocus genotypes were at a mean distance of less than 1cm from each other (and maximum of 6.55cm), we can suppose that the dispersal capacity of asexual fragments in *C. aurea* is extremely low. Although we cannot discard the possibility that fragments can disperse longer distances, the present observation is important to population genetic studies, since the sampling of clonal individuals must be avoided. In the present study, few events of fragmentation (eight) and fusion (six) were observed among individuals of *C. aurea*; however, we cannot discard that these numbers are underestimated, since more of such events may have happened between the sampling periods and, therefore, were not accounted.

Size and longevity

The dynamics of increase and reduction in size of individuals of calcareous sponges was already documented (Johnson, ´79; Gaino et al., ´91; ´96; Cavalcanti et al., 2013). In *C. clathrus* it can vary in an order of 50% (increase or decrease) in less than five days and also in different parts of the same individual (Gaino et al., ´91). In *C. blanca, C. coriacea* and *B. cerebrum*, size reduction was attributed to the reproductive effort (Johnson, ´79; Gaino et al., ´96), but in *C. aurea* we cannot make such suggestion because reproductive elements are not even known in this species (Lanna and Klautau, 2015). In the present work, we did not observe a seasonal trend in the size variation of *C. aurea* nor was it associated only with fragmentation (as many individuals that did not fragment, also reduced in size). This observation suggests that this characteristic may be controlled by an internal regulation of the individual or by external ecological factors or interactions. In *Paraleucilla magna* and *C. clathrus* (in the example above), the causes of size reduction were also not clear (Gaino et al., ´91; Cavalcanti et al., 2013), although seawater temperature is considered the main factor triggering reproductive effort and metabolic activities in the class (Orton, ´14; Johnson, ´79; Burlando et al., ´92; Lanna et al., 2014). Intra-specific growth rate variability is commonly reported for encrusting demosponges (Fell and Lewandowsky, ´81; Turon et al., ´98; Blanquer et al., 2008) and is influenced by environmental parameters such as habitat differences, depth, intra- and interspecific competition and temperature in organisms with indeterminate growth (Sebens, ´87; Turon et al., ´98; Duckworth et al., 2004).

Our results indicate that *C. aurea* has a mean life span of 4.5 months, ranging from one to at least 16 months. Other authors have already observed short life spans for calcareous sponges, ranging from few weeks to one year (Orton, ´14, ´20; Johnson, ´79;

Cavalcanti et al., 2013). Compared to calcareous sponges, demosponges have usually a longer life span that can range from months (Ereskovsky, 2000) to years (Turon et al., ´98), decades (Mercado-Molina et al., 2011) or even hundreds to thousands of years (McMurray et al., 2008). The interindividual variation in size observed during the present study could not be correlated with the age of individuals and the same was observed with the demosponge *Crambe crambe* (Turon et al., ´98) and the calcareous *P. magna* (Cavalcanti et al., 2013). General life-history theories predict that small species are expected to have shorter life spans allied with faster growth rates than larger species (Hernaman and Munday, 2005). Moreover, it was already suggested for sessile marine invertebrates that a higher survivorship could compensate sporadic or low recruitment rates (Silvertown et al., ´93; Linares et al., 2007; Mercado-Molina et al., 2011). Considering the literature concerning calcareous sponges (Orton, ´14, ´20; Johnson, ´79; Gaino et al., ´91; ´96; Cavalcanti et al., 2013; Padua et al., 2013b), the general condition seems to be a short life span associated with high recruitment rates for species of this class.

It is not possible to discard that interactions such as competition for space with other sessile species may have influenced the area coverage variations and survival of individuals. Thus, more studies are necessary to evaluate which environmental characteristics or interactions may influence the survival and growth rates of *C. aurea* and other calcareous sponges.

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Tables

Table 1: Multilocus genotypes of three fragments from each individual of *Clathrina aurea* analyzed for IGH in the present study. (--) means that the *locus* could not be successfully genotyped.

Ind.	Frag.	Cau_A7	Cau_B2	Cau_C2	Cau_D1	Cau_D8	Cau_E6	Cau_G3
1	1	336/344	326/328	404/404	476/480	366/368	186/186	154/156
	2	336/344	326/328	404/404	476/480	366/368	186/186	154/156
	3	336/344	326/328	404/404	476/480	366/368	186/186	154/156
2	1	336/336	330/332	404/410	462/476	392/394	160/160	156/156
	2	336/336	330/332	404/410	462/476	392/394	160/160	156/156
	3	336/336	330/332	404/410	462/476	392/394	160/160	156/156
3	1	338/338	334/334	404/410	476/480	397/399	160/184	156/156
	2	338/338	334/334	404/410	476/480	397/399	160/184	156/156
	3	338/338	334/334	404/410	476/480	397/399	160/184	156/156
4	1	336/338	326/328	398/418	462/476	398/400	160/160	156/156
	2	336/338	326/328	398/418	462/476	398/400	160/160	156/156
	3	336/338	326/328	398/418	462/476	398/400	160/160	156/156
5	1	336/338	336/338	392/410	476/476	--	160/160	156/156
	2	336/338	336/338	392/410	476/476	--	160/160	156/156
	3	336/338	336/338	392/410	476/476	--	160/160	156/156
6	1	336/338	326/328	404/410	476/480	407/409	160/160	156/156
	2	336/338	326/328	404/410	476/480	407/409	160/160	156/156
	3	336/338	326/328	404/410	476/480	407/409	160/160	156/156
7	1	338/340	332/334	392/404	476/476	399/401	160/160	154/156
	2	338/340	332/334	392/404	476/476	399/401	160/160	154/156
	3	338/340	332/334	392/404	476/476	399/401	160/160	154/156
8	1	338/340	324/336	404/418	476/476	--	160/160	156/156
	2	338/340	324/336	404/418	476/476	--	160/160	156/156
	3	338/340	324/336	404/418	476/476	--	160/160	156/156
9	1	338/340	330/332	412/418	476/476	--	160/160	156/156
	2	338/340	330/332	412/418	476/476	--	160/160	156/156
	3	338/340	330/332	412/418	476/476	--	160/160	156/156
10	1	338/340	330/332	402/412	462/462	389/391	160/186	156/156
	2	338/340	330/332	402/412	462/462	389/391	160/186	156/156
	3	338/340	330/332	402/412	462/462	389/391	160/186	156/156

Figures

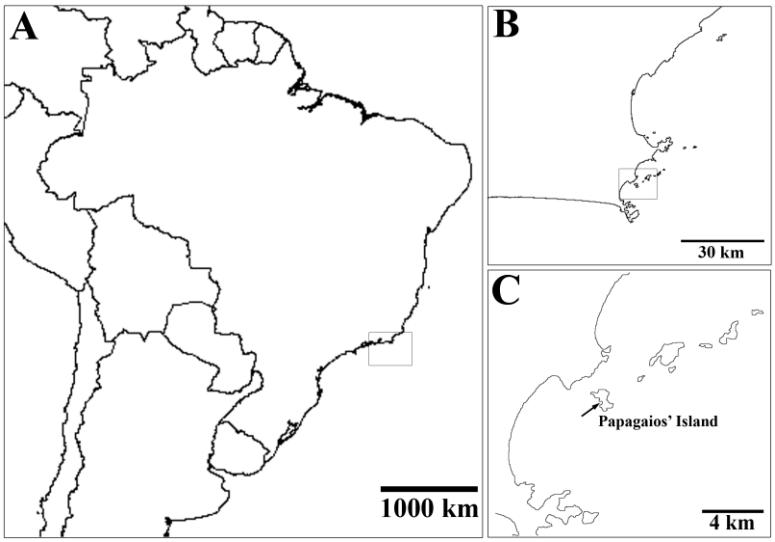

Fig. 1. Map showing where the present study was undertaken: (A) Brazil; (B) Cabo Frio region; (C) Papagaios' Island with an arrow pointing to the location of the study.

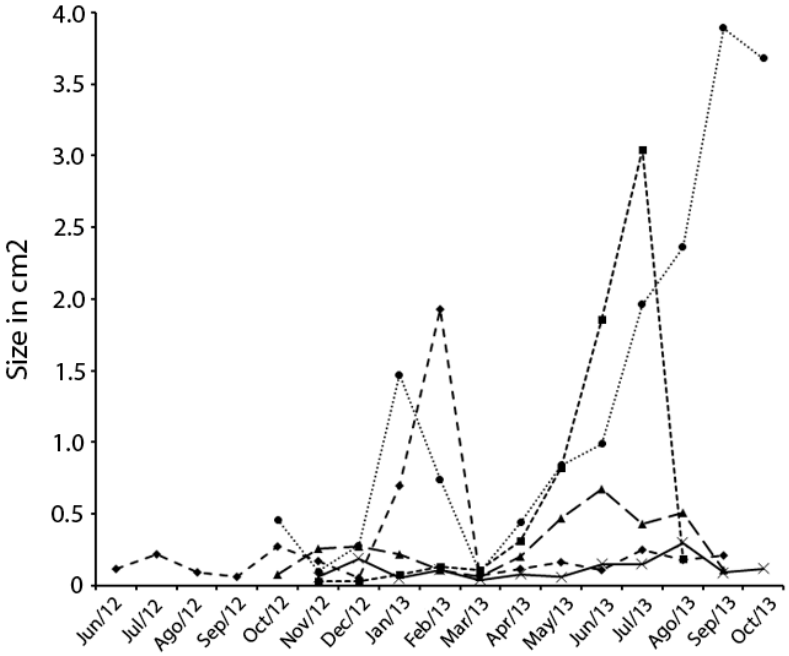

Fig. 2. Size variations (increase and reduction) in cm^2 of five selected individuals that lived more than 10 months. Each line represents an individual.

