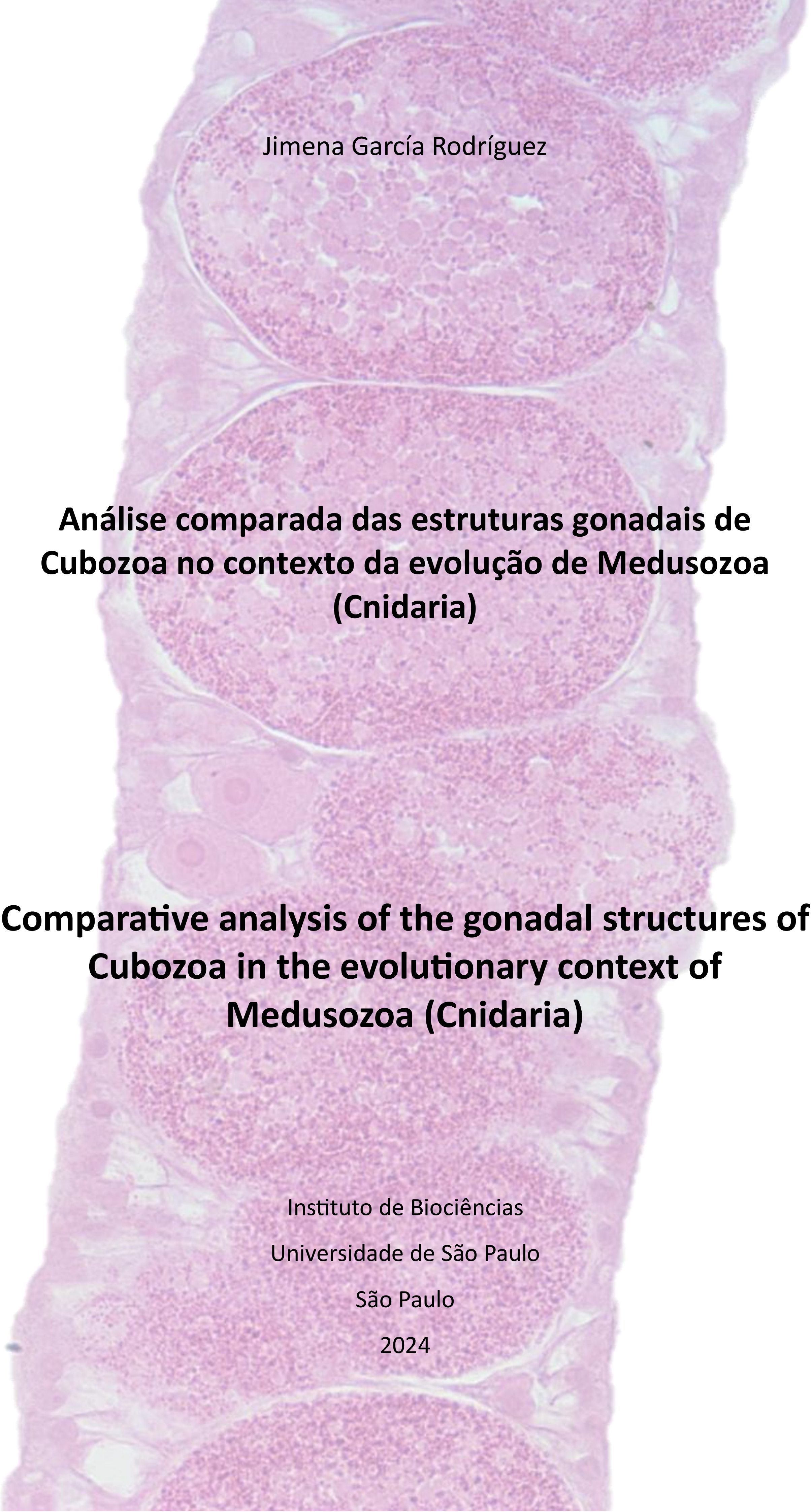

Jimena García Rodríguez

Análise comparada das estruturas gonadais de Cubozoa no contexto da evolução de Medusozoa (Cnidaria)

Comparative analysis of the gonadal structures of Cubozoa in the evolutionary context of Medusozoa (Cnidaria)

Instituto de Biociências

Universidade de São Paulo

São Paulo

2024

Jimena García Rodríguez

Análise comparada das estruturas gonadais de Cubozoa no contexto da evolução de Medusozoa (Cnidaria)

Comparative analysis of the gonadal structures of Cubozoa in the evolutionary context of Medusozoa (Cnidaria)

Tese apresentada ao Instituto de
Biociências da Universidade de São Paulo,
para a obtenção de Título de Doutora em
Biologia, na Área de Zoologia.

Orientador: Antonio Carlos Marques
Co- orientadora: Amanda Ferreira e Cunha

São Paulo

2024

Ficha catalográfica

Ficha catalográfica elaborada pelo Serviço de Biblioteca do Instituto de Biociências da USP,
com os dados fornecidos pelo (a) autor (a) no formulário:
'https://ib.usp.br/ficha-catalografica/src/ficha.php'

Garcia Rodriguez, Jimena
Análise comparada das estruturas gonadais de Cubozoa no contexto da evolução de Medusozoa (Cnidaria) / Garcia Rodriguez Jimena ; orientador Carlos Marques Antonio ; coorientadora Ferreira e Cunha Amanda -- São Paulo, 2024.
146 p. + anexo

Tese (Doutorado) -- Instituto de Biociências da Universidade de São Paulo. Ciências Biológicas (Zoologia).

1. Cubozoa. 2. Medusozoa. 3. Sexual Reproduction. 4. Cnidaria. 5. Gonads. I. Carlos Marques, Antonio , orient. II. Ferreira e Cunha, Amanda, coorient. Título.

Bibliotecária responsável pela catalogação:
Elisabete da Cruz Neves - CRB - 8/6228

Comissão Julgadora:

____

Prof(a). Dr(a).

____

Prof(a). Dr(a).

____

Prof(a). Dr(a).

____

Prof. Dr. Antonio Carlos Marques
(Orientador)

A mis padres

por ser unos luchadores

y a mi hermana por estar en el ring.

Happiness is only real when shared

Christopher McCandless, 1992

Agradecimentos

Em primeiro lugar, gostaria de agradecer as agências de fomento, à FAPESP (#2019/01370-2 e #2021/09739-5) e o CNPq (#140235/2018-3), que ajudaram a execução desse trabalho. Graças ao dinheiro investido em pesquisa que essas agências oferecem, além de desenvolver esse trabalho, pude ter a oportunidade de viajar ao exterior para um congresso em Cape Town (South Africa), e fazer um estágio no National Museum of Natural History (NMNH), Smithsonian Institution, Washington DC, USA. Devido a essas experiências eu desenvolvi dois dos capítulos desta tese, o Capítulo 2 e o Capítulo 3.

Obrigada aos meus orientadores e coorientadores. Obrigada Tim pelas orientações nas minhas ideias perdidas, pela confiança desde o primeiro dia, pela parceria no trabalho e pela amizade e paciência durante esses quase 8 anos. Obrigada Amanda por ser dura e séria comigo (quando tinha que ser). Obrigada por me hospedar na sua nova e linda cidade. Obrigada por lutar comigo e me animar quando estava com pouca força. Obrigada pelas revisões e, sempre certas, sugestões. Obrigada mesmo pela tranquilidade, pela confiança, pela coragem, pelas marmitas e cervejas e sobretudo pela paciência e por me ensinar "que tudo pode" nesse caminho que você fez comigo. Thanks Allen, for making me feel comfortable with science, for being my support far away from my home, for teaching me what is the favorite menu for a jellyfish, for the confidence and for all the funny moments during these months of pandemic. Thanks Cheryl, for your smile and infinite knowledge, and for being the fastest person who answers my emails full of questions. With you, everything is easier.

Obrigada aos professores e colaboradores que me ajudaram com a tese. Obrigada Zé, meu mestre de histologia, tudo o que eu sei foi você que me ensinou. Obrigada André por me emprestar o material que precisei e pela confiança. Obrigada Lu pelas colaborações, pela confiança e por esses dias em BH.

Obrigada aos meus companheiros de vida. Gracias Adri por no haberte mudado a otra casa y haberme soportado durante todo un año de convivencia (y más). Gracias Adriana por acompañarme en los ejercicios fitness, por escucharme y nunca haber *surtado* en mi desesperación. Adris, gracias por esa eterna *parceria* y convivencia por tantos años. Los mejores *realitys* con vosotros (de protagonistas). Saudades. Obrigada Ju, a menina que começou sendo apenas uma companheira de pandemia amiga de amigos (me apresentando inumeráveis vezes) e terminou sendo a minha parceira de vida. Obrigada pela cumplicidade, pelos almoços e jantas de eternos aniversários, por *quarentenar* comigo na melhor covid que eu poderia ter, pelas músicas tocadas lembrando a nossa juventude, pelos viajes á Rio, por ter me deixado entrar na sua maravilhosa família, por ser intermediária com a *Alexa* e por dividir comigo o nosso melhor lugar, a praia. Obrigada aos três, vocês foram, são e serão a minha família mais querida.

Gracias a la familia Delval. Gracias Lino y Oli por vuestra inocencia, por hacer que nuestros días tengan sentido y darnos rumbo en la vida. Por esas sonrisas y hacernos partícipes de vuestra alegría. Gracias Ire, la super mami y supermujer más *corajosa* que conozco, por las *fofocas* =), las incansables bohemias *geladas*, las *corridas y nadadas*. Gracias por toda la fuerza, el apoyo y tener tanta confianza en mí y en mi trabajo.

Obrigada à minha família de pandemia. Gracias Andresito y Ad, por vuestra compañía, por los planes que salían en días sin sentido, por todos los memes que hacen que mi día sea más entretenido, por las figuriñas y por vuestra amistad. Gracias Fani por compartir conmigo los días de caos, los famosos días de la marmota, los *por de sol*, los karaokes y por los productos ibéricos=). Obrigada Lê pela compreensão, pelas escutas, por ser tão doce e pelo cuidado. Gracias Agus por las miradas de complicidad, por las risas de carnaval, por los días de fiesta, por acompañarme al *bandejão* y por tu sonriente amistad.

Obrigada às minhas companheiras e amigas do MEL. Obrigada Lu e Ma pelo conhecimento e pela ajuda que precisei durante o desenvolvimento do meu trabalho.

Thanks to the *USA crew*. Thanks to Vanessa, for being my partner in crime during my fellowship. Thanks for not making me feel bad buying everything that I didn't need ;). Gracias Juanita por ese *match*, por acogerme en tu vida de DC y presentarme a nuestra gente. Obrigada João e Nat por ser esse pedaço de Brasil que eu precisava estando longe. Gracias Juanmi y Dani por haber concordado siempre en todos los planes y haber formado parte de esta experiencia. Gracias Maria Vizeu por enseñarme DC, por Oli, por haberme dejado entrar en tu vida sin dudar, por tu comprensión y por la rápida amistad que comenzó al otro lado del mundo.

Also, thanks to the *NMNH crew*. Thanks to MC, to Bred, to Prof. Karen Osborn for being nice and showing me amazing works. Thank you to Yolanda Villacampa for guiding me through the labyrinths of the building. Thanks, Freya, for your advice in histology, for your attention and for your patience during my fellowship. Thanks Prof. Hannah Wood for borrowing the microtome and for your confidence. Thanks to the MSC (Museum Support Center) crew for your patience and always being nice: Bill (William Moser), Lisa Comer and Katie Ahlfeld.

Obrigada aos meus lindos vizinhos da Vila Felicitá por cuidar do Cosme e por serem os melhores vizinhos.

Gracias a todos mis amigos que están en la distancia. Gracias Angelito, por las video llamadas de desespero, por los cotilleos, por hacerme siempre reir, por tus "sabios" consejos, por escucharme, por ser *mititi* y mejor compañero. Gracias mi Ser, por hacerme partícipe de tus mejores días, por comer kilos de comida conmigo y por ser tan chulo. Gracias mi Al y Guille, por vuestra compañía aquí en SP, por sacarme siempre una sonrisa en mis peores días, por las baladas y los bailes, por las aventuras y por ser tan eruditos. Gracias también a los de siempre, Ainhoa, Angel, Lolo, CrisGar, CrisGon, Yoko, Rafa, Ol, Lour, Vir, Lau, Huelin, Saro, Patrol por todos vuestros mensajes y por toda la fuerza de toda una vida. Gracias a mis Erasmus, por contarme aventuras y hacerme reír. Gracias tía Sonia por todo el apoyo, por las risas, por tu visita a USA, por las cremas, por estar ahí cuidándome y cuidando de los míos. Gracias también Andrés por cuidar a mi familia. Obrigada Ma por ter sido meu companheiro nesse final, por ter me deixado entrar na sua família, pela sua paciência e tranquilidade eterna, pela confiança e pelo amor. Muito obrigada a família da cunha Laste por ter dado todo o suporte, pelo carinho e por deixar as coisas mais fáceis.

No tengo cómo agradecer a los cuidadores en un tiempo de caos. Gracias Melisa, Felipito, Lu, Ire, Andrés, Ju. Gracias por ese apoyo incondicional tan importante y necesario cuando más lo necesitaba. Gracias por cuidar de mi familia y por cuidar de mi.

Gracias papá y mamá. Gracias por hacerme grande, por confiar, por acompañarme en mi aventura, por el silencio necesario, por respetar mis decisiones, por todo el apoyo, por dejarme libre. Gracias Eli por cuidar de ellos, gracias por creer en mí.

Gracias a todos los que me acompañaron en este camino, tropezando conmigo y empujándome hasta llegar al día de hoy.

Sumário

Introdução Geral

Introdução Geral

Cnidários como organismos “simples”

Uma compreensão atávica do processo evolutivo faz com que uma série de organismos sejam qualificados como “simples”. Porém, há de se questionar o que significa ser “simples”? Intuitivamente, em uma escala de diversidade ampliada, um organismo simples pode ser considerado aquele formado por uma célula, como as bactérias ou os protistas, ou aqueles formados por várias células idênticas ou similares, como ocorre em alguns fungos e algas (Brusca & Brusca 2002). Mas essa visão também ocorre em referência aos animais, predominando uma compreensão que animais não-bilaterais (i.e., Porifera, Cnidaria, Ctenophora e Placozoa) são ... “simples”. Isso a despeito da natureza animal que, por si só, já implica em haver uma multicelularidade, inclusive com vários tipos celulares diferentes interconectados entre si por junções variadas. Tal visão provavelmente resulta do fato dos animais não-bilaterais terem sido historicamente estudados sob a lente de conhecimento baseada na biologia dos animais bilaterais (veja Dunn et al. 2015). Nesse sentido, o foco em caracteres compartilhados por animais bilaterais e não-bilaterais acaba por ocultar os caracteres únicos e complexos desses filos de origem mais basal como, por exemplo, as células totipotentes de Porifera que podem se diferenciar em qualquer outro tipo celular, ou as comunicações intercelulares que podem ser precursoras de sistemas sinápticos (Hooper et al. 2002; Dunn et al. 2015; Musser et al. 2021).

Nesse contexto, os cnidários também são considerados como organismos simples (e.g., (Satterlie & Spencer 1987; Galliot & Schmid 2002; Ball et al. 2004) por causa de sua simetria “radial” e de sua organização tecidual diploblástica (com ectoderme e endoderme) (Chapman et al. 1962). Entretanto, cnidários são frequentemente usados como modelos experimentais devido à relativamente fácil manipulação de suas células e tecidos (e.g., Prasher et al. 1992; Amiel & Houliston 2009; Ambrosone & Tortiglione 2013), além de possuírem genes e várias vias regulatórias de desenvolvimento semelhantes àquelas dos animais bilaterais (Kortschak et al. 2003). A pressuposição de simplicidade dirigida aos grupos basais de animais desconsidera a grande diversidade que eles encerram em determinados sistemas. Para Cnidaria, por exemplo, há enorme diversidade de planos corpóreos bem como na variedade de embriologias e dos ciclos de vida que apresentam. Essas características combinadas conferem ao grupo um grande potencial em termos de complexidade estrutural.

Os cnidários compreendem três clados, Endocnidozoa, Anthozoa e Medusozoa (Kayal et al. 2018), que podem apresentar comportamentos e características complexas, frequentemente observadas em grupos particulares menos inclusivos. Apresentam sistema nervoso e muscular desenvolvidos (Chapman et al. 1962; Ball et al. 2004) o que lhes permite um movimento ativo de natação e, como requinte em algumas espécies, uma predação ativa na coluna d’água (Colin et al. 2013) ou evadirem-se de obstáculos (Garm et al. 2007; Bielecki et al. 2023). Os cnidários também são a linhagem peçonhenta mais antiga dentre os animais, com a inoculação de toxinas realizada por organelas intracelulares complexas, as cnidas, consideradas uma das sinapomorfias do grupo (Marques & Collins 2004). As cnidas, que incluem os nematocistos, são produtos de uso único secretados pelo complexo de Golgi, com funções primárias tais como defesa, captura de alimento e/ou adesão (e.g., Mariscal 1974; Morandini et al. 2014). Os nematocistos são ativados por meio de diferentes estímulos de natureza química, elétrica e/ou mecânica (Mariscal 1974). A composição de seu veneno é variável dependendo da espécie, do tipo de nematocisto, e até mesmo de fatores bióticos e abióticos (Columbus-Shenkar et al. 2018; Jaimes-Becerra et al. 2019), sendo que algumas espécies podem levar vítimas humanas a óbito (Bengston et al. 1991). A água viva *Chironex fleckeri* (Cubozoa), por exemplo, é considerado o animal mais venenoso do mundo (Calder & Peters 1975).

Outra estrutura complexa de alguns cnidários, dessa vez sensorial, é o ropálio, com a função de orientar o posicionamento de medusas de Scyphozoa e Cubozoa na coluna d’água (Sötje 2011). Em Cubozoa, especificamente, o ropálio apresenta olhos complexos com córnea, lente e retina (Fig. 1), comparáveis aos olhos do tipo câmera que ocorrem em outros invertebrados bilaterais (Berger 1898; Coates 2003). Consequentemente, a visão espacial nos Cubozoa permite uma interação com seu ambiente a ponto de proporcionar-lhes a seleção de habitats (Coates 2003), ou até mesmo comportamentos mais complexos, como o de corte, em que o macho reconhece a fêmea para a transferência indireta do esperma (Lewis & Long 2005). Há também outros níveis de complexidade observados em várias características dos cnidários, como por exemplo em seu ciclo de vida metagenético e plástico, em que uma mesma espécie pode apresentar variações de estratégias de vida, como a produção ou não de uma medusa (Prudkovsky et al. 2019). De fato, a reprodução em Medusozoa é um dos seus sistemas mais elaborados e de compreensão mais desafiadora (Migotto 1998; Jarms & Morandini 2019).

Reprodução em Medusozoa

A reprodução em Cnidaria é muito diversificada (Fautin, 2002), certamente uma das mais variadas no reino animal, e isso fica evidente a partir da observação da vasta gama de padrões diferentes de ciclos de vida que ocorre entre os hidrozoários (Boero & Bouillon 1993). Medusozoa se reproduz tanto assexuadamente, por processos como brotamento (‘*budding*’) e fissão, geralmente ocorrentes na fase do pólipo (e.g., Berrill 1949), como sexualmente, envolvendo gametas masculinos e femininos, geralmente expressados na fase medusa (e.g., Schiariti et al. 2012). Esse tipo de ciclo de vida, denominado metagenético (Campbell 1974; Collins 2002; Morandini et al. 2016), pode também incluir outras fases larvais, como a plânula livre-natante (Müller & Leitz 2002), caracterizando seu desenvolvimento do tipo indireto. No entanto, há algumas espécies com desenvolvimento direto (sem fase do pólipo), com ciclo holoplantônico, provavelmente como resultado de adaptações fisiológicas ao ambiente, como no caso da espécie *Periphylla periphylla* (Scyphozoa, Jarms et al. 2002), que ocorre em grandes profundidades. Um ciclo de vida mais diversificado evidentemente influencia na biologia das espécies, tanto em sua distribuição por meio das fases advectivas de medusa e plânula (Sommer 1992), como nas interações bióticas diversas com comunidades do bentos e/ou do pelagial. Outra peculiaridade de algumas espécies de Medusozoa é a capacidade única de reverter seu desenvolvimento larval, como no caso da medusa de *Turritopsis nutricula* (Hydrozoa), a qual pode retornar ao estágio de pólipo colonial e vice-versa, sendo assim chamada de “a medusa imortal” (Piraino et al. 1996).

A fertilização em Cnidaria pode ocorrer tanto de maneira externa como interna, mas a fertilização externa, em que os gametas masculinos e femininos são liberados no meio, é observada com mais frequência (Marques et al. 2015; Orr & Brennan 2015). Há adaptações relacionadas exclusivamente à fertilização externa, como por exemplo a sincronia na liberação dos gametas (e.g., Uye 2014), em que medusas encontram-se em águas superficiais para liberar seus gametas (e.g., Mills 2001). No entanto, dentre os Medusozoa há espécies em que a fertilização interna ocorre e, em alguns casos, isso pode envolver comportamentos sexuais muito elaborados (Hartwick 1991; Lewis & Long 2005).

Estratégias reprodutivas em Hydrozoa

A grande variedade de estratégias reprodutivas de Medusozoa tem os fenômenos de redução ou perda de um dos estágios do ciclo de vida (pólipo ou medusa) como um de seus principais componentes. Dentre os Hydrozoa, as modificações no ciclo de vida metagenético típico resultaram em vários graus de redução da medusa (Teissier 1926; Boero & Sarà 1987; Schuchert & Brinckmann-Voss 2006) e, frequentemente, ocorre perda completa desse estágio, expressado múltiplas vezes ao longo da evolução do grupo (Leclère et al. 2009). Por exemplo, o gonóforo fixo ou esporosaco (e.g., em *Turritopsoides marhei*, Maggioni et al. 2017), que é considerado o mais alto grau de redução das medusas (Teissier 1926; Rees 1957), está ligado ao hidrocaule, e produz os óvulos ou espermatozoides, funcionando como a própria gônada (Berril & Liu 1948). As espécies com um gonóforo fixo exibem baixa capacidade de dispersão, muitas vezes ainda mais limitada pela ocorrência de incubação larval na colônia polipoide (Miller 1973; Burmistrova et al. 2018). Por outro lado, o estágio de medusoide (e.g., em *Macrorynchia philippina*, Bourmaud & Gravier-Bonnet 2004), às vezes referido como um “gonóforo livre-natante” (Boero & Bouillon 1989), é compreendido como uma bolsa transportadora sem tentáculos que é liberada da colônia quando os gametas estão maduros, tendo uma vida planctônica breve (e.g., Migotto 1998; Gravier-Bonnet & Migotto 2000; Bourmaud & Gravier-Bonnet 2004). Por outro lado, estruturas como os meconídios, que ocorrem em espécies de *Gonothyraea*, representam uma forma medusoide reduzida, mas que permanece ligada à gonoteca, desempenhando assim o papel duplo de estrutura produtora de gametas e de câmara incubadora para a fertilização e desenvolvimento das larvas plânulas (Miller 1973). Embora vários estágios de redução da medusa já tenham sido observados e descritos em Hydrozoa, pouco se sabe sobre as possíveis influências dessas variações nos padrões reprodutivos do grupo.

Ciclo reprodutivo em Scyphozoa

Em Scyphozoa, a fase de pólipo (cifopólipo ou cifístoma) se reproduz assexuadamente por brotamento, gerando clones (Littleford 1939). Posteriormente, o pólipo passa por estrobilação (divisão assexuada transversal repetida do cifístoma), um processo no qual ele se divide em segmentos da região oral chamados éfiras, e em que cada éfira se desenvolve em uma medusa jovem. As éfiras podem ser produzidas uma por vez (estrobilação monodisco) (e.g., Rhizostoma luteum, Kienberger et al., 2018) ou várias éfiras podem se acumular antes de serem liberadas conforme amadurecem (estrobilação polidisco) (e.g., *Lychnorhiza lucerna* Schiariti et al., 2008).

Ciclo reprodutivo em Cubozoa

O ciclo de vida em Cubozoa apresenta um padrão em que um cubopólipo solitário se metamorfoseia em uma medusa (e.g. *Tripedalia binata*, Toshino et al., 2017). Esse padrão foi a justificativa para o estabelecimento da classe Cubozoa como distinta de Scyphozoa (Werner 1973). Há, entretanto, uma compreensão alternativa desse desenvolvimento, em que haveria um processo semelhante ao de estrobilação monodisco, onde o resquício do pólipo se regenera antes a medusa se desprender (e.g., *Carybdea marsupialis* e *Morbakka virulenta*, Toshino et al., 2015). De qualquer maneira, a medusa é gonocórica e, em alguns casos, apresenta dimorfismo sexual na morfologia gonadal (Lewis & Long 2005; Straehler-Pohl et al. 2014; Garm et al. 2015).

Ciclo de vida em Staurozoa

Em Staurozoa, a metamorfose é principalmente observada na região apical (ou oral) do estauropólipo, quando essa parte do cálice adquire as caraterísticas típicas de uma estauromedusa (Wietrzykowski 1912; Miranda et al. 2018). A estauromedusa permanece fixa ao substrato por um pedúnculo, e é uma mistura de características polipoides (mais basais) e medusoides (mais apicais) (Collins et al., 2006; Miranda et al., 2018).

Objetivos do trabalho

A partir da construção de uma base de referência de dados realizada com análises histológicas e informações sobre a diversidade de reprodução sexuada obtidas da literatura, os objetivos deste estudo foram: (1) avaliar a variação do tamanho do ovo em espécies de Medusozoa a partir de uma abordagem comparativa (Capítulo 1); (2) inferir padrões histomorfológicos e ultraestruturais das gônadas femininas em Cubozoa com relação ao tipo de fertilização e ecologia, estabelecendo uma base comparativa e modelar a partir da descrição da oogênese da espécie brasileira *Chiropsalmus quadrumanus* (Capítulo 2); e (3) agregar mais dados de caracteres reprodutivos sobre uma base taxonômica ampliada e representativa de Cubozoa, permitindo inferir padrões evolutivos em um contexto filogenético mais amplo, além de caracterizar processos gerais como a oogênese e seus elementos constitutivos , assim como a nematogênese e as relações dos nematocistos com a reprodução de algumas espécies (Capítulo 3).

Organização da tese

Este documento está organizado em cinco partes: (1) uma introdução geral onde se contextualiza os pontos teóricos relacionados aos tópicos investigados nessa tese e seus objetivos gerais; (2-4) três capítulos (enumerados como 1, 2 e 3) apresentados em maior detalhe e na forma de manuscritos científicos (ver abaixo); e, para finalizar, (5) as considerações finais do estudo, que buscam fazer uma síntese contextualizada das principais contribuições dessa tese para sua área de estudo.

Nas partes 2-4 está o *Capítulo 1*, em que apresentamos uma síntese da variação do tamanho do ovo entre as quatro classes de Medusozoa, usando uma abordagem comparativa e tendo como padrão principal o fato que, embora a variação do tamanho do ovo seja relativamente conservada ao longo da maior parte da filogenia, ela pode ser explicada por características biológicas/sexuais e ambientais. Este estudo foi publicado na revista *Proceedings of the Royal Society B: Biological Sciences*.
Citação: García-Rodríguez J, Cunha AF, Morales-Guerrero A, González-Chaves A, Guerrero AC, Miranda LS, Serrano FC, Jaimes-Becerra A, Marques AC. 2023. Reproductive and environmental traits explain the variation in egg size among Medusozoa (Cnidaria). Proc. R. Soc. B 290: 20230543. https://dx.doi.org/10.1098/rspb.2023.0543

O *Capítulo 2* aborda aspectos da oogênese em *Chiropsalmus quadrumanus*, desvelando pela primeira vez dados dessa natureza para um táxon representante de Chirodropida e ampliando, assim, os subsídios para compreensão da diversidade reprodutiva em Cubozoa como um todo. O padrão principal que emerge desse estudo é a reprodução iterópara nesta espécie, que ocorre no Brasil, sugerida pela ooogênese assincrônica, com espécimes maduros apresentando todos os estágios de ovócitos. Da mesma forma, nossas descobertas destacam a presença de quatro estágios diferentes de ovócitos, de acordo com o tamanho e a quantidade de proteína do vitelo e inclusões desconhecidas nos ovócitos em desenvolvimento. O estudo foi publicado na revista *Diversity*.

Citação: García-Rodríguez, J., Ames, C. L., Jaimes-Becerra, A., Tiseo, G. R., Morandini, A. C., Cunha, A. F., & Marques, A. C. (2023). Histological Investigation of the Female Gonads of Chiropsalmus quadrumanus (Cubozoa: Cnidaria) Suggests Iteroparous Reproduction. *Diversity*, *15*(7), 816. https://doi.org/10.3390/d15070816

O *Capítulo 3* foi desenvolvido a partir de uma extensa base de dados histológicos construída ao longo do doutorado, a maior para o grupo até o momento, com base em estudos de materiais do todo o globo trabalhados a partir das coleções no National Museum of Natural History – Smithsonian Institution e materiais próprios do Brasil. O trabalho intitulado “Novel features and role of sexual reproduction in jellyfishes of the class Cubozoa (Cnidaria: Medusozoa)” envolveu a análise de gônadas de machos e fêmeas de Cubozoa em diferentes estágios de desenvolvimento para a caracterização da gametogênese em 15 espécies diferentes. O padrão principal encontrado mostra novas características da oogênese para esta pequena classe; a descrição de ovócitos hexagonais; a caraterização de inclusões com origem no núcleo em alguns ovócitos em desenvolvimento sugerindo a autossíntese de vitelo; e a presença de nematocistos e nematoblastos em não-tripedaliídeos.

Referências

Ambrosone A, Tortiglione C (2013) Methodological approaches for nanotoxicology using cnidarian models. *Toxicology Mechanisms and Methods*, *23*, 207–216. doi: 10.3109/15376516.2012.747117

Amiel A, Houliston E (2009) Three distinct RNA localization mechanisms contribute to oocyte polarity establishment in the cnidarian *Clytia hemisphaerica*. *Developmental Biology*, *327*, 191–203. doi: 10.1016/j.ydbio.2008.12.007

Ball EE, Hayward DC, Saint R, Miller DJ (2004) A simple plan - Cnidarians and the origins of developmental mechanisms. *Nature Reviews Genetics*, *5,* 567–577. doi: 10.1038/nrg1402

Bengston K, Nichols MM, Schnadig V, Ellis MD (1991) Sudden death in a child following jellyfish envenomation by *Chiropsalmus quadrumanus*: case report and autopsy findings. JAMA: *The Journal of the American Medical Association, 266*, 1404–1406. doi: 10.1001/jama.1991.03470100096040

Berger EW (1898) The histological structure of the eyes of Cubomedusae. *Journal of Comparative Neurology, 8*, 223–230. doi: 10.1002/cne.910080317

Berrill NJ, Liu CK (1948) Germplasm, Weismann, and Hydrozoa. *The Quarterly Review of Biology 2,* 124–132. doi: 10.1086/396241

Berrill NJ (1949) Developmental analysis of Scyphomedusae. *Biological Reviews*, *24*, 393–409. doi: 10.1111/j.1469-185X.1949.tb00581.x

Boero F, Bouillon J (1989) An evolutionary interpretation of anomalous medusoid stages in the life cycles of some Leptomedusae (Cnidaria). *In* Ryland JS, Tyler PA (eds) Reproduction, genetics and distribution of marine organisms. Olsen and Olsen, Fredensborg, Denmark, pp 37–41

Boero F, Bouillon J (1993) Zoogeography and life cycle patterns of Mediterranean hydromedusae (Cnidaria). *Biological Journal of the Linnean Society*, *48*, 239–266. doi: 10.1111/j.1095-8312.1993.tb00890.x

Boero F, Sarà M (1987) Motile sexual stages and evolution of Leptomedusae (Cnidaria). *Bolletino di zoologia 54*, 131–139. doi: 10.1080/11250008709355572

Bourmaud C, Gravier-Bonnet N (2004) Medusoid release and spawning of *Macrorynchia philippina* Kirchenpauer, 1872 (Cnidaria, Hydrozoa, Aglaopheniidae). *Hydrobiologia 530–531,* 365–372. doi: 10.1007/s10750-004-2665-5

Brusca RC, Brusca GJ (2002) Invertebrates (No. Ed. 2). Sinauer Associates Incorporated.