CAPÍTULO 4

TÍTULO: Genetic homogeneity in nature *versus* chimerism *in vitro* in calcareous sponges (Porifera, Calcarea).

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Genetic homogeneity in nature *versus* chimerism *in vitro* in calcareous sponges (Porifera, Calcarea).

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Abstract

Adult sponges are able to fuse their bodies with genetically identical conspecifics in nature and to aggregate their isolated cells, developing a complete individual after cell dissociation *in vitro*. Both abilities require a system of self-recognition. The absence or loss of such system may originate chimeric individuals or, in the case of dissociated cells, may hamper the formation of functional sponges. In the class Demospongiae, although the presence of a recognition system has already been proposed in adults, chimeric individuals were recently reported in a species with frequent fusion events. In dissociated cells experiments, allogeneic mixtures are usually present at the beginning, being followed by sorting out of cells or cytotoxic reactions and cell death. In the class Calcarea, chimeras were never found *in situ*, although observations of allogeneic mixtures *in vitro* have already been demonstrated. A previous study showed that fusion in *Clathrina aurea* occurred only

between genetically identical individuals, suggesting that chimeric sponges did not occur in nature. In the present work, the existence of naturally occurring chimeric individuals of the calcareous sponge *Paraleucilla magna* was investigated. Besides, the formation of allo- and xenogeneic cell aggregates (primmorphs) after dissociation of the calcareous sponges *Clathrina aurea* and *Paraleucilla magna* was investigated. The genotypic identity of the individuals was confirmed by microsatellite markers and the cells from each individual were stained with different fluorescent dyes. Chimeric individuals of *P. magna* were not found in nature, but primmorfs of *P. magna* and of *C. aurea* were formed by cells from genetically different individuals. In *C. aurea*, these aggregates lasted for six days and died upon adhesion to the substrate, but in *P. magna*, in one third of the experiments undertaken, chimeric aggregates adhered to the substrate and started the reorganization, but never reached the state of functional sponges. Xenogeneic cell mixtures of *C. aurea* and another *Clathrina* species were most species-specific, but bi-species aggregates were also observed. We raise the hypothesis that, after dissociation, the undiferentiated cells of calcareous sponges may lose their capacity of self-recognition. Future studies will allow the understanding of the molecular mechanisms underlying the recognition system of Calcarea.

Keywords: Allorecognition, Cell aggregation, Chimerism, IGH, Xenorecognition

Introduction

Genetic homogeneity is traditionally considered the rule within a living organism; however, several examples showing the opposite have been reported across all life kingdoms (Pineda-Krch & Lehtilä 2004). Intraorganismal genetic heterogeneity (IGH) can result in internal cellular conflicts and even cell parasitism, but it can also lead to a higher genetic variability of the individual and its population. Mosaicism and chimerism are the main types of IGH but they differ on their origins. Mosaicism arises through genetic changes in a single organism. It has been reported especially in clonal organisms, such as marine invertebrate taxa, plants and fungi, and does not seem to be a rare event as previously considered (Loxdale & Lushai 2003; Pineda-Krch & Lehtilä 2004; Maier et al. 2012; Schweinsberg et al. 2015). Chimeric organisms, on the other hand, are formed by contact followed by fusion of genetically different individuals and is a widespread phenomenon in animals, fungi and plants (Pineda-Krch & Lehtilä 2004). Mechanisms that avoided fusion upon body contact emerged in all animal phyla, mainly in sessile marine adult animals, and especially in those living in highly dynamic environments hampering the formation of chimeras. The sessile condition was probably responsible for imposing the emergence of a recognition system upon contact to maintain the integrity of the individual and avoid the deleterious effects of fusion (Buss 1987; Grosberg 1988; Brusini et al. 2013).

Sponges (phylum Porifera) are among the most abundant elements of the marine benthos and represent the earliest branch of extant metazoans. They are sedentary, filter feeding organisms possessing a simple body organization characterized by a great morphological plasticity and high mobility and totipotency of cellular elements (van Soest

et al. 2012). As most sessile invertebrate taxa, sponges are capable of body fragmentation and fusion and such events are probably influenced by their external morphology (Wulff 1991; Tsurumi & Reiswig 1997). *In situ* observations and graft experiments show that fusion in sponges does not occur between different species and is observed mostly between genetically identical conspecifics or kin (van de Vyver et al. 1985; Amano 1990; Gaino et al. 1999; Chapter 3). Although not allowed between genetically different adults, fusion is possible between larvae or juveniles derived from different individuals (Ilan & Loya 1990; Maldonado 1998; Gauthier & Degnan 2008). Therefore, allorecognition seems to be the rule in adult sponges, precluding fusion between genetically different individuals and the formation of chimeras. Nevertheless, a case of natural IGH was recently described for the first time in adult individuals of the demosponge *Scopalina lophyropoda*, a species with frequent fragmentation and fusion events (Blanquer & Uriz 2011).

The recognition capacity of sponges is present even after their body organization is totally disarranged. The discovery of this morphogenetic capacity of sponges, discovered by Wilson (1907), led to several works on the complexity of aggregation and recognition behaviour of sponge cells (van de Vyver et al. 1985). Xenogeneic (different species) sponge cell mixtures result in aggregates composed of cells of only one species (Wilson 1907; Curtis 1970a; Humphreys 1970; Johnston 1990), although some works have already shown bi-species aggregations (de Laubenfels 1928; Curtis 1962; Sarà 1956; Sarà et al. 1966a; Curtis 1970a,b). When cells from different conspecifics are mixed, allogeneic cell aggregates are formed, but do not develop into functional sponges, as observed in autogeneic cell aggregations (Johnston 1990; Yin & Humphreys 1996).

Studies concerning adult or cellular allorecognition and the presence of natural IGH in sponges are almost restricted to the class Demospongiae. In adult individuals of the calcareous sponges *Leucandra abratsbo* HOZAWA 1929 and *Clathrina aurea* SOLÉ-CAVA et al. 1991, for example, IGH was not observed and fusion was restricted to genetically identical individuals or close kin (Amano 1990; Chapter 3). On the other hand, when dissociated cells of different species were put into contact, bi-species aggregates were observed (Sarà 1956). Therefore, in the present study, we reported the occurrence of natural IGH *in situ* and investigated if there is cell allorecognition after dissociation in two calcareous sponge species.

Methods

Species studied

Two species of calcareous sponges were used as models in the present study: *Clathrina aurea* and *Paraleucilla magna* KLAUTAU ET AL. 2004. In the former, a high genetic diversity has already been reported (Padua et al. 2013) and IGH was not detected, since fusion *in situ* happened only between genetically identical adult individuals (Chapter 3). *Paraleucilla magna* also presents high genetic diversity, but events of fragmentation and fusion were never observed in this species (Guardiola et al. 2011; Cavalcanti, 2013; Cavalcanti et al. 2013).

Natural IGH

The natural occurrence of IGH was investigated for *C. aurea* in a previous study (Chapter 3). Here, we accessed this information for *P. magna* with the same methodology previously used: 10 specimens were collected in Cabo Frio, Southeastern Brazil (22°53'50"S, 41°58'56"W) and three fragments from different parts of the same individual were taken and genotyped for nine microsatellite *loci* formerly developed for the species. Unioscular and multioscular individuals were considered. The fragments were fixed in 93% ethanol and DNA extractions were performed by the phenol-chloroform protocol (Hillis *et al*., 1996). The PCR conditions and genotyping procedures for each *locus* followed the conditions described by Cavalcanti (2013).

Dissociated cell recognition

Dissociated cell recognition experiments were undertaken with *C. aurea* and *P. magna*. Individuals of each species were collected in Cabo Frio, Southeastern Brazil (*C. aurea*) or in Vermelha Beach, Rio de Janeiro (22°57'18"S, 43°09'42"W) (*P. magna*) and brought to the laboratory in aerated vials and seawater temperature as close as possible to that of the sponge environment to minimize stress to the sponges. Individuals were collected as far as possible from each other to avoid sampling clones and siblings. To confirm that the individuals collected were genetically different, a fragment was removed and genotyped for microsatellite *loci* (Cavalcanti 2013; Padua et al. 2013). Each experiment involved two genetically different individuals (Supplementary Material 1) and was performed in a six-well plate containing: two controls for individual 1 (individual 1 x 1; stained and unstained), two controls for individual 2 (individual 2 x 2; stained and

unstained) and two allogeneic cell mixtures (individual 1 x 2; stained and unstained). For this experiment six replicates were made for *C. aurea* and 12 for *P. magna*. An additional experiment for xenorecognition was made with *C. aurea* and a undescribed species of *Clathrina* (n=two).

At the laboratory, the sponges were washed with 0.22 µm-filtered seawater (FSW) and softly cleaned with a brush and forceps to remove epibionts. Specimens were always kept in FSW to avoid air contact. The sponges were cut into fragments of about 2 mm^2 and transferred to falcon tubes containing calcium and magnesium-free seawater with EDTA (CMFSW-EDTA: NaCl: 0.4585 mol/L; $NaSO_4$: 0.0082 mol/L; KCl: 0.0099 mol/L; Hepes buffer: 0.0099 mol/L; EDTA: 0.0032 mol/L; pH 8.0-8.2), in a proportion of 1:10 (1 part of sponge for 10 parts of CMFSW-EDTA). For one hour the fragments in CMFSW-EDTA were gently homogenized every 5 minutes. After dissociation, cells were spinned for 10 seconds and the supernatant was transferred to another tube, which was centrifuged for 5 minutes at 1200 rpm. After centrifugation, the supernatant was discarded and the cells were gently resuspended in 2 mL FSW. Half of this cell suspension (one millilitre) was used for the unstained isogeneic controls, while the other half was incubated with a fluorescent dye at a final concentration of 10 µM. Cells from one individual were always incubated with CMFDA (green) while those from the other were incubated with CMTMR (orange) (Life Technologies). Incubation time was of 30 minutes and occurred in the dark. After incubation, the cells were submitted to two washing rounds consisting of 5 minutes centrifugation at 1200rpm. The supernatant was discarded and the cells were resuspended in 2mL FSW and distributed in a six-well plate as described above. The plate was

maintained at 18°C in the dark and aggregates were observed through an epifluorescence inverted microscope (Olympus).

Results

Natural IGH

Within the successfully genotyped *loci*, we observed that all fragments collected in the 10 individuals of *P. magna* presented the same MLG, indicating genetic homogeneity in adult individuals of the species (Supplementary Material 2).

Dissociated cell recognition

After sedimentation, single cells and aggregates of various sizes were observed (Fig. 1A). Single cells assumed mainly two forms: rounded or amoeboid, with the latter actively exploring the surroundings with pseudopods. The events occurred in control and mixed cell suspensions and were similar in both species, although some differences were observed and will be explained later. Initially, cell aggregates had an amorphous shape (primary aggregates) and increased in size due to additions of single cells or even aggregates. In both species, within 24 hours after the beginning of the experiment, aggregates took a rounded shape culminating in the formation of primmorphs (aggregates delimited by a layer of pinacocytes) of variable sizes (Fig. 1C, 2A). In allogeneic cell mixtures of both species, aggregates formed by cells from genetically different individuals were observed since the beginning of the experiment and fusion between primary aggregates with cells from only one individual was observed (Fig. 1, 2). All allogeneic

experiments of both species originated chimeric primmorphs within 24 hours (Fig. 1F, 2D). Fusions between isogeneic primmorphs occurred, but were not observed among allogeneic primmorphs.

By the fourth day after dissociation, spicules were observed in both controls and chimeric primmorphs of *P. magna*, while in *C. aurea* spicule formation was never observed. In all *P. magna* primmorphs, the first spicules secreted were triactines, and it usually occurred before adhesion to the substrate (Fig. 2F). When the primmorphs tried to adhere at the substrate, we observed a high mortality rate in both stained and unstained isogeneic and allogeneic wells in both species. Despite this, in 33% of the *P. magna* experiments, by the fourth day, we observed successful adhesion of both isogeneic and allogeneic primmorphs, followed by formation of choanocyte chambers and beginning of the skeleton organization (Fig. 2G, H). Both stained and unstained isogeneic mixtures led to the complete formation of functional sponges by the eigth day with the appearance of the osculum (Fig. 2H), while allogeneic young sponges formed choanocyte chambers and secreted the skeleton, but did not develop an osculum (Fig. 2G). Cell cultures could be followed by a maximum of six days for *C. aurea* due to primmorph death upon adhesion and 11 days for *P. magna* due to sponge's death or dye fading.

In *Clathrina* xenogeneic mixtures, although in rare cases we observed the formation of aggregates and even primmorphs composed of cells from two different species, most aggregates and primmorphs were species-specific (Fig. 1G). We did not observe sorting out of cells in xenogeneic primmorphs and both xenogeneic and mono-specific primmorphs lasted for six days (Fig. 1H).

Discussion

Natural IGH in sponges

Results from the present and previous studies (Amano 1990; Cavalcanti et al. 2013; Guardiola et al. 2013; Chapter 3) showed that fusion (when it occurs) in adult calcareous sponges is possible only between genetically identical parts or kin and, thus, naturally formed chimeras have never been observed in this class. In the class Demospongiae, although multiple genotypes were observed within single individuals of *S. lophyropoda* (Blanquer & Uriz 2011), most species studied showed allogeneic rejection between adult sponges (Gaino et al. 1999). In the exceptional case of *S. lophyropoda*, multiple somatic mutations or fusion between genetically different larvae, juveniles or adults were considered putative causes of the IGH found in this species (Blanquer & Uriz 2011).

Fusion in early stages of sponge development was already detected between larvae or juveniles from different individuals of demosponges (Ilan & Loya 1990; Maldonado, 1998; McGhee 2006; Gauthier & Degnan 2008). Among adults, despite the great inter- and intraspecific variability of response processes, allogeneic rejection is considered the rule (Hildemann 1980; Buscema & van de Vyver 1983; Amano 1990; Fernandez-Busquets & Burger 1999). In the species studied in the present work, IGH was not observed in nature but chimeras were formed after the sponge body had been dissociated (Chapter 2). In fact, in *P. magna*, fusion among adult individuals was never observed (Cavalcanti et al. 2013; Guardiola et al. 2013). In *C. aurea*, fusion occurs only between genetically identical adult individuals (Chapter 3). The mechanisms of recognition in adults are attributed to start with the pinacoderm, as it is the most superficial cell layer (Buscema & van de Vyver 1984),

followed by movement of other cell types to the contact zone (van de Vyver & Buscema 1990) and the formation of a collagen barrier or cytotoxic reaction in allogeneic grafts (Humphreys, 1994). Despite this, Amano (1990) suggested that the protruding spicules of the calcareous sponge *L. abratsbo* would make a physical barrier preventing contact between adult individuals and hampering fusion. As allogeneic fusions also did not occur in calcareous sponges with a smooth surface (such as *C. aurea*; Chapter 3), we suggest that a cellular process, and not a physical process (such as spicules), is the mechanism responsible for self-recognition and the absence of natural chimeras in calcareous sponges.

Dissociated cell recognition

Chimerism was not observed in adult calcareous sponges, but when body organization is disrupted by cell dissociation, chimeric cell aggregates are formed, develop into primmorphs and adhere to the substrate starting the development into functional sponges. According to previous authors, after dissociation, all or at least some specific types of cells suffer dedifferentiation attaining an undifferentiated state (Wilson 1907; Huxley 1912; Galtsoff 1925; Ganguly 1960). If dissociation indeed leads the cells to an undifferentiated state, it may, perhaps, lose its self-recognition capacity. The loss of self-recognition capacity apparently is not followed by cellular conflicts between genetically different lineages after allogeneic aggregation, as observed in demosponges (Johnston 1990; Humphreys 1994; Yin & Humphreys 1996). The capacity of achieving some critical points necessary to rebuild a functional sponge from dissociated cells, such as the development of a pinacoderm in the primmorph, spicule secretion and skeleton

organization, adhesion to the substrate and development of choanocyte chambers (Chapter 2), exemplifies this statement. In the present work, the fate of each cell lineage (individual) in the primmorph and subsequent morphogenetic events was not followed. However, in the demosponge *Amphimedon queenslandica* HOOPER & VAN SOEST, 2006, after allogeneic larval fusion, there is a partitioning of the cell lineages with each one giving rise to different cell types in the juvenile (Gauthier & Degnan 2008). The partitioning of cell lineages into different cell populations in the juvenile has important evolutionary significance, as a single genotype will give rise to the future germ cell lineage.