Burmistrova YA, Osadchenko B V., Bolshakov F V., Kraus YA, Kosevich IA (2018) Embryonic development of thecate hydrozoan *Gonothyraea loveni* (Allman, 1859). *Development Growth and Differentiation*, *60*, 483–501. doi: 10.1111/dgd.12567

Calder DR, Peters EC (1975) Nematocysts of *Chiropsalmus quadrumanus* with comments on the systematic status of the Cubomedusae. *Helgoländer Wissenschaftliche Meeresuntersuchungen*, *27*, 364–369. doi: 10.1007/BF01611701

Chapman DM, Pantin, CF, Robson EA (1962). Muscle in coelenterates. *Revue canadienne de biologie*, *21*, 267-278

Coates MM (2003) Visual ecology and functional morphology of Cubozoa (Cnidaria). *Integrative and Comparative Biology*, *43*, 542–548. doi: 10.1093/icb/43.4.542

Colin SP, Costello JH, Katija K, Seymour J, Kiefer K (2013) Propulsion in Cubomedusae: Mechanisms and utility. *PLoS One, 8*. doi: 10.1371/journal.pone.0056393

Collins A, Schuchert P, Marques A, Jankowski T, Medina M, Schierwater B (2006) Medusozoan phylogeny and character evolution clarified by new large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models. *Systematic Biology*, *55*, 97–115. doi: 10.1080/10635150500433615

Columbus-Shenkar YY, Sachkova MY, Macrander J, Fridrich A, Modepalli V, Reitzel AM, Sunagar K, Moran Y (2018) Dynamics of venom composition across a complex life cycle. *ELife*, *7*. doi: 10.7554/eLife.35014.001

Dunn CW, Leys SP, Haddock SHD (2015) The hidden biology of sponges and ctenophores. *Trends in Ecology and Evolution, 30*, 282–291. doi: https://doi.org/10.1016/j.tree.2015.03.003

Fautin DG (2002) Reproduction of Cnidaria. *Canadian Journal of Zoology*, *80*, 1735–1754. doi: 10.1139/z02-133

Galliot B, Schmid V (2002) Cnidarians as a model system for understanding evolution and regeneration. *International Journal of Developmental Biology*, *46*, 39–48. doi: 10.1387/ijdb.11902686

Garm A, O'Connor M, Parkefelt L, Nilsson DE (2007) Visually guided obstacle avoidance in the box jellyfish *Tripedalia cystophora* and *Chiropsella bronzie*. *Journal of Experimental Biology, 210*, 3616–3623. doi: 10.1242/jeb.004044

Garm A, Lebouvier M, Tolunay D (2015) Mating in the box jellyfish *Copula sivickisi* -Novel function of cnidocytes. *Journal of Morphology*, *276*, 1055–1064. doi: 10.1002/jmor.20395

Gravier-Bonnet N, Migotto AE (2000) Gonangium development and medusoid of *Nemalecium lighti* (Hargitt, 1924) (Cnidaria: Hydrozoa, Haleciidae). *Scientia Marina*, *64*, 207–213. doi: 10.3989/scimar.2000.64s1207

Hartwick RF (1991) Observations on the anatomy, behaviour, reproduction and life cycle of the cubozoan Carybdea sivickisi. *Hydrobiologia, 216–217*, 171–179. doi: 10.1007/BF00026459

Hooper JNA, Van Soest RWM, Debrenne F (2002) Phylum Porifera Grant, 1836. *In* Systema Porifera: a guide to the classification of sponges. Kluwer Academic/Plenum Publishers, New York, pp 9–13.

Jaimes-Becerra A, Gacesa R, Doonan LB, Hartigan A, Marques AC, Okamura B, Long PF (2019) "Beyond Primary Sequence" - Proteomic Data Reveal Complex Toxins in Cnidarian Venoms. *Integrative and Comparative Biology*, *59*, 777–785. doi: 10.1093/icb/icz106

Jarms G, Morandini AC (2019) World Atlas of Jellyfish. Abhandlungen des Naturwissenschaftlichen Vereins, Hamburg, Special Edition, English Edition. Hamburg: Dölling und Galitz Verlag

Jarms G, Tiemann H, Båmstedt U (2002) Development and biology of *Periphylla periphylla* (Scyphozoa: Coronatae) in a Norwegian fjord. *Marine Biology*, *141*, 647–657. doi: 10.1007/s00227-002-0858-x

Kayal E, Bentlage B, Sabrina Pankey M, Ohdera AH, Medina M, Plachetzki DC, Collins AG, Ryan JF (2018) Phylogenomics provides a robust topology of the major cnidarian lineages and insights on the origins of key organismal traits. *BMC Evolutionary Biology*, *18*. doi: 10.1186/s12862-018-1142-0

Kienberger K, Riera-Buch M, Schönemann AM, Bartsch V, Halbauer R, Prieto L (2018) First description of the life cycle of the jellyfish *Rhizostoma luteum* (Scyphozoa: Rhizostomeae). *PLoS ONE*, *13*. doi: 10.1371/journal.pone.0202093

Kortschak RD, Samuel G, Saint R, Miller DJ (2003) EST Analysis of the cnidarian *Acropora millepora* reveals extensive gene loss and rapid sequence divergence in the model invertebrates. *Current Biology*, *13,* 2190–2195. doi: 10.1016/j.cub.2003.11.030

Leclère L, Schuchert P, Cruaud C, Couloux A, Manuel M (2009) Molecular phylogenetics of Thecata (Hydrozoa, Cnidaria) reveals long-term maintenance of life history traits despite high frequency of recent character changes. *Systematic Biology*, *58*, 509–526. doi: 10.1093/sysbio/syp044

Lewis C, Long TAF (2005) Courtship and reproduction in *Carybdea sivickisi* (Cnidaria: Cubozoa). *Marine Biology*, *147*, 477–483. doi: 10.1007/s00227-005-1602-0

Littleford RA (1939) The life cycle of *Dactylometra quinquecirrha*, L. Agassiz in the Chesepeake Bay. *The Biological Bulletin*, *77*, 368–381. doi: https://doi.org/10.2307/1537646

Maggioni D, Puce S, Galli P, Seveso D, Montano S (2017) Description of *Turritopsoides marhei* sp. nov. (Hydrozoa, Anthoathecata) from the Maldives and its phylogenetic position. *Marine Biology Research, 13,* 983–992. doi: 10.1080/17451000.2017.1317813

Mariscal RN (1974) Nematocyst. In: Coelenterate biology: Reviews and new perspectives. New York: Academic Press. pp 129–178

Marques AC, Collins AG (2004) Cladistic analysis of Medusozoa and cnidarian evolution. *Invertebrate Biology, 123,* 23–42. doi: 10.1111/j.1744-7410.2004.tb00139.x

Marques AC, García J, Lewis Ames C (2015) Internal fertilization and sperm storage in cnidarians: a response to Orr and Brennan. *Trends in Ecology & Evolution*, *30*(8), 435-436. doi: 10.1016/j.tree.2015.06.002

Migotto AE (1998) The life cycle of *Sertularia marginata* Kirchenpauer, 1864 (Cnidaria: Hydrozoa): A medusoid-producing sertulariid. *Journal of Natural History*, *32*, 1–12. doi: 10.1080/00222939800770011

Miller R (1973) The role of the gonomedusa and gonangium in the sexual reproduction (fertilization) of the Hydrozoa. *Publications of the Seto Marine Biological Laboratory, 20,* 367–400. doi: 10.5134/175769

Mills CE (2001) Jellyfish blooms: Are populations increasing globally in response to changing ocean conditions? *Hydrobiologia, 451,* 55–68. doi: 10.1023/A:1011888006302

Miranda LS, Mills CE, Hirano YM, Collins AG, Marques AC (2018) A review of the global diversity and natural history of stalked jellyfishes (Cnidaria, Staurozoa). *Marine Biodiversity, 48,*1695–1714. doi: 10.1007/s12526-017-0721-4

Morandini AC, Stampar SN, Kubota S (2014) Mass occurrence of the cubomedusa *Copula sivickisi* (Cnidaria: Cubozoa) at Seto Harbor, Shirahama, Wakayama, Japan, in summer of 2013; a possible recent example of global warming. *Publications of the Seto Marine Biological Laboratory, 42,* 108–111. doi: 10.5134/185694

Musser JM, Schippers KJ, Nickel M, Mizzon G, Kohn AB, Pape C, Ronchi P, Papadopoulos N, Tarashansky AJ, Hammel JU, Wolf F, Liang C, Hernández-Plaza A, Cantalapiedra CP, Achim K, Schieber NL, Pan L, Ruperti F, Francis WR, Vargas S, Kling S, Renkert M, Polikarpov M, Bourenkov G, Feuda R, Gaspar I, Burkhardt P, Wang B, Bork P, Beck M, Schneider TR, Kreshuk A, Wörheide G, Huerta-Cepas J, Schwab Y, Moroz LL, Arendt D (2021) Profiling cellular diversity in sponges informs animal cell type and nervous system evolution. *Science, 374,* 717–723. doi: https://doi.org/10.1101/758276

Orr TJ, Brennan PLR (2015) Sperm storage: distinguishing selective processes and evaluating criteria. *Trends in Ecology & Evolution*, *30*, 261–272. doi: 10.1016/j.tree.2015.03.006

Piraino S, Boero F, Aeschbach B, Schmid V (1996) Reversing the life cycle: Medusae transforming into polyps and cell transdifferentiation in *Turritopsis nutricula* (Cnidaria, Hydrozoa). *Biological Bulletin, 190,* 302–312. doi: 10.2307/1543022

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene, 111,* 229–233. doi: 10.1016/0378-1119(92)90691-H

Prudkovsky AA, Ekimova IA, Neretina T V. (2019) A case of nascent speciation: unique polymorphism of gonophores within hydrozoan *Sarsia lovenii*. *Scientific Reports*, *9*, 1–13. doi: 10.1038/s41598-019-52026-7

Rees WJ (1957) Evolutionary trends in the classification of Capitate hydroids and medusae. *Bulletin of the British Museum (Natural History) Zoology, 4,* 453–534.

Satterlie RA, Spencer AN (1987) Organization of conducting systems in “simple” invertebrates: Porifera, Cnidaria and Ctenophora. *In:* Ali, M.A. (eds) Nervous Systems in Invertebrates. Springer, Boston, MA. doi: 10.1007/978-1-4613-1955-9_9

Schiariti A, Kawahara M, Uye S, Mianzan HW (2008) Life cycle of the jellyfish *Lychnorhiza lucerna* (Scyphozoa: Rhizostomeae). *Marine Biology*, *156*, 1–12. doi: 10.1007/s00227-008-1050-8

Schiariti A, Christiansen E, Morandini AC, da silveira FL, Giberto DA, Mianzan HW (2012) Reproductive biology of *Lychnorhiza lucerna* (Cnidaria: Scyphozoa: Rhizostomeae): Individual traits related to sexual reproduction. *Marine Biology Research, 8,* 255–264. doi: 10.1080/17451000.2011.616897

Schuchert P, Brinckmann-Voss A (2006) The true and false gonophores of *Coryne epizoica* and its shell colonizing mechanisms. *Italian Journal of Zoology, 73,* 319–324. doi: 10.1080/11250000600881340

Sommer C (1992) Larval biology and dispersal of *Eudendrium racemosum* (Hydrozoa, Eudendriidae). *Scientia Marina*, *56*, 205–211

Sötje I, Neues F, Epple M, Ludwig W, Rack A, Gordon M , Boese R, Tiemann H (2011). Comparison of the statolith structures of *Chironex fleckeri* (Cnidaria, Cubozoa) and *Periphylla periphylla* (Cnidaria, Scyphozoa): a phylogenetic approach. *Marine Biology*, *158*, 1149-1161. doi: 10.1007/s00227-011-1637-3

Straehler-Pohl I, Garm A, Morandini AC (2014) Sexual dimorphism in Tripedaliidae (Conant 1897) (Cnidaria, Cubozoa, Carybdeida). *Zootaxa, 3785,* 533–549. doi: 10.11646/zootaxa.3785.4.3

Teissier G (1926) Notes critiques sur la morphologie des gonophores chez les hydraires. I. Caractères distinctifs des hétéromédusoïdes et des cryptomédusoïdes. *Archives de Zoologie expérimentale et générale, 65, 75–86*

Toshino S, Miyake H, Ohtsuka S, Adachi A, Kondo Y, Okada S, Hirabayashi T, Hiratsuka T (2015) Monodisc strobilation in Japanese giant box jellyfish *Morbakka virulenta* (Kishinouye, 1910): a strong implication of phylogenetic similarity between Cubozoa and Scyphozoa. *Evolution & Development*, *17*, 231–239. doi: 10.1111/ede.12127

Toshino S, Miyake H, Srinui K, Luangoon N, Muthuwan V, Sawatpeera S, Honda S, Shibata H (2017) Development of *Tripedalia binata* Moore, 1988 (Cubozoa: Carybdeida: Tripedaliidae) collected from the eastern Gulf of

Thailand with implications for the phylogeny of the Cubozoa. *Hydrobiologia, 792*, 37–51. doi: 10.1007/s10750-016-3022-1

Uye SI (2014) The giant jellyfish *Nemopilema nomurai* in east Asian marginal seas. *In* Jellyfish Blooms. Springer Netherlands, pp 185–205.

Werner B (1973) New Investigations on systematics and evolution of the class Scyphozoa and the phylum Cnidaria. *Publications of the Seto Marine Biological Laboratory, 20,* 35–61.

Wietrzykowski W (1912) Recherches sur le développement des Lucernaires. *Archives de zoologie expérimentale et Générale, 10,* 1–95

Figuras

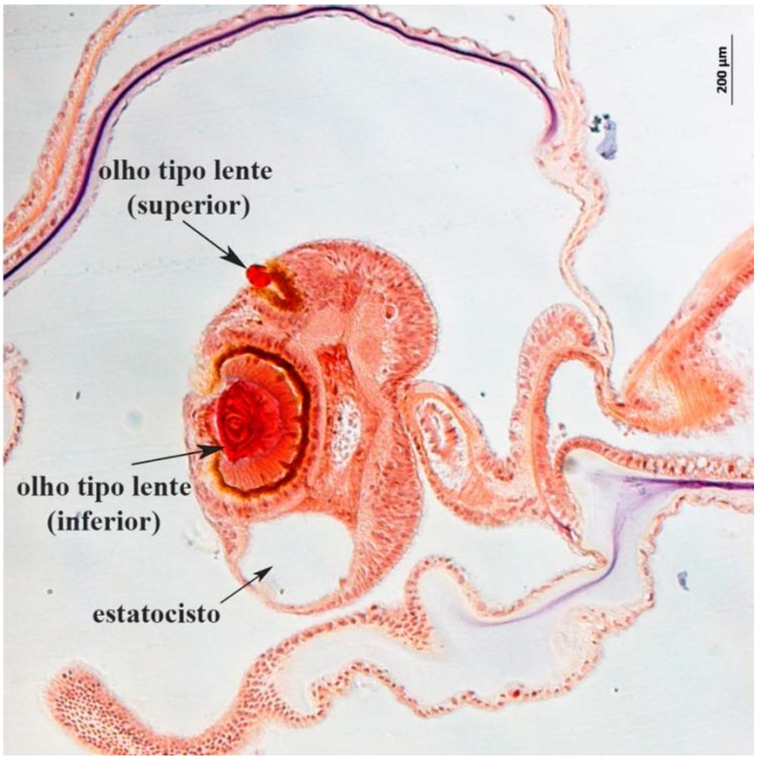

Figura 1. Corte histológico do ropálio de *Copula sivickisi* (Cubozoa). Método de coloração Tricrômico de Malory + hematoxilina.

Capítulos

Capitulo 1

Reproductive and environmental traits explain the variation in egg size among Medusozoa (Cnidaria)

Jimena García-Rodríguez[1], Amanda Ferreira Cunha[1,3], Adriana Morales-Guerrero[1], Adrian González-Chaves[2], Agustín Camacho[4], Lucília Souza Miranda[5], Filipe C. Serrano[2], Adrian Jaimes-Becerra[1,6] and Antonio Carlos Marques[1]

[1] Department of Zoology, Institute of Biosciences and [2] Department of Ecology, Institute of Biosciences, University of São Paulo, R. Matão, Tv. 14, 101, 05508-090 São Paulo, Brazil

[3] Departamento de Biologia Animal, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa, Av. P.H. Rolfs, s/n, 36570-900 Viçosa, Brazil

[4] Departamento de Ecología Evolutiva, Estación Biológica de Doñana, CSIC, Av. Américo Vespucio s/n, 41092 Sevilla, Spain

[5] Department of Zoology, Institute of Biological Sciences, Federal University of Minas Gerais, Av. Presidente Antônio Carlos 6627, 31270-901 Belo Horizonte, Brazil

[6] Department of Ecology, Evolution and Behavior, Alexander Silberman Institute of Life Sciences, Faculty of Science, Hebrew University of Jerusalem, 9190401 Jerusalem, Israel

https://dx.doi.org/10.1098/rspb.2023.0543 (Anexo I)

Abstract

Medusozoa (Cnidaria) are characterized by diverse life cycles, with different semaphoronts (medusa, medusoid, fixed gonophore, polyp) representing the sexual phase and carrying the gametes. Although egg size is often considered a proxy to understand reproductive and developmental traits of medusozoans, understanding of the processes influencing egg size variation in the group under an evolutionary context is still limited. We carried out a comprehensive review of the variation of egg size in Medusozoa to test whether this variation is related to biological/sexual or environmental traits. Egg size presents a strong phylogenetic signal (λ = 0.79, K = 0.67), explaining why closely related species with different reproductive strategies and different individual sizes have similar egg sizes. However, variation in egg size is influenced by the number of eggs, depth and temperature, with larger eggs frequently present in species with few eggs (1–15), in deep-sea species and in cold-water species. Conversely, the production of small eggs among cold-water species of Staurozoa might be associated with the development of a small benthic larvae in this group. Our study reinforces that egg sizes respond to reproductive and environmental traits, although egg size is highly conserved within medusa classes.

Keywords: oocyte, reproduction, medusa, phylogenetic signal, depth, temperature

Introduction

Interspecific comparison of sexual traits helps to understand the diversity and evolution of reproductive patterns, for example, why species living in shallow and deep waters have different reproductive performances [1,2]. Some frequently studied sexual traits are gonadal morphology [3], fecundity (e.g., number of eggs) [4], gametogenesis [5], reproductive cycles (e.g., phenology) [6] and sexual strategies (e.g., predominance of asexual or sexual reproduction) [7]. Egg size has raised interest because it is considered a proxy to infer characteristics of the reproductive cycle of marine species [8]. Variation in egg size has been found to predict variation in larval and female size [9,10], developmental mode (lecithotrophic larval species have larger eggs in many taxa) [11], reproductive cycle (e.g., deep-sea echinoderms show egg size variation depending on their reproductive biology) [6] and fertilization success (e.g., larger eggs have a higher chance of fertilization in marine invertebrates with broadcast-spawning) [12]. Egg size was also demonstrated to respond to environmental conditions [13,14], with larger eggs present in species living at low salinity (e.g., in estuarine amphipods [15]), in deep-sea habitats (e.g., in deep-sea fishes) [13], in low temperatures (with lipid-rich yolk stocks) [14] and in food-rich environments (e.g., in echinoderms) [16]. Similarly, a correlation between egg size and a given environmental factor may also depend on the species and latitude [14,17].

Sexual reproduction in Cnidaria (including Anthozoa, Endocnidozoa and Medusozoa [18]) is highly diverse [19]. Among the major cnidarian lineages, Medusozoa (a subphylum including the classes Hydrozoa, Scyphozoa, Cubozoa and Staurozoa) is particularly interesting because of its unique metagenetic life cycle [20]. The life cycle includes a swimming larva or planula, a sedentary polyp, and a free-living medusa [20], and the two main stages (polyp and medusa) may undertake sexual and asexual reproduction [21,22]. The medusa, when present, is the sexual adult phase carrying the gonads and gametes [22,23]. The development of the medusae is particularly plastic in the class Hydrozoa, where it may show several degrees of morphological reduction, even among species of the same genus [24]. Some species have medusoids (i.e., a short-lived reduced medusae [25,26]) that may remain attached to the gonotheca in a structure named a meconidium [27]. Other species have the medusa stage reduced to a fixed gonophore or sporosac [28], which is attached to the hydroid and considered to be the most reduced state of a medusa [29].

Medusozoan gonads are usually the focus in studies concerning sexual reproduction [30], and have been considered either as the regions where gametes are formed (therefore not true ‘gonads’, or not even an organ [31,32]), or true reproductive individualized organs with meiotic cells, sperm, and vitellogenic oocyte stages [33]. Gonadal development, location, and shape are also distinctive characters among medusozoans [34]. Scyphozoa, Staurozoa and Cubozoa are predominantly gonochoristic with gonads developing from the endodermal tissue of the bell [31,35,36]. Meanwhile

Hydrozoa have gonads of ectodermic origin developing either in the region of the radial canals (e.g., in the leptothecate *Mitrocoma cellularia* [37]), around the manubrium (e.g., in the 'anthoathecate' *Sarsia lovenii* [38]) or spadix (in fixed gonophores such as in the 'anthoathecate' Eudendrium [39]). Gametes (egg and sperm) can be liberated directly into the water for external fertilization (broadcasting as in *Nemalecium lighti* [40]), which is the presumed ancestral state in marine invertebrates [41], or only the sperm is liberated and fertilizes the eggs inside the gastrovascular cavity (e.g., *Alatina alata* [42]), sometimes including brooding (e.g., *Eudendrium* [39]).

Some topics of the sexual reproduction in Medusozoa have been relatively well studied, such as gametogenesis [43] and fertilization [44]. However, there is little information concerning egg morphology and nutrient content in Medusozoa [45]. Egg size data are also scarce, and comparative studies focusing on its variation within the group have not yet been published, despite the importance and high variability of this reproductive trait among Medusozoa. For instance, the contribution of historical processes and environmental pressures to Medusozoa reproductive patterns is limited, with no estimates of the relative influence of phylogeny and environment in reproductive trait variation. In this study we use comparative phylogenetic methods to understand the evolution of egg size in Medusozoa, considering the phylogenetic relationships within Medusozoa, and the relative contributions of reproductive (viz. egg number, individual size, reproductive strategies [polyp, medusa, medusoid], fertilization mode [internal, external]) and environmental (viz. depth range, water temperature) traits.

Material and methods

Oocyte and egg

We highlight that the technical literature generally uses egg (or ovum, pl. ova) and oocyte (or ovocyte) as synonymous, referring to stages of differentiation of the female gametes from the primary oocyte during the oogenesis [46]. The egg is derived from the maturation of the ootid that resulted from the meiosis II of the secondary oocyte. Ovum is considered the mature female gamete after meiosis is completed, whereas the secondary oocyte is an immature egg cell produced by the meiosis I of the primary oocyte [47,48]. Therefore, strictly, the terms egg and oocyte are not synonymous. However, the use of the term egg to refer to a late vitellogenic oocyte stage (Oiii) is widespread, and we adopt the term herein. The largest oocyte in the gonad is considered to represent the late vitellogenic stage [49] and is therefore the one usually measured to obtain the egg size.

Reproductive and ecological traits

Information on egg size was obtained for a total of 187 species of Medusozoa (136 Hydrozoa, 8 Staurozoa, 32 Scyphozoa and 11 Cubozoa), for which the taxonomy was standardized according to the World Register of Marine Species (WoRMS, 2021). Most of the species had their measurement surveyed in the literature, either by collecting data directly as provided in the paper or by measuring the eggs from photos or drawings available in species descriptions using ImageJ [50] (electronic supplementary material, table S1). We complemented the data by measuring eggs from histological preparations of gonads of hydrozoans (*Liriope tetraphylla, Orthopyxis crenata, Turritopsis nutricula*) and cubozoans (*Alatina alata, Carybdea marsupialis, Chiropsalmus quadrumanus, Copula sivickisi, Tamoya haplonema*) (electronic supplemental methods). When measuring eggs both from literature illustrations and histological preparations, we selected the largest eggs, considered to be in late vitellogenic stage (Oiii). For histological preparations, we measured up to 20 Oiii and used the average size value in the analyses. When egg size data available from the literature were given in ranges (n = 90), we used the average size value for subsequent analyses.

Information of reproductive traits was obtained for each species based on a literature review. We considered the following traits: (1) fertilization mode (external or internal), (2) number of eggs (1–15, 16–50, 51–100 and ≥101), (3) reproductive strategy (medusa, medusoid, fixed gonophore and polyp), and (4) individual size (see supplemental methods for further details). We also obtained information on the following environmental traits: (1) the mean sea surface temperature data for each species (n = 185; electronic supplementary material, table S1), and (2) the maximum depth of occurrence (n = 187; electronic supplemental material, methods).

Phylogenetic signal

In order to evaluate if the evolution of egg size in Medusozoa is constrained by phylogeny, we tested for phylogenetic signal (statistical dependence among species' trait values due to their phylogenetic relatedness [51]) of egg size with Blomberg's K and Pagel's lambda (λ) [52], with the 'phylosig' function in the package 'phytools' in R [52]. Both use Brownian motion (BM) evolutionary process in their implementation but differ in their approach. Blomberg's K compares the variance of a trait with the variance from a BM model. Values of K can take higher than 1 representing more phylogenetic signal than expected (more variance between clades) and K lower than 1 representing less phylogenetic signal than expected (more variance within clades) [51]. Pagel's λ, on the other hand, illustrates the transformation of the phylogeny that fits a BM model so when λ equals 1, the phylogeny can explain changes in traits, indicating a high phylogenetic signal and when λ equals 0 the trait is evolving independently of the phylogeny [51]. We therefore use high indices (λ close to 1 and p-values significative) to assess that closely related species are similar in egg size [53]. In addition, we tested the phylogenetic signal for environmental traits which turned out significant in the analysis of model selection and phylogenetic ANCOVA. This was done in order to test the pattern of phylogenetic niche conservatism.

Ancestral state reconstruction

To investigate the evolutionary history of egg size, egg number, temperature and depth we reconstructed ancestral states of these variables using the tree topology of 134 species (see supplemental methods for further details). Briefly, a composite phylogenetic tree including sequences of mitochondrial (16S and COI) and nuclear genes (18S and 28S) of 134 species was constructed based on the available published molecular phylogenies [54–59], and species for which we did not have egg size data were later removed from the tree. Bayesian estimates of divergence times were obtained using BEAST2 [53].

The ancestral state of egg size and temperature (continuous variable) was estimated using the ‘fastAnc’ function in the R package ‘phytools’ [53]. The model of evolution (Brownian motion, Ornstein–Uhlenbeck or early-burst) was selected using the R package ‘geiger’ [60] based on AIC scores. The ancestral states were mapped on the phylogeny using the ‘contMap’ function in R package ‘phytools’ [53]. We used maximum likelihood to compare models of traits evolution: Brownian motion (BM), Ornstein– Uhlenbeck (OU) and early burst (EB) models of trait evolution [61]. The ancestral state of egg number, and depth (categorical traits) were inferred by using stochastic character mapping implemented in R [62]. Transitions from one state to another can occur at different rates and in different directions in the phylogeny. For that, we first chose the best-fitting model of evolution for the reconstruction of this categorical trait among equal-rates (ER), symmetrical (SYM) and all-rates-different (ARD), identified by the lowest AICc score, calculated with the ‘ace’ function in the package ‘APE’ in R [63]. The uncertainty on the value of the trait at each ancestral node was described with the ‘make.simmap’ function (in ‘phytools’) after 1000 generations, resulting in a posterior probability distribution of character histories. The posterior distribution of character state history along each branch was summarized by using the ‘describe.simmap’ function [53].

Model selection

We used PGLS to investigate the influence of phylogeny, reproductive (number of eggs, individual size, reproductive strategies), and ecological (temperature and depth) traits’ on egg size. For this analysis, phylogenetic distances among species were obtained from our phylogenetic hypothesis based on molecular data (*n* = 134; see electronic supplementary material, methods), the same one used for the ancestral state reconstructions.

We assumed a Gaussian error distribution for the log transformed egg size variable, which seemed appropriated given the high number of species with data (134). Using the full model (constructing with all the variables), we compared different covariance structures corresponding to alternative models of evolution based on the lowest AIC value [64]. The tested covariance structures were BM, Martins (equivalent to OU model), Grafen (incorporates a calculation of branch lengths based on number of descendants), Blomberg (assumes that traits evolve under a BM model which rates either accelerate or slow through time) and Pagel (a modification of a BM model that incorporates phylogenetic signal). Between the plausible models we tested the multicollinearity using the 'vif' function in the R package 'car' [65].

We use the 'vif' function to remove the variable (greater than 3) to obtain our full model without multicollinearity. Then, we used the function 'model.sel' to identify which factors led to the lowest AIC. After building the full model considering additive effects among the predictive variables, we tested all possible combinations (between the variables number of eggs, individual size, reproductive strategies, class and temperature) using 'dredge' function from 'MuMIn' packages [66]. All models with delta AIC lower than 2 were considered equally plausible (see electronic supplementary material, table S3).

Model validation was performed with residual analyses (standardized residuals versus fitted values and Q-Q plots) (see the script in electronic supplementary material).

Finally, we tested the association of egg number and both reproductive strategy and fertilization mode fitting a phylogenetic generalized linear squares model (PGLS) [67]. Since evolutionary correlation of discrete characters is prone to reporting spurious significant associations [68], we transformed 'egg number' from a categorical to a continuous variable by using the mean number of each interval. Furthermore, has visual inspection of data is likely better to interpret these results [68,69], we mapped these traits using boxplots (see electronic supplementary material, figures S1 and S2, and script).

Phylogenetic ANCOVA

In addition to model selection and in order to test the hypothesis that egg number, individual size, reproductive strategy, depth, or temperature affects egg size in Medusozoa, we implement an ANCOVA model using phylogenetic generalized least squares (PGLS). This was done because we have a combination of continuous and discrete traits as independent variables [70].

Results

Phylogenetic signal

There is strong phylogenetic signal related to egg size across Medusozoa (Pagel's $\lambda = 0.79$, $p < 0.001$; $K = 0.67$, $p < 0.001$), considering that related species have similar egg sizes. The λ and K values (0.79 and 0.67 respectively) (close to 1) correspond to similar egg sizes between close taxa (figure 1), even though $K < 1$ shows that there is also moderate intra-clade variance. Likewise, it was also found that there is phylogenetic signal for temperature (Pagel's $\lambda = 0.80$, $p < 0.001$ and $K = 0.60$, $p < 0.001$) and depth ($p < 0.05$—rejects no signal model). This means that related species in the phylogeny have similar temperature values and depth ranges.

Ancestral reconstructions of egg size and related reproductive/environmental traits

Reconstruction of the ancestral state of egg size in Medusozoa shows that intermediate egg sizes are plesiomorphic and highly conserved within the group (figure 1; best-fit was OU model, AIC = 1750.46). The smallest eggs occur in Staurozoa (18–72 µm), intermediate in Cubozoa and Hydrozoa, and some of the largest in Scyphozoa (figure 1).

A higher number of eggs ($n \geq 101$) is the ancestral condition in Medusozoa, conserved in Staurozoa, Scyphozoa, and Cubozoa (figure 2; best-fit was symmetrical model, AIC = 135.43) ($n = 103$). The ancestral condition changed to fewer eggs ($n = 1$–15) in the origin of Hydroidolina. However, an intermediate number of eggs ($n = 16$–50 and 51–100) has evolved multiple times in less inclusive groups of Hydroidolina, such as Siphonophorae (e.g., *Muggiaea atlantica*, *Nanomia cara*), Capitata (e.g., Coryne fucicola, Coryne epizoica) and Macrocolonia (e.g., *Dentitheca dendritica, Sertularella polyzonias*). In addition, a reversal to a higher number of eggs (n ≥ 101) originated independently in Eirenida (e.g., *Aequorea victoria*), Campanulinida (e.g., *Earleria corachloeae*), Hebellidae (e.g., *Anthohebella parasitica*) and Bougainvilliidae (*Bougainvillia superciliaris*).

Reconstruction of the ancestral state of temperature (figure 3; best-fit was OU model, AIC = 885.96) shows that the Medusozoa ancestor (≈ 680 millions of years) probably lived in temperate waters (≈ 14°C). There was an increase in the average temperature (Devonian/Carboniferous period) occupied by the species throughout the evolution of Cubozoa and Scyphozoa (Kolpophorae) in tropical waters, as well as Hydrozoa, with some decreases to cold temperatures in several clades (Campanulariida, Haleciida, Bougainvillia, Aplanulata). It is important to note that Staurozoa and a few Scyphozoa species currently occurring in cold waters present contrasting egg sizes (small eggs in Staurozoa and large eggs in deep sea Scyphozoa species, compare figure 1 and figure 3).