In our experiments, genetically different cells were able to aggregate and form primmorphs, but we never observed fusion between chimeric primmorphs. Although we did not follow from which individual, the pinacocyte layer of primmorphs originated, this result may indicate that pinacocytes are responsible for self-recognition in Calcarea, as already suggested for adult individuals of other sponges (van de Vyver 1970; Buscema & van de Vyver 1984).

Previous works also observed aggregation of dissociated cells from conspecifics in demosponges. Johnston (1990) and Yin & Humphreys (1996) observed cell degeneration followed by death starting between 24 and 48 hours after cell mixture and aggregates did not form primmorphs or functional sponges, while Humphreys (1994) noted cytotoxic reactions beginning within 2 to 4 hours, which resulted in a separation of allogeneic aggregates. Custódio et al. (2004) observed the initial adhesion followed by a collagen deposition at the contact zone in allogeneic cellular assays and pairs of primmorphs. Differently from these studies, *Clathrina aurea* allogeneic (and isogeneic) primmorphs

lasted for six days, when died upon adhesion and, in one third of *P. magna* isogeneic and allogeneic cell mixtures, primmorphs settled and developed into young sponges (Fig. 2G, H). Chimeric aggregates were able to develop a pinacocyte layer (primmorph formation) and adhered chimeric primmorphs developed choanocyte chambers, started to develop a skeleton organization similar to that of an adult, but never developed oscules (Fig. 2).

In xenogeneic cell mixtures of *Clathrina*, most aggregates were species-specific, but xenogeneic aggregates were formed at the beginning of the experiment and developed into primmorphs. Between genetically different conspecifics, allogeneic aggregation is allowed, but it is not (in most cases) when cell suspensions from different species are mixed. This may suggest that, even in undifferentiated state, dissociated cells from *C. aurea* maintain their capacity to recognize different species although they do not recognize different individuals of the same species. The recognition studies of sponge cells show a great variability of responses. Classical sponge aggregation and subsequent works studied initially the species-specific recognition capacity of sponge dissociated cells, based mainly on the different pigments each species produced. Most studies observed mono-species cell aggregations (Wilson 1910; Galtsoff 1923; de Laubenfels 1927; Curtis 1962; Humphreys 1963; 1970; Johnston 1990), usually with initial, but temporary non-specific aggregations and xenorecognition acquired over time. However, bi-species cell aggregates (de Laubenfels 1928; Curtis 1962; Sarà 1956; Sarà et al. 1966a; Curtis 1970a,b) and even aggregates made up of cells from different sponge classes (Demospongiae and Calcarea; Sarà et al. 1966a) or of different phyla (Porifera and Cnidaria; Sarà et al. 1966b) have also been reported. These last were not followed further than 24 hours, although de Laubenfels

(1927) reported bi-species aggregates maintained for 10 days. Sarà (1956), working with calcareous sponges, observed that cells from *Leucosolenia complicata* (MONTAGU 1814) and *L. botryoides* (ELLIS & SOLANDER 1786) grouped and originated stable bi-species aggregates for, at least 48 hours, while cell mixtures of different genera resulted only in mono-species aggregates. Species-specific cell recognition in sponges is mediated by molecular complexes called aggregation factors (Fernandez-Busquets & Burger 1999) and the finding of stable bi-species cell mixtures in the present work raise questions about the specificity of such molecular complexes in calcareous sponges.

Previous studies using dissociated cells for allorecognition essays did not use molecular markers to confirm if the individuals used were indeed genetically different. In the present work, variable molecular markers (microsatellites) were used for the first time to prove the genetic identity of each individual used and the differences between them. Thus, the results of the present and a previous study (Chapter 3), using variable molecular markers to confirm the genetic identity of individuals, indicate the absence of naturally occurring IGH (mosaicism or chimerism) in adult calcareous sponges (at least in *Clathrina aurea* and *Paraleucilla magna*), suggesting an allorecognition system in adults. However, when the body organization of those sponges is disrupted, allogeneic fusion and formation of chimeric aggregates and primmorphs are allowed. This indicates, perhaps, that dissociation leads the cells to an undifferentiated state (as stated by previous authors) and, consequently, to a loss of the allorecognition capacity in calcareous sponges. However, the loss of the self-recognition capacity occurs between conspecifics and recognition of different species is maintained. Further studies are necessary to investigate the recognition

mechanism and the fate of both cell lineages in calcareous sponges' allogeneic and xenogeneic cell mixtures after aggregation *in vitro*.

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Figures

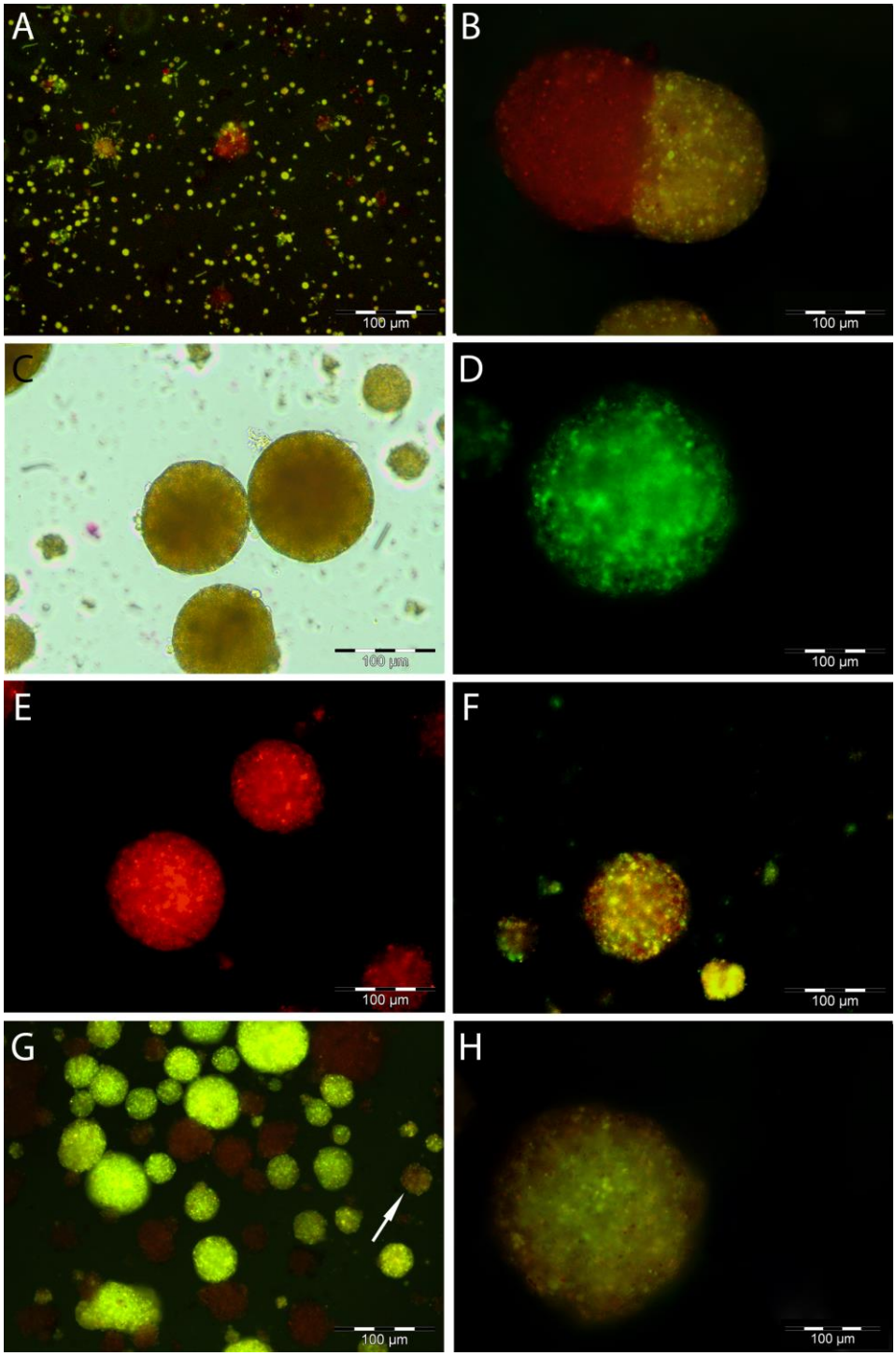

Fig. 1: *Clathrina aurea* cell recognition experiment. (A) Dissociated cells and early aggregates within allogeneic cell mixture, just after cell dissociation. (B) Two genetically different aggregates (green and orange) fusing, around two hours after beginning of the

experiment (ABE). (C) Unstained *C. aurea* primmorphs 24 hours ABE. (D) Stained control 1 primmorphs 24 hours ABE. (E) Stained control 2 primmorphs 24 hours ABE. (F) Chimeric *C. aurea* primmorph, three days ABE. (G) Xenogeneic cell mixture of *Clathrina* species showing species-specific and rare bi-species (arrowhead) primmorphs, 24 hours ABE. (H) Xenogeneic *Clathrina* spp. primmorph six days ABE.

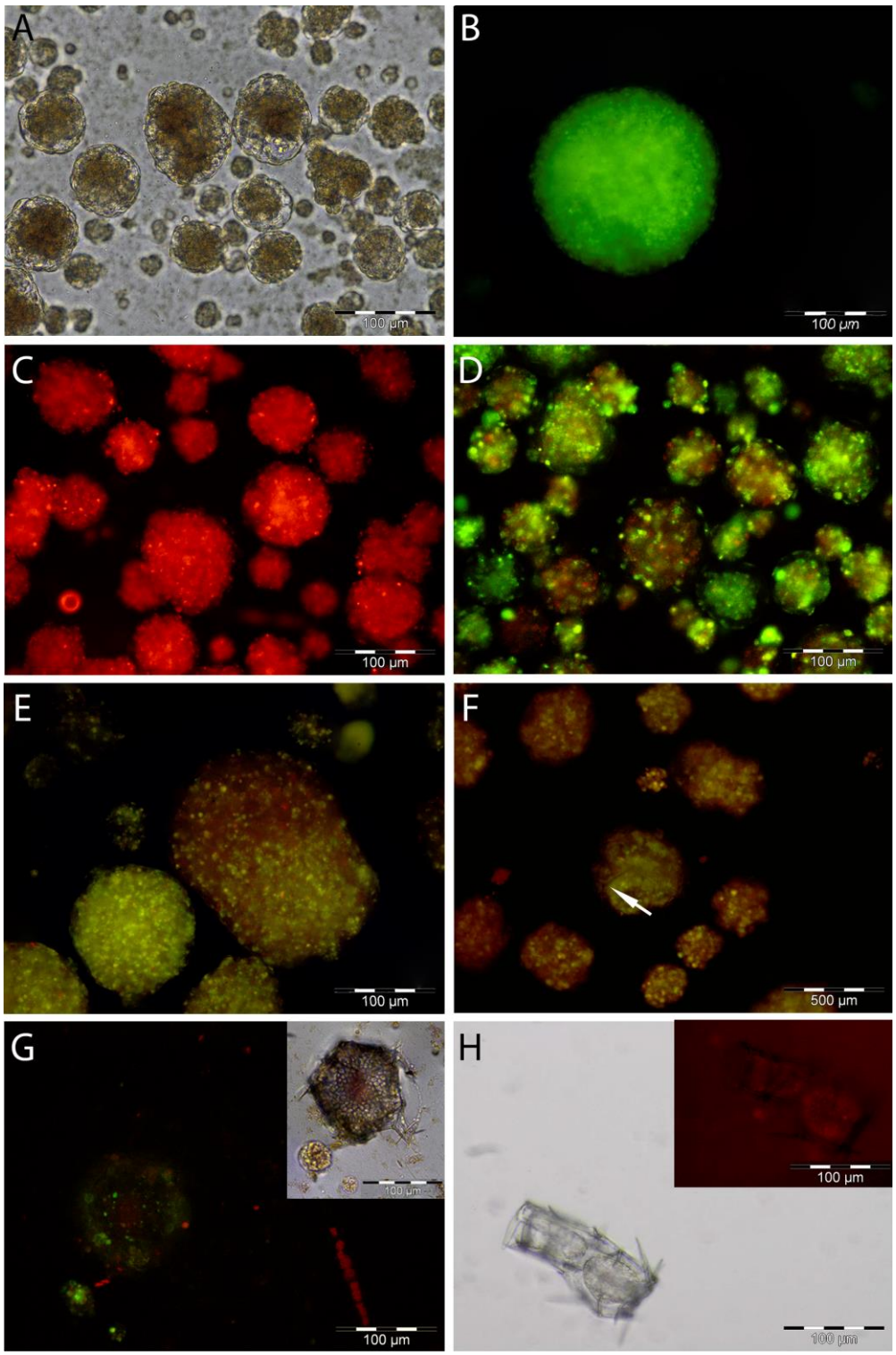

Fig. 2: *Paraleucilla magna* cell recognition experiment. (A) Unstained (control) primmorphs 24 hours after beginning of the experiment (ABE). (B) Stained control 1 primmorphs 24 hours ABE. (C) Stained control 2 primmorphs 24 hours ABE. (D, E)

Chimeric primmorphs 24 hours ABE. (F) Chimeric primmorphs not adhered to the substrate with triactine spicule (arrowhead) four days ABE (G) Adhered chimeric young sponge, in detail the same individual without fluorescence, four days ABE. (H) Functional young sponge from isogeneic cell mixture, in detail the same sponge in fluorescence, 10 days ABE.

Supplementary Material 1: Microsatellite genotypes of each experimental set of the dissociated cell experiment of *Paraleucilla magna* and *Clathrina aurea*. ExpS= experimental set; Ind= individual; (--) means that the locus was not successfully genoyped.

Paraleucilla magna:

Ind.	Frag.	PMA2	PMA4	PMA103	PMA105	PMA106	PMA113	PMB6	PMB101	PMB118
1	1	294/310	160/174	228/228	240/240	218/226	--	180/180	110/110	194/194
	2	315/323	160/166	252/252	250/255	200/224	--	206/206	--	194/194
2	1	310/314	160/165	212/212	246/246	232/244	160/160	222/240	148/172	192/194
	2	314/334	160/162	252/252	242/248	216/222	--	200/202	110/110	198/200
3	1	294/300	160/174	245/261	238/242	228/232	263/263	206/248	110/110	192/192
	2	290/310	160/162	248/248	246/248	226/228	164/164	194/194	110/136	192/192
4	1	294/310	160/176	230/252	236/236	230/244	--	220/222	100/100	194/194
	2	290/306	168/180	190/226	240/250	222/244	174/174	192/194	110/110	192/194
5	1	300/315	168/174	212/212	240/250	225/227	--	220/222	--	192/194
	2	294/310	176/176	228/252	242/250	218/226	--	260/262	153/173	192/194
6	1	310/314	162/184	188/246	242/246	228/230	--	--	--	192/194
	2	304/304	172/184	252/252	240/248	216/228	160/164	180/218	110/110	192/194

Clathrina aurea:

Ind.	Frag.	Cau_A7	Cau_B2	Cau_C2	Cau_D1	Cau_D8	Cau_E6	Cau_G3
1	1	356/356	364/364	426/432	470/490	411/423	202/204	174/174
	2	358/360	346/346	416/426	494/494	423/425	178/178	174/174
2	1	362/364	346/348	418/426	472/472	417/419	198/204	174/174
	2	358/358	350/350	426/438	496/496	409/411	178/178	174/174
3	1	358/360	346/348	426/434	466/474	417/419	178/178	174/174
	2	354/356	346/352	430/430	494/494	419/421	178/178	174/174
4	1	358/360	346/348	426/434	466/474	417/419	178/178	174/174
	2	354/356	348/350	422/428	--	419/421	178/178	174/174
5	1	354/356	346/352	430/430	494/494	419/421	178/178	174/174
	2	356/356	346/346	420/430	494/494	417/419	204/204	174/174
6	1	356/356	346/346	420/430	494/494	417/419	204/204	174/174
	2	354/356	348/350	422/428	--	419/421	178/178	174/174

Supplementary Material 2: Microsatellite genotypes of nine *loci* of each fragments taken from different parts of the body of 10 specimens of *Paraleucilla magna*. Ind= individual; Frag= fragment; (--) means that the locus was not successfully genoyped.