The ancestral state of the depth (best-fit was ER model, AIC = 118.8) shows that Medusozoa inhabited shallow waters (0–50 m), independently appearing in only a few species in deep waters (greater than 101 m) and intermediate waters (51–100 m) (electronic supplementary material, figure S3).

Reproductive and environmental predictors of egg size

The evolutionary model based on AIC was for Grafen correlation (electronic supplementary material, table S2). Prior to model selection, multicollinearity was tested from the first model with six predictors (egg number, reproductive strategy, individual size, depth, and temperature). All predictors lower than 3, are showed on the final model. The best 5 models resulted under dredge function were compared using AIC (electronic supplementary material, table S3) and the best-fit model show that egg number, temperature and depth had an effect on egg size (AIC = 166.1, SE = 0.68, d.f. = 103) (figure 4 and electronic supplementary material, tables S3 and S4). The phylogenetic ANCOVA analyses show the same significant variables explaining the variation in egg size (depth, $p = 0.003$; egg number, $p = 0.037$; temperature, $p = 0.045$) as the results obtained with model selection.

Results from the phylogenetic generalized estimating equations analyses revealed a positive and significant correlation between the number of eggs and the ‘medusa’ reproductive strategy of Medusozoa (medusa: 1797.76, $t = 16.03$, $p < 0.001$) while not being significant for the other reproductive strategies (gonophore: 15.85, $t = 0.08$, $p = 0.93$; medusoid: 242.44, $t = 0.91$, $p = 0.37$; polyp: 7.12, $t = 0.008$, $p = 0.99$) (electronic supplementary material, figure S1). Likewise, a significant correlation with the number of eggs was found for external fertilization (1626.54, $t = 8.71$, $p > 0.001$) but not for internal fertilization (−393.09, $t = -1.39$, $p = 0.174$) (electronic supplementary material, figure S2).

Discussion

Evolution of egg size and its relationship with reproductive traits

Although small egg sizes are presumably a basal condition for animals [71], our analyses support that intermediate egg size is the ancestral condition in Medusozoa, being conserved along most of its phylogenetic history (figure 1). Among Medusozoa classes, however, Staurozoa and the cubozoan Tripedaliidae differ by having a relatively early transition (Carboniferous and Jurassic, respectively) to smaller eggs, while transitions to larger eggs in the scyphozoan Coronatae occurred since the Cretaceous (figure 1). Both genetic and environmental factors may explain egg size variation in marine invertebrates at different evolutionary levels, from individuals to populations, or even in species and more inclusive taxonomic groups [72]. Several medusozoan lineages have independent evolutionary shifts in egg size, but overall variation in egg size is correlated to the number of eggs related to different reproductive strategies (electronic supplementary material, figure S1). This suggests that egg size may be modulated by life-history tradeoffs and/or selective pressures unique to the evolutionary history of each taxa. Thus, for instance, species from different classes (e.g., Hydro-zoa and Scyphozoa) living in similar habitats (e.g., deep sea species) are prone to have similar egg size.

Several models for the evolution of egg size in marine invertebrates have been suggested, often involving tradeoffs between egg size and egg number (e.g. [73]). For instance, larger eggs require a lower concentration of sperm for fertilization, and are related to reduced developmental time and offspring mortality, but have the disadvantage of an increase in resources allocated to each offspring, leading to a reduction in fecundity (or number of eggs) [12,73]. In Medusozoa, a higher number of eggs (*n* ≥ 101) is the ancestral condition for the group (figure 2; see also [54]). In addition, a higher number of eggs is characteristic of species that show small to intermediate egg sizes (figure 4). A clear shift to a smaller number of eggs was observed in the appearance of Hydroidolina (Hydrozoa), which also presents a higher frequency of occurrence of fixed gonophores (electronic supplementary material, table S1). Indeed, our results show that species with medusa produce more eggs (*n* ≥101) (electronic supplementary material, figure S1) and also show a tendency to have smaller eggs than species with fixed gonophores (*n* = 1–15) (e.g., *Eudendrium bentart*, *Eudendrium klausi*, *Laomedea flexuosa*, *Synthecium flabellum*; figure 4; electronic supplementary material, table S1).

The relationship between egg size, egg number and reproductive strategy in Hydrozoa was proposed by Teissier [71], who suggested that the reduction of the medusa in some lineages of Hydrozoa could explain the fewer and larger eggs in species with fixed gonophores. This explanation was subsequently corroborated in differences concerning egg number and egg size among hydrozoans with different reproductive strategies (e.g. [26,74]). For instance, most species with a fixed gonophore strategy (in which eggs are produced by the polyp stage) are colonial hydroids that, as modular organisms, compartmentalize the functions in different modules [75] (polymorphs) [76,77] (see supplemental methods). This compartmentalization, allow the resources to be shared among the modules, with growth and reproduction often proceeding simultaneously [77,78]. Differently, the medusa, as a unique individual, has to balance growth and reproduction [79]. In modular colonies, however the eggs are produced in small numerous modules called gonozoids, which are distributed along the colony [78,80] and may also contribute to the existence of larger eggs. In fact, brooding in medusozoans is often associated with modular colonies, which present smaller and more sparsely distributed masses of eggs when compared to medusae. This is consistent with the view that the cost of brooding is associated with oxygen provision [81], as smaller and more spaced embryos facilitate oxygen supply (e.g., [82]), and in the case of modular colonies, could favour the occurrence of brooding [83]. These different patterns would have consequences from basic physiology (e.g., energetic demand of the different reproductive system and trade off with other systems) to species interaction (e.g., competition for substrate or prey) and biogeography (e.g., advective events and range distribution). Among species with a fixed gonophore (within Hydro-zoa), the relationship of brooders producing fewer and larger eggs, as well as larger planulae, than non-brooder shave also been suggested elsewhere [26]. Our results statistically corroborate and extend part of this hypothesis, showing that egg number could be related to fertilization mode in Medusozoa (electronic supplementary material, figure S2). Indeed, our study corroborates that species with external fertilization more often produces a higher number of eggs [84], while we found no support for internal fertilization being associated with the production of fewer eggs, as suggested before [85]. This, however, may reflect insufficient data on egg size of species with internal fertilization, and highlights the need for additional studies on reproductive patterns in Medusozoa, especially considering that several species are thought to present internal fertilization, even though most inferences are based on indirect evidence [86,87]. In a scenario in which external fertilization is usually considered as a primitive character [41], Medusozoa presents a high diversity and complexity of reproductive traits when compared toother basal Metazoa, and, given the few studies, a high potential for the discovery of additional complex reproductive traits. For instance, it includes viviparous species, as *Crossota millsae* [34], as well as elaborate sexual behaviors, such as the courtship and sperm transfer via spermatophore in *Copula sivickisi* [88].

Egg size and environmental conditions in Medusozoa

Environmental conditions are often correlated with the evolution of morphological characters (e.g., different egg morphologies depending on temperature or salinity in copepods [89]) and with reproductive traits in marine invertebrates [90]. Several environmental variables are suggested to trigger variation in the morphology of Medusozoa as a result of phenotypic plasticity [91]. For instance, species reared under laboratory conditions, such as *Millepora complanata* [92] and *Pennaria disticha* [93], produced larger eggs (both 600 µm) than wild collected specimens, suggesting that a greater investment of energy in reproduction under controlled environment with higher food supply increments the female gamete size [94].

Temperature plays an important role in reproduction, triggering mass reproductive events based on synchronized gametogenetic cycles [95], and affecting egg number [96] and egg size [97]. Many marine taxa (e.g., amphipods, echinoids, fishes) generally produce larger eggs in colder temperatures (e.g. [98]). Within Medusozoa, large eggs are known to be related to direct development, in which the developing medusa benefits from the high amount of energy stored in the yolk of these eggs [99–101]. Direct development mainly occurs in species adapted to colder temperatures in deep-sea species [3]. Indeed, we found that the largest eggs among medusozoans are in deep-sea species, *Atolla wyvillei* (403 µm), and *Periphylla periphylla* (1480 µm), that also present direct development [102] and are associated with lower temperatures [14].

Besides, temperature increases in ectothermic animals, leading to increased energetic costs of development which could explain for the smaller egg sizes [103]. However, the relationship between temperature and egg size may also reflect adaptive strategies shaped throughout the evolutionary history of the group [14,104], which could be the case of Staurozoa that mainly occurs in intertidal cold waters [105] and has small eggs.

According to the results obtained from the phylogenetic signal for temperature and depth, we recognize the possible existence of a pattern of phylogenetic niche conservatism (PNC) that may arise from multiple process including phylogenetic constraint where a common selective environment is shared in related species [106].

Staurozoa, the class with smaller eggs

Staurozoa showed an early shift to smaller eggs in the ancestral state reconstruction, coinciding with their colonization of colder waters [105] (figure 1). In Cnidaria, the planula stage follows egg fertilization and embryonic development. The planula and egg in Medusozoa are similar in size, as described for eggs and larvae of other organisms (e.g., echinoderms [107]). It is possible that the conspicuous difference between the egg size of Staurozoa and other medusozoans is related to the staurozoan unique planula [108]. The diminutive eggs of Staurozoa (18–72 µm recorded in this study) are followed by diminutive (e.g., 100 µm long and 20 µm wide in *Haliclystus* sp.), non-swimming (benthic) planulae, with a constant number of 16 endodermal cells [109] and apparently able to feed when attached [110] (although staurozoan eggs were reported with yolk substances [108,109,111]). Moreover, these small planulae have limited movement ability, crawling on the substrate (i.e., vermiform movements of elongation and retraction) [108,109]. Indeed, staurozoan planulae may settle in groups, living side-by-side, apparently maximizing the efficiency in prey capture [112,113] and maybe in defense, and promoting genetically homogeneous populations [112]. Finally, their small eggs and planulae dimensions and habit would also be associated with lower metabolic demands, as suggested for other marine invertebrates [114].

Conclusion

This is the first comparative study addressing egg size in Medusozoa and we show the importance of evaluating this trait and its relationship with historical, sexual, and ecological traits. Egg size in Medusozoa has a strong phylogenetic signal, explaining why this group with many different reproductive strategies and different individual sizes tend to have eggs of similar dimensions. Indeed, egg size is considerably conserved, with intermediate egg sizes as the ancestral condition in the group. Egg size is also related to sexual traits, such as the number of eggs, with larger eggs frequently associated with species with few eggs (1–15); and to environmental traits as depth and temperature. On the other hand, Staurozoa has small eggs probably associated with their small benthic crawling planulae, which therefore would have presumably lower metabolic demands. After this study we can conclude that variation in egg size in Medusozoa is explained by bio-logical/sexual and environmental factors, stressing the role of past ecological and genetic processes in Medusozoa evolution. Further information on deep sea species, as well as data on fertilization mode and egg size of more species of Medusozoa, shall contribute to provide a broader understanding of the evolutionary patterns of sexual reproduction in this group, as well as in other marine taxa.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material [115].

Authors' contributions. J.G.-R.: conceptualization, data curation, formal analysis, investigation, project administration, resources, software, supervision, validation, visualization, writing—original draft, writing—review and editing; A.F.C.: conceptualization, supervision, validation, writing—review and editing; A.M.-G.: data curation, formal analysis, investigation, methodology, resources, software, vali-dation, visualization, writing—review and editing; A.G.-C.: data curation, formal analysis, methodology, resources, software, vali-dation, writing—review and editing; A.C.G.: data curation, formal analysis, methodology, software, supervision, validation, writing—review and editing; L.S.M.: validation, writing—review and editing; F.C.S.: formal analysis, methodology, software, writing—review and editing; A.J.-B.: data curation, formal analysis, investigation, methodology, resources, software, validation, visualization, writing—review and editing; A.C.M.: supervision, validation, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. The authors have no competing of interests.

Funding. This study was supported by São Paulo Research Foundation (FAPESP) (grants 2011/50242-5 to A.C.M., 2017/00641-7 to A.F.C.,2017/00312-3 to A.M.-G., 2017/19411-1 to A.G.-C., 2019/01370-2and 2021/09739-5 to J.G.-R.), CNPq (grants 140235/2018-3 to J.G.-R., 316095/2021-4 to A.C.M.), CAPES (grant 23650751852 to A.J.B.).

Acknowledgements. We are grateful to Prof. Alvaro Migotto, Prof. André Morandini and Dr Marina O. Fernandez for suggestions in response to a previous version of the manuscript. We thank Dr Edgar Gamero-Mora for providing pictures to measure egg size of *Cassiopeia* spp, and Prof. José Eduardo Marian for helping with the histological procedures. We also thank Mr. Enio Matos and Mr Phillip Lenktaitis for their support in the histology lab. We thank the four anonymous reviewers who improved the manuscript.

References

1. Shlesinger T, Loya Y. 2019 Sexual reproduction of Scleractinian corals in mesophotic coral ecosystems vs. shallow reefs. In Mesophotic coral ecosystems (eds Y Loya, KA Puglise, TCL Bridge), pp. 653–666.Berlin, Germany: Springer.

2. Waller RG. 2006 Deep-water Scleractinia (Cnidaria: Anthozoa): current knowledge of reproductive processes. In Cold-water corals and ecosystems (eds A Freiwald, JM Roberts), pp. 691–700. Berlin, Germany: Springer.

3. Lucas CH, Reed AJ. 2010 Gonad morphology and gametogenesis in the deep-sea jellyfish *Atolla wyvillei* and *Periphylla periphylla* (Scyphozoa: Coronatae) collected from Cape Hatteras and the Gulf of Mexico. J. Mar. Biol. Assoc. U. K. 90,1095–1104. (doi:10.1017/S0025315409000824)

4. Costa EFS, Dias JF, Murua H. 2015 Reproductive strategy and fecundity of the keystone species *Paralonchurus brasiliensis* (Teleostei, Sciaenidae): an image processing techniques application. Environ. Biol. Fishes 98,2093–2108. (doi:10.1007/s10641-015-0432-2)

5. Waller RG, Tyler PA, Gage JD. 2005 Sexual reproduction in three hermaphroditic deep-sea *Caryophyllia species* (Anthozoa: Scleractinia) from the NE Atlantic Ocean. Coral Reefs 24, 594–602. (doi:10.1007/s00338-005-0031-3)

6. Gage JD, Tyler PA. 1991 Reproduction, recruitment and growth of deep-sea organisms. In Deep-sea biology: a natural history of organisms at the deep-sea floor, pp. 301–336. Cambridge, UK: Cambridge University Press.

7. Fischer EA, Petersen CW. 1987 The evolution of sexual patterns in the seabasses. Bioscience 37,482–489. (doi:10.2307/1310420)

8. Grant A, Tyler PA. 1983 The analysis of data in studies of invertebrate reproduction. II. The analysis of oocyte size/frequency data, and comparison of different types of data. Int. J. Invertebr. Reprod. 6,271–283. (doi:10.1080/01651269.1983.10510053)

9. Johnston TA, Leggett WC. 2002 Maternal and environmental gradients in the egg size of an iteroparous fish. Ecol. Soc. Am. 83, 1777–1791.

10. McEdward LR. 1986 Comparative morphometrics of echinoderm larvae. I. Some relationships between egg size and initial larval form in echinoids. J. Exp. Mar. Biol. Ecol. 96, 251–265. (doi:10.1016/0022-0981(86)90206-6)

11. Thorson G. 1950 Reproductive and larval ecology of marine bottom invertebrates. Biol. Rev. 25, 1–45. (doi:10.1111/j.1469-185X.1950.tb00585.x)

12. Levitan DR. 2006 The relationship between egg size and fertilization success in broadcast-spawning marine invertebrates. Integr. Comp. Biol. 46,298–311. (doi:10.1093/icb/icj025)

13. Marshall NB. 1953 Egg size in Artic, Antartic and deep-sea fishes. Evolution7, 328–341. (doi:10.1111/j.1558-5646.1953.tb00096.x)

14. Laptikhovsky V. 2006 Latitudinal and bathymetric trends in egg size variation: A new look at Thorson's and Rass's rules. Mar. Ecol. 27, 7–14. (doi:10.1111/j.1439-0485.2006.00077.x)

15. Steele DH, Steele VJ. 1991 Effects of salinity on the survival, growth rate, and reproductive output of *Gammarus lawrencianus* (Crustacea, Amphipoda). Mar. Ecol. Prog. Ser. 78, 49–56. (doi:10.3354/meps078049)

16. George SB. 1996 Echinoderm egg and larval quality as a function of adult nutritional state. Oceanolog. Acta 19, 297–308.

17. Fleming IA, Gross MR. 1990 Latitudinal clines: A trade-off between egg number and size in Pacific salmon. Ecol. Soc. Am. 71, 1–11. (doi:10.2307/20167213)

18. Kayal E, Bentlage B, Sabrina Pankey M, Ohdera AH, Medina M, Plachetzki DC, Collins AG, Ryan JF. 2018 Phylogenomics provides a robust topology of the major cnidarian lineages and insights on the origins of key organismal traits. BMC Evol. Biol. 18. (doi:10.1186/s12862-018-1142-0)

19. Fautin DG. 2002 Reproduction of Cnidaria. Can. J. Zool. 80, 1735–1754. (doi:10.1139/z02-133)

20. Müller WA, Leitz T. 2002 Metamorphosis in the Cnidaria. Can. J. Zool. 80, 1755–1771. (doi:10.1139/z02-130)

21. Morandini AC, Schiariti A, Stampar SN, Maronna MM, Straehler-Pohl I, Marques AC. 2016 Succession of generations is still the general paradigm for scyphozoan life cycles. Bull. Mar. Sci. 92, 343–351. (doi:10.5343/bms.2016.1018)

22. Collins AG. 2002 Phylogeny of Medusozoa and the evolution of cnidarian life cycles. J. Evol. Biol. 15, 418–432. (doi:10.1046/j.1420-9101.2002.00403.x)

23. Werner B. 1973 New Investigations on systematics and evolution of the class Scyphozoa and the phylum Cnidaria. Publ. Seto Mar. Biol. Lab. 20,35–61. (doi:10.5134/175791)

24. Boero F, Sarà M. 1987 Motile sexual stages and evolution of Leptomedusae (Cnidaria). Bolletino di zoologia 54, 131–139. (doi:10.1080/11250008709355572)

25. Cornelius P. 1992 Medusa loss in leptolid Hydrozoa (Cnidaria), hydroid rafting, and abbreviated life-cycles among their remote-island faunae: an interim review. Sci. Mar. 56, 245–261.

26. Migotto A. 1998 The life cycle of *Sertularia marginata* Kirchenpauer, 1864 (Cnidaria: Hydrozoa): a medusoid-producing sertulariid. J. Nat. Hist. 32,1–12. (doi:10.1080/00222939800770011)

27. Cornelius PFS. 1995 North-west European thecate hydroids and their medusae, part 2: Sertulariidae to Campanulariidae. London, UK: Field Studies Council.

28. Allman GJ. 1983 Report on the present state of our knowledge of the reproductive system in the Hydroida. Report of the Thirty-third Meeting of the British Association for the Advancement of Science.

29. Teissier G. 1926 Notes critiques sur la morphologie des gonophores chez les hydraires. Archives de Zoologic Expdrimentale, notes et reveu 65, 75–86.

30. Eckelbarger KJ, Larson RJ. 1993 Ultrastructural study of the ovary of the sessile scyphozoan, *Haliclystus octoradiatus* (Cnidaria: Stauromedusae). J. Morphol.218, 225–236. (doi:10.1002/jmor.1052180210)

31. Campbell RD. 1974 Cnidaria. Reprod. Mar. Invertebr.1, 133–199.

32. Eckelbarger KJ, Hodgson AN. 2021 Invertebrate oogenesis – a review and synthesis: comparative ovarian morphology, accessory cell function and the origins of yolk precursors. Invertebr. Reprod. Dev.65, 71–140. (doi:10.1080/07924259.2021.1927861)

33. Schiariti A, Kawahara M, Uye S, Mianzan HW. 2008 Life cycle of the jellyfish *Lychnorhiza lucerne* (Scyphozoa: Rhizostomeae). Mar. Biol. 156, 1–12. (doi:10.1007/s00227-008-1050-8)

34. Thuesen E v. 2003 *Crossota millsae* (Cnidaria: Trachymedusae: Rhopalonematidae), a new species of viviparous hydromedusa from the deep sea off California and Hawaii. Zootaxa 309, 1. (doi:10.11646/zootaxa.309.1.1)

35. Widersten B. 1965 Genital organs and fertilization some Scyphozoa. Zoological Institute, Uppsala 37,45–58. (doi:10.1016/0011-7471(66)90698-x)

36. Hyman LH. 1940 Metazoa of the tissue grade of construction: the radiate phylum Cnidaria. In The invertebrates: Protozoa through Ctenophora. New York, NY: McGraw-Hill.

37. Arai MN, Brinckmann-Voss A. 1980 Hydromedusae of British Columbia and Puget Sound. Can. Bull.Fish. Aquat. Sci. 204, 1–192.

38. Edwards C. 1978 The hydroids and medusae *Sarsia occulta* sp. nov., *Sarsia tubulosa* and *Sarsia loveni*. J. Mar. Biol. Assoc. U. K. 58, 291–311. (doi:10.1017/S0025315400027995)

39. Marques A, Peña CA, Vervoort W. 2000Mediterranean species of *Eudendrium* Ehrenberg, 1834 (Hydrozoa, Anthomedusae, Eudendriidae) with the description of a new species. J Zool. 252,197–213. (doi:10.1111/j.1469-7998.2000.tb00615.x)

40. Gravier-Bonnet N, Migotto AE. 2000 Gonangium development and medusoid of *Nemalecium lighti* (Hargitt, 1924) (Cnidaria: Hydrozoa, Haliciidae). SciMar 64, 207–213. (doi:10.3989/scimar.2000.64s1207)

41. Subramoniam T. 2018 Mode of reproduction: Invertebrate animals. In Encyclopedia of reproduction (ed. MK Skinner), pp. 32–40. New York, NY: Elsevier.

42. Lewis C, Bentlage B, Yanagihara A, Gillan W, van Blerk J, Keil DP, Bely AE, Collins AG. 2013 Redescription of *Alatina alata* (Reynaud, 1830) (Cnidaria: Cubozoa) from Bonaire, Dutch Caribbean. Zootaxa 3737, 473–487. (doi:10.11646/zootaxa.3737.4.8)

43. Morandini AC, da Silveira FL. 2001 Sexual reproduction of *Nausithoe aurea* (Scyphozoa, Coronatae): gametogenesis, egg release, embryonic development, and gastrulation. Sci Mar 65,139–149. (doi:10.3989/scimar.2001.65n2139)

44. Freeman G, Miller RL. 1982 Hydrozoan eggs can only be fertilized at the site of polar body formation. Dev. Biol. 94, 142–152. (doi:10.1016/0012-1606(82)90077-X)

45. Beckwith CJ. 1914 The genesis of the plasma-structure in the egg of *Hydractinia echinata*. J. Morphol. 25, 189–251. (doi:10.1002/jmor.1050250202)

46. Gilbert SF. 2000 Oogenesis. In Developmental biology (ed. SF Gilbert), pp. 453–454. Sunderland, MA: Sinauer Associates.

47. Sagata N. 1996 Meiotic metaphase arrest in animal oocytes: its mechanisms and biological significance. Trends Cell Biol. 6, 22–28. (doi:10.1016/0962-8924(96)81034-8)

48. Taieb F, Thibier C, Jessus C. 1997 On cyclins, oocytes, and eggs. Mol. Reprod. Dev. 48, 397–411. (doi:10.1002/(SICI)1098-2795(199711)48:3<397::AID-MRD14>3.0.CO;2-T)

49. Schiariti A, Christiansen E, Morandini AC, da Silveira FL, Giberto DA, Mianzan HW. 2012 Reproductive biology of *Lychnorhiza lucerna* (Cnidaria: Scyphozoa: Rhizostomeae): Individual traits related to sexual reproduction. Marine Biology Research 8, 255–264. (doi:10.1080/17451000.2011.616897)

50. Schneider CA, Rasband WS, Eliceiri KW. 2012 NIH Image to ImageJ: 25 years of image analysis. Nat. Methods. 9, 671–675. (doi:10.1038/nmeth.2089)

51. Münkemüller T, Lavergne S, Bzeznik B, Dray S, Jombart T, Schiffers K, Thuiller W. 2012 How to measure and test phylogenetic signal. Methods Ecol Evol 3,743–756. (doi:10.1111/j.2041-210X.2012.00196.x)

52. Blomberg SP, Garland T, Ives AR. 2003 Testing for phylogenetic signal in comparative data: behavioral traits are more labile. Evolution 57, 717–745. (doi:10.1111/j.0014-3820.2003.tb00285.x)

53. Revell LJ. 2012 Phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. 3, 217–223. (doi:10.1111/j.2041-210X.2011.00169.x)

54. Collins A, Schuchert P, Marques A, Jankowski T, Medina M, Schierwater B. 2006 Medusozoan phylogeny and character evolution clarified by new large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models. Syst. Biol. 55, 97–115. (doi:10.1080/10635150500433615)

55. Miranda LS, Hirano YM, Mills CE, Falconer A, Fenwick D, Marques AC, Collins AG. 2016 Systematics of stalked jellyfishes (Cnidaria: Staurozoa). PeerJ 4, e1951. (doi:10.7717/peerj.1951)

56. Collins AG. 2009 Recent insights into cnidarian phylogeny. Smithson Contrib Mar Sci 38, 139–149. (doi:10.5479/si.1943667X.0)

57. Cartwright P, Evans NM, Dunn CW, Marques AC, Miglietta MP, Schuchert P, Collins AG. 2008 Phylogenetics of Hydroidolina (Hydrozoa: Cnidaria). J. Mar. Biol. Assoc. U. K. 88, 1663–1672. (doi:10.1017/S0025315408002257)

58. Maronna MM, Miranda TP, Peña Cantero ÁL, BarbeitosMS, Marques AC. 2016 Towards a phylogenetic classification of Leptothecata (Cnidaria, Hydrozoa). Sci. Rep. 6, 18075. (doi:10.1038/srep18075)

59. Kayal E, Bentlage B, Cartwright P, Yanagihara AA, Lindsay DJ, Hopcroft RR, Collins AG. 2015 Phylogenetic analysis of higher-level relationships within Hydroidolina (Cnidaria: Hydrozoa) using mitochondrial genome data and insight into their mitochondrial transcription. PeerJ 3, e1403. (doi:10.7717/peerj.1403)

60. Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W. 2008 GEIGER: Investigating evolutionary radiations. Bioinformatics 24, 129–131. (doi:10.1093/bioinformatics/btm538)

61. Cooper N, Jetz W, Freckleton RP. 2010 Phylogenetic comparative approaches for studying niche conservatism. J. Evol. Biol. 23, 2529–2539. (doi:10.1111/j.1420-9101.2010.02144.x)

62. Huelsenbeck JP, Nielsen R, Bollback JP. 2003 Stochastic mapping of morphological characters. Syst. Biol. 52, 131–158. (doi:10.1080/10635150390192780)

63. Paradis E, Claude J, Strimmer K. 2004 APE: Analyses of phylogenetics and evolution in R language. Bioinformatics 20, 289–290. (doi:10.1093/bioinformatics/btg412)

64. Grafen A. 1989 The phylogenetic regression. Phil. Trans. R. Soc. Lond. B 326, 119–157. (doi:10.1098/rstb.1989.0106)

65. Kassambara A. 2018 Multicollinearity essentials and VIF in R. See http://www.sthda.com/english/articles/39-regression-model-diagnostics/160-multicollinearity-essentials-and-vif-in-r.

66. Barton K. 2020 MuMIn: multi-model inference. R package version 1.43.17. See https://cran.r-project.org/web/packages/MuMIn/MuMIn.pdf.

67. Paradis E, Claude J. 2002 Analysis of comparativedata using generalized estimating equations.J. Theor. Biol. 218, 175–185. (doi:10.1006/jtbi.2002.3066)

68. Maddison WPM, Fitzjohn RG. 2015 The unsolved challenge to phylogenetic correlation tests for categorical characters. Syst. Biol. 64, 127–136. (doi:10.1093/sysbio/syu070)

69. Rabosky DL, Goldberg EE. 2015 Model inadequacy and mistaken inferences of trait-dependent speciation. Syst. Biol. 64, 340–355. (doi:10.1093/sysbio/syu131)

70. Smaers JB, Rohlf FJ. 2016 Testing species' deviation from allometric predictions using the phylogenetic regression. Evolution 70, 1145–1149. (doi:10.1111/evo.12910)

71. Teissier G. 1922 Observation des médusoides libres et des planulas de *Sertularia operculata* L. Bull. Soc.Zool. Fr. 47, 357–361.

72. Lessios HA. 1990 Adaptation and phylogeny as determinants of egg size in echinoderms from the two sides of the isthmus of Panama. Am. Nat. 135. (doi:10.1086/285028)

73. Podolsky RD, Strathmann RR. 1996 Evolution of egg size in free-spawners: consequences of the fertilization-fecundity trade-off. Am. Nat. 148,160–173. (doi:10.1086/285916)

74. Bourmaud C, Gravier-bonnet N. 2005 Non-released cryptomedusoid in the life cycle of *Antennella* sp. (Cnidaria, Hydrozoa, Halopterididae) from La Réunion, Indian Ocean. Invertebr. Reprod. Dev. 48,55–62. (doi:10.1080/07924259.2005.9652170)

75. Rosen BR. 1986 Modular growth and form of corals: a matter of metamers? Phil. Trans. R. Soc. Lond. B313, 115–142. (doi:10.1098/rstb.1986.0029)

76. Mackie GO. 1986 From aggregates to integrates: physiological aspects of modularity in colonial animals. Phil. Trans. R. Soc. Lond. B 313, 175–196. (doi:10.1098/rstb.1986.0032)

77. Harvell CD. 1994 The evolution of polymorphism in colonial invertebrates and social insects. Q Rev. Biol.69, 155–185. (doi:10.1086/418538)

78. Hughes RN. 1989 Modular colonies. In A functional biology of clonal animals, pp. 231–275. London, UK: Chapman and Hall.

79. Gershwin L. 2005 Taxonomy and phylogeny of Australian Cubozoa. Thesis, James Cook University, Townsville, Australia.

80. Millard NAH. 1975 Monograph on the Hydroida of Southern Africa. Annals of the South African Museum 68, 1–513.

81. Fernández M, Calderón R, Cifuentes M, Pappalardo P. 2006 Brooding behaviour and cost of brooding in small body size brachyuran crabs. Mar. Ecol. Prog. Ser. 309, 213–220. (doi:10.3354/meps309213)

82. Lee CE, Strathmann RR. 1998 Scaling of gelatinous clutches: Effects of siblings' competition for oxygen on clutch size and parental investment per offspring. Am. Nat. 151, 293–310. (doi:10.1086/286120)

83. Strathmann RR. 2020 The association of coloniality with parental care of embryos. J Exp Zool. 336,221–230.

84. Szmant-Froelich A, Yevich P, Pilson MEQ. 1980 Gametogenesis and early development of the temperate coral *Astrangia danae* (Anthozoa: Scleractinia). Biol. Bull.158, 257–269. (doi:10.2307/1540935)

85. Fadlallah YH, Pearse JS. 1982 Sexual reproduction in solitary corals: overlapping oogenic and brooding cycles, and benthic planulas in Balanophylliaelegans. Mar. Biol. 71.

86. Toshino S, Hiroshi M, Ohtsuka S, Okuizumi K, AdachiA, Hamatsu Y, Urata M, Nakaguchi K, Yamaguchi S. 2013 Development and polyp formation of the giant box jelly fish *Morbakka virulenta* (Kishinouye,1910) (Cnidaria: Cubozoa) collected from the Seto Inland Sea, western Japan. Plankton Benthos Res. Benthos Res. 8, 1–8. (doi:10.3800/pbr.8.1)

87. García-Rodríguez J, Ames CL, Marian JEAR, Marques AC. 2020 Histomorphological comparison of testes in species of box jellyfish (Cnidaria; Cubozoa): does morphology differ with mode of reproduction and fertilization? Org Divers Evol 20, 25–36. (doi:10.1007/s13127-019-00427-x)

88. Lewis C, Long TAF. 2005 Courtship and reproduction in *Carybdea sivickisi* (Cnidaria: Cubozoa). Mar. Biol.147, 477–483. (doi:10.1007/s00227-005-1602-0)

89. Castro-Longoria E, Williams JA. 1999 The production of subitaneous and diapause eggs: a reproductive strategy for *Acartia bifilosa* (Copepoda: Calanoida) in Southampton Water, UK. J Plankton Res 21, 65–84. (doi:10.1093/plankt/21.1.65)

90. Stimson JS. 1978 Mode and timing of reproduction in some common hermatypic corals of Hawaii and Enewetak. Mar. Biol. 48, 173–184. (doi:10.1007/ BF00395017)

91. Cunha AF, Maronna MM, Marques AC. 2016 Variability on microevolutionary and macroevolutionary scales: a review on patterns of morphological variation in Cnidaria Medusozoa. Org Divers Evol. 16, 431 442. (doi:10.1007/s13127-016- 0276-4)

92. Lewis JB. 1991 The ampullae and medusae of the calcareous hydrozoan *Millepora complanata*. Hydrobiologia 216–217, 165–169. (doi:10.1007/ BF00026458)

93. Genzano GN, Kubota S. 2003 Synchronous mass release of mature medusae from the Hydroid *Halocordyle disticha* (Hydrozoa, Halocordylidae) and experimental induction of different timing by light changes. Publ. Seto Mar. Biol. Lab. 39, 221–228. (doi:10.5134/176311)

94. Chester CM. 1996 The effect of adult nutrition on the reproduction and development of the estuarine nudibranch, *Tenellia adspersa* (Nordmann, 1845). J. Exp. Mar. Biol. Ecol. 198, 113–130. (doi:10.1016/ 0022-0981(95)00197-2)

95. Keith SA et al. 2016 Coral mass spawning predicted by rapid seasonal rise in ocean temperature. Proc. R. Soc. B 283, 20160011. (doi:10.1098/rspb. 2016.0011)

96. Galea HR, Ferry R, Bertot JM. 2012 Medusoids in the life cycle of *Dentitheca dendritica* (Nutting, 1900) and *Nemalecium gracile* sp. nov. (Cnidaria: Hydrozoa). Zootaxa 3527, 43–54. (doi:10.11646/ zootaxa.3527.1.3)

97. Pitt KA, Kingsford MJ. 2000 Reproductive biology of the edible jellyfish *Catostylus mosaicus* (Rhizostomeae). Mar. Biol. 137, 791–799. (doi:10.1007/s002270000399)

98. Skadsheim A. 1989 Regional variation in amphipod life history: effects of temperature and salinity on breeding. J. Exp. Mar. Biol. Ecol. 127, 25–42. (doi:10.1016/0022-0981(89)90207-4)

99. Hargitt GT. 1919 Germ cells of coelenterates. VI. General considerations, discussion, conclusions. J. Morphol. 33, 1–59. (doi:10.1002/jmor.1050330102)

100. Berrill NJ. 1949 Developmental analysis of Scyphomedusae. Biol. Rev. 24, 393–409. (doi:10. 1111/j.1469-185X.1949.tb00581.x)

101. Larson RJ. 1986 Pelagic scyphomedusae (Scyphozoa: Coronatae and Semaeostomeae) of the Southern Ocean. In Biology of the antarctic seas XVI (ed. LS Kornicker), pp. 59–165. Washington, DC: American Geophysical Union.

102. Jarms G, Båmstedt U, Tiemann H, Martinussen MB, Fosså JH, Høisoeter T. 1999 The holopelagic life cycle of the deep-sea medusa *Periphylla periphylla* (Scyphozoa, Coronatae). Sarsia 84, 55–65. (doi:10. 1080/00364827.1999.10420451)

103. Parry GD. 1983 The influence of the cost of growth on ectotherm metabolism. J. Theor. Biol. 101, 453–477. (doi:10.1016/0022-5193(83)90150-9)

104. Lardies MA, Medina MH, Correa JA. 2008 Intraspecific biogeographic pattern breakage in the snapping shrimp *Betaeus emarginatus* caused by coastal copper mine tailings. Mar. Ecol. Prog. Ser. 358, 203–210. (doi:10.3354/meps07347)

105. Miranda LS, Mills CE, Hirano YM, Collins AG, Marques AC. 2018 A review of the global diversity and natural history of stalked jellyfishes (Cnidaria, Staurozoa). Mar. Biodivers. 48, 1695–1714. (doi:10. 1007/s12526-017-0721-4)

106. Losos JB. 2011 Seeing the forest for the trees: The limitations of phylogenies in comparative biology. Am. Nat. 177, 709–727. (doi:10.1086/660020)

107. Rahman MA, Uehara T, Rahman M. 2002 Effects of egg size on fertilization, fecundity and offspring performance: a comparative study between two sibling species of tropcial sea urchins (Genus Echinometra). Pak. J. Biol. Sci. 5, 114–121. (doi:10.3923/pjbs.2002.114.121)

108. Mayorova TD, Osadchenko B, Kraus Y. 2020 How to build a larval body with less than a hundred cells? Insights from the early development of a stalked jellyfish (Staurozoa, Cnidaria). Org Divers Evol 20, 681–699. (doi:10.1007/s13127-020-00459-8)

109. Otto JJ. 1976 Early development and planula movement in Haliclystus (Scyphozoa: Stauromedusae). In Coelenterate ecology and behavior (ed. GO Mackie), pp. 319–329. Berlin, Germany: Springer.

110. Jarms G, Tiemann H. 1996 On a new hydropolyp without tentaeles, *Microhydrula limopsicola* n. sp., epibiotic on bivalve shells from the Antarctic. Sci Mar 60, 109–115.

111. Hanaoka K-I. 1934 Notes on the Early Development of a Stalked Medusa. Proc. Imp. Acad. 10, 117 120. (doi:10.2183/pjab1912.10.117)

112. Miranda LS, Collins AG, Marques AC. 2010 Molecules clarify a cnidarian life cycle: the 'hydrozoan' *Microhydrula limopsicola* is an early life stage of the staurozoan *Haliclystus antarcticus*. PLoS ONE 5, 1–9. (doi:10.1371/journal.pone.0010182)

113. Wietrzykowski W. 1912 Recherches sur le développement des Lucernaires. Archives de zoologie expérimentale et générale 10, 1–95.

114. McEdward LR, Morgan KH. 2001 Interspecific relationships between egg size and the level of parental investment per offspring in echinoderms. Biological Bulletin 200, 33–50. (doi:10.2307/1543083)

115. García-Rodríguez J, Cunha AF, Morales-Guerrero A, González-Chaves A, Camacho A, Miranda LS, Serrano FC, Jaimes-Becerra A, Marques AC. 2023 Reproductive and environmental traits explain the variation in egg size among Medusozoa (Cnidaria). Figshare. (doi:10.6084/m9.figshare.c.6730050)

Figures

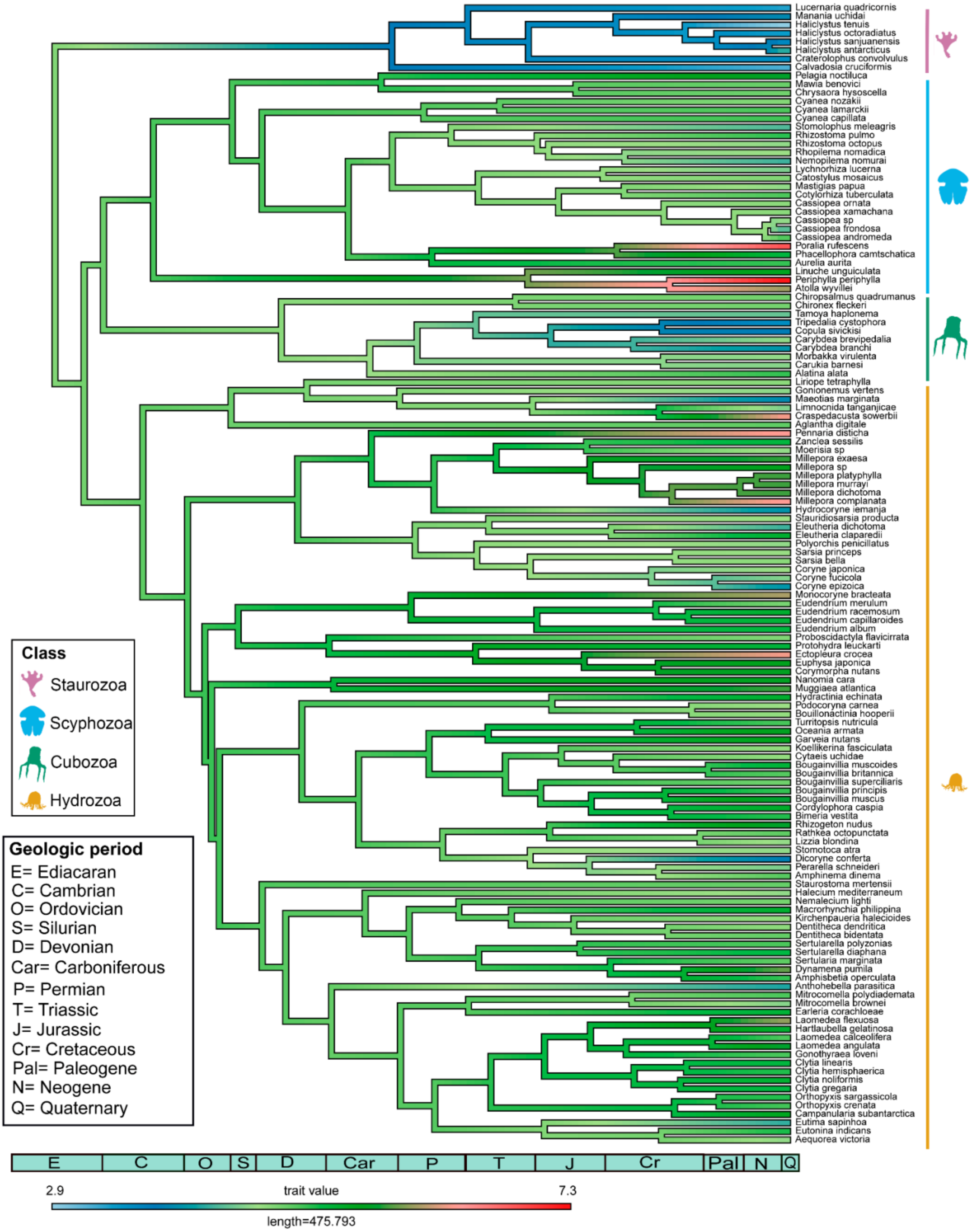

Figure 1. Reconstruction of ancestral egg size (log) in Medusozoa for the 134 species analysed in this study. Colour on branches indicates egg size along the topology. Trait value = min and max egg size (log) (2.9–7.3).

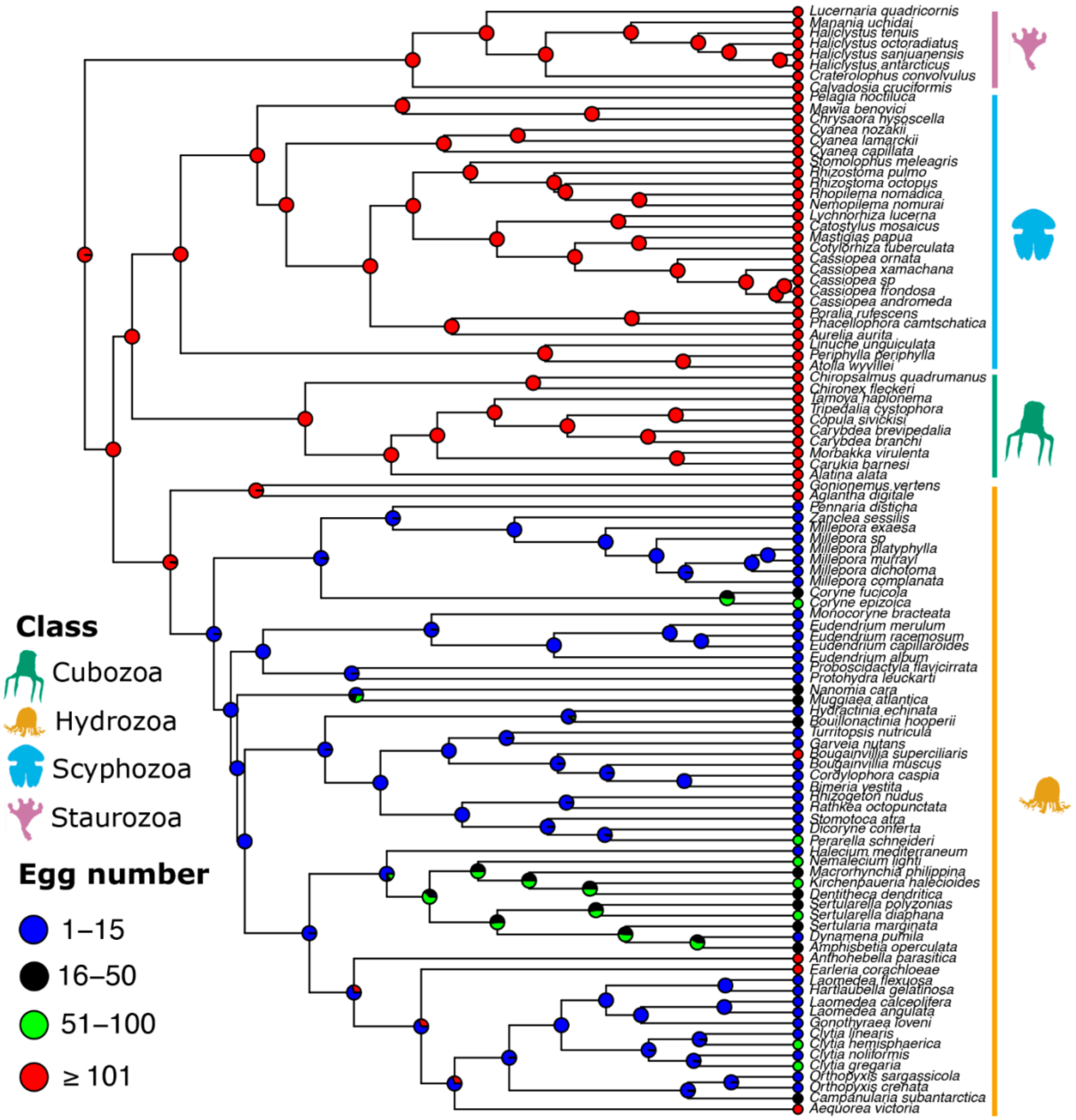

Figure 2. Inferred reconstruction of the number of eggs (1–15, 16–50, 51–100, ≥ 101) within Medusozoa, for 103 species included in the analysis. Colours inside the pie charts at the internal nodes represent the posterior probability of alternative categories of egg number.

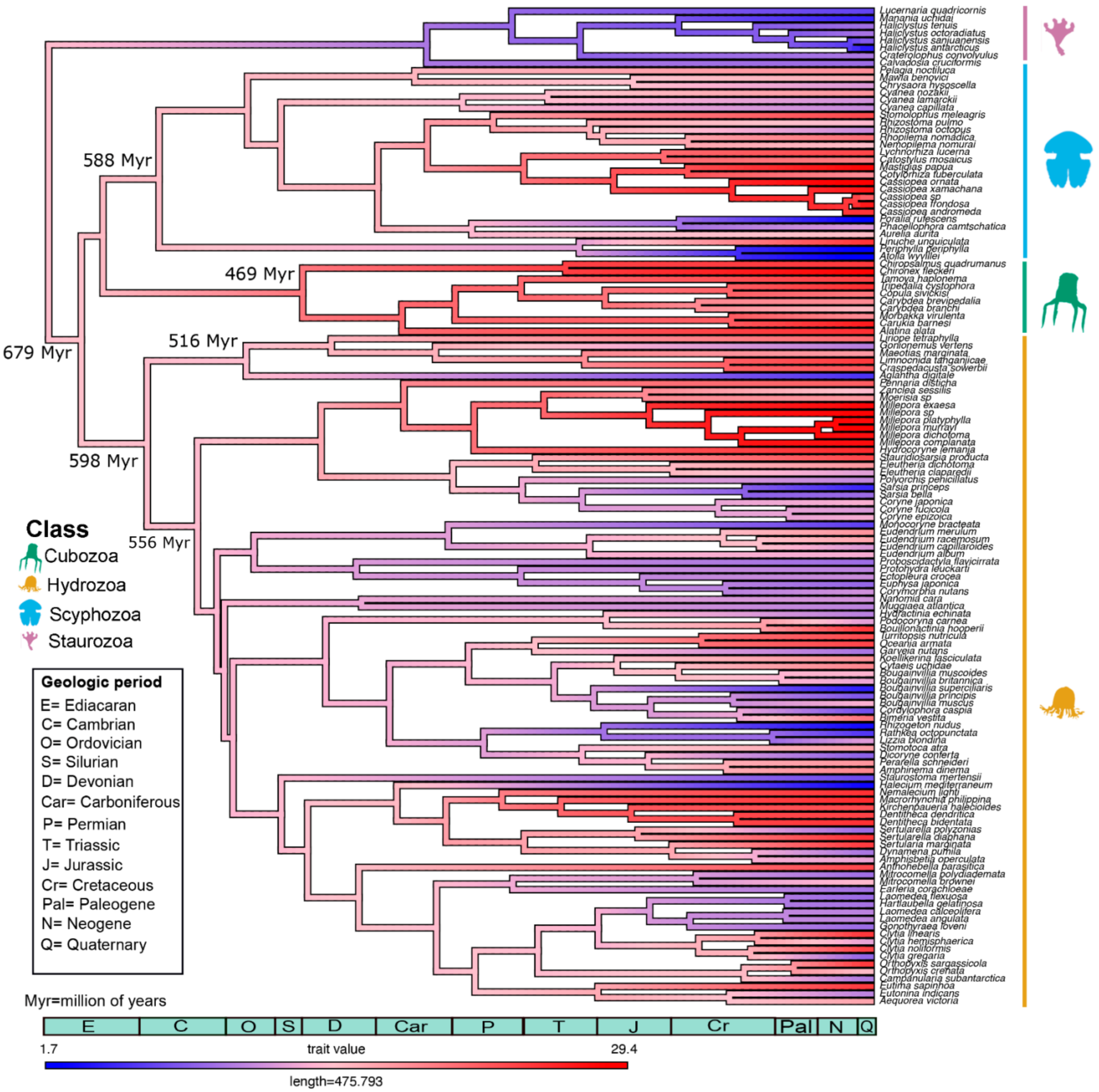

Figure 3. Ancestral reconstruction with time (in millions of years corresponding with geological periods) for temperature in Medusozoa. This graph assumes that the thermal niche of each lineage has not changed across the years. Colour figures: yellow = Hydrozoa, green = Cubozoa, blue = Scyphozoa, pink = Staurozoa. Trait value = min and max temperature (1.7–29.4°C).

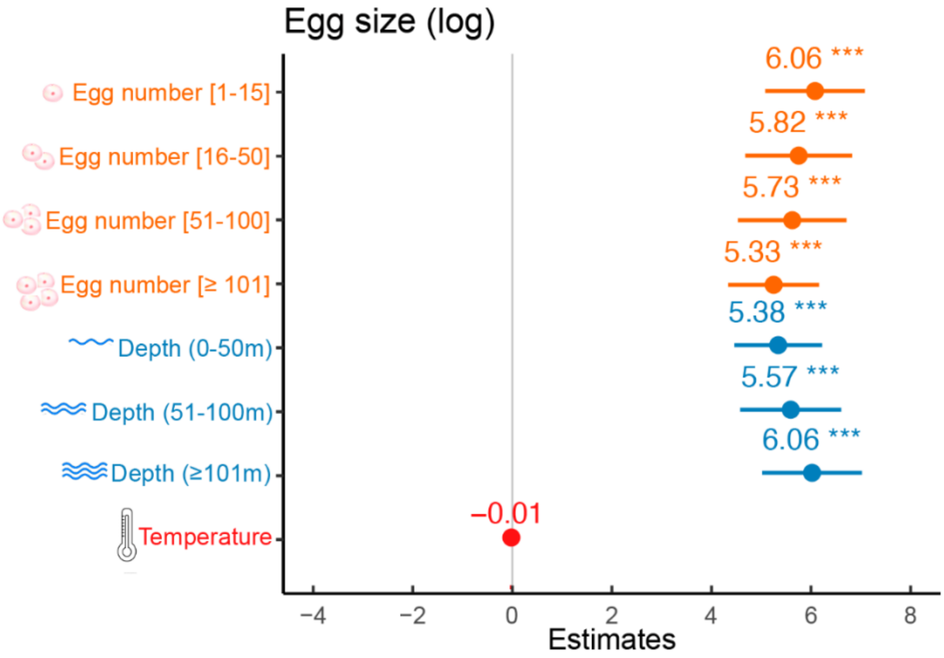

Figure 4. Forest plot of estimates of the best model with 95% confidence intervals. Variables that estimate overlap the line of null effect are not statistically significant while asterisks highlight significant variables (***p > 0.001). Values on the right side of the grey line represent positive estimates while values on the left side of the line (red) represent negative estimates.

Electronic Supplementary Materials

Proceedings of the Royal Society B: Biological Sciences

Reproductive and environmental traits explain the variation in egg size among Medusozoa (Cnidaria)

Jimena García-Rodríguez, Amanda Ferreira Cunha, Adriana Morales-Guerrero, Adrian González-Chaves, Agustín Camacho Guerrero, Lucília Souza Miranda, Filipe C. Serrano, Adrian Jaimes-Becerra and Antonio Carlos Marques

<u>Supporting Information</u>:

Supplemental Methods

Histological preparations

Female specimens included in histological analyses were fixed in 4% paraformaldehyde/seawater solution. The samples were dehydrated in alcohol series and embedded in glycol methacrylate following the instructions furnished with the embedding kits (“Leica Historesin Embedding Kit, Leica Microsystems Nussloch GmbH, Germany”) [1]. The sections were longitudinally cut in 5 µm with a Leica RM2255 microtome. The slides were stained with Hematoxylin-Eosin (HE), Toluidine Blue (TB) and Gomori´s Trichrome (TG) (according to [2–7]and cover-slipped using Entellan. The samples were analyzed using a Zeiss Axio Imager.M2. The images were edited with Adobe Photoshop CC 2017.

Description of reproductive traits

In order to compare the egg size among the four different medusozoan classes, we standardized the following concepts:

1. Gonads

In this study, the gonads are considered as the area where gametes are formed [8]. For example, in Cubozoa the gonad is interpreted as the summation area of each hemigonad (they have a total of 8). The same with the portion of gonads in radial canals in some Hydrozoa species (e.g., *Aequorea Victoria*, *Benthocodon hyalinus*, *Clytia hemisphaerica*).

2. Definition of modules

Medusozoa is a group with a wide variation of life cycles patterns [9]. Within the different reproductive strategies, we compare solitary or individual organisms (such as medusae, some solitary hydroids or polyps) with modular organisms, such as colonial hydroids. The colony of hydroids are made up of zooids that are considered different modules [10] or polymorphs[11] :hydranths or gastrozooids (feeding polyps), gonozooids (reproductive polyps bearing gonophores) and dactylozooids (protective polyps) [12]. Considering the different types of organization among species with different reproductive strategies, is the individual organism who carry the gametes, the medusa or polyp, comparable with the gonophore in a modular organism?

For comparisons between modular and individual organisms, we follow the concept proposed by the authors Vuorisalo and Tuomi in two of their works [13,14], which defines module “as a repeated multicellular part, partially self-maintaining of structural individual”. They also defined structural individual in a modular organism as a “physically separate ecological unit”. In this sense, we regarded the medusa and medusoid as modules from a structural individual, and thus their size (individual size) as well as egg size and egg number comparable with the fixed gonophore in a modular organism.

Moreover, Rosen [10] described the internal organization of a coral (modular organism) as analogous to an individual organism.

3. Reproductive strategies

Hydrozoa is the class with most variation in reproductive strategies [9]. Within the four different classes we considered four strategies in this study:

- Medusa: sexual adult life stage that typically follows an intermediate polyp stage and can have free-swimming, benthic, or crawling life habits [15].
- Medusoid or eumedusoid, free and short-lived pelagic stage that produces the gametes. Present in some Hydrozoa species (e.g., *Nemalecium lighti*, [16]).
- Fixed gonophore or sporosac, which produces the gametes, formed directly from the body of the hydranth (gastrozooid) in several Anthoatheacata, or as a separate module (gonozooid) in remaining species of hydroids [17,18].
- Polyp, benthic individual carrying gametes. We considered this strategy when the gametes were formed on the polyp wall, with no special structure for carrying the gametes (the Hydrozoa *Protohydra leuckarti* and the Scyphozoa *Thecoscyphus zibrowii*).

The Hydrozoa *Gonothyraea loveni* originally described with fixed medusoid (or meconidia) is here considered analogous to a fixed gonophore and was coded as such for the analyses, since they remain attached to the hydroid colony.

4. Individual/module size

In order to compare the size between an individual organism (medusa, medusoid or polyp) and a module from a colonial organism (fixed gonophore), we considered the medusa stage as a gastrozooid, corresponding to a feeding polyp (module) of a hydroid colony. The following measures were taken for each reproductive strategy: maximum diameter of the hydranth or hydrotheca (measured in the body region just below the tentacles) in hydroid species with fixed gonophores; maximum diameter of the hydranth in species with a solitary polyp; maximum diameter of the umbrella in species with medusa and medusoid. In staurozoan species, individual size was measured as the maximum diameter of the calyx.

5. Egg number

In Hydrozoa species with fixed gonophores, the total number of eggs were counted on two different ways depending if 1) the gonophore grows in a gonozooid, independently from the gastrozooid in the colony (e.g., *Bimeria vestita*), or if 2) in a solitary polyp or colony, the fixed gonophore grows on the gastrozooid (e.g., *Acaulis primarius*, *Ectopleura crocea*). The number of eggs in growing from solitary polyps or gastrozooids were the result of the product between the number of gonophores for each solitary polyp/gastrozooid and the number of eggs on each gonophore. For example, *Coryne* *fucicola*has approximately two sporosacs with 25 eggs each per hydranth (gastrozooid) [19], totaling 50 (25x2) eggs. Alternatively, the number of eggs in colonial species with fixed gonophores formed on a gonozooid was considered to be the total number of eggs inside one gonozooid, since that each gonozooid, or module, was regarded as comparable with solitary individuals (polyp, medusa, medusoid), growing independently from each other in the colony.

For the analyses, the number of eggs was divided in four categories: 1-15, 16-50, 51-100 and ≥101 eggs, since the exact number of eggs was not always available from the literature and often varied within the species.

Finally, as the exact number of eggs for species with a medusa in the classes Cubozoa, Scyphozoa, Staurozoa and some Hydrozoa were often not available but are known to vary from hundreds to thousands for the majority of species (for Hydrozoa see [20]; for Cubozoa, see [21]; for Scyphozoa, see [22]; for Staurozoa, see [23], all these species were classified as having ≥101 eggs.

6. Fertilization

Fertilization is also described by some authors as reproductive mode (brooding or broadcasting) [24]. This information is not available for most species in this study.

General remarks on trait information

For some species, mean trait values were not recorded due to the absence of additional records of the species apart from the paper in which the information on egg size was obtained. In these cases, we used single trait values obtained directly from that paper: 1) individual size, maximum depth, temperature and geographical distribution data for unidentified specimens (*Cassiopea* sp., *Moerisia* sp. and *Millepora* sp.); 2) temperature and maximum depth for the hydrozoan *Limnocnida tanganjicae* (freshwater species).

Tree topology and divergence-time estimation

We constructed a phylogeny based on previously published studies [25–29]. We used the topology of Collins et al. 2006 as the backbone topology of the Medusozoa tree used in this work. The relationships within Hydrozoa were based on the work of Cartwright et al. 2008, Kayal et al. 2015 and Maronna et al. 2016. In addition, the relationships within Scyphozoa and Cubozoa were based on the work of Collins 2009 and the relationships within Staurozoa were based on the work of Miranda et al 2016. Sequences of two mitochondrial (16S and COI) and two nuclear genes (18S and 28S) (GenBank accession numbers in electronic supplementary material, Table S5) were aligned using the L-INS-i algorithm in MAFFT version 7.453 [30]. The alignments were used to estimate the branch lengths on the constrained topology with IQ-TREE version 2.0 [31].

The divergence times of phylogeny were obtained with BEAST2 [32]. We used BEAUti 2.4.7 to create the BEAST2 input file. All four genes were analyzed under the GTR+I+Γ4 model. We fixed the starting topology based on the relationships that we obtained previously. We employed an uncorrelated lognormal relaxed clock model [33] and provided a priori age distributions for five calibration points from the fossil record (Table S6, electronic supplementary material). The age distributions were log-normal and the minimum age of each fossil was used as an offset value. The MCMC length was 15 × 107 sampled every 10,000 generations, with two parallel running performed. BEAST log files were analyzed with Tracer v.1.7.1 [34]. We checked that the effective sample size (ESS) for all parameters in the log file were more than 200. The maximum clade credibility tree with median heights was generated by TreeAnnotator in the BEAST package, while the initial 10% trees were discarded as burn-in.

Ancestral state reconstruction

Models of traits evolution were compared using maximum likelihood. Brownian Motion (BM), implies that traits vary proportionally with time, so that the more phylogenetically closer two species are the more similar that trait is. Ornstein-Uhlenbeck (OU) models, on the other hand, assume that a trait is evolving towards an optimum. Finally, Early Burst (EB) models usually reflect adaptive radiations and are characterized by slowing rate of evolution through time [35].

Supplemental Results

Egg size variation

Egg size among cnidarian classes vary from 20 to 160 µm for the majority of species with molecular data, except by some species as the intertidal stauromedusa *Haliclystus tenuis* (18 µm) [35], the fixed gonophore hydroid *Ectopleura crocea* (550 µm, shallow waters) [36], the hydromedusa *Pennaria disticha* (600 µm, shallow waters) [37] and the coronate medusa *Periphylla periphylla* (maximum of 1,480 µm, specimen collected at 450 m deep) [38] (Figs. S4, S5, electronic supplementary material).

Supplementary Figures and Tables

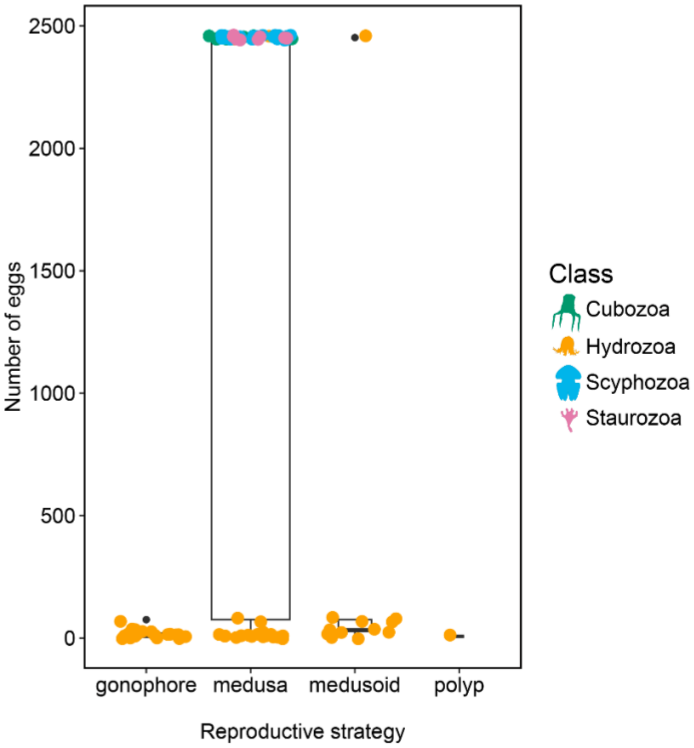

Figure S1. Boxplot of number of eggs for reproductive strategies (gonophore, medusa, medusoid and polyp), colored according to class (Cubozoa, Hydrozoa, Scyphozoa and Staurozoa).