Ind.	Frag.	PMA2	PMA4	PMA103	PMA105	PMA106	PMA113	PMB6	PMB101	PMB118
1	1	--	146/148	--	224/234	208/226	146/146	--	172/192	166/166
	2	271/271	146/148	--	224/234	208/226	146/146	226/228	172/192	166/166
	3	271/271	146/148	--	224/234	208/226	146/146	226/228	172/192	166/166
2	1	270/290	148/148	208/210	224/230	213/225	--	202/204	172/192	152/152
	2	270/290	148/148	208/210	224/230	213/225	--	202/204	172/192	152/152
	3	270/290	148/148	208/210	224/230	213/225	--	202/204	172/192	152/152
3	1	--	--	210/210	224/230	183/183	146/146	--	107/107	174/174
	2	271/271	144/166	210/210	224/230	183/183	146/146	--	107/107	174/174
	3	271/271	144/166	210/210	224/230	183/183	146/146	--	107/107	174/174
4	1	270/272	144/166	208/226	222/232	208/212	146/146	162/162	172/192	172/174
	2	270/272	144/166	208/226	222/232	208/212	146/146	162/162	172/192	172/174
	3	270/272	144/166	208/226	222/232	208/212	146/146	162/162	172/192	172/174
5	1	294/294	146/146	208/208	224/234	182/182	146/146	160/160	172/192	--
	2	294/294	146/146	208/208	224/234	182/182	146/146	160/160	172/192	--
	3	294/294	146/146	208/208	224/234	182/182	146/146	160/160	172/192	--
6	1	260/260	144/166	376/378	222/224	200/214	142/142	228/230	92/92	166/166
	2	260/260	144/166	376/378	222/224	--	--	228/230	92/92	166/166
	3	260/260	144/166	376/378	222/224	200/214	142/142	228/230	92/92	166/166
7	1	270/300	146/158	208/208	224/232	178/178	146/146	234/236	172/192	166/166
	2	270/300	146/158	208/208	224/232	178/178	146/146	234/236	172/192	166/166
	3	270/300	146/158	208/208	224/232	178/178	146/146	234/236	172/192	166/166
8	1	271/285	144/144	210/210	224/246	182/214	146/146	160/160	172/192	176/176
	2	271/285	144/144	210/210	224/246	182/214	146/146	160/160	172/192	176/176
	3	271/285	144/144	210/210	224/246	182/214	146/146	160/160	172/192	176/176
9	1	271/285	142/144	272/274	222/224	192/214	--	176/176	172/192	--
	2	271/285	142/144	272/274	222/224	192/214	--	176/176	172/192	--
	3	271/285	142/144	272/274	222/224	192/214	--	176/176	172/192	--
10	1	263/263	148/156	210/216	224/224	208/208	146/148	162/162	128/128	174/174
	2	263/263	148/156	210/216	224/224	208/208	146/148	162/162	128/128	174/174
	3	263/263	148/156	210/216	224/224	208/208	146/148	162/162	128/128	174/174

DISCUSSÃO GERAL

A busca por uma definição do que representa e define um indivíduo não é recente. Pelo menos no mundo ocidental, tais questionamentos podem ser traçados até o Período Clássico da Grécia Antiga (cerca de 2500 anos atrás). Aristóteles reconhecia cada indivíduo como uma entidade com traços biopsicossociais particulares que eram formados ou influenciados pela hereditariedade, pelas experiências emocionais e pela influência da sociedade (Ierodiakonou, 2012). Mais recentemente, o movimento filosófico e literário existencialista, representado por Søren Kierkegaard e Jean-Paul Sartre, por exemplo, centrava e enfatizava suas ideias na existência humana pela liberdade do indivíduo em definir quem ele é a partir das suas vivências, da sua responsabilidade e capacidade de definir sua própria essência e dar significado à sua vida apesar dos obstáculos que ela possa apresentar (Flynn, 1999).

Na verdade, o termo indivíduo pode ser abordado de vários pontos de vista, dependendo da disciplina: filosofia, sociologia, biologia evolutiva, ecologia, biologia do desenvolvimento, medicina, entre outras.

O indivíduo biológico

Charles Darwin, em seu *A Origem das Espécies*, descreve que as forças da seleção natural atuam sobre indivíduos e que estes precisam ter variações no seu *fitness* que sejam herdáveis (Darwin, 1859). Entretanto, autores mais recentes defendem que outros níveis de organização biológica, como moléculas, células, grupos, populações e espécies, possuem, em algum grau, estas características e, portanto, podem atuar como unidades de seleção ou como indivíduos em um senso evolutivo (Lewontin, 1970; Hull, 1980; Michod & Roze, 1997; Dawkins, 2007).

No entanto, saindo do escopo da biologia evolutiva, o termo indivíduo é, muitas vezes, tratado como um sinônimo ou uma aproximação de "organismo", sendo entendido de forma geral como uma entidade discreta e integrada, com limites espaço-temporais bem definidos (Hull, 1980; Santelices, 1999). Discussões acerca desta definição surgiram ao longo do século XX. Indivíduos biológicos são entidades organizadas (mesmo que não seja durante todo o ciclo de vida), compostas por partes que devem atuar coordenadamente e em harmonia umas com as outras gerando integração fisiológica e autonomia (Clarke, 2010). Vários atributos já foram considerados para a definição de um indivíduo: capacidade de reprodução (sexuada), genótipo único, apresentar separação entre linhagens germinativa e somática, histocompatibilidade, mecanismos de cooperação, controle e

mediação de conflitos, limite espaço-temporal, entre outras (Huxley, 1912b; Hull, 1980; Buss, 1983; 1987; Fagerström, 1992; Maynard-Smith & Szathmáry, 1995; Santelices, 1999; Queller & Strassmann, 2009; Clarke, 2010). No entanto, dependendo do organismo, muitos desses atributos são violados ou considerados não essenciais e, por isso, muitos autores defendem a necessidade de uma definição mais ampla da individualidade (Fagerström, 1992; Santelices, 1999; Pineda-Krch & Lehtilä, 2004; Tuomi, 2004; Queller & Strassmann, 2009; Clarke, 2010).

O indivíduo de esponja

Dois dos mais frequentes assuntos de debate na história na espongologia foram a natureza animal destes organismos e, depois, o que representaria um indivíduo. Aristóteles já considerava esponjas como animais, ao incluir esses organismos em seus tratados de zoologia (como *História dos Animais* e *Partes dos Animais*). Apesar disso, o filósofo grego reconhecia a natureza ambígua destes organismos, por possuírem algumas características animais (como resposta a estímulos) e outras vegetais (não se moverem) (Voultisiadou, 2007; Perrier *et al.*, 2009). Tal "natureza ambígua" foi responsável pela classificação das esponjas como plantas ou mesmo como Zoophytas (animais-planta) por muitos séculos. Contrariando a visão que dominava naquele momento, por volta da metade do século XVIII, John Ellis propôs que esponjas fossem, na verdade, animais. A condição animal foi confirmada por Robert Grant e outros naturalistas no início do século XIX, a partir da constatação da criação de fluxo de água no interior da esponja por células flageladas (Hartman & Reiswig, 1973; Levi, 1999).

Após a sua aceitação como um animal, proposições acerca da concepção do indivíduo entre as esponjas foram o foco de muitos naturalistas. Em meados do século XIX, o francês Isidore Geoffroy de Saint-Hillaire, trabalhando no Museu Nacional de História Natural de Paris sobre a transição de colônias para indivíduos verdadeiros, apesar de não ser categórico em suas conclusões, reconhecia a dificuldade de identificar indivíduos ("pólipos") dentro de uma "colônia" de esponja (Perrier *et al*., 2009). Ao mesmo tempo, o francês Felix Dujardin e o alemão Lieberkühn, consideravam todo o corpo da esponja como um indivíduo. No entanto, naturalistas contemporâneos e outros que se seguiram no mesmo século, olhavam para as esponjas como um "amontoado" de células ameboides e flageladas. Estes, como Henry Carter, atribuíam às células isoladas (amebócitos ou coanócitos), a unidade básica da individualidade das esponjas devido, principalmente, à sua alta capacidade de migração e independência de função. Ainda no

século XIX, outros naturalistas, como o alemão Ernst Haeckel, desenvolveram a ideia de diferentes graus de complexidade de indivíduos de esponjas de acordo com a organização do sistema aquífero. Esta ideia considerava a "cavidade gástrica" como um indivíduo. Assim, espécies asconóides de forma tubular (por exemplo, *Leucosolenia*) representariam o tipo mais simples de um indivíduo de esponja, enquanto que as câmaras coanocitárias de espécies siconóides e leuconóides eram consideradas os verdadeiros indivíduos dentro de uma colônia. Mesmo um exemplar do gênero asconóide *Clathrina* era considerado uma colônia formada por vários tubos anastomosados (Hartman & Reiswig, 1973; Levi, 1999; Ereskovsky, 2003).

Outra visão levantada durante o século XIX foi a do também alemão Oscar Schmidt que colocava o ósculo no centro do seu conceito, sendo cada indivíduo constituído por um ósculo e todas as câmaras e canais que se conectavam a este. Esta visão foi seguida e desenvolvida no início e decorrer do século XX, pelo inglês Edward A. Minchin, por autores russos (como Beklemishev, Koltun e Korotkova) e pelo belga Paul Brien, que diziam que existiam diferentes graus de individualidade desde uma esponja com apenas um ósculo até uma "colônia" contendo vários ósculos e, consequentemente, formada por vários indivíduos (Brien, 1967; Hartman & Reiswig, 1973; Ereskovsky, 2003). Brien dizia que o indivíduo básico de esponja era o *olynthus*, que ao crescer, atingiria um grau mais elevado no espectro da individualidade, as colônias (Brien, 1967).