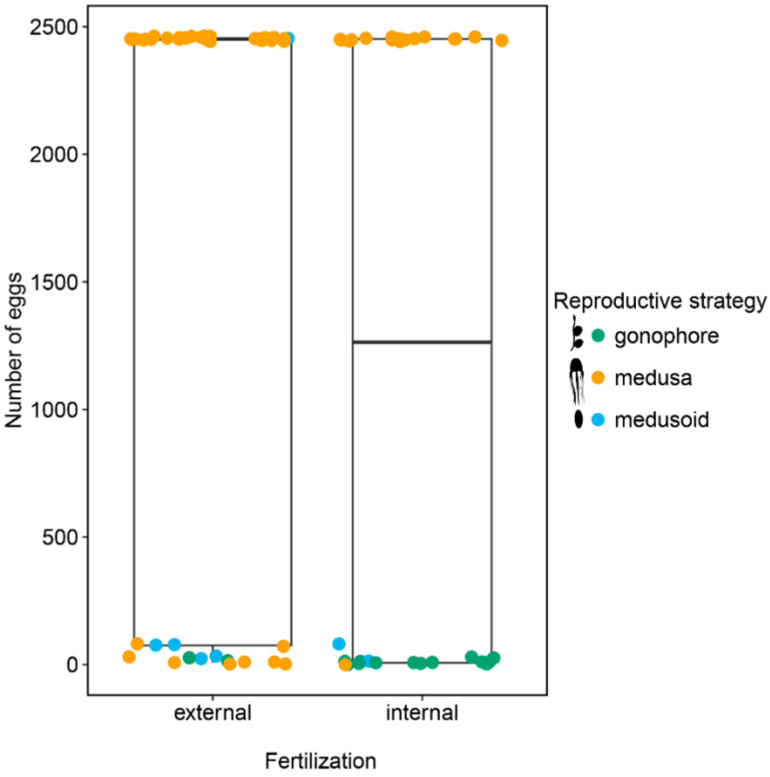

Figure S2. Boxplot of number of eggs for species with external (left) and internal (right) fertilization, colored according to reproductive strategy (gonophore, medusa and medusoid). Line represents the median value.

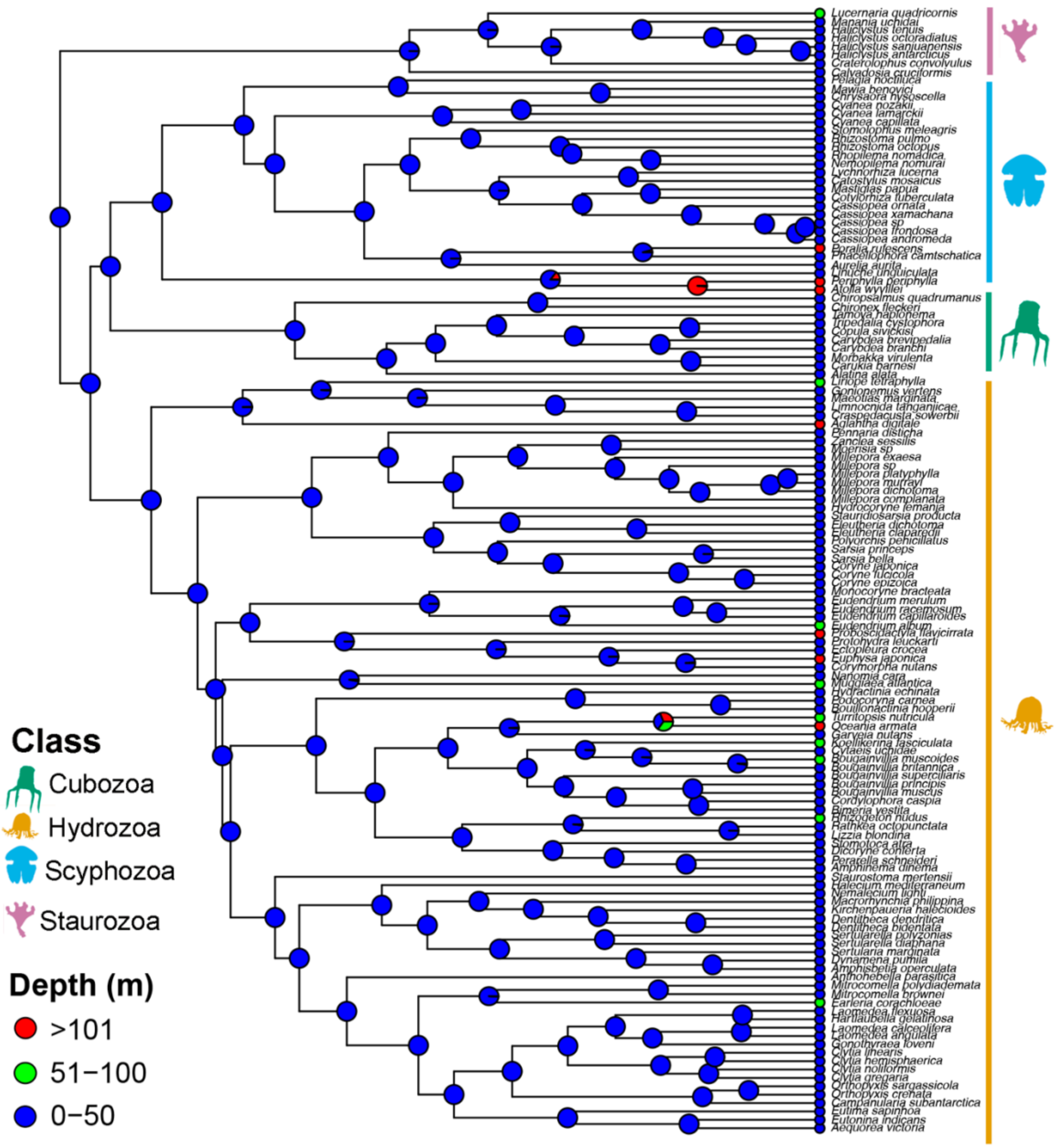

Figure S3. Ancestral reconstruction for depth (in metres) in Medusozoa (n=134). The depth niche of medusozoans has not changed across the years. The most species lived in superficial waters (0 -50 m).

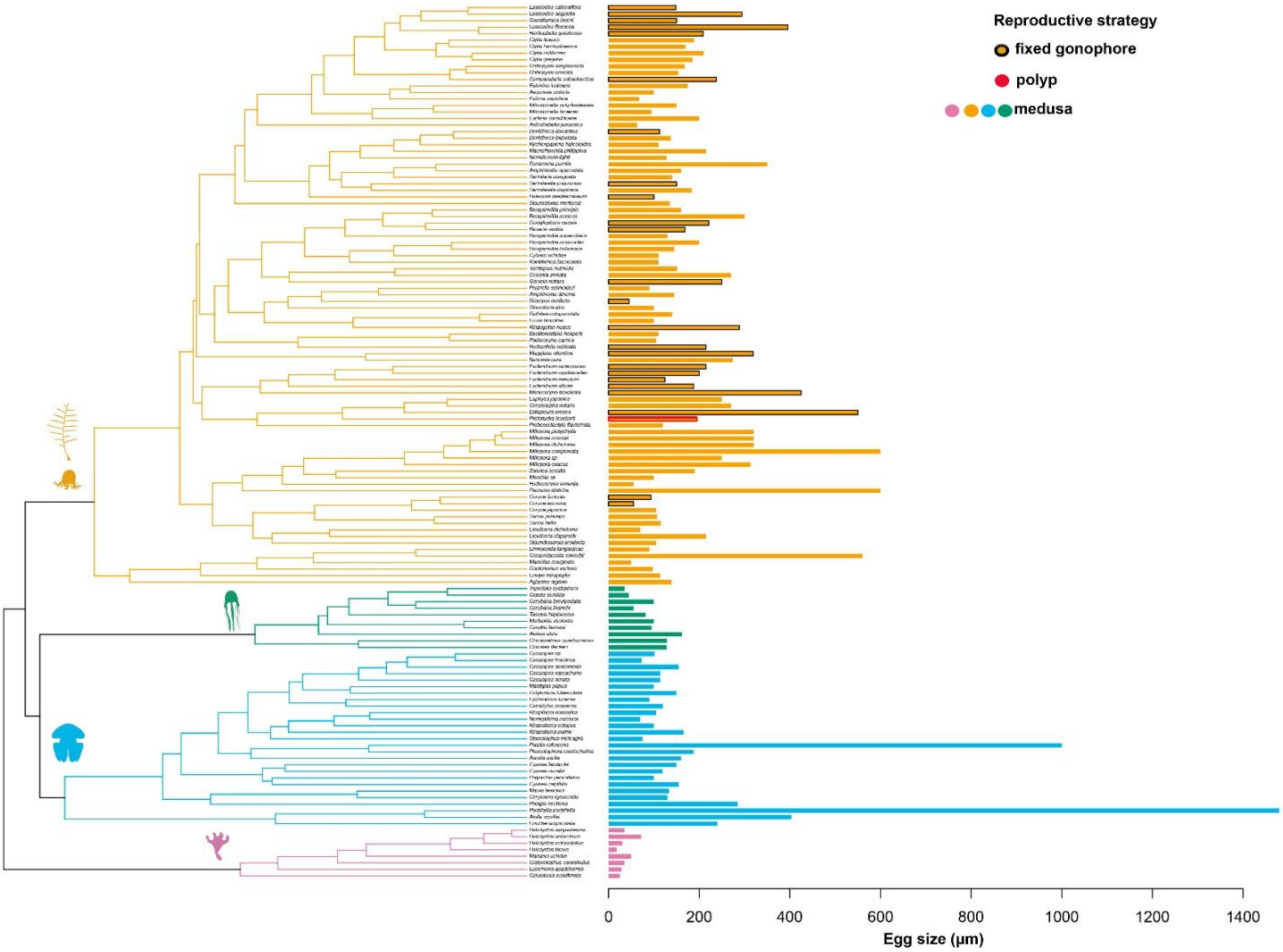

Figure S4. Medusozoa phylogeny with egg size (right panel in µm) for each species analyzed in this study (N=134). Color bars indicate egg size for each medusozoan species according with the classes (yellow=Hydrozoa, green=Cubozoa, blue=Scyphozoa, pink=Staurozoa) and reproductive strategies (fixed gonophore, medusa, polyp).

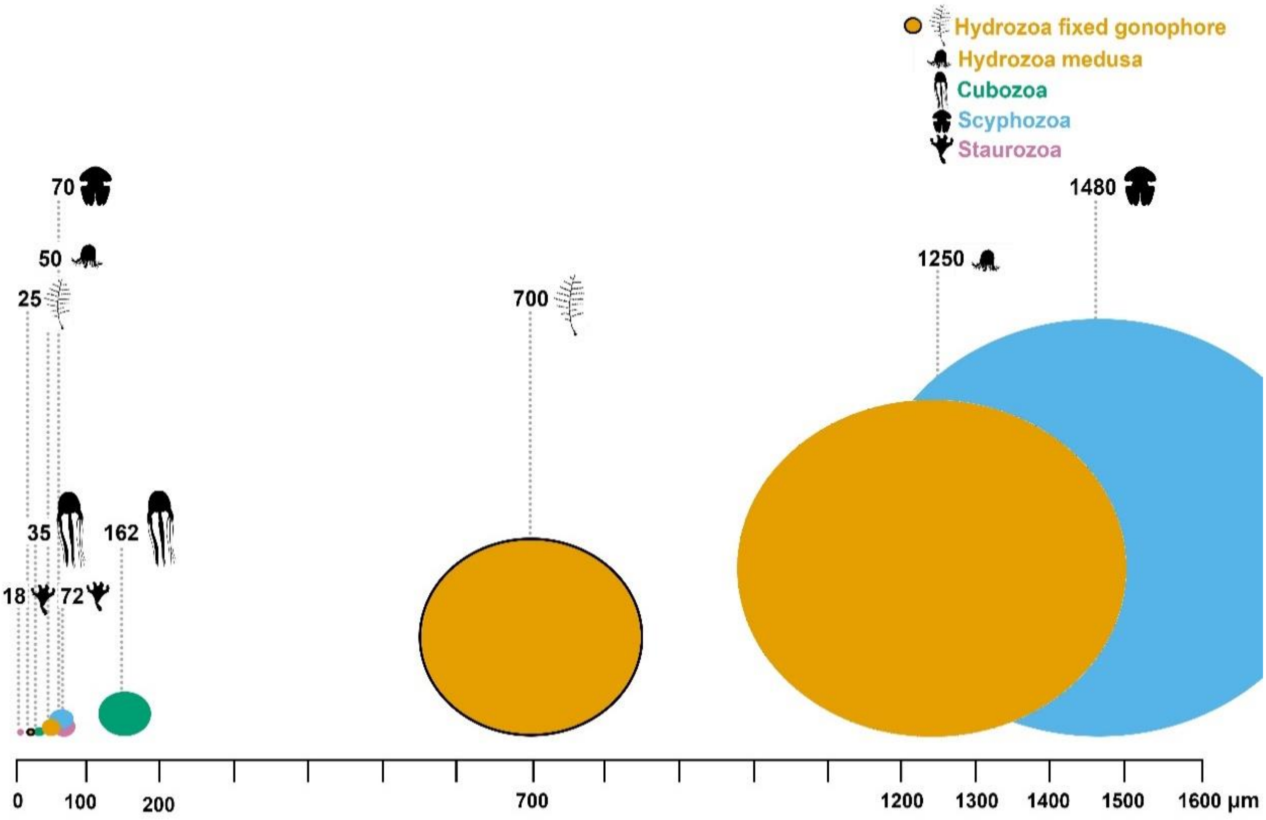

Figure S5. Maximum and minimum value of egg size registered for each class (Hydrozoa, Cubozoa, Scyphozoa, Staurozoa) across Medusozoa species analyzed in this study.

Table S1. Sexual and ecological traits collected for species with egg size. Species* with molecular data, included in the phylogeny; Class, H=Hydrozoa, St=Staurozoa, Scy=Scyphozoa, C=Cubozoa; Egg size*, mean previously in ranges collected from the literature; Temperature data from Bio-Oracle environmental layers; Bio-Oracle* distribution records for species with no data registered in OBIS were documented from Google Earth; Depth* registered from OBIS; ImageJ, eggs measured for this study; freshwater species in blue.

Species	Class	Egg size (µm)	Egg number	Fertilization	Reproductive strategy	Individual size (mm)	Temperature (° C)	Bio-Oracle	Depth (m)	ImageJ	References
Acaulis primarius	H	225*	51-100		gonophore	2.4	7.61	yes	0-50	yes	[36,37]
Acauloides ilonae	H	150		external	gonophore	2	14.47	yes*	0-50		[37,38]
*Aequorea victoria**	H	100	≥101		medusa	50	17.75	yes	0-50*		[39–41]
*Aglantha digitale**	H	139	≥101	external	medusa	7.5	5.67	yes	≥101		[41,42]
*Amphinema dinema**	H	145*			medusa	4	19.74	yes	0-50*		[43]
Amphinema rollinsi	H	187.5*			medusa	1.45	8		≥101		[44]
*Amphisbetia operculata**	H	160	16-50		medusoid	2.4	13.57	yes	0-50*		[45]
*Anthohebella parasítica**	H	63.3	≥101	external	medusoid	0.5	24.10	yes	0-50*	yes	[46]
Benthocodon hyalinus	H	1250*			medusa	40	-1.45	yes*	≥101		[40]
*Bimeria vestita**	H	168.6	1-15	internal	gonophore	0.302	21.29	yes	0-50		[47,48]
Bythotiara huntsmani	H	77.5*		external	medusa	6.25	9.77	yes	0-50		[49]
Bythotiara stilbosa	H	75		external	medusa	5.4	12.35	yes	0-50		[50]
*Bougainvillia britannica**	H	145*			medusa	6.5	13.64	yes	0-50		[51]
*Bougainvillia muscoides**	H	200			medusa	4	14.67	yes	51-100		[52]
*Bougainvillia muscus**	H	300	1-15		medusa	1.6	14.49	yes	0-50		[53]
*Bougainvillia principis**	H	160			medusa	6.19	7.92	yes	0-50*		[41,54]
Bougainvillia rugosa	H	115*			medusa	1.09	24.71	yes	0-50*		[55]
*Bougainvillia superciliaris**	H	130	≥101	external	medusa	10	4.17	yes	0-50		[56]
*Bouillonactinia hooperii**	H	110*	16-50	external	medusa	0.75	25.33	yes	0-50*	yes	[57]
*Campanularia subantarctica**	H	238	16-50	internal	gonophore	0.225	10.36	yes	0-50		[58]
Cladocarpus salix	H	173.07	1-15		gonophore	0.157	8.90	yes*	0-50	yes	[59]
Climacocodon ikarii	H	180	1-15	internal	medusa	3	12.16	yes*	0-50*		[60]
*Clytia gregaria**	H	185*	51-100	external	medusa	6	8.83	yes	0-50*		[41,61]
*Clytia hemisphaerica**	H	170*	51-100	external	medusa	10	13.27	yes	0-50*		[62,63]
*Clytia linearis**	H	188.5*	1-15		medusa	3.05	25.38	yes	0-50		[64]

Species	Class	Egg size (µm)	Egg number	Fertilization	Reproductive strategy	Individual size (mm)	Temperature (° C)	Bio-Oracle	Depth (m)	ImageJ	References
Clytia lomae	H	175.5	>101		medusa	12.5	10.88	yes	0-50*		[20]
*Clytia noliformis**	H	210*	1-15		medusa	4	24.70	yes	0-50*		[64]
*Cordylophora caspia**	H	221	1-15	internal	gonophore	0.323	7.38	yes	0-50	yes	[65]
*Corymorpha nutans**	H	270*		internal	medusa	3	12.14	yes	0-50*		[66]
*Coryne epizoica**	H	55	51-100		gonophore	0.153	14.15	yes*	0-50*	yes	[67]
*Coryne fucicola**	H	93.8	16-50		gonophore	0.3	12.66	yes	0-50*	yes	[68]
*Coryne japonica**	H	105			medusa	3.45	14.29	yes	0-50*		[69]
*Craspedacusta sowerbii**	H	560*		external	medusa	25	22.99	yes	0-50*		[70]
*Cytaeis uchidae**	H	110		external	medusa	1.286	19.72	yes	0-50*	yes	[71,72]
*Dentitheca bidentata**	H	137.5*		external	medusoid	0.61	25.55	yes	0-50		[73]
*Dentitheca dendrítica**	H	112.5*	16-50	internal	gonophore	0.342	24.37	yes	0-50		[74]
*Dicoryne conferta**	H	45.3	1-15		gonophore	0.514	8.71	yes	0-50	yes	[75]
Diphasia scalariformis	H	25	16-50		gonophore	0.47	27.77	yes*	0-50		[76]
*Dynamena pumila**	H	350*	1-15	internal	medusoid	0.66	10.13	yes	0-50*	yes	[45]
*Earleria corachloeae**	H	200	>101	external	medusa	10	9.92	yes*	51-100		[77]
*Ectopleura crocea**	H	550		internal	gonophore	0.853	11.86	yes	0-50*	yes	[78,79]
Ectopleura radiata	H	550*	16-50	internal	gonophore	0.5	4.27	yes*	0-50*		[80]
Ectopleura venusta	H	500*	1-15	internal	gonophore	0.55	4.34	yes	0-50*	yes	[80]
*Eleutheria claparedii**	H	215*		internal	medusa	0.45	12.98	yes	0-50*		[37]
*Eleutheria dichotoma**	H	70*		internal	medusa	0.6	18.54	yes	0-50		[37]
*Eudendrium álbum**	H	188	1-15		gonophore	0.189	14.10	yes	51-100	yes	[81]
Eudendrium bentart	H	430	1-15		gonophore	0.327	-1.37	yes	51-100		[82]
*Eudendrium capillaroides**	H	200	1-15	internal	gonophore	0.275	12.69	yes*	0-50		[83]
Eudendrium klausi	H	700	1-15		gonophore	0.55	28.01	yes*	0-50		[84]
*Eudendrium merulum**	H	124.5	1-15	internal	gonophore	0.5	18.74	yes	0-50	yes	[81,85]
Eudendrium moulouyensis	H	225*	1-15	internal	gonophore	0.26	14.11	yes*	0-50		[86,87]
Eudendrium pocaruquarum	H	270*	1-15		gonophore	0.285	21.35	yes	0-50		[88]
*Eudendrium racemosum**	H	215*	1-15		gonophore	0.535	17.55	yes	0-50		[84]

Species	Class	Egg size (µm)	Egg number	Fertilization	Reproductive strategy	Individual size (mm)	Temperature (° C)	Bio-Oracle	Depth (m)	ImageJ	References
Eudendrium vervoorti	H	250*	1-15	internal	gonophore	0.17	11.77	yes*	0-50*		[89]
Eugymnanthea inquilina japonica	H	51*			medusa	1.02	20.32	yes*	0-50*		[90]
*Euphysa japonica**	H	250			medusa	8.77	9.16	yes	≥101	yes	[41,91]
Eutima japonica	H	73*		external	medusa	14.6	10.83	yes	0-50*		[92]
*Eutima sapinhoa**	H	68*		external	medusa	13.3	24.93	yes	0-50		[93]
*Eutonina indicans**	H	175*			medusa	30	11.08	yes	0-50*		[41,94]
Garveia franciscana	H	125*	1-15	internal	gonophore	0.5	15.72	yes	0-50	yes	[52,95]
*Garveia nutans**	H	250	1-15		gonophore	0.35	10.13	yes	0-50		[52]
*Gonionemus vertens**	H	97.5*	>101	external	medusa	20	10.90	yes	0-50*		[41]
*Gonothyraea loveni**	H	150	1-15	internal	gonophore	0.388	9.55	yes	0-50	yes	[96]
Halecium annuliforme	H	50	1-15		gonophore	0.222	4.45	yes*	0-50		[97]
Halecium corrugatissimum	H	197.5*	1-15		gonophore	0.125	10.45	yes*	0-50		[98,99]
Halecium delicatulum	H	190*	1-15		gonophore	0.215	7.89	yes	0-50		[99]
Halecium humeriformis	H	170*	1-15		gonophore	0.165	8.96	yes	0-50		[99]
Halecium labiatum	H	210	1-15	internal	gonophore	0.12	14.24	yes*	0-50		[100]
Halecium lightbourni	H	170*	1-15		gonophore	0.145	25.74	yes	0-50		[100]
Halecium maximum	H	267.5*	1-15		gonophore	0.22	10.84	yes*	0-50		[99]
*Halecium mediterraneum**	H	100	1-15	internal	gonophore	0.8	2.82	yes	0-50		[98]
Halecium modestum	H	215*	1-15		gonophore	0.23	11.23	yes*	0-50		[99]
Halecium platythecum	H	170*	1-15		gonophore	0.222	9.73	yes*	0-50		[99]
Halecium plicatum	H	176	1-15		gonophore	0.142	24.96	yes*	0-50	yes	[100]
Halecium tristaniensis	H	142.5*	1-15		gonophore	0.18	2.71	yes*	0-50*		[99]
Halimedusa typus	H	115*	>101	external	medusa	13	8.65	yes	0-50*		[101]
*Hartlaubella gelatinosa**	H	209	1-15	internal	gonophore	0.175	10.07	yes	0-50	yes	[102]
*Hydractinia echinata**	H	215*	1-15	external	gonophore	0.45	10.98	yes	0-50*		[57,103,104]
*Hydrocoryne iemanja**	H	55*		external	medusa	0.7	26.01	yes*	0-50*		[105]
*Kirchenpaueria halecioides**	H	110	51-100		medusoid	0.08	23.81	yes	0-50		[106]
*Koellikerina fasciculata**	H	110			medusa	12	19.04	yes	51-100		[52]

Species	Class	Egg size (µm)	Egg number	Fertilization	Reproductive strategy	Individual size (mm)	Temperature (° C)	Bio-Oracle	Depth (m)	ImageJ	References
*Laomedea angulata**	H	294.5	1-15	internal	gonophore	0.325	11.33	yes	0-50	yes	[107]
*Laomedea calceolifera**	H	148.2	1-15	internal	gonophore	0.35	11.06	yes	0-50		[107]
*Laomedea flexuosa**	H	396	1-15	internal	gonophore	0.205	9.43	yes	0-50*	yes	[108,109]
*Limnocnida tanganjicae**	H	90		external	medusa	22	25.04	yes*	0-50		[110]
*Lizzia blondina**	H	100*			medusa	1	13.04	yes	0-50*		[78,111]
*Liriope tetraphylla**	H	114.02		external	medusa	3	21.72	yes	51-100	yes	[51]
*Macrorhynchia philippina**	H	215.5	16-50	external	medusoid	0.667	25.92	yes	0-50	yes	[112]
*Maeotias marginata**	H	50			medusa	45	18.26	yes	0-50		[113]
Margelopsis haeckelii	H	160*	16-50	internal	medusa	1.25	12.24	yes	0-50*		[37]
Merga tergestina	H	76*			medusa	4	25.74	yes	0-50*		[52,114]
Merona ibera	H	48.5*	51-100		gonophore	0.36	13.06	yes	0-50		[115]
*Moerisia sp**	H	100			medusa	4.25	18.50		0-50		[113]
*Monocoryne bracteata**	H	425*	1-15		gonophore	3	6.56	yes	0-50		[116,117]
*Millepora sp**	H	250	1-15	external	medusa	0.6	28.58	yes	0-50*		[118]
*Millepora complanata**	H	600*	1-15		medusa	0.6	27.86	yes	0-50*		[119]
*Millepora dichotoma**	H	320*	1-15	external	medusa	0.84	27.66	yes	0-50		[120]
*Millepora exaesa**	H	313.75	1-15		medusa	0.55	28.15	yes	0-50*	yes	[121]
*Millepora murrayi**	H	320*	1-15	external	medusa	0.84	29.28	yes	0-50		[120]
*Millepora platyphylla**	H	320*	1-15	external	medusa	0.84	27.92	yes	0-50		[120]
Mitrocoma cellularia	H	150	>101	external	medusa	45	13.09	yes	≥101		[41]
*Mitrocomella brownei**	H	95			medusa	6	15.05	yes	0-50*		[122]
*Mitrocomella polydiademata**	H	150*		external	medusa	12.5	9.70	yes	0-50*		[41,123]
*Muggiaea atlántica**	H	319	16-50	external	gonophore	0.05	11.22	yes	51-100		[41,42,124]
*Nanomia cara**	H	274	16-50		medusoid	0.488	12.18	yes	0-50*		[41,42,125]
Nemalecium gracile	H	174.5	51-100		medusoid	0.162	27.50	yes*	0-50	yes	[74]
*Nemalecium lighti**	H	127.5*	51-100	external	medusoid	0.317	25.59	yes	0-50*	yes	[16,126]
*Oceania armata**	H	270			medusa	7	24.22	yes	≥101		[115]
Orthopyxis compressa	H	200*	1-15		medusoid	0.547	18.44	yes	0-50*		[101,127]

Species	Class	Egg size (µm)	Egg number	Fertilization	Reproductive strategy	Individual size (mm)	Temperature (° C)	Bio-Oracle	Depth (m)	ImageJ	References
*Orthopyxis crenata**	H	154	1-15		medusoid	1	15.80	yes	0-50	yes	[75]
Orthopyxis everta	H	101.5	1-15	internal	medusoid	2	20.54	yes	0-50*	yes	[128]
*Orthopyxis sargassicola**	H	168*	1-15		medusoid	0.161	25.25	yes	0-50		[55]
Pachycordyle michaeli	H	100	1-15	internal	gonophore	0.35	8.59	yes*	0-50*		[115]
Pachycordyle napolitana	H	70	>101	external	medusoid	0.3	22.41	yes*	0-50		[115]
*Pennaria disticha**	H	600*	1-15		medusa	0.72	22.54	yes	0-50*		[129]
*Perarella schneideri**	H	90*	51-100	internal	medusoid	0.86	18.72	yes	0-50	yes	[130]
*Podocoryna carnea**	H	105*		internal	medusa	1	12.93	yes	0-50*		[131]
*Polyorchis penicillatus**	H	100			medusa	25	11.55	yes	0-50*		[41]
*Proboscidactyla flavicirrata**	H	120	1-15		medusa	7	9.61	yes	≥101		[41]
*Protohydra leuckarti**	H	195	1-15		polyp	0.27	11.17	yes	0-50		[37,132]
Ralpharia magnifica	H	300	16-50	external	medusoid	0.9	16.46	yes	0-50		[133]
*Rathkea octopunctata**	H	140	1-15		medusa	4	5.79	yes	0-50*		[43,134]
*Rhizogeton nudus**	H	288.5	1-15	internal	gonophore	0.172	3.04	yes*	51-100	yes	[115]
*Sarsia bella**	H	115*			medusa	7.5	9.56	yes*	0-50*		[135]
*Sarsia princeps**	H	107		external	medusa	2.3	4.68	yes	0-50*		[41]
*Sertularella diaphana**	H	184	51-100	external	medusoid	0.211	25.58	yes	0-50*	yes	[136]
*Sertularella polyzonias**	H	150	16-50		gonophore	0.38	9.44	yes	0-50		[137]
*Sertularia marginata**	H	140*	16-50	external	medusoid	0.114	24.94	yes	0-50*	yes	[138,139]
Stauridiosarsia nipponica	H	125*			medusa	1.96	26.09	yes	0-50		[140]
*Stauridiosarsia producta**	H	105*			medusa	2.5	24.16	yes	0-50		[55]
*Staurostoma mertensii**	H	135*			medusa	150	7.19	yes	0-50*		[131,141]
*Stomotoca atra**	H	100	1-15	external	medusa	4.5	18.06	yes	0-50*		[41]
Synthecium flabellum	H	550*	1-15		gonophore	0.27	8.79	yes*	0-50		[76]
Synthecium samauense	H	550*	1-15		gonophore	0.18	20.15	yes	0-50		[76]
*Turritopsis nutricula**	H	151.36	1-15	internal	medusa	4.5	23.76	yes	51-100	yes	[142]
*Zanclea sessilis**	H	190*	1-15		medusa	1.33	15.09	yes*	0-50		[143]
*Calvadosia cruciformis**	St	25	≥101	external	medusa	5.6	9.59	yes*	0-50	yes	[144]

Species	Class	Egg size (µm)	Egg number	Fertilization	Reproductive strategy	Individual size (mm)	Temperature (° C)	Bio-Oracle	Depth (m)	ImageJ	References
*Craterolophus convolvulus**	St	35	≥101	external	medusa	21.9	10.30	yes*	0-50	yes	[144]
*Haliclystus antarcticus**	St	72	≥101	external	medusa	13	3.44	yes*	0-50	yes	[145]
*Haliclystus octoradiatus**	St	30	≥101	external	medusa	15	10.15	yes*	0-50	yes	[146]
*Haliclystus sanjuanensis**	St	35	≥101	external	medusa	26	8.78	yes*	0-50		[147]
*Haliclystus tenuis**	St	18	≥101	external	medusa	9.6	9.83	yes*	0-50	yes	[144]
*Lucernaria quadricornis**	St	29	≥101	external	medusa	14.9	6.78	yes*	51-100	yes	[145]
*Manania uchidai**	St	50	≥101	external	medusa	13.8	4.64	yes*	0-50	yes	[148]
Atolla parva	Scy	750	≥101		medusa	18	2.03	yes	≥101		[149]
*Atolla wyvillei**	Scy	403*	≥101		medusa	20	1.73	yes	≥101		[150]
*Aurelia aurita**	Scy	160	≥101	internal	medusa	40	15.82	yes	0-50*		[151]
*Cassiopea andromeda**	Scy	155*	≥101	internal	medusa	150	25.39	yes	0-50		[152]
*Cassiopea frondosa**	Scy	73.08	≥101	internal	medusa	76.7	27.84	yes	0-50	yes	Gamero-Mora in prep
*Cassiopea ornata**	Scy	113.69	≥101	internal	medusa	71	29.37	yes	0-50*	yes	Gamero-Mora in prep
*Cassiopea sp.**	Scy	101.54	≥101	internal	medusa	200	21.67	yes	0-50*	yes	[153]
*Cassiopea xamachana**	Scy	114.6	≥101	internal	medusa	84.6	27.38	yes	0-50	yes	Gamero-Mora in prep
*Catostylus mosaicus**	Scy	120	≥101		medusa	250	20.33	yes	0-50*		[154]
*Chrysaora hysoscella**	Scy	130	≥101	internal	medusa	275	12.73	yes	0-50*		[151,155,156]
*Cyanea capillata**	Scy	155*	≥101	internal	medusa	150	11.63	yes	0-50*		[151]
*Cyanea lamarckii**	Scy	150	≥101		medusa	105	12.20	yes	0-50*		[131]
*Cyanea nozakii**	Scy	119	≥101	external	medusa	500	18.72	yes*	0-50*	yes	[157,158]
*Cotylorhiza tuberculata**	Scy	150*	≥101	internal	medusa	340	19.14	yes	0-50		[159]
Discomedusa lobata	Scy	165*	≥101	external	medusa	260	19.77	yes*	0-50*		[160]
*Linuche unguiculata**	Scy	240	≥101	external	medusa	17	25.51	yes	0-50		[155,156]
*Lychnorhiza lucerna**	Scy	90*	≥101	external	medusa	148	24.20	yes	0-50*		[161]
*Mastigias papua**	Scy	100	≥101	internal	medusa	140	28.35	yes	0-50*		[155]
*Mawia benovici**	Scy	133.14	≥101		medusa	50	16.96	yes*	0-50*	yes	[162]

Species	Class	Egg size (µm)	Egg number	Fertilization	Reproductive strategy	Individual size (mm)	Temperature (° C)	Bio-Oracle	Depth (m)	ImageJ	References
Nausithoe aurea	Scy	175	≥101	external	medusa	5	24.42	yes	0-50*		[163]
Nausithoe globifera	Scy	250	≥101		medusa	22	2.14	yes	≥101		[131]
Nausithoe punctata	Scy	230	≥101	external	medusa	15	24.52	yes	0-50*		[155]
*Nemopilema nomurai**	Scy	70*	≥101	external	medusa	1230	15.83	yes*	0-50*		[164]
*Pelagia noctiluca**	Scy	285*	≥101	external	medusa	43	19.16	yes	0-50		[160,165–167]
*Periphylla periphylla**	Scy	1480*	≥101		medusa	13	2.16	yes	≥101		[168,169]
*Phacellophora camtschatica**	Scy	187.5*	≥101	internal	medusa	425	12.98	yes	0-50		[170]
*Poralia rufescens**	Scy	1000	≥101		medusa	55	2.07	yes	≥101		[171]
*Rhizostoma octopus**	Scy	100	≥101	external	medusa	190	11.81	yes	0-50*		[172]
*Rhizostoma pulmo**	Scy	165*	≥101	internal	medusa	300	14.92	yes	0-50		[160]
*Rhopilema nomadica**	Scy	105*	≥101	external	medusa	450	21.32	yes	0-50		[173]
*Stomolophus meleagris**	Scy	75*	≥101	external	medusa	180	23.99	yes	0-50*		[174,175]
Thecoscyphus zibrowii	Scy	100	≥101	internal	polyp	1.237	17.26	yes*	0-50		[176]
*Alatina alata**	C	162.34	≥101	internal	medusa	20	25.66	yes	0-50	yes	[1]
*Carybdea branchi**	C	55	≥101	internal	medusa	36.83	16.16	yes*	0-50*		[177]
*Carybdea brevipedalia**	C	100	≥101	external	medusa	33	19.47	yes	0-50*		[178,179]
Carybdea marsupialis	C	56.56	≥101	internal	medusa	31.5	18.09	yes	0-50	yes	[180]
*Carukia barnesi**	C	95*	≥101	external	medusa	25	26.88	yes	0-50*		[181]
*Chironex fleckeri**	C	128	≥101	external	medusa	100	28.53	yes	0-50*		[182]
*Chiropsalmus quadrumanus**	C	129	≥101	external	medusa	43	24.86	yes	0-50		[183]
*Copula sivickisi**	C	44.58	≥101	internal	medusa	6	20.64	yes	0-50	yes	[184,185]
*Morbakka virulenta**	C	100	≥101	external	medusa	70	19.12	yes	0-50*		[21]
*Tamoya haplonema**	C	82.4	≥101	external	medusa	45	24.44	yes	0-50		[183]
*Tripedalia cystophora**	C	35*	≥101	internal	medusa	8.5	25.70	yes	0-50		[186]

Table S2. Model selection results for the four different evolutionary models. Values of AIC, delta AIC (dAIC), degrees of freedom (df) and weight. Equally plausible model (dAIC <2) is in bold.

Evolutionary model	AIC	dAIC	df	weight
Grafen	**170.2**	**0**	**13**	**0.997**
Martins	184.2	14.05	12	0.001
Brownian	184.2	14.05	12	0.001
Blomberg	184.2	14.05	12	0.001
Pagel	208.4	38.21	13	0

Table S3. The best five fit-models with "dredge" function with all the predictors (class, egg number, individual size, reproductive strategy and temperature), degrees of freedom (df), AIC, delta AIC (dAIC) and weight. Equally plausible model (dAIC <2) is in bold.

Model	Depth (m)	Egg number	Individual size	Reproductive strategy	Temperature	df	AIC	dAIC	weight
20	**+**	**+**			**-0.013**	**9**	**166.1**	**0**	**0.306**
4	+	+				8	166.8	0.70	0.215
24	+	+	-0.002		-0.013	10	168.1	1.99	0.113
28	+	+		+	-0.018	12	168.2	2.12	0.106
8	+	+	-0.004			9	168.7	2.66	0.081

Table S4. Model selection table with estimates of predictors. CI= intervals of confiance, p=p-values. Bold p-values indicate significance ($p < 0.05$).

Predictors	Estimates	CI	*p*
Egg number (1-15)	6.06	5.33 – 6.80	**<0.001**
Egg number (16-50)	5.82	5.03 – 6.61	**<0.001**
Egg number (51-100)	5.73	4.92 – 6.53	**<0.001**
Egg number (≥101)	5.33	4.66 – 6.01	**<0.001**
Depth (0-50m)	6.06	5.33 – 6.80	**<0.001**
Depth (51-100m)	5.38	4.73 – 6.04	**<0.001**
Depth (≥101m)	5.57	4.82 – 6.32	**<0.001**
Temperature (°C)	-0.01	-0.03 – 0.00	0.098

Table S5. GenBank accession numbers of mitochondrial (16S and COI) and nuclear genes (18S and 28S) included in the tree topology obtained for this study.

	16S	**18S**	**28S**	**COI**
Species				
Outgroup				
Anemonia viridis	EU190760.1	EU190849.1	KJ483095.1	
Aiptasia mutabilis	KP761256.1	FJ489438.1	KJ483115.1	
Anthopleura elegantissima	U40292.1	EU190844.1	KJ483104.1	KM611880.1
Stylophora pistillata		FR819686.1		EF633532.1
Ingroup				
Aequorea victoria	EU305469.1	AF358077.1	AY920799.1	
Aglantha digitale	EU293985.1	EU247821.1	AY920791.1	GQ120073.1
Amphinema dinema	KP776816.1			JQ716085.1
Amphisbetia operculata	FJ550489.1	FJ550561.1	FJ550418.1	
Anthohebella parasitica	AY787918.1	EU272603.1	EU272545.1	
Bimeria vestita	KT266604.1	KT722386.1	EU272548.1	MG791809.1
Bougainvillia britannica	AM183127.1			
Bougainvillia muscoides	AM411412.1			
Bougainvillia muscus	KT266606.1	KT722388.1	KT757176.1	MG791811.1
Bougainvillia principis	MK139154.1			
Bougainvillia superciliaris				GQ120052.1
Bouillonactinia hooperii		JQ407395.1	JQ410726.1	
Campanularia subantarctica	KM405575.1	KX665450.1		KM405568.1
Corymorpha nutans	EU876532.1	EU876558.1	EU879931.1	JX121586
Cytaeis uchidae	LC439504.1	JQ407405.1	JQ410764.1	
Clytia gregaria	MF000540.1			MF000499.1
Clytia hemisphaerica	KX665296.1	KX665379.1	KX665495.1	MF000504.1
Clytia linearis	KX665343.1	AY789747.1	KX665519.1	AY789897.1
Clytia noliformis	KX665350.1	KX665453.1	KX665526.1	KX665247.1
Cordylophora caspia	KC489508.1	EU272612.1	EU272556.1	KC489509.1
Coryne epizoica	GQ395314.1		GQ424295.1	
Coryne fucicola	AM084259.1	GQ424326.1		
Coryne japonica	AJ878719.1			
Craspedacusta sowerbyi	EU293971.1	MG979336	MG979301	MK600509.1
Dentitheca bidentata	DQ855942.1	KT722390.1	KT757143.1	
Dentitheca dendritica	MH212656.1			MH282671.1
Dicoryne conferta		JQ407404.1	JQ410763.1	
Dynamena pumila	AY787902.1	FJ550558.1	FJ550415.1	MG935408.1
Earleria corachloeae		FJ843103.1		
Ectopleura crocea	EU876533.1	EU876559.1	EU879932.1	JX121589
Eleutheria claparedii	AM088486.1	GQ424320.1	GQ424292.1	
Eleutheria dichotoma	KP776785.1	GQ424321.1	GQ424291.1	
Eudendrium album	KP776818.1			
Eudendrium capillaroides	KP776804.1			
Eudendrium merulum	KP776770.1			

Eudendrium racemosum	KP776820.1	AF358094	EU272562.1	JN109192.1
Euphysa japonica	JX122503.1	EU301605.1	JX122505.1	JX121577.1
Eutima japonica				AB458502.1
Eutima sapinhoa		EU305493.1	EU305515.1	
Eutonina indicans	KT266615.1	KY363971.1	KT757190.1	GQ120070.1
Garveia nutans	KP776768.1			
Gonionemus vertens	EU293976	MG979338	MG979303	KY451454.1
Gonothyraea loveni	KX665257.1	KX665455.1	KT757149.1	KX665161.1
Halecium mediterraneum	FJ550492.1	FJ550566.1	FJ550423.1	
Hartlaubella gelatinosa	KX665339.1	KX665436.1		KX665236.1
Hydractinia echinata	KX355405.1	AY920763.1	JQ410733.1	JN109196.1
Hydractinia hooperi	FJ214537.1			
Hydrocoryne iemanja	GQ389713.1			
Kirchenpaueria halecioides	KT266623.1	FJ550530.1	FJ550385.1	MH282704.1
Koellikerina fasciculata	AM183129.1	EU272623.1	EU272571.1	
Laomedea angulata	KX665293.1	KX665404.1	KX665501.1	KX665196.1
Laomedea calceolifera	KX665344.1	KX665446.1	KX665496.1	KX665202.1
Laomedea flexuosa	KX665346.1	KX665448.1		JN109190.1
Limnocnida tanganjicae	EU293972.1	AY920755.1	AY920795.1	
Lizzia blondina	LS974807.1	EU272625.1	EU272574.1	KC440074.1
Liriope tetraphylla	KJ859220.1	AY920756.1	KT757195.1	KU364622.1
Macrorhynchia philippina	KT266625.1	KT722407.1	KT757153.1	MH282740.1
Maeotias marginata	AY512508.1	AF358056	EU247810.1	AF383926.1
Moerisia sp.	EU876555.1	AF358083.1	AY920801.1	
Monocoryne bracteata	LN898143.1			
Millepora sp	EU876551.1	AF358088.1	EU879950.1	
Millepora complanata				KC570841.1
Millepora dichotoma	MH825204.1			MH825616.1
Millepora exaesa	MH825173.1			MH825585.1
Millepora murrayi	KP776778.1			
Millepora platyphylla	MH825207.1			MH825619.1
Mitrocomella brownei	KX355404.1	FJ550521.1	FJ550374.1	MF000485.1
Mitrocomella polydiademata	KY363949.1	KY363979.1		MF000508.1
Muggiaea atlantica	KJ801943.1	AY937337.1		KF977299.1
Nanomia cara				GQ120028.1
Nemalecium lighti	MG811616.1	KT722410.1	KT757146.1	
Oceania armata	KP776815.1			KX712145.1
Orthopyxis crenata	KM405593.1	KX665366.1	KX665510.1	KX665238.1
Orthopyxis everta	AY789793.1	AY789728.1		KT981919.1
Orthopyxis sargassicola	KT266633.1	KT722416.1	KT757155.1	AY789883.1
Pennaria disticha	KT984741.1	GQ424342.1	EU272581.1	
Perarella schneideri	AM411414.1	HM357626.1	HM357628.1	
Podocoryna carnea	FJ214469.1	JQ407393.1	JQ410752.1	
Polyorchis penicillatus	KX355412.1	AF358090.1		KM347973.1
Proboscidactyla flavicirrata	AM183137.1	AY920768.1	EU305527.1	JX121600.1
Protohydra leuckarti	KU721827.1	KU721835.1		KU721814.1

Rathkea octopunctata	EU305483.1	EU272634.1	EU272591.1	KC440083.1
Rhizogeton nudus	KX355436.1	EU272635.1	EU272592.1	
Sarsia bella	MF000538.1			MF000497.1
Sarsia princeps	EU876549.1	EU876575.1	EU879947.1	GQ120061.1
Sertularella diaphana	MH034131.1	KT722430.1	KT757131.1	
Sertularella polyzonias	MG811635.1			
Sertularia marginata	KT266642.1	KT722435.1	KT757150.1	
Stauridiosarsia producta	KX355421.1			KT981911.1
Staurostoma mertensii	KY363948.1	KY363978.1		MF000507.1
Stomotoca atra	MG136734.1			MG136786.1
Turritopsis nutricula	KT266646.1	KT722442.1	KT757169.1	JQ716083.1
Zanclea sessilis	KX355442.1			MG811652.1
Calvadosia cruciformis	KU257514.1	KU308576.1	KU308608.1	KU257487.1
Craterolophus convolvulus	MF322746.1	AF099104.1	AY920781.1	GQ120102.1
Haliclystus antarcticus	EU294003.1	EU247811.1	KU308588.1	
Haliclystus octoradiatus	KU257501.1	AY845346.1	AH014894.2	KU257476.1
Haliclystus sanjuanensis	MN329738.1	AF358102.1	AY920782.1	MH242772.1
Haliclystus tenuis	MF322742.1	KU308564.1	KU308595.1	KU257479.1
Lucernaria quadricornis	KU257506.1	KU308568.1	KU308601.1	MG422933.1
Manania uchidai	KU257513.1	KU308575.1	KU308607.1	
Atolla wyvillei	JX393251.1	HM194788.1	HM194841.1	GQ120088.1
Aurelia aurita	KC767898.1	AY039208.1	HM194866.1	MG935022.1
Cassiopea andromeda	KY610612.1	HM194818.1	KY611006.1	LC198773.1
Cassiopea frondosa	KY610615.1	HM194819.1	KY611004.1	HF930520.1
Cassiopea ornata	KY610616.1	HM194785.1	HM194838.1	LC198737.1
Cassiopea sp.	U19374.1	AF099675.1		GQ120100.1
Cassiopea xamachana		AY920771.1		AY319468.1
Catostylus mosaicus	KY610585.1	HM194779.1	HM194832.1	AY737198.1
Chrysaora hysoscella	MF141696.1	KY610811.1	KM651815.1	MF141611.1
Cyanea capillata	MF141641.1	HM194820.1	HM194873.1	KM281995.1
Cyanea lamarckii		JX995326.1		JX995362.1
Cyanea nozakii	JX845345.1	JX845355.1	KY655781.1	JX845350.1
Cotylorhiza tuberculata	KY610619.1	AY920773.1	HM194839.1	HF930535.1
Linuche unguiculata		HM194806.1	HM194859.1	
Lychnorhiza lucerna	KY610593.1	HM194807.1	KY610908.1	KY611035.1
Mastigias papua	KY610621.1	HM194796.1	HM194849.1	KU901464.1
Mawia benovici	MF141692.1		MF141631.1	MF141603.1
Nemopilema nomurai	AB720950.1	HQ413771.1	LK392317.1	JX845348.1
Pelagia noctiluca	JX235418.1	HE591464.1	KM651810.1	HM358403.1
Periphylla periphylla		HM194789.1	HM194842.1	MF742347.1
Phacellophora camtschatica	JX393261.1	HM194822.1	AY920778.1	MF742348.1
Poralia rufescens	KY610659.1	HM194792.1	HM194845.1	
Rhizostoma octopus		KT962256.1		HQ425479.1
Rhizostoma pulmo	KY610632.1	HM194795.1	HM194848.1	HF536559.1
Rhopilema nomadica		HE591465.1	HG931672.1	HG931668.1
Stomolophus meleagris	KY610640.1	HM194814.1	HM194867.1	MH087556.1

Alatina alata	KU707333.1	GQ849082.1	GQ849058.1	KM200330.1
Carybdea branchi	KT288232.1	GQ849089.1	GQ849066.1	
Carybdea brevipedalia	KT288255.1	KY212121.1	KY212122.1	
Carybdea marsupialis	KT288234.1	AF358106	GQ849072.1	
Carukia barnesi	GQ849097.1	AF358107.1	GQ849059.1	
Chironex fleckeri	GQ849103.1	GQ849074.1	GQ849051.1	FJ665181.1
Chiropsalmus quadrumanus	GQ849109.1	GQ849079.1	GQ849056.1	GQ120103.1
Copula sivickisi	GQ849113.1	GQ849087.1	GQ849064.1	
Morbakka virulenta	GQ849121.1	GQ849083.1	GQ849060.1	
Tamoya haplonema	HQ824527.1	GQ849085.2	GQ849062.1	HQ824531.1
Tripedalia cystophora	KM200334.1	GQ849088.1	GQ849065.1	

Table S6. Information of clade ages obtained from the fossil record. Fossil constraints for five nodes.

MRCA of clades	Fossil	Lower limit	Upper limit	References
Cnidaria	Conulariid	543	635	[182]
Hexacorallia	*†Eolympia pediculata*	538	542	[187]
Medusozoa	*†Yunnanoascus haikouensis*	521	529	[183]
Trachylina	unnamed fossil	505	509	[184,185]
Discomedusa	unnamed fossil	505	509	[184,185]

Supplemental References

1. García-Rodríguez J, Lewis Ames C, Marian JEAR, Marques AC. 2018 Gonadal histology of box jellyfish (Cnidaria: Cubozoa) reveals variation between internal fertilizing species *Alatina alata* (Alatinidae) and *Copula sivickisi* (Tripedaliidae). *J Morphol* **279**, 841–856. (doi:10.1002/jmor.20815)
2. Humason GL. 1962 *Animal tissue techniques*. San Francisco, CA: W.H. Freeman and Company.
3. Behmer OA, Tolosa EMC, Freitas Neto AG. 1976 *Manual de tecnicas para histologia normal e patologica*. São Paulo: Livraria Editora.
4. Bancroft J, Stevens A. 1982 *Theory and practice of histological techniques*. London, UK: Churchill Livingstone.
5. Pearse AGE. 1985 Histochemistry theoretical and Applied vol. 2. In *Analytical Tehnology*, Churchill Livingstone.
6. Junqueira LC. 1995 Histology revisited - Technical improvement promoted by the use of hydrophilic resin embedding. *Cienc Cult* **47**, 92–95.
7. Mendoza-Becerril MA, Marian JEAR, Migotto AE, Marques AC. 2017 Exoskeletons of Bougainvilliidae and other Hydroidolina (Cnidaria, Hydrozoa): structure and composition. *PeerJ* **5**, e2964. (doi:10.7717/peerj.2964)
8. Campbell RD. 1974 Cnidaria. *Reproduction of Marine invertebrates* **1**, 133–199.
9. Collins AG. 2002 Phylogeny of Medusozoa and the evolution of cnidarian life cycles. *J Evol Biol* **15**, 418–432. (doi:10.1046/j.1420-9101.2002.00403.x)
10. Rosen BR. 1986 Modular growth and form of corals: a matter of metamers? *Philosophical Transactions - Royal Society of London, Series B* **313**, 115–142. (doi:10.1098/rstb.1986.0029)
11. Hiebert LS, Simpson C, Tiozzo S. 2020 Coloniality, clonality and modularity in animals: The elephant in the room. *J. Exp. Zool. (Mol. Dev. Evol.)*
12. Bouillon J, Gravili C, Pagès F, Gili J-M, Boero F. 2006 *An introduction to Hydrozoa*. See http://www.mnhn.fr/publication/.
13. Tuomi J, Vuorisalo T. 1989 What are the units of selection in modular organisms? *Oikos* **54**, 227–233.
14. Vuorisalo T, Tuomi J. 1986 Unitary and modular organisms: criteria for ecological division. *Oikos* **47**, 382–385.
15. Marques AC, Collins AG. 2004 Cladistic analysis of Medusozoa and cnidarian evolution. *Invertebrate Biology* **123**, 23–42. (doi:10.1111/j.1744-7410.2004.tb00139.x)
16. Gravier-Bonnet N, Migotto AE. 2000 Gonangium development and medusoid of *Nemalecium lighti* (Hargitt, 1924) (Cnidaria: Hydrozoa, Haliciidae). *Sci Mar* **64**, 207–213. (doi:10.3989/scimar.2000.64s1207)
17. Cornelius PFS (Paul FS. 1995 North-west European thecate hydroids and their medusae. Part 1. Introduction, Laodiceidae to Haleciidae. *Synopses of the British fauna* **50**, 1–347.
18. Schuchert P. 2012 North-West European Athecate hydroids and their medusae. *Synopses of the British fauna (New Series)* **59**.
19. Schuchert P. 2005 Rediscovery of *Coryne fucicola* (de Filippi, 1866) (Cnidaria: Hydrozoa). *Cah Biol Mar* **46**, 305–310.
20. Larson RJ. 1986 Ova production by hydromedusae from the NE Pacific. *J Plankton Res* **8**, 995–1002. (doi:10.1093/plankt/8.5.995)

21. Toshino S, Hiroshi M, Ohtsuka S, Okuizumi K, Adachi A, Hamatsu Y, Urata M, Nakaguchi K, Yamaguchi S. 2013 Development and polyp formation of the giant box jelly fish *Morbakka virulenta* (Kishinouye, 1910) (Cnidaria: Cubozoa) collected from the Seto Inland Sea, western Japan. *Plankton and Benthos Research Benthos Research* **8**, 1–8. (doi:10.3800/pbr.8.1)

22. Morandini AC, Silveira FL da. 2001 Sexual reproduction of *Nausithoe aurea* (Scyphozoa, Coronatae). Gametogenesis, egg release, embryonic development, and gastrulation. *Sci Mar* **65**, 139–149. (doi:10.3989/scimar.2001.65n2139)

23. Miranda LS, Morandini AC, Marques AC. 2009 Taxonomic review of *Haliclystus antarcticus* Pfeffer, 1889 (Stauromedusae, Staurozoa, Cnidaria), with remarks on the genus Haliclystus Clark, 1863. *Polar Biol* **32**, 1507–1519. (doi:10.1007/s00300-009-0648-8)

24. Shlesinger Y, Goulet TL, Loya Y. 1998 Reproductive patterns of scleractinian corals in the northern Red Sea. *Mar Biol* **132**, 691–701. (doi:10.1007/s002270050433)

25. Kayal E, Bentlage B, Cartwright P, Yanagihara AA, Lindsay DJ, Hopcroft RR, Collins AG. 2015 Phylogenetic analysis of higher-level relationships within Hydroidolina (Cnidaria: Hydrozoa) using mitochondrial genome data and insight into their mitochondrial transcription. *PeerJ* **3**, e1403. (doi:10.7717/peerj.1403)

26. Maronna MM, Miranda TP, Peña Cantero ÁL, Barbeitos MS, Marques AC. 2016 Towards a phylogenetic classification of Leptothecata (Cnidaria, Hydrozoa). *Sci Rep* **6**. (doi:10.1038/srep18075)

27. Cartwright P, Evans NM, Dunn CW, Marques AC, Miglietta MP, Schuchert P, Collins AG. 2008 Phylogenetics of Hydroidolina (Hydrozoa: Cnidaria). *Journal of the Marine Biological Association of the United Kingdom* **88**, 1663–1672. (doi:10.1017/S0025315408002257)

28. Collins AG. 2009 Recent insights into cnidarian phylogeny. *Smithson Contrib Mar Sci* **38**, 139–149. (doi:10.5479/si.1943667X.0)

29. Collins A, Schuchert P, Marques A, Jankowski T, Medina M, Schierwater B. 2006 Medusozoan phylogeny and character evolution clarified by new large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models. *Syst Biol* **55**, 97–115. (doi:10.1080/10635150500433615)

30. Katoh K, Standley DM. 2013 MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol Biol Evol* **30**, 772–780. (doi:10.1093/molbev/mst010)

31. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. 2015 IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* **32**, 268–274. (doi:10.1093/molbev/msu300)

32. Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu CH, Xie D, Suchard MA, Rambaut A, Drummond AJ. 2014 BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *PLoS Comput Biol* **10**, 1–6. (doi:10.1371/journal.pcbi.1003537)

33. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. 2006 Relaxed phylogenetics and dating with confidence. *PLoS Biol* **4**, 699–710. (doi:10.1371/journal.pbio.0040088)

34. Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018 Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst Biol* **67**, 901–904. (doi:10.1093/sysbio/syy032)

35. Cooper N, Jetz W, Freckleton RP. 2010 Phylogenetic comparative approaches for studying niche conservatism. *J Evol Biol* **23**, 2529–2539. (doi:10.1111/j.1420-9101.2010.02144.x)

36. Berrill NJ. 1952 Growth and form in gymnoblastic hydroids. II. Sexual and asexual reproduction in Rathkea. III. Hydranth and gonophore development in Pennaria and Acaulis. IV. Relative growth in Eudendrium. *J Morphol* **90**, 1–32.

37. Schuchert P. 2006 The European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Capitata Part 1. *Revue Suisse de Zoologie* **113**, 325–410. (doi:10.5962/bhl.part.117779)

38. Brinckmann-Voss A. 1966 The morphology and development of *Acaulis ilonae* sp. n. (order Anthomedusae/Athecatae, Fam. Acaulidae). *Can J Zool* **44**, 291–301.

39. Strong LH. 1925 Development of certain Puget Sound hydroids and medusae. *Publ. Pug. Sound Biol. Sta.* **3**, 383–400.

40. Larson RJ, Harbison GR. 1990 Medusae from McMurdo Sound, Ross Sea including the descriptions of two new species, *Leuckartiara brownei* and *Benthocodon hyalinus*. *Polar Biol*. **11**.

41. Strathmann MF. 1987 *Reproduction and development of marine invertebrates of the northern Pacific coast: data and methods for the study of eggs, embryos, and larvae.* University of Washington Press.

42. Freeman G. 1983 Experimental studies on embryogenesis in Hydrozoans (Trachylina and Siphonophora) with direct development. *Biol Bull* **165**, 591–618. (doi:10.2307/1541468)

43. Rees WJ, Russell FS. 1937 On rearing the hydroids of certain medusae, with an account of the methods used. *Journal of the Marine Biological Association of the United Kingdom* **22**, 61–82. (doi:10.1017/S0025315400011875)

44. Widmer CL. 2007 The hydroid and medusa of *Amphinema rollinsi* (Cnidaria: Hydrozoa), a new species of pandeid from the Monterey Bay Submarine Canyon, USA. *Zootaxa* **1595**, 53–59.

45. Teissier G. 1922 Observation des médusoides libres et des planulas de *Sertularia operculata* L. *Bull. Soc. Zool. Fr.* **47**, 357–361.

46. Boero F. 1980 *Hebella parasitica* (Cnidaria, Hydroida): A thecate polyp producing an anthomedusa. *Mar Biol* **59**, 133–136. (doi:10.1007/BF00396859)

47. Kelmo F, de la Santa-Isabel LM. 1998 The athecatae hydroids (Cnidaria, Hydrozoa) from Northern Bahia, Brazil. *Rev Biol Trop* , 61–71.

48. Genzano GN, Zamponi MO. 1999 Historia natural de *Bimeria vestita* Wright, 1859 (Hydrozoa, Bougainvillidae) en el intermareal rocoso de Mar del Plata (Argentina). *Cienc Mar* **25**, 63–74.

49. Brinckmann-Voss A. 1979 The life-cycle of *Bythotiara huntsmani* (Fraser 1911) (Calycopsidae, Hydrozoa, Cnidaria). *Can J Zool* **57**, 1226–1231. (doi:10.1139/z79-157)

50. Mills C, Rees JT. 1979 *Bythotiara stilbosa,* new species, (Anthomedusae: Calycopsidae) from neritic waters in central California. *J Nat Hist* **13**, 285–293.

51. Russell FS. 1953 *The medusae of the British Isles: anthomedusae, leptomedusae, limnomedusae, trachymedusae and narcomedusae*. University Press.

52. Schuchert P. 2007 The European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Filifera part 2. *Revue Suisse de Zoologie* **114**, 195–396. (doi:10.5962/bhl.part.80395)

53. Denitto F, Miglietta MP, Boero F. 2007 Life cycle of *Bougainvillia nana* (Cnidaria: Hydrozoa: Bougainvilliidae) from Italy, including a discussion of *Bougainvillia muscus* in the Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom* **87**, 853–857. (doi:10.1017/S0025315407054574)
54. Edwards C. 1966 The hydroid and the medusa *Bougainvillia principis,* and a review of the British species of Bougainvillia. *Journal of the Marine Biological Association of the United Kingdom* **46**, 129–152.
55. Migotto AE. 1996 Benthic shallow-water hydroids (Cnidaria, Hydrozoa) of the coast of Sao Sebastiao, Brazil, including a checklist of Brazilian hydroids. *Zoologische Verhandelingen* **306**, 3–125.
56. Berrill NJ. 1949 Growth and form in gymnoblastic hydroids. I. Polymorphic development in Bougainvillia and Aselomaris. *J Morphol* **84**, 1–30.
57. Berrill NJ. 1953 Growth and form in gymnoblastic hydroids. VI. Polymorphism within the Hydractiniidae. *J Morphol* **92**, 241–272. (doi:10.1002/jmor.1050920203)
58. Galea HR, Häussermann V, Försterra G. 2009 New additions to the hydroids (Cnidaria: Hydrozoa) from the fjords region of Southern Chile. *Zootaxa* **28**, 1–28. (doi:10.11646/zootaxa.2019.1.1)
59. di Camillo CG, Puce S, Bavestrello G. 2011 Lytocarpia and Cladocarpus (Cnidaria: Hydrozoa, Aglaopheniidae) from the Bunaken National Marine Park (North Sulawesi, Indonesia). *Marine Biodiversity* **41**, 517–536. (doi:10.1007/s12526-011-0081-4)
60. Kubota S. 1979 Morphological notes on the polyp and medusa of *Climacocodon ikarii* Uchida (Hydrozoa, Margelopsidae) in Hokkaido. *Journal of the Faculty of Science Hokkaido University Series* VI. *Zoology* **22**, 122–136.
61. Roosen-Runge EC. 1970 Life cycle of the hydromedusa *Phialidium gregarium* (A. Agassiz, 1862) in the laboratory. *Biol Bull* **139**, 203–221.
62. Carré D, Carré C. 2000 Origin of germ cells, sex determination, and sex inversion in medusae of the genus Clytia (Hydrozoa, Leptomedusae): The influence of temperature. *Journal of Experimental Zoology* **287**, 233–242. (doi:10.1002/1097-010X(20000801)287:3<233::AID-JEZ5>3.3.CO;2-6)
63. Amiel A, Houliston E. 2009 Three distinct RNA localization mechanisms contribute to oocyte polarity establishment in the cnidarian *Clytia hemisphærica*. *Dev Biol* **327**, 191–203. (doi:10.1016/j.ydbio.2008.12.007)
64. Lindner A, Migotto AE. 2002 The life cycle of *Clytia linearis* and *Clytia noliformis*: Metagenic campanulariids (Cnidaria: Hydrozoa) with contrasting polyp and medusa stages. *Journal of the Marine Biological Association of the United Kingdom* **82**, 541–553. (doi:10.1017/S0025315402005866)
65. Muskó IB, Bence M, Balogh. 2008 Occurrence of a new Ponto-Caspian invasive species, *Cordylophora caspia* (Pallas, 1771) (Hydrozoa: Clavidae) in Lake Balaton (Hungary). *Acta Zoologica Academiae Scientiarum Hungaricae* **54**, 169–179.
66. Rees WJ. 1937 The development of the hydroid *Corymorpha nutans* M. Sars from the egg. *Journal of the Marine Biological Association of the United Kingdom* **21**, 743–746.
67. Schuchert P, Brinckmann-Voss A. 2006 The true and false gonophores of Coryne epizoica and its shell colonizing mechanisms. *Italian Journal of Zoology* **73**, 319–324. (doi:10.1080/11250000600881340)

68. Schuchert P. 2005 Rediscovery of Coryne fucicola (de Filippi, 1866) (Cnidaria: Hydrozoa). *Cah Biol Mar* **46**, 305–310.

69. Kubota S, Takashima Y. 1992 Redescription of Sarsia japonica (Nagao) (Hydrozoa: Corynidae) from Hokkaido, Japan. *Publications of the Seto Marine Biological Laboratory* **35**, 371–381.

70. Beadle L, Thomas I, Poole D. 1960 Early development of *Limnocnida victoriae* Gunther (Limnomedusae). *Proceedings of the Zoological Society of London* **134**, 217–219.