Em seu livro de 1912, *The Individual in the Animal Kingdom*, Julian Huxley admite a independência estrutural e funcional das células de esponja, mas sugere a existência de uma individualidade maior que a das células, a qual estas fazem parte mesmo quando dissociadas, por exemplo, e assim, reconhece e considera que "*the whole sponge is a true individual*" (Huxley, 1912b). Alguns anos depois, Tuzet, Pavans de Ceccatty e Paris (1963) utilizam-se de argumentos morfológicos, embriológicos e fisiológicos para afirmar que uma unidade anatomicamente isolada representa um indivíduo de esponja. Posteriormente, Borojevic *et al.* (1968), em concordância com as ideias de seus antecessores, e reconhecendo a plasticidade tanto de forma quanto de organização do sistema aquífero, definem um indivíduo como a massa de esponja delimitada por uma pinacoderme contínua e abandonam de vez o termo colônia. Embora esta definição tenha sido largamente aceita, o termo colônia continuou sendo usado para as esponjas em alguns casos, principalmente pela sua capacidade de duplicação de "unidades funcionais" (Simpson, 1973). Posteriormente, um fator imunológico pôde ser acrescentado à individualidade das esponjas, uma vez descoberta a capacidade que

indivíduos adultos possuem de reconhecer outros indivíduos da mesma espécie e de espécies diferentes, evitando a fusão e mantendo a integridade do indivíduo (van de Vyver, 1970; Curtis, 1979).

Esponjas não seguem alguns dos atributos sugeridos e mostrados anteriormente que comporiam um indivíduo como, por exemplo, o sequestro de uma linhagem germinativa e a homogeneidade e singularidade do genótipo. Portanto, tendo em vista o escopo e os resultados desta tese, discutiremos brevemente como as esponjas se inserem em alguns atributos classicamente atribuídos a um indivíduo biológico, resumidos sob três termos: singularidade genética, homogeneidade genética e unidade anatômica e fisiológica.

Singularidade genética

A singularidade genética representaria a existência de um único genótipo dentro de um mesmo organismo e, portanto, diferenciaria indivíduos dentro de uma mesma espécie (Santelices, 1999). No entanto, este atributo é violado por diversos organismos, uma vez que a reprodução assexuada e a geração de organismos clonais são observadas em plantas, fungos, protozoários e diversos grupos animais, especialmente em metazoários basais e animais marinhos sésseis (Buss, 1983, 1987; Blackstone & Jasker, 2003; Brusini *et al.*, 2013). As esponjas não fogem a essa exceção. Apesar de eventos de reprodução assexuada não terem sido reportados ainda em algumas espécies (inclusive da classe Calcarea), esta é considerada um atributo presente, comum e amplamente reportado ao longo do ciclo de vida de praticamente todas as espécies de esponja (Reiswig, 1973; Wulff, 1991, 1995; Zilberberg *et al*., 2006a; Blanquer *et al.*, 2008), permitindo a colonização de novos ambientes e a dispersão de genótipos bem adaptados (Maldonado & Uriz, 1999; Zilberberg *et al*., 2006a). Assume-se, portanto, que a fragmentação gere indivíduos independentes, mas clonais. No Capítulo 3, confirmamos essa afirmativa para a esponja calcárea *Clathrina aurea*, mostrando que a característica da singularidade genética, ou seja, um genótipo por indivíduo, não é seguida pelas esponjas.

Homogeneidade genética

A ausência de homogeneidade genética dentro de um organismo é amplamente reportada na natureza, mostrando-se presente em protistas, plantas e animais (Buss, 1982; Gill *et al*., 1995; Rinkevich, 2011). Entre os animais, diversos estudos mostram que heterogeneidade genética intraorganísmica (IGH) pode se originar durante a maturação do

sistema imune através de uma janela do desenvolvimento, onde a fusão de tecidos diferentes é possível (Rinkevich, 2004, 2011). Em linhagens que realizam reprodução assexuada, o IGH também pode se originar durante a fase adulta, caso não exista tal sistema que permita o reconhecimento do indivíduo e impeça a fusão (alorreconhecimento; Grosberg, 1988; de Tomaso, 2006) ou a partir de alterações genéticas durante a vida do organismo ou a replicação assexuada (Gill *et al*., 1995; Loxdale & Lushai, 2003; Lushai & Loxdale, 2002).

Em esponjas, um sistema de alorreconhecimento é amplamente reportado entre indivíduos adultos (Gaino *et al.*, 1999). No entanto, comportamento gregário seguido de fusão é comumente observado *in vitro* entre larvas provenientes tanto do mesmo indivíduo como de indivíduos diferentes (Levi, 1956; Borojevic, 1967; Ilan & Loya, 1990; Maldonado, 1998; McGhee, 2006; Gauthier & Degnan, 2008) mas, não parece resultar em uma vantagem ecológica relacionada ao aumento de tamanho e maior taxa de sobrevivência (Maldonado, 1998). Em uma espécie de Demospongiae, a fusão entre larvas provenientes de indivíduos diferentes pode resultar em parasitismo de linhagens celulares (Gauthier & Degnan, 2008). Teorias de seleção de parentes (*kin selection*) argumentam que os riscos da fusão diminuem quanto mais relacionados são os organismos e explicam a possibilidade de fusão entre indivíduos geneticamente próximos ou parentes (Aanen *et al*., 2008). A seleção para a fusão entre organismos relacionados pode se dar pela presença de alelos específicos em *loci* de alorreconhecimento. Tais *loci* apresentam enorme variabilidade e já foram observados em ascídias que é possível a fusão entre indivíduos geneticamente diferentes, mas que portavam pelo menos um alelo idêntico em tais *loci* (Grosberg & Quinn, 1986, 1988; Rinkevich *et al.*, 1995; de Tomaso, 2006).

Entre as esponjas calcáreas já estudadas, a fusão na fase adulta, quando ocorre, parece estar restrita a indivíduos geneticamente idênticos (Capítulo 3) ou, em raros casos, entre indivíduos supostamente relacionados (Amano, 1990). Portanto, diferentemente do que já foi encontrado em uma espécie de Demospongiae (Blanquer & Uriz, 2011), nesta tese não observamos a existência de IGH via quimerismo em adultos da classe Calcarea, sugerindo que indivíduos dessa classe possuam mecanismos que evitem a fusão entre genótipos diferentes. No entanto, observamos que, quando as células de dois indivíduos geneticamente diferentes são dissociadas e misturadas, pode ocorrer a fusão e posterior formação de um indivíduo quimérico *in vitro*. A formação *in vitro* de juvenis quiméricos a partir da larva foi observada na demosponja *Amphimedon queenslandica* e, nesta espécie, ao longo do desenvolvimento, ocorre uma separação das linhagens celulares, cada uma

dando origem a um tipo celular no juvenil (Gauthier & Degnan, 2008). Esse fato tem importantes implicações evolutivas, pois, apesar de possuir dois genótipos no indivíduo, apenas um dará origem às células germinativas. Exemplos de sinergismo, parasitismo celular, bem como de morte e reabsorção por parte de uma das linhagens que fusionaram são observados também em outros invertebrados marinhos, tais como ascídias e corais (Rinkevich & Weissman, 1987, 1992; Rinkevich & Yankelevich, 2004). Apesar de observar a formação de quimeras *in vitro*, nós não seguimos o destino de cada linhagem celular, sendo necessários outros estudos para tentar entender o que acontece com cada uma delas após a fusão e durante a reconstituição da esponja.

Outros pontos interessantes sobre a homogeneidade genética em esponjas são: (1) a existência de micro-organismos simbiontes em vários estágios do ciclo de vida e; (2) a existência de células maternas nas larvas. Em relação ao primeiro tópico, as relações mutualísticas entre esponjas e bactérias fotossintetizantes e heterotróficas são amplamente descritas para várias espécies (Ereskovsky, 2010). Já foram encontrados representantes de quase metade dos filos de bactérias conhecidos e de várias linhagens de Archaea (Hentshel *et al.*, 2006; Taylor *et al.*, 2007) atuando tanto na síntese de compostos utilizados como proteção química (Shigemori *et al*., 1992; Jayatilake *et al*., 1996) como fonte de alimento pelas esponjas, seja via fagocitose ou pela utilização de produtos resultantes da atividade bacteriana (Vacelet *et al.*, 1996; Fromont *et al*., 2015). As esponjas podem obter as bactérias simbiontes tanto do ambiente em que vivem (Reiswig, 1971), quanto pela transmissão vertical por meio dos ovócitos e larvas (Maldonado, 2007; Ereskovsky & Boury-Esnault, 2002; Ereskovsky *et al.*, 2005). Nas espécies estudadas nesta tese, principalmente em *P. magna*, uma grande quantidade de micro-organismos simbiontes (tais como dinoflagelados e diatomáceas) que estavam presentes na própria esponja, foram observados nas culturas de células dissociadas (Figura 1). Associações simbióticas foram responsáveis por grandes transições evolutivas da vida na Terra (a origem de mitocôndrias e cloroplastos, por exemplo) e continuam sendo uma grande fonte de inovações evolutivas, permitindo uma rápida diversificação dos organismos e o acesso dos hospedeiros a novas fontes de energia (Kiers & West, 2015). Organismos simbiontes presentes em todos os animais atuam integradamente de forma fisiológica e metabólica, além de constituírem uma fonte de variação e herança genética e interagirem com o desenvolvimento de seus hospedeiros. As íntimas associações entre simbiontes e seus hospedeiros mostram que organismos holobiontes são difundidos na natureza e desafiam

as definições e os limites da individualidade (Gilbert *et al*., 2012; Booth, 2014; Gilbert, 2014).