71. Takeda N, Nakajima Y, Koizumi O, Fujisawa T, Takahashi T, Matsumoto M, Deguchi R. 2013 Neuropeptides trigger oocyte maturation and subsequent spawning in the hydrozoan jellyfish *Cytaeis uchidae*. *Mol Reprod Dev* **80**, 223–232. (doi:10.1002/mrd.22154)

72. Takeda N, Deguchi R, Itabashi T. 2018 Reproductive strategies in marine hydrozoan jellyfish: sexual medusae and asexual polyps. In *Reproductive and Developmental Strategies*, pp. 157–174. Springer, Tokyo. (doi:10.1007/978-4-431-56609-0_8)

73. Migotto AE, Marques AC. 1999 Redescription of *Dentitheca bidentata* (Cnidaria: Hydrozoa, Plumulariidae), with notes on its life cycle. *J Nat Hist* **33**, 949–960. (doi:10.1080/002229399300029)

74. Galea HR, Ferry R, Bertot JM. 2012 Medusoids in the life cycle of *Dentitheca dendritica* (Nutting, 1900) and *Nemalecium gracile* sp. nov. (Cnidaria: Hydrozoa). *Zootaxa* , 43–54. (doi:10.11646/zootaxa.3527.1.3)

75. Gravili C, de Vito D, di Camillo CG, Martell L, Piraino S, Boero F. 2015 The non-Siphonophoran Hydrozoa (Cnidaria) of Salento, Italy with notes on their life-cycles: An illustrated guide. *Zootaxa* **3908**, 1–187. (doi:10.11646/zootaxa.3908.1.1)

76. Schuchert P. 2003 Hydroids (Cnidaria, Hydrozoa) of the Danish expedition to the Kei Islands. *Steenstrupia* **27**, 137–256.

77. Widmer CL, Cailliet G, Geller J. 2010 The life cycle of *Earleria corachloeae* n. sp. (Cnidaria: Hydrozoa) with epibiotic hydroids on mid-water shrimp. *Mar Biol* **157**, 49–58. (doi:10.1007/s00227-009-1294-y)

78. Rees WJ. 1957 Evolutionary trends in the classification of Capitate hydroids and medusae. *Bulletin of the British Museum (Natural History) Zoology* **4**, 453–534.

79. Seavolt H. 2001 ‘*Ectopleura crocea*’ (On-line), Animal Diversity Web. Accessed January 04, 2020 at https://animaldiversity.org/accounts/Ectopleura_crocea/.

80. Nagao Z. 1965 Studies on the development of *Tubularia radiata* and *Tubularia venusta* (Hydrozoa). *Publications from the Akkeshi Marine Biological Station* **15**, 9–35.

81. Marques AC, Mergner H, Höinghaus R, Santos CMD, Vervoort W. 2000 Morphological study and taxonomical notes on Eudendriidae (Cnidaria: Hydrozoa: Athecatae/Anthomedusae). *Zool. Med. Leiden* **5**, 75–118.

82. Peña Cantero ÁL. 2013 Benthic hydroids from off Low Island (Southern Ocean, Antarctica). *Marine Ecology* **34**, 123–142. (doi:10.1111/maec.12031)

83. Schuchert P. 2008 The European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Filifera Part 4. *Revue Suisse de Zoologie* **115**, 677–757. (doi:10.5962/bhl.part.117793)

84. Puce S, Cerrano C, di Camillo C, Bavestrello G, Marques AC. 2006 Eudendrium (Cnidaria: Hydrozoa) from Bunaken Marine Park, Sulawesi Sea, Indonesia. *Zool Stud* **45**, 616–625.

85. Bavestrello G, Piraino S. 1991 On two Eudendrium (Cnidaria, Hydrozoa) species from the Mediterranean Sea. *Oebalia* **17**, 197–207.

86. Marques A, Peña Cantero A, Vervoort W. 2000 Mediterranean species of Eudendrium Ehrenberg, 1834 (Hydrozoa, Anthomedusae, Eudendriidae) with the description of a new species. *J Zool* **252**, 197–213. (doi:10.1111/j.1469-7998.2000.tb00615.x)

87. de Vito D, Boero F, di Camillo CG, Megina C, Piraino S. 2008 Redescription of the zooxanthellate *Eudendrium moulouyensis* (Eudendriidae: Hydrozoa) from the Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom* **88**, 1655–1662. (doi:10.1017/S0025315408002142)

88. Marques AC. 1995 *Eudendrium pocaruquarum* n. sp. (Hydrozoa, Eudendriidae) from the southeastern coast of Brazil, with remarks on taxonomic approaches to the family Eudendriidae. *Bijdragen tot de Dierkunde* **65**, 35–40.

89. Marques AC, Migotto AE. 1998 A new species of Eudendrium (Hydrozoa: Anthomedusae: Eudendriidae) from the Netherlands. *Zool. Verh. Leiden* **323**, 149–154.

90. Kubota S. 1985 Systematic study on a bivalve-inhabiting hydroid *Eugymnanthea inquilina japonica* Kubota from Central Japan. *Journal of the Faculty of Science Hokkaido University Series ＶＩ. Zoology* **24**, 70–85.

91. Miller RL. 1979 Sperm chemotaxis in the hydromedusae. I. Species-specificity and sperm behavior. *Mar Biol* **53**, 99–113. (doi:10.1007/BF00389182)

92. Kubota S. 1983 Studies on life history and systematics of the japanese commensal hydroids living in bivalves, with some reference to their evolution. *Journal of The Faculty of Science Hokkaido University Series VI. Zoology* **23**, 296–402.

93. Migotto AE, Caobelli JF, Kubota S. 2004 Redescription and life cycle of *Eutima sapinhoa* Narchi and Hebling, 1975 (Cnidaria: Hydrozoa, Leptothecata): A hydroid commensal with *Tivela mactroides* (Born) (Mollusca, Bivalvia, Veneridae). *J Nat Hist* **38**, 2533–2545. (doi:10.1080/00222930310001647316)

94. Peter S, Manojkumar B, Pillai D. 2018 First record of *Eutonina indicans* (Romanes, 1876) from Kerala waters, India. *Indian Journal of Geo-Marine Sciences* **47**, 1636–1639.

95. Vervoort W. 1964 Note on the distribution of *Garveia franciscana* (Torrey, 1902) and *Cordylophora caspia* (Pallas, 1771) in the Netherlands. *Zoologische Mededelingen* **39**, 125–146.

96. Burmistrova YA, Osadchenko B v., Bolshakov F v., Kraus YA, Kosevich IA. 2018 Embryonic development of thecate hydrozoan *Gonothyraea loveni* (Allman, 1859). *Dev Growth Differ* **60**, 483–501. (doi:10.1111/dgd.12567)

97. Galea HR, Schories D. 2012 Some hydrozoans (Cnidaria) from Central Chile and the Strait of Magellan. *Zootaxa* , 19–67. (doi:10.11646/zootaxa.3296.1.2)

98. Schuchert P. 2005 Taxonomic revision and systematic notes on some Halecium species (Cnidaria, Hydrozoa). *J Nat Hist* **39**, 607–639. (doi:10.1080/00222930400001319)

99. Galea HR, Schories D, Försterra G, Häussermann V. 2014 New species and new records of hydroids (Cnidaria: Hydrozoa) from Chile. *Zootaxa* **3852**, 1–50. (doi:10.11646/zootaxa.3852.1.1)

100. Galea HR, Ferry R. 2015 Notes on some hydroids (Cnidaria) from Martinique, with descriptions of five new species. *Revue Suisse de Zoologie* **122**, 213–246. (doi:10.5281/zenodo.29998)

101. Mills CE. 2000 The life cycle of *Halimedusa typus,* with discussion of other species closely related to the family Halimedusidae (Hydrozoa, Capitata, Anthomedusae. *Sci Mar* **64**, 97–106. (doi:10.3989/scimar.2000.64s197)

102. Vervoort W. 1981 Note on the distribution of Garveia franciscana (Torrey, 1902) and Cordylophora caspia (Pallas, 1771) in the Netherlands. *Feestbundel bij de opening van het Kolveniershof en het Rubenianum* , 125–146.

103. Freeman G, Miller RL. 1982 Hydrozoan eggs can only be fertilized at the site of polar body formation. *Dev Biol* **94**, 142–152.

104. Freeman G. 1987 The role of oocyte maturation in the ontogeny of the fertilization site in the hydrozoan *Hydractinia echinata*. *Roux's archives of developmental biology* **196**, 83–92.

105. Morandini AC, Stampar SN, Migotto AE, Marques AC. 2009 *Hydrocoryne iemanja* (Cnidaria), a new species of Hydrozoa with unusual mode of asexual reproduction. *Journal of the Marine Biological Association of the United Kingdom* **89**, 67–76. (doi:10.1017/S0025315408002968)

106. Galea HR. 2018 A swimming medusoid gonophore in the life cycle of *Ventromma halecioides* (Alder, 1859) (Hydrozoa: Leptothecata: Kirchenpaueriidae). *Revue Suisse de Zoologie* **125**, 137–147. (doi:10.5281/zenodo.l196038)

107. Cornelius PFS. 1995 North-west European thecate hydroids and their medusae. Part 2. Sertulariidae to Campanulariidae. In *Synopses of the British Fauna (New Series)*, Published for the Linnean Society of London and the Estuarine and Coastal Services Association by Field Studies Council.

108. Knight DP. 1970 Sclerotization of the perisarc of the calyptoblastic hydroid, *Laomedea Flexuosa:* 1. The identification and localization of dopamine in the hydroid. *Tissue Cell* **2**, 467–477.

109. O'Rand MG, Miller RL. 1974 Spermatozoan vesicle loss during penetration of the female gonangium in the hydroid. *Campanularia flexuosa*. *Journal of Experimental Zoology* **188**, 179–193. (doi:10.1002/jez.1401880206)

110. Beadle LC, Thomas IF. 1957 The African freshwater jellyfish *Limnocnida tanganyicae*. *Nature*. **179**, 110. (doi:10.1038/179110a0)

111. Schuchert P. 2019 The hydroid of the medusa *Lizzia blondina* Forbes, 1848. *Marine Biodiversity* **49**, 1683–1693. (doi:10.1007/s12526-019-00936-0)

112. Bourmaud C, Gravier-Bonnet N. 2004 Medusoid release and spawning of *Macrorynchia philippina* Kirchenpauer, 1872 (Cnidaria, Hydrozoa, Aglaopheniidae). *Hydrobiologia* **530–531**, 365–372. (doi:10.1007/s10750-004-2665-5)

113. Rees JT, Gershwin LA. 2000 Non-indigenous hydromedusae in California's upper San Francisco Estuary: Life cycles, distribution, and upper potential environmental impacts. *Sci Mar* **64**, 73–86. (doi:10.3989/scimar.2000.64s173)

114. Vannucci M, Yamada M. 1959 The life cycle of *Merga tergestina* (Anthomedusae, Pandeidae). *Pubblicazioni della Stazione Zoologica di Napoli* **31**, 320–333.

115. Schuchert P. 2004 Revision of the European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Families Oceanidae and Pachycordylidae. *Revue Suisse de Zoologie* **111**, 315–369. (doi:10.5962/bhl.part.80242)

116. Stepanjants SD, Christiansen BO, Svoboda A, Anokhin BA. 2003 The genus Monocoryne (Hydrozoa, Capitata): Peculiarities of morphology, species composition, biology and distribution. *Sarsia: North Atlantic Marine Science* **88**, 97–106. (doi:10.1080/00364820310001714)

117. Schuchert P, Sanamyan N, Sanamyan K. 2016 Observations on two large athecate hydroids (Cnidaria: Hydrozoa) from the Kamchatka Peninsula (NW Pacific). *Revue suisse de Zoologie* **123**, 165–178. (doi:10.5281/zenodo.46301)

118. Hickson SJ. 1900 The medusae of Millepora. *Proceedings of the Royal Society of London* **66**, 3–10.

119. Lewis JB. 1991 The ampullae and medusae of the calcareous hydrozoan *Millepora complanata*. *Hydrobiologia* **216–217**, 165–169. (doi:10.1007/BF00026458)

120. Soong K, Cho LC. 1998 Synchronized release of medusae from three species of hydrozoan fire corals. *Coral Reefs* **17**, 145–154. (doi:10.1007/s003380050109)

121. Bourmaud CAF, Leung JKL, Bollard S, Gravier-Bonnet N. 2013 Mass spawning events, seasonality and reproductive features in Milleporids (Cnidaria, Hydrozoa) from Reunion Island. *Marine Ecology* **34**, 14–24. (doi:10.1111/maec.12024)

122. Russell FS, Rees WJ. 1936 On rearing the hydroid *Zanclea implexa* (Alder) and its medusa *Zanclea gemmosa* McCrady, with a review of the genus Zanclea. *Journal of the Marine Biological Association of the United Kingdom* **21**, 107–130. (doi:10.1017/S002531540001122X)

123. Martin V, Chia F -S, Koss R. 1983 A fine structural study of metamorphosis of the hydrozoan *Mitrocomella polydiademata*. *J Morphol* **176**, 261–287. (doi:10.1002/jmor.1051760303)

124. Blackett M. 2015 Biology and ecology of the siphonophore *Muggiaea atlantica* in the northeast Atlantic. University of Southampton.

125. Mapstone GM. 2014 Global diversity and review of Siphonophorae (Cnidaria: Hydrozoa). *PLoS One* **9**. (doi:10.1371/journal.pone.0087737)

126. Bouillon J. 1986 Nemalecium gen. nov., genre nouveau de Haleciidae (Thecata-Leptomedusae, Hydrozoa, Cnidaria). *Indo-Malayan Zoology* **3**, 71–80.

127. Vervoort W, Watson JE. 2003 *The marine fauna of New Zealand: Leptothecata (Cnidaria: Hydrozoa) (Thecate Hydroids)*.

128. Coma R. 1996 Quantification of sexual reproduction in the marine benthic hydroid *Campanularia everta*. *Mar Biol* **125**, 365–373. (doi:10.1007/BF00346317)

129. Genzano GN, Kubota S. 2003 Synchronous mass release of mature medusae from the Hydroid *Halocordyle disticha* (Hydrozoa, Halocordylidae) and experimental induction of different timing by light changes. *Publications of the Seto Marine Biological Laboratory* **39**, 221–228.

130. Bavestrello G, Puce S, Cerrano C, Balduzzi A. 2000 Life history of *Perarella schneideri* (Hydrozoa, Cytaeididae) in the Ligurian Sea. *Sci Mar* **64**, 141–146. (doi:10.3989/scimar.2000.64s1141)

131. Russell FRS. 1970 The Medusae of the British Isles. Vol 2. *Cambiridge University Press* , 284.

132. Westblad E. 1930 Ueber die Geschlechtsorgane und die systematische Bedeutung von *Protohydra Leuckarti* Greeff. *Arkiv för Zoologi* **21**, 1–13.

133. Watson JE. 1980 The identity of two tubularian hydroids from Australia with a description and observations on the reproduction of *Ralpharia magnifica* gen. et sp. nov. *Memoirs of the National Museum of Victoria* **41**, 53–63.

134. Kulikova V. 1957 A case of hermaphroditism of the gonade in *Rathkea octopunctata* . *Dokl Akad Nauk SSSR* **114**, 899–900.

135. Brinckmann-Voss A. 2000 The hydroid and medusa of *Sarsia bella* sp. nov. (Hydrozoa, Anthoathecatae, Corynidae), with a correction of the ‘life cycle’ of *Polyorchis penicillatus* (Eschscholtz). *Sci Mar* **64**, 189–195. (doi:10.3989/scimar.2000.64s1189)

136. Gravier-Bonnet N, Lebon ML. 2002 Swimming medusoid gonophores in two species of sertularella (cnidaria, hydrozoa, sertulariidae) from réunion Island, Indian Ocean. *Invertebr Reprod Dev* **41**, 101–108. (doi:10.1080/07924259.2002.9652740)

137. Honegger TG. 1980 Gametogenesis of *Sertularella polygons* with special reference to vitellogenesis (Hydrozoa, Atacata). In *Developmental and cellular biology of Coelenterates*, pp. 67–73. Amsterdam, New York.

138. Migotto A. 1998 The life cycle of Sertularia marginata Kirchenpauer, 1864 (Cnidaria: Hydrozoa): a medusoid-producing sertulariid. *J Nat Hist* **32**, 1–12.

139. González-Duarte MM, Megina C, Bethencourt M. 2013 *Sertularia marginata* (Cnidaria: Hydrozoa) in the Mediterranean: An alien species in expansion? *Mediterr Mar Sci* **14**, 384–389. (doi:10.12681/mms.445)

140. Kubota S. 1991 Taxonomic notes on polyp and medusa of *Sarsia nipponica* Uchida (Hydrozoa: Corynidae) from the type locality in Japan. *Publications of the Seto Marine Biological Laboratory* **35**, 17–23. (doi:10.5134/176173)

141. Gauld DT. 1952 *Staurophora mertensii* Brandt in the Firth of Clyde. *Nature* **169**, 752.

142. Ma H, Yang Y. 2010 *Turritopsis nutricula* . *Nat Sci (East Lansing)* **8**, 15–20.

143. Altuna Á. 2016 The life cycle of symbiotic *Zanclea sessilis* (Hydrozoa: Zancleidae) colonies with polymorphic hydranths from the Bay of Biscay. *Marine Biodiversity* **46**, 901–910. (doi:10.1007/s12526-016-0456-7)

144. Miranda LS, Collins AG, Hirano YM, Mills CE, Marques AC. 2016 Comparative internal anatomy of Staurozoa (Cnidaria), with functional and evolutionary inferences. *PeerJ* **4**, e2594. (doi:10.7717/peerj.2594)

145. Miranda LS, Collins AG, Marques AC. 2013 Internal anatomy of *Haliclystus antarcticus* (Cnidaria, Staurozoa) with a discussion on histological features used in staurozoan taxonomy. *J Morphol* **274**, 1365–1383. (doi:10.1002/jmor.20185)

146. Wietrzykowski W. 1912 Recherches sur le développement des Lucernaires. *Archives de zoologie expérimentale et générale* **10**, 1–95.

147. Otto JJ. 1976 Early development and planula movement in Haliclystus (Scyphozoa: Stauromedusae). In *Coelenterate ecology and behavior*, pp. 319–329.

148. Hanaoka K-I. 1934 Notes on the early development of a stalked medusa. *Proceedings of the Imperial Academy* **10**, 117–120. (doi:10.2183/pjab1912.10.117)

149. Russell FS. 1959 Some observations on the scyphomedusa Atolla. *Journal of the Marine Biological Association of the United Kingdom* **38**, 33–40. (doi:10.1017/S0025315400015563)

150. Lucas CH, Reed AJ. 2010 Gonad morphology and gametogenesis in the deep-sea jellyfish *Atolla wyvillei* and *Periphylla periphylla* (Scyphozoa: Coronatae) collected from Cape Hatteras and the Gulf of Mexico. *Journal of the Marine Biological Association of the United Kingdom* **90**, 1095–1104. (doi:10.1017/S0025315409000824)

151. Widersten B. 1965 Genital organs and fertilization in some Scyphozoa. *Zoologiska Bidrag fran Uppsala* **37**, 45–58. (doi:10.1016/0011-7471(66)90698-x)

152. Gohar HAF, Eisawy AM. 1960 The biology of *Cassiopea andromeda* (from the Red Sea) (With a note on the species problem). *Publ Mar Biol Stat Ghardaqa* **11**, 3–39.

153. Hofmann DK, Hadfield MG. 2002 Hermaphroditism, gonochorism, and asexual reproduction in Cassiopea sp.—an immigrant in the islands of hawai'i. *Invertebr Reprod Dev* **41**, 215–221. (doi:10.1080/07924259.2002.9652754)

154. Pitt K a., Kingsford MJ. 2000 Reproductive biology of the edible jellyfish *Catostylus mosaicus* (Rhizostomeae). *Mar Biol* **137**, 791–799. (doi:10.1007/s002270000399)

155. Berrill NJ. 1949 Developmental analysis of Scyphomedusae. *Biological Reviews* **24**, 393–409. (doi:10.1111/j.1469-185X.1949.tb00581.x)

156. Eckelbarger KJ, Larson R. 1992 Ultrastructure of the ovary and oogenesis in the jellyfish *Linuche unguiculata* and *Stomolophus meleagris,* with a review of ovarian structure in the Scyphozoa. *Mar Biol* **114**, 633–643. (doi:10.1007/BF00357260)

157. Dong J, Sun M, Wang B, Liu H. 2008 Comparison of life cycles and morphology of *Cyanea nozakii* and other scyphozoans. *Plankton Benthos Res* **3**, 118–124. (doi:10.3800/pbr.3.118)

158. Liu C sheng, Zhuang Z meng, Chen S qing, Yan J ping, Liu C lin, Sun J ming. 2015 A large-scale breeding experiment and observations on development of *Cyanea nozakii* (Scyphozoa, Cyaneidae) from fertilized eggs to larval medusae. *Hydrobiologia* **754**, 113–123. (doi:10.1007/s10750-014-1883-8)

159. Kikinger R. 1992 *Cotylorhiza tuberculata* (Cnidaria: Scyphozoa) - Life history of a stationary population. *Marine Ecology* **13**, 333–362. (doi:10.1111/j.1439-0485.1992.tb00359.x)

160. Avian M, Sandrini LR. 1991 Oocyte development in four species of scyphomedusa in the northern Adriatic Sea. *Hydrobiologia* **216**, 189–195.

161. Schiariti A, Kawahara M, Uye S, Mianzan HW. 2008 Life cycle of the jellyfish *Lychnorhiza lucerna* (Scyphozoa: Rhizostomeae). *Mar Biol* **156**, 1–12. (doi:10.1007/s00227-008-1050-8)

162. Piraino S, Aglieri G, Martell L, Mazzoldi C, Melli V, Milisenda G, Scorrano S, Boero F. 2014 *Pelagia benovici* sp. nov. (Cnidaria, Scyphozoa): A new jellyfish in the Mediterranean Sea. *Zootaxa* **3794**, 455–468. (doi:10.11646/zootaxa.3794.3.7)

163. da Silveira FL, Morandini AC. 1997 *Nausithoe aurea* n. sp. (Scyphozoa: Coronatae: Nausithoidae), a species with two pathways of reproduction after strobilation: sexual and asexual. *Contributions to Zoology* **66**, 235–246.

164. Kawahara M, Uye SI, Ohtsu K, Iizumi H. 2006 Unusual population explosion of the giant jellyfish *Nemopilema nomurai* (Scyphozoa: Rhizostomeae) in East Asian waters. *Mar Ecol Prog Ser* **307**, 161–173. (doi:10.3354/meps307161)

165. Goette A. 1893 Vergleichende entwicklungsgeschichte von *Pelagia noctiluca* Per. *Zeitschrift fur wissenschaftliche Zoologie* **55**, 645–695.

166. Rottini-Sandrini L, Avian M, Axiak V, Malej A. 1983 The breeding period of *Pelagia noctiluca* (Scyphozoa, Semaeostomeae) in the Adriatic and Central Mediterranean Sea. *Nova Thalassia* **6**, 65–75.

167. Avian M. 1983 Tecniche istologiche per lo studio delle gonadi nei Celenterati. *Boll. Soc. adriat. Sci.* **67**, 89–98.

168. Jarms G, Båmstedt U, Tiemann H, Martinussen MB, Fosså JH, Høisœter T. 1999 The holopelagic life cycle of the deep-sea medusa *Periphylla periphylla* (Scyphozoa, Coronatae). *Sarsia* **84**, 55–65. (doi:10.1080/00364827.1999.10420451)

169. Tiemann H, Jarms G. 2010 Organ-like gonads, complex oocyte formation, and long-term spawning in *Periphylla periphylla* (Cnidaria, Scyphozoa, Coronatae). *Mar Biol* **157**, 527–535. (doi:10.1007/s00227-009-1338-3)

170. Widmer CL. 2006 Life cycle of *Phacellophora camtschatica* (Cnidaria: Scyphozoa). *Invertebrate Biology* **125**, 83–90. (doi:10.1111/j.1744-7410.2006.00043.x)

171. Russell FS. 1962 On the scyphomedusa *Poralia rufescens* Vanhöffen. *Journal of the Marine Biological Association of the United Kingdom* **42**, 387–390.

172. Holst S, Sötje I, Tiemann H, Jarms G. 2007 Life cycle of the rhizostome jellyfish *Rhizostoma octopus* (L.) (Scyphozoa, Rhizostomeae), with studies on cnidocysts and statoliths. *Mar Biol* **151**, 1695–1710. (doi:10.1007/s00227-006-0594-8)

173. Lotan A, Ben-Hillel R, Loya Y. 1992 Life cycle of *Rhopilema nomadica:* a new immigrant scyphomedusan in the Mediterranean. *Mar Biol* **112**, 237–242. (doi:10.1080/03078698.1994.9674073)

174. Calder DR. 1982 Life history of the cannonball jellyfish, *Stomolophus meleagris* L. Agassiz, 1860 (Scyphozoa, Rhizostomida). *Biological Bulletin* **162**, 149–162.

175. Carvalho-Saucedo L, García-Domínguez F, Rodríguez-Jaramillo C, López-Martínez J. 2010 Variación lipídica en los ovocitos de la medusa *Stomolophus meleagris* (Scyphozoa: Rhizostomeae), durante el desarrollo gonádico, en la laguna Las Guásimas, Sonora, México. *Rev Biol Trop* **58**, 119–130.

176. Sötje I, Jarms G. 2009 Derivation of the reduced life cycle of *Thecoscyphus zibrowii* Werner, 1984 (Cnidaria, Scyphozoa). *Mar Biol* **156**, 2331–2341. (doi:10.1007/s00227-009-1261-7)

177. Mohamed R, Skrypzeck H, Gibbons MJ. 2019 Describing gonad development and gametogenesis in southern Africa's endemic box jellyfish *Carybdea branchi* (Cubozoa, Carybdeidae). *Afr J Mar Sci* **41**, 83–91. (doi:10.2989/1814232X.2019.1575282)

178. Okada YK. 1927 Note sur l'ontogenie de *Carybdea rastonii* Haacke. *Bull Biol* **3**, 241–249.

179. Toshino S, Miyake H, Shibata H. 2018 Development of *Carybdea brevipedalia* Kishinouye, 1891 (Cnidaria: Cubozoa: Carybdeida: Carybdeidae) collected from northern Japan. *Plankton Benthos Res* **13**, 116–128. (doi:10.3800/pbr.13.116)

180. Acevedo MJ *et al.* 2019 Revision of the genus Carybdea (Cnidaria: Cubozoa: Carybdeidae): Clarifying the identity of its type species *Carybdea marsupialis*. *Zootaxa* **4543**, 515–548. (doi:10.11646/zootaxa.4543.4.3)

181. Courtney R, Browning S, Seymour J. 2016 Early life history of the 'irukandji' jellyfish *Carukia barnesi*. *PLoS One* **11**, 1–13. (doi:10.1371/journal.pone.0151197)

182. Yamaguchi M, Hartwick R. 1980 Early life history of the sea wasp *Chironex fleckeri* (class Cubozoa). *Development and cellular biology of coelenterates* , 11–16.

183. Tiseo GR, García-Rodríguez J, Zara FJ, Ames CL, Marques AC, Morandini AC. 2019 Spermatogenesis and gonadal cycle in male *Tamoya haplonema* and *Chiropsalmus quadrumanus* (Cnidaria, Cubozoa). *Zool Anz* **279**, 59–67. (doi:10.1016/j.jcz.2018.12.008)

184. Lewis C, Long TAF. 2005 Courtship and reproduction in *Carybdea sivickisi* (Cnidaria: Cubozoa). *Mar Biol* **147**, 477–483. (doi:10.1007/s00227-005-1602-0)

185. Garm A, Lebouvier M, Tolunay D. 2015 Mating in the box jellyfish *Copula sivickisi* -Novel function of cnidocytes. *J Morphol* **276**, 1055–1064. (doi:10.1002/jmor.20395)

186. Helmark S, Garm A. 2019 Gonadal cnidocytes in the cubozoan *Tripedalia cystophora* Conant, 1897 (Cnidaria: Cubozoa). *J Morphol* **280**, 1530–1536. (doi:10.1002/jmor.21046)

187. Han J, Kubota S, Uchida HO, Stanley GD, Yao X, Shu D, Li Y, Yasui K. 2010 Tiny sea anemone from the Lower Cambrian of China. *PLoS One* **5**, 1–10. (doi:10.1371/journal.pone.0013276)

Medusozoa egg size code

https://drive.google.com/file/d/11lVM1te1bdbS3idlj2u3FR8m_fjVL5gz/view?usp=sharing

Capítulo 2

Histological investigation of the female gonads of *Chiropsalmus quadrumanus* (Cubozoa: Cnidaria) suggests iteroparous reproduction

Jimena Garcia-Rodriguez[1], Cheryl Lewis Ames[2,3], Adrian Jaimes-Becerra[1,4], Gisele Rodrigues Tiseo[1], André Carrara Morandini[1,5], Amanda Ferreira Cunha[6], Antonio Carlos Marques[1]

[1] Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil

[2] Graduate School of Agricultural Science, Faculty of Agriculture, Tohoku University, Sendai, 980-8572, Japan

[3] Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington DC, 20013-7012, USA

[4] Department of Ecology, Evolution and Behavior, Alexander Silberman Institute of Life Sciences, Faculty of Science, Hebrew University of Jerusalem, 9190401 Jerusalem, Israel

[5] Centro de Biologia Marinha, Universidade de São Paulo (CEBIMar-Usp), Brazil

[6] Departamento de Biologia Animal, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa, Av. P.H. Rolfs, s/n, 36570-900, Viçosa, Brazil

https://doi.org/10.3390/d15070816 (Anexo II)

Abstract

The box jellyfish *Chiropsalmus quadrumanus* (Chirodropida: Cubozoa: Cnidaria) is common in warm waters. Although it is assumed that external fertilization is a characteristic of Chirodropida, the life history of *C. quadrumanus* is not yet known since its reproductive behavior has never been described, nor has the polyp has been found in nature. As a result, in the absence of documentation of reproductive behavior, we sought to test the hypothesis of external fertilization through a histological analysis of the female gonads. Herein, we analyze ten females collected in São Paulo and Rio de Janeiro (Brazil) and describe the gonadal organization and pattern of oocyte development. The discovery of four distinct stages of oocyte differentiation augments the scant existing reports of the structural and functional maturation of sex cells in Cubozoa species. Furthermore, the gonads of mature females comprise both mature (average diameter of 122 µm) and immature oocytes, suggesting that *C. quadrumanus* is iteroparous and exhibits multiple reproductive cycles during its life. Medusa bell size was not found to correlate with maturity state as even small females possessed a high percentage of oocytes in late vitellogenesis, suggesting that sexual maturation occurs rapidly in *C. quadrumanus* females.

Keywords: oocyte; medusa; box jellyfish; fertilization; oogenesis; differentiation gradient; seasonality

Introduction

Reproductive studies in Cubozoa (box jellyfish) are hampered because reports of their jellyfish (cubomedusae) or medusoid form often refer to sightings of a single individual [1] or a limited (seasonal) reproductive period [2,3], and many species are notoriously venomous to humans, making cubomedusae challenging to collect [4]. Cubozoans, like many medusozoans (jellyfishes), have complex metagenetic life cycles, metamorphosing from a sessile polyp stage (asexual larval stage) into the characteristic, free-swimming medusa (sexual adult stage) [5,6,7]. Asexual polypoid reproduction is favorable under certain environmental conditions because it allows for a rapid increase in the number of clonal medusozoan individuals; this is also the case for other bi- or multiphasic marine invertebrates [8,9]. Meanwhile, in marine invertebrates, sexual reproduction, which generates genetic novelties via mixing genotypes, is triggered by specific environmental and genetic factors [8], many of which are undetermined. Unfortunately, female gonadal maturation (oogenesis), reproductive strategies and dynamics, and fertilization modes are particularly understudied in cubozoans [10]. This lack of knowledge limits actions to monitor cubozoan populations with the aim of managing the influence of cubomedusae on tourism and recreation while at the same time considering biodiversity conservation and public safety.

The accumulated knowledge on the sexual reproduction of cubomedusae is derived from seminal lab studies on five species of Carybdeida (*Alatina alata* (Reynaud, 1830) [3,11,12]; *Carybdea marsupialis* (Linnaeus, 1758) [13]; *Copula sivickisi* (Stiasny, 1926) [12,14,15,16,17]; *Morbakka virulenta* (Kishinouyea, 1910) [18]; *Tamoya haplonema* F. Müller, 1859 [19,20]) and two species of Chirodropida (*Chironex fleckeri* Southcott, 1956 [21,22], and *Chiropsalmus quadrumanus* (F. Müller, 1859) [19]). The sexual behaviors of a few species have been described from the field (e.g., [3,23,24]) or from tank observations (e.g., [15,25]), proving the difficulty of performing in situ observations as cubomedusae are active swimmers [26,27] with long tentacles and the ability to avoid obstacles [27] using their complex eyes [28,29]. Accordingly, only *Tripedalia cystophora* Conant, 1897, a mild stinger and the second smallest cubomedusan species, has been reared to sexual maturity in vitro (cf. [30]). Therefore, mechanisms of gametogenesis and reproductive behaviors in this small class are often inferred via methods of histomorphology rather than relying exclusively on in situ observations.

The mode of fertilization in cubomedusae can be external or internal depending on the species, but external fertilization is considered plesiomorphic in marine invertebrates (cf. [31]). External fertilization is considered typical for Chirodropida based on scant reports for just two species, *C. fleckeri* [21] and *C. quadrumanus* [19]. In contrast, internal fertilization is considered a synapomorphy of Carybdeida [2] and is well documented for two species of Tripedaliidae (*C. sivickisi* and *T. cystophora*) that exhibit complex reproductive behaviors involving spermatophore transfer [14,15,16,23,32,33].

However, reports of external fertilization for *M. virulenta* (Carukiidae) [18] cast doubts on the universality of internal fertilization in Carybdeida. Regarding periodicity, most cubomedusae are considered seasonal spawners based on the few reports of reproductive events witnessed in the field (e.g., *C. marsupialis* [34]; *C. fleckeri* [35]; *C. sivickisi* [15]). However, the scattered knowledge about carybdeid species makes it difficult to establish either semelparity or iteroparity as a universal pattern at the level of class or even order. For example, *A. alata* medusae reproduce during monthly spermcasting aggregations in which the entire gonad tissue ruptures and the reproductive cells are released [36]; it may be unique among cubozoans as a semelparous species (for references on iteroparous cubomedusae, see [19,20]).

In many gonochoristic marine organisms, sexual dimorphism can be exhibited in the form of marked phenotypic differences between males and females (e.g., in body size, color, and shape), but a disproportionate focus on a handful of "model" species has led to a skewed representation of the underlying mechanisms of the evolution of different modes of reproduction (reviewed by [8]). In cubozoans, although all species are gonochoristic, only species of Tripedaliidae exhibit sexual dimorphism with respect to gonadal shape, development, and/or color (e.g., *C. sivickisi* [15,16,17,37,38] and *T. cystophora* [23,32,37]). During gametogenesis, male and female cubomedusae develop their gonads (defined as the "area where gametes are formed" by [39], p. 142) from endodermal tissue in the bell [39,40,41,42,43]. In most cubomedusae, subtle phenotypic variation between males and females typically occurs at the level of reproductive tissues and in germ cell morphology [19,37,44]. However, reports of sexually dimorphic gonadal morphologies and patterns of gametogenesis are limited to cubomedusae that exhibit internal fertilization and exhibit sperm "packet" transfer [12], suggesting the potential for gonad morphology to infer reproductive modes in Cubozoa.

Recent histomorphological studies have elucidated spermatogenesis in several species of cubomedusae [19,20], but studies concerning oogenesis are limited to *C. marsupialis* [13] and *C. sivickisi* (as *Carybdea sivickisi* [15,17]). Most recently, oogenesis was reported in *Carybdea murrayana* Haeckel, 1880 (as *Carybdea branchi* [45]) from southern African waters, and a "maturation scale" for female sex cells was established for the first time in the class. During the process of vitellogenesis, oocytes accumulate yolk protein granules and subsequently increase in diameter. Oocyte maturation patterns corresponded to significant egg size differences documented during the oogenesis of a single cubozoan species, viz. *C. murrayana* [45]. Nevertheless, scarce information on egg size and its relation to cubomedusae maturity precludes the ability to establish a baseline of sexual maturation related to reproductive season for the 50 estimated species.

Herein, we report on the hitherto obscure reproductive strategy of the chirodropid species *C. quadrumanus* (Chirodropida, Chiropsalmidae), a relatively common species in the western tropical Atlantic from Brazil to the USA [46,47,48,49,50]. *Chiropsalmus quadrumanus* is represented by conspicuous cubomedusae (with a bell height of 10 cm and a width of 12 cm on average) which are poorly studied; for instance, a mere three sightings have been reported in the literature in the past decade [19,20,51]. This species' mode of fertilization was previously suggested to be external [19,20], but no evidence supports this claim as the life cycle and cubopolyp location in nature remain undetermined. Herein, we carry out a histolomorphological analysis to elucidate an oocyte structural maturation "scale" in a chirodropid species for the first time while aiming to infer the sexual reproductive strategy and contemplate previous reports speculating on the fertilization mode of this species. We also refute the hypothesis that sexual maturity in all cubomedusae can be inferred accurately by bell size [15,16], as our outcomes for *C. quadrumanus* fail to align with previous findings on carybdeid species.

Materials and Methods

Material Samples

Medusae of *C. quadrumanus* have been collected in the field at typical marine ecosystem salinities (20–30‰) and at shallow depths ranging from 5 m to 10 m. In the northern hemisphere, specimens have been found during the summer (May–August) [52,53] and sometimes in the fall (September) (e.g., Matagorda Bay, Texas [54]). Conversely, in the southern hemisphere (e.g., Brazil), specimens have been collected during the winter, in the dry season (July–August) [20], and sometimes during spring (March–April) [19].

In this study, we analyzed 10 females (Table 1) collected via trawling from a depth of 5 m to 40 m during the dry season (April to September) in 2008, 2010, and 2014. The specimens were collected from the São Sebastião Channel (São Paulo State, n = 2), Santos Bay and São Vicente (São Paulo State, n = 2), and Macaé (Rio de Janeiro State, n = 6) (Table 1). The medusae were fixed in 10% formaldehyde solution in seawater. The bell height (BH, from the apex of the umbrella to the margin) and interpedalial distance (IPD, distance along the bell margin between alternate pedalia) were measured for all specimens (cf. [45,55]) (Figure 1A) (Table 1). All necessary approvals for the sampling of specimens were obtained (sampling permit 16802 SISBIO/ICMBIO—Instituto Chico Mendes de Conservação da Biodiversidade). The specimens from Macaé were deposited in the collection of the Museum of Zoology (MZ), and the remaining specimens were deposited in the Marine Evolution Lab (MEL), both belonging to the University of São Paulo (Table 1).

Maturity of Specimens

Chiropsalmus quadrumanus has eight hemi-gonads arranged in four pairs, with each pair located at interradial septa [50]. Mature female medusae were distinguished based on the microscopic observation of oocytes, characterized by white spheres in the female gonads. Sexual maturity was inferred by the presence of more than 15% of gonadal oocytes in the late vitellogenic stage (Oiii) (cf. [45,56]). We propose a maturity scale for the different oocyte developmental states observed in our samples, following the scale proposed for *Carybdea murrayana* (as *Carybdea branchi* [45]), based on oocyte diameter and quantity of yolk.

Histological Analysis

Several fragments over the entire length of each gonad were dissected individually using forceps. The samples were fixed with formalin 4%, dehydrated in an ethanol series, and embedded in glycol methacrylate using the Leica Historesin Embedding Kit (Leica Microsystems Nussloch GmbH, Germany), following the manufacturer's protocol. The embedded material was cut into 5 µm transversal sections with a Leica RM2255 microtome, with ca. 12 sections set per slide (ca. 60 µm of gonad tissue); each gonad subsample was accommodated on 6–8 slides (i.e., ca. 480 µm of gonadal tissue). The slides were stained with hematoxylin–eosin (HE), toluidine blue (TB) and Gomori's trichrome (GT) (according to [57,58,59,60,61,62]) and cover-slipped using Entellan. The slides were subsequently analyzed using a Zeiss Axio Imager.M2 microscope, images enhanced with Adobe Photoshop CC 2017 version 24.0.1, and the oocytes were measured using the software ImageJ 1.53s [63].

This study is the first reported attempt to document oogenesis in jellyfish using a glycol methacrylate (historesin) method. This methodology has been successfully used in previous studies on other taxonomic groups [62,64] to obtain high-quality results.

Statistical Analysis

Several slides per specimen (ca. one third) were selected to measure the oocytes with a 4x objective lens on a compound microscope, and 12 oocytes (per tissue sample) were measured for each of the four established oogenesis stages. The maximum and minimum oocyte diameters of each specimen were averaged, and the standard deviations were calculated (Table 2). Average diameters for each stage were compared using the R package dplyr (v.1.0.7, function "summarise") to discriminate between the different maturation stages based on size, and the results were visualized using R package ggplot2 (v.3.3.3). One slide per specimen was selected to calculate the percentage of oocytes present at each stage of oogenesis. Furthermore, evidence for a correlation between cubomedusa size (interpedalial distance) and maturity (percentage of oocyte stage) was tested using the Spearman correlation method with the R package ggpubr (v.0.4.0, function "ggscatter"). The code is available as Supplementary Material.

Results

In *Chiropsalmus quadrumanus*, oogenesis presented a pattern of increased size and yolk density during the process of sexual maturation. Though late-vitellogenic oocytes were large (on average, 122 µm in diameter), all gonads were intact with no sign of ovulation underway in the cubomedusae examined.

Four different oocyte states (Figure 2 and Figure 3) are defined for *C. quadrumanus* females according to their diameter and vitellogenic content, following the scale previously established by [45]: (1) pre-vitellogenic oocytes (p), round-shaped, average diameter of 21 ± 6.88 µm, without vitellogenic content and basophil cytoplasm; (2) early-vitellogenic oocytes (Oi) with some yolk granules, average diameter of 42 ± 13.5 µm; (3) mid-vitellogenic oocytes (Oii), average diameter of 84 ± 21.6 µm; (4) late-vitellogenic oocytes (Oiii), rich in yolk granules, average diameter of 122 ± 39.1 µm. The average oocyte size differs significantly among the stages ($\chi2 = 355.82$; $df = 3$; $p < 2.2 \times 10{-}16$), although their ranges overlap (Figure 2).

Oogenesis in *C. quadrumanus* was found to be asynchronous, and oocytes of all four stages of development (p—Oiii) were observed in almost all specimens analyzed (Figure 1B, C and Table 2). All the specimens possessed pre-vitellogenic oocytes, but late-vitellogenic oocytes (Oiii) were not present in four specimens from Macaé, suggesting that they were immature despite their relatively large bells (IPD 4–5 mm). Conversely, a small specimen (IPD 3.8 mm) from the São Sebastião Channel had late-vitellogenic oocytes (Oiii), suggesting a non-linear relationship between sexual maturity and bell size. Although all females examined possessed gonads, the maximum oocyte diameter observed for each of the four stages varied between specimens collected at different geographic localities, with maximum diameters of 235 µm for the São Sebastião Channel and 113 µm for Macaé, despite the similarity of the bell sizes (IPD 4.7 mm) (Table 2).

Female gonads are composed of an epithelial bilayer corresponding to the external gastrodermal epithelium and an internal gonadal layer within the mesoglea composed of oocytes (Figure 4A). Epithelial gonadal cells are columnar with basal vacuoles (Figure 4B). While there is no developmental gradient seen along the length of the gonad, oocytes increase in size due to the accumulation of yolk (vitellogenesis) during maturation (Figure 1 and Figure 2). Histological cross-sections revealed that late-vitellogenic oocytes (Oiii) were found across the entire length of the gonad within the gonadal epithelium (Figure 1C). Neither trophocytes (specialized nutritive gastrodermal cells) nor nurse cells were found in contact with the oocytes and gastrodermal epithelium (Figure 1 and Figure 4), suggesting that mature oocytes develop freely in the mesoglea (in direct contact with the gastrodermis) (cf. [65]). However, we also report here that some specimens did show inclusions of unknown natures within the oocytes (Figure 5).

We defined mature females by the presence of gonads with >15% of oocytes in the late-vitellogenic stage [45]. Accordingly, there were four females with this pattern; therefore, they were considered sexually mature (Table 3). Although the ten female specimens had IPD values ≥ 4.4 mm, not all were found to be mature. Two specimens from Macaé (IPDs = 4.5 and 5 mm) had no late-vitellogenic oocytes. One specimen from Macaé (MA57, IPD = 4 mm) had pre-vitellogenic (14%) and early-vitellogenic (86%) oocytes, representing the most immature female studied herein (Figure 4). The Spearman correlation test indicates that the bell size of C. quadrumanus is not correlated with the proportion of late-vitellogenic oocytes present (r = 0.019; p = 0.96).

Discussion

Patterns of female sex cell maturation (oogenesis) and the reproductive strategy of the cubomedusa *C. quadrumanus* are presented for the first time based on our histological approach. Females have four different stages of oocyte development, as determined via a comparative analysis of vitellogenic content, which was present in most specimens examined (Figure 2 and Figure 3). The presence of pre-vitellogenic oocytes even in mature female gonads is interpreted as an indication of asynchronous oogenesis in which a mature female develops immature oocytes in order to reproduce more than once in her lifetime. This pattern diverges from the pattern reported for *C. murrayana* in which only late-vitellogenic oocytes were observed in mature females, suggesting a single spawning event occurring upon cubomedusae maturity [45]. Several reports of oogenesis in non-cubozoan jellyfishes (e.g., *Cassiopea andromeda*, cf. [66]) have mentioned trophocytes associated with mature oocytes during ovulation; the absence of these specialized nutritive cells in *C. quadrumanus* corroborates findings reported by [45] for the cubomedsusa *C. murayana*. The absence of embryos or planulae within the gastrovascular cavity in females, taken together with knowledge that sperm are released via the rupture of the follicle wall in males of this species [19], strongly indicates that *C. quadrumanus* exhibits external fertilization as a reproductive strategy.

Seasonality

Chiropsalmus quadrumanus is a shallow-water species inhabiting the Atlantic coast of the Americas. Its seasonality is marked by the presence of mature medusae from April to September in the Southern Hemisphere (e.g., [19,20] and this study). To date, the most sexually mature female (>37% of Oiii) was trawled in São Paulo in August (Table 2 and Table 3), suggesting that the reproductive season occurs in the dry season in the Southern Hemisphere. Occurrence data for *C. quadrumanus* for the Northern Hemisphere recorded “blooms” in Georgia in July of 1971 [52], a “predominance” in the Mississippi Sound in August of 1968 [53], and floating dead specimens in the fall (September–November) after a Texas rainy season [54]. However, a broader understanding of the distribution and seasonality of this species requires phylogenetic studies to determine whether specimens in Brazil, the Gulf of Mexico, and North Carolina [46] belong to the same species or if a complex of cryptic species exists instead (cf. [1] for a similar western Atlantic cubomedusae). Elucidating the taxonomy of the species will provide a better understanding beyond seasonality, for example, in determining possible differences in relation to its venom, an important public safety area given the case of the death of a child in the Gulf of Mexico associated with *C. quadrumanus* [67].

The female cubomedusae studied herein presented oocytes at different stages of development, but none of the females was undergoing ovulation. The presence of pre-vitellogenic oocytes in all specimens (4–22%, Table 2 and Table 3) supports the existence of continuous oogenesis, making it likely that *C. quadrumanus* cubomedusae are iteroparous (shedding their gametes more than once during their lifetime), a pattern observed in a few cubomedusae (e.g., [68]) and the tripedaliid cubozoans [15]. Although the histological data are robust, in situ data on spawning females are needed to corroborate our findings of a maturation scale and its persistence within the population in order to fully validate *C. quadrumanus* as a truly iteroparous species. Large and predictable spermcasting aggregations of the semelparous *A. alata* demonstrated that the leaf-like hemi-gonads visible on radial septa break, becoming a thin line after spawning, and stranded medusae along the rocks on the beach have varying degrees of gonadal rupture (from partial to almost complete) (more details in [3]). However, as is the case for most cubozoan species due to a dearth of histological data on cubomedusa gonad maturation, it remains difficult to corroborate observations on cubomedusae sexual reproduction in situ.

Fertilization mode and oocyte nutrition

The challenges inherent in rearing cubomedusae for observing sexual reproduction in vitro hamper the experimental study of the modes of fertilization in this class. Even without visual confirmation of sexual reproduction, the presence of fertilized eggs or planulae in the gastric cavity of collected females has served as evidence of internal fertilization in some medusozoan species [15,69]. Additionally, external fertilization in Chirodropida is inferred for *C. fleckeri* [21] and *C. quadrumanus* based on observations of sperm release via the rupture of the follicle wall [19]. Our data corroborate the external fertilization theory for *C. quadrumanus* due to the absence of sperm or embryos within the female gastrovascular cavity, but as no samples had signs of insufficient spawning, gonadal maturity may have precluded the females from signaling males of an ensuing spawning event (for a review of such genetic signals, see [25]).

The presence of inclusions of unknown nature in some oocytes (Figure 5) may indicate that the yolk is supplied to the oocytes by phagocytic bodies, a mode of vitellogenesis previously reported in at least one medusozoan (viz., *Hydra*, [70,71]). However, we avoid further speculation on their origin or function prior to conducting further investigations.

Individual size and maturity

Egg size can indicate the degree of sexual maturity [72,73,74], defined as the period in which a female is able to reproduce sexually [75]. The average oocyte diameter in *C. quadrumanus* (122 µm) is considerably larger than that reported for the cubomedusa *C. murrayana* (55 µm). This difference may be related to general medusa size, since the bell height of a mature *C. quadrumanus* specimen (>4.4 cm) is greater than that of a mature *C. murrayana* (3.68 cm, as *C. branchi* in [45]). Aside from indicating sexual maturity, egg size is often considered a proxy for understanding the reproductive and developmental traits of medusozoans, as large eggs are related to direct development [76,77,78] and are found in some deep-sea scyphomedusae [79].

Sexual maturity in medusozoans has previously been connected with bell size [15,80], but these studies have the caveat of defining the sexual maturity of a female cubomedusae simply through the presence of visible oocytes [3], during the act of ovulation [25], or stating that the gonads are full of “mature eggs” [81]. However, our maturation scale and statistical analysis thereof demonstrate that bell size is not necessarily correlated with gonadal maturity in *C. quadrumanus*. A possible explanation for the lack of correlation between bell size and sexual maturity could be that small individuals with high percentages of late-vitellogenic oocytes (e.g., specimen MA52, Table 3) might have undergone more rapid development under different environmental parameters such as temperature, food availability, or breeding period, even within the same population (cf. [3,56,80] for other medusozoan examples). Future in situ studies documenting the reproductive behavior of cubomedusae that are carried out in conjunction with histological studies of the gonads should further elucidate and complement the patterns revealed in this study.

Conclusions

In this study, we presented a female oocyte maturation scale based on histological data for the cubozoan *Chiropsalmus quadrumanus*, making it the first of its kind presented for a chirodropid. We demonstrated the existence of four distinct stages of oogenesis, congruent with an iteroparous mode of sexual reproduction with external fertilization. This framework shall serve as a standard for future investigations into sexual maturation and reproductive strategies in cubomedusae. Although large and unpredictable aggregations or “blooms’’ of *C. quadrumanus* have not been recorded on the Brazilian coast, it is important to be vigilant for potential events related to the emergence of these cubomedusae at the surface to understand the environmental conditions that favor both the sexual and asexual proliferation of this species. Thus, year-round local surveys along the Brazilian coast are needed to fully elucidate the reproductive season of *C. quadrumanus* and corroborate our hypotheses of its iteroparity. Furthermore, additional specimen collections directed at molecular phylogenetic analyses will be important to validate whether the widely distributed *C. quadrumanus* lineage (Gulf of Mexico, North Carolina, Brazil) correspond, in fact, to the same species examined in this work. We expect our findings will serve as a baseline for understanding the structural and functional maturation of female sex cells and the evolution of sexual reproductive strategies within species of the order Chirodropida.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15070816/s1.

Author Contributions. A.C.M. (André Carrara Morandini) and A.C.M. (Antonio Carlos Marques): Conceptualization, Writing—review & editing. All authors contributed to the study conception and design. J.G.-R. and A.J.-B. conceived the ideas; Material preparation, data collection, and analysis were performed by J.G.-R., A.J.-B. and G.R.T. The first draft of the manuscript was written by J.G.-R. and C.L.A., and all authors reviewed and commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding. This study was supported by the São Paulo Research Foundation (FAPESP) (grants 2011/50242-5 to A.C.M. (Antonio Carlos Marques), 2017/12970-5 to G.R.T., 2019/01370-2 and 2021/09739-5 to J.G.R.), CNPq (grants 140235/2018-3 to J.G.R., 481399/2007-0 and 309440/2019-0 to A.C.M. (André Carrara Morandini), 316095/2021-4 to A.C.M. (Antonio Carlos Marques)), and CAPES (grant 23650751852 to A.J.B.) and FAPERJ (grant E-26/171.150/2006 to A.C.M. (André Carrara Morandini)).

Data Availability Statement. Our manuscript has data included in the electronic Supplementary Material (DOI 10.5281/zenodo.7901541).

Acknowledgments. We thank R. Mohamed (University of the Western Cape, Cape Town, South Africa) for sharing oocyte data from his study with *Carybdea murrayana* (as *Carybdea branchi*) and Fernando Zara (Univiversidade Estadual Paulista, Brazil) for assistance with field samples. This manuscript was improved due to thorough review and kind feedback by external reviewers.

Conflicts of Interest. The authors declare no conflict of interest.

References

1. Collins, A.G.; Bentlage, B.; Gillan, W.; Lynn, T.H.; Morandini, A.C.; Marques, A.C. Naming the Bonaire Banded Box Jelly, *Tamoya Ohboya*, n. sp. (Cnidaria: Cubozoa: Carybdeida: Tamoyidae). Zootaxa 2011, 68, 53–68.

2. Bentlage, B.; Cartwright, P.; Yanagihara, A.A.; Lewis, C.; Richards, G.S.; Collins, A.G. Evolution of Box Jellyfish (Cnidaria: Cubozoa), a Group of Highly Toxic Invertebrates. Proc. R. Soc. B Biol. Sci. 2010, 277, 493–501.

3. Lewis, C.; Bentlage, B.; Yanagihara, A.; Gillan, W.; van Blerk, J.; Keil, D.P.; Bely, A.E.; Collins, A.G. Redescription of *Alatina alata* (Reynaud, 1830) (Cnidaria: Cubozoa) from Bonaire, Dutch Caribbean. Zootaxa 2013, 3737, 473–487.

4. Baxter, E.H.; Marr, A.G.M. Sea Wasp (*Chironex fleckeri*) Venom: Lethal, Haemolytic and Dermonecrotic Properties. Toxicon 1969, 7, 195–210.

5. Werner, B.; Cutress, C.E.; Studebaker, J.P. Life Cycle of *Tripedalia cystophora*, Conant (Cubomedusae). Nature 1971, 232, 655–657.

6. Ceh, J.; Gonzalez, J.; Pacheco, A.S.; Riascos, J.M. The Elusive Life Cycle of Scyphozoan Jellyfish—Metagenesis Revisited. Sci. Rep. 2015, 5, 12037.

7. Morandini, A.C.; Schiariti, A.; Stampar, S.N.; Maronna, M.M.; Straehler-Pohl, I.; Marques, A.C. Succession of Generations Is Still the General Paradigm for Scyphozoan Life Cycles. Bull. Mar. Sci. 2016, 92, 343–351.

8. Picard, M.A.L.; Vicoso, B.; Bertrand, S.; Escriva, H. Diversity of Modes of Reproduction and Sex Determination Systems in Invertebrates, and the Putative Contribution of Genetic Conflic. Genes 2021, 12, 1136.

9. Siebert, S.; Juliano, C.E. Sex, Polyps, and Medusae: Determination and Maintenance of Sex in Cnidarians. Mol. Reprod. Dev. 2017, 84, 105–119.

10. Eckelbarger, K.J.; Hodgson, A.N. Invertebrate Oogenesis—A Review and Synthesis: Comparative Ovarian Morphology, Accessory Cell Function and the Origins of Yolk Precursors. Invertebr. Reprod. Dev. 2021, 65, 71–140.

11. Arneson, A.C.; Cutress, C.E. Life History of *Carybdea alata* Reynaud, 1830 (Cubomedusae). In Coelenterate Ecology and Behavior; Mackie, G.O., Ed.; Springer: New York, NY, USA, 1976; pp. 227–236.

12. García-Rodríguez, J.; Lewis Ames, C.; Marian, J.E.A.R.; Marques, A.C. Gonadal Histology of Box Jellyfish (Cnidaria: Cubozoa) Reveals Variation between Internal Fertilizing Species *Alatina alata* (Alatinidae) and *Copula sivickisi* (Tripedaliidae). J Morphol 2018, 279, 841–856.

13. Avian, M.; Bonivento, P.; Montanari, G.; Rinaldi, A.; Rottini Sandrini, L. *Carybdea marsupialis* L. (Cubozoa), Istologia Delle Gonadi. Biol. Mar. Suppl. Notiz. SIBM 1993, 1, 75–78.

14. Hartwick, R.F. Observations on the Anatomy, Behaviour, Reproduction and Life Cycle of the Cubozoan *Carybdea sivickisi*. Hydrobiologia 1991, 216–217, 171–179.

15. Lewis, C.; Long, T.A.F. Courtship and Reproduction in *Carybdea sivickisi* (Cnidaria: Cubozoa). Mar. Biol. 2005, 147, 477–483.

16. Lewis, C.; Kubota, S.; Migotto, A.E.; Collins, A.G. Sexually Dimorphic Cubomedusa *Carybdea sivickisi* in Seto, Wakayama, Japan. Publ. Seto Mar. Biol. Lab. 2008, 40, 1–8. Available online: https://repository.si.edu/handle/10088/6250.

17. Garm, A.; Lebouvier, M.; Tolunay, D. Mating in the Box Jellyfish *Copula sivickisi*—Novel Function of Cnidocytes. J. Morphol. 2015, 276, 1055–1064.

18. Toshino, S.; Hiroshi, M.; Ohtsuka, S.; Okuizumi, K.; Adachi, A.; Hamatsu, Y.; Urata, M.; Nakaguchi, K.; Yamaguchi, S. Development and Polyp Formation of the Giant Box Jelly Fish *Morbakka virulenta* (Kishinouye, 1910) (Cnidaria: Cubozoa) Collected from the Seto Inland Sea, Western Japan. Plankton Benthos Res. Benthos Res. 2013, 8, 1–8.

19. Tiseo, G.R.; García-Rodríguez, J.; Zara, F.J.; Ames, C.L.; Marques, A.C.; Morandini, A.C. Spermatogenesis and Gonadal Cycle in Male *Tamoya haplonema* and *Chiropsalmus quadrumanus* (Cnidaria, Cubozoa). Zool. Anz. 2019, 279, 59–67.

20. García-Rodríguez, J.; Ames, C.L.; Marian, J.E.A.R.; Marques, A.C. Histomorphological Comparison of Testes in Species of Box Jellyfish (Cnidaria; Cubozoa): Does Morphology Differ with Mode of Reproduction and Fertilization? Org. Divers. Evol. 2020, 20, 25–36.

21. Yamaguchi, M.; Hartwick, R. Early Life History of the Sea Wasp *Chironex fleckeri* (Class Cubozoa). In Development and Cellular Biology of Coelenterates; Elsevier: Amsterdam, The Netherlands, 1980; pp. 11–16.

22. Hartwick, R.F. Distributional Ecology and Behaviour of the Early Life Stages of the Box-Jellyfish *Chironex fleckeri*. Hydrobiologia 1991, 216–217, 181–188.

23. Stewart, S. Field Behavior of *Tripedalia cystophora* (Class Cubozoa). Mar Freshw. Behav. Physiol. 1996, 27, 175–188.

24. Carrette, T.; Straehler-Pohl, I.; Seymour, J. Early Life History of *Alatina cf. moseri* Populations from Australia and Hawaii with Implications for Taxonomy (Cubozoa: Carybdeida, Alatinidae). PLoS ONE 2014, 9, e84377.

25. Lewis Ames, C.; Ryan, J.F.; Bely, A.E.; Cartwright, P.; Collins, A.G. A New Transcriptome and Transcriptome Profiling of Adult and Larval Tissue in the Box Jellyfish *Alatina alata*: An Emerging Model for Studying Venom, Vision and Sex. BMC Genom. 2016, 17, 650.

26. Hamner, W.M.; Jones, M.S.; Hamner, P.P. Swimming, Feeding, Circulation and Vision in the Australian Box Jellyfish, *Chironex fleckeri* (Cnidaria: Cubozoa). Mar. Freshw. Res. 1995, 46, 985–990.

27. Garm, A.; O'Connor, M.; Parkefelt, L.; Nilsson, D.E. Visually Guided Obstacle Avoidance in the Box Jellyfish *Tripedalia cystophora* and *Chiropsella bronzie*. J. Exp. Biol. 2007, 210, 3616–3623.

28. Berger, E.W. Physiology and Histology of the Cubomedusae, including Dr. F. S. Conant's Notes on the Physiology; Memoirs from the Biological Laboratory of the Johns Hopkins University; The Johns Hopkins Press: Baltimore, MD, USA, 1900; Volume 4, 84p.

29. Coates, M.M. Visual Ecology and Functional Morphology of Cubozoa (Cnidaria). Integr. Comp. Biol. 2003, 43, 542–548.

30. Werner, B. Killermedusen und Ihr Liebesspiel. Umschau 1976, 76, 80–81.

31. Subramoniam, T. Mode of Reproduction: Invertebrate Animals. In Encyclopedia of Reproduction; Elsevier: Amsterdam, the Netherlands, 2018; pp. 32–40. ISBN 9780128151457.

32. Werner, B. Spermatozeugmen und Paarungsverhalten Bei *Tripedalia cystophora* (Cubomedusae). Mar. Biol. 1973, 18, 212–217.

33. Helmark, S.; Garm, A. Gonadal Cnidocytes in the Cubozoan *Tripedalia cystophora* Conant, 1897 (Cnidaria: Cubozoa). J. Morphol. 2019, 280, 1530–1536.

34. Bordehore, C.; Fuentes, V.L.; Atienza, D.; Barberá, C.; Fernandez-Jover, D.; Roig, M.; Acevedo-Dudley, M.J.; Canepa, A.J.; Gili, J.M. Detection of an Unusual Presence of the Cubozoan *Carybdea marsupialis* at Shallow Beaches Located near Denia, Spain (South-Western Mediterranean). Mar. Biodivers. Rec. 2011, 4, E69.

35. Keesing, J.K.; Strzelecki, J.; Stowar, M.; Wakeford, M.; Miller, K.J.; Gershwin, L.A.; Liu, D. Abundant Box Jellyfish, *Chironex* sp. (Cnidaria: Cubozoa: Chirodropidae), Discovered at Depths of over 50 m on Western Australian Coastal Reefs. Sci. Rep. 2016, 6, 22290.

36. Lawley, J.W.; Ames, C.L.; Bentlage, B.; Yanagihara, A.; Goodwill, R.; Kayal, E.; Hurwitz, K.; Collins, A.G. Box Jellyfish *Alatina alata* Has a Circumtropical Distribution. Biol. Bull. 2016, 231, 152–169.

37. Straehler-Pohl, I.; Garm, A.; Morandini, A.C. Sexual Dimorphism in Tripedaliidae (Conant 1897) (Cnidaria, Cubozoa, Carybdeida). Zootaxa 2014, 3785, 533–549.

38. Toshino, S.; Miyake, H.; Iwanaga, S. Development of *Copula sivickisi* (Stiasny, 1926) (Cnidaria: Cubozoa: Carybdeidae: Tripedaliidae) Collected from the Ryukyu Archipelago, Southern Japan. Plankton Benthos Res. 2014, 9, 32–41.

39. Campbell, R.D. Cnidaria. Reprod. Mar. Invertebr. 1974, 1, 133–199.

40. Hyman, L.H. Metazoa of the Tissue Grade of Construction—The Radiate Phyla—Phylum Cnidaria. In The Invertebrates: Protozoa through Ctenophora; McGraw-Hill: New York, NY, USA; London, UK, 1940; Volume 1.

41. Widersten, B. Genital Organs and Fertilization in Some Scyphozoa. Zool. Bidr. Fran Upps. 1965, 37, 45–58.

42. Miller, R.L. Cnidaria. Reprod. Biol. Invertebr. 1983, 2, 23–73.

43. Marques, A.C.; Collins, A.G. Cladistic Analysis of Medusozoa and Cnidarian Evolution. Invertebr. Biol. 2004, 123, 23–42.

44. Southcott, R.V. Studies on Australian Cubomedusae, Including a New Genus and Species Apparently Harmful to Man. Mar. Freshw. Res. 1955, 7, 254–280.

45. Mohamed, R.; Skrypzeck, H.; Gibbons, M.J. Describing Gonad Development