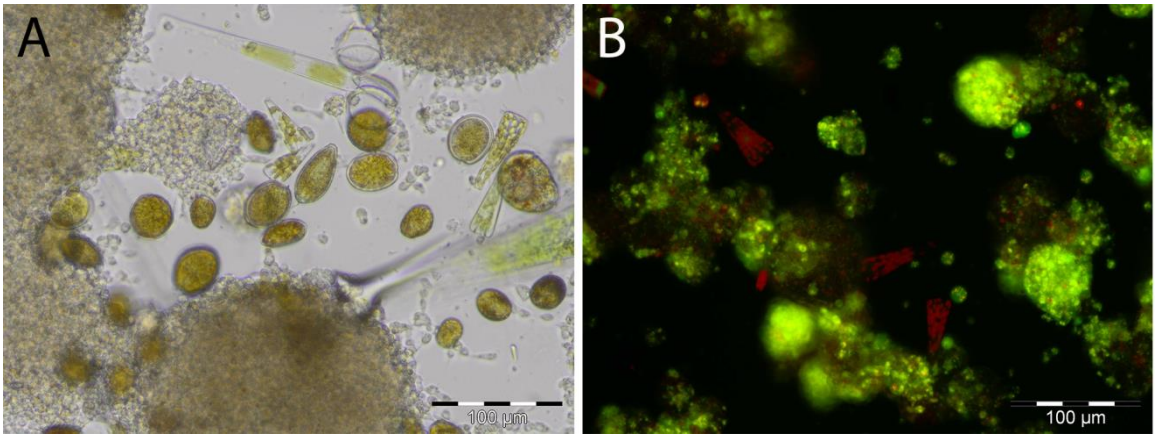

Figura 1: Imagens dos organismos associados (dinoflagelados e diatomáceas) às esponjas presentes nas culturas de célula. (A) com luz branca e (B) utilizando filtro azul (ca. 450nm) para mostrar a fluorescência dos organismos.

A presença de células esferulosas de origem materna no interior das larvas é conhecida em várias espécies de Demospongiae e Homoscleromorpha. Tais tipos celulares apresentam as mais diversas funções: imune, secretora, excretora, armazenamento de toxinas, metabólitos e produção de matriz extracelular (Ereskovsky, 2010). Apesar de não se ter maiores evidências da atuação das células maternas no desenvolvimento das esponjas, a sua permanência nos estágios subsequentes poderia ser uma fonte de IGH em indivíduos adultos. Já foi sugerido também que a reabsorção por parte da mãe de larvas não liberadas durante o ciclo reprodutivo pudesse ser outra possível fonte de IGH nos adultos (Maldonado & Uriz, 1999; Blanquer & Uriz, 2011). No entanto, isso nunca foi demonstrado em esponjas, mas sabe-se, por exemplo, que o quimerismo materno-fetal é o tipo mais comum em mamíferos (Rinkevich, 2004, 2011).

Unidade anatômica e fisiológica

Esta talvez seja a característica em que as esponjas melhor se enquadrem. Um mesmo indivíduo revestido por uma camada única de pinacoderme e contínua de esqueleto é capaz de coordenação e sincronização nos eventos reprodutivos (Maldonado & Riesgo, 2008), na resposta a estímulos (Leys *et al.*, 1999), na contração dos ósculos e no controle do fluxo de água (Reiswig, 1971). No entanto, a unidade anatômica e fisiológica coesa do indivíduo adulto pode ser corrompida ou totalmente desfeita, por exemplo, em casos de regeneração e dissociação celular podendo, em alguns casos, ser reconstituída.

Durante o processo de regeneração após a perda de parte do corpo, sabe-se que esponjas com forma definida e polarizada apresentam maior capacidade de reconstituir a parte perdida, especialmente quando o ferimento ocorre em uma parte importante do corpo do indivíduo, como o ósculo (Jackson, 1979; Bell, 2002; Padua & Klautau, 2015). Por outro lado, em espécies sem polarização do corpo, eventos de embriogênese somática costumam ocorrer, como a formação de um novo ósculo no local do ferimento ou a inversão da orientação do corpo em relação à forma original (Korotkova, 1961, 1963; Korotkova & Gelihovskaia, 1963).

O exemplo máximo de ruptura da unidade anatômica e fisiológica das esponjas talvez seja a dissociação de suas células. Após a dissociação, as células são capazes de se reagregar e reconstituir um novo indivíduo, readquirindo a unidade do organismo. No entanto, vários fatores podem influenciar nessa reagregação e poucas espécies estudadas até aqui foram capazes de fato de reconstruir um indivíduo funcional (Lavrov & Kosevich, 2014). Interessante notar que, em *P. magna*, a reconstituição de um indivíduo funcional a partir de suas células dissociadas apresenta diferenças temporais e de eventos em comparação com o desenvolvimento normal a partir da larva, atingindo o estágio de adulto funcional de forma mais rápida (Lanna, 2012; Capítulo 2).

Outro caso extremo de desestruturação que pode ocorrer em indivíduos adultos sob estresse ambiental, por exemplo, também foi reportado. Huxley (1912), Wilson (1932) e Borojevic (1971) relataram a formação de "corpos de redução" que poderiam reconstituir uma esponja quando as condições voltassem a ser favoráveis. No Capítulo 3 desta tese, reportamos dois casos onde indivíduos de *C. aurea* haviam desaparecido e, alguns meses depois, um novo indivíduo com o mesmo genótipo apareceu no mesmo lugar. Uma possibilidade de explicação para isso poderia ser, por exemplo, a formação desses "corpos de redução" nos indivíduos estudados.

Todo conceito apresenta limitações em sua definição. O mesmo acontece com a definição de indivíduo biológico e as características comumente atreladas a ele. Nesta tese, observamos que alguns desses atributos (tais como a unidade fisiológica e o alorreconhecimento mantendo a homogeneidade genética no adulto) são observados nas esponjas da classe Calcarea, enquanto outros, como a singularidade genética e homogeneidade genética quando a unidade é desfeita, não são. Portanto, a grande variabilidade morfológica e celular das esponjas se reflete na forma como a individualidade é expressa nos indivíduos das diferentes espécies do filo.

CONCLUSÕES

O desenvolvimento desta tese nos permitiu concluir os seguintes pontos em relação às esponjas da classe Calcarea:

- A morfologia externa definida e polarizada exerce grande influência sobre a capacidade de regeneração de partes perdidas do corpo;
- As células dissociadas de *P. magna* e *C. aurea* são capazes de reagregação e formação de primorfos;
- A formação do primorfo é essencial para a reconstituição do indivíduo funcional;
- A reconstituição de indivíduos funcionais a partir de suas células dissociadas apresenta diferenças tanto no tempo quanto na sequência de eventos em relação ao desenvolvimento normal a partir da larva em *Paraleucilla magna*;
- Indivíduos adultos de *Clathrina aurea* fusionam apenas com outros indivíduos geneticamente idênticos, sugerindo a existência de um sistema de alorreconhecimento;
- Indivíduos de *Clathrina aurea* apresentam uma longevidade média curta, onde eventos de fragmentação e fusão são comuns, bem como a variação de tamanho.
- Não foram encontrados indícios de quimeras naturalmente formadas nas espécies *C. aurea* e *P. magna*;
- A capacidade de alorreconhecimento parece ser perdida quando as células de esponjas calcáreas são dissociadas;
- Células dissociadas de *C. aurea* mantêm sua capacidade de reconhecimento interespecífico mesmo quando dissociadas;
- A grande variabilidade genética observada em *C. aurea* e *P. magna* não parece resultar da formação de quimeras;

PERSPECTIVAS FUTURAS

Apesar dos resultados obtidos, muitas questões ainda faltam ser respondidas. Trabalhos futuros poderão focar, por exemplo, nas alterações celulares que ocorrem durante a regeneração de esponjas calcáreas. Outros pontos interessantes seriam: (1) o estudo da expressão de genes e fatores de transcrição durante o processo de dissociação celular e reorganização de um novo indivíduo em comparação com o desenvolvimento normal a partir da larva; (2) bem como durante a possível dediferenciação, agregação e

formação de indivíduos quiméricos *in vitro*; (3) o destino das linhagens celulares de cada indivíduo quimérico após a formação do primorfo e da esponja juvenil; (4) se ocorre e qual o resultado da fusão entre larvas e juvenis geneticamente diferentes; (5) utilização de enxertos de indivíduos geneticamente diferentes nas espécies aqui estudadas para observar a reação ao contato.

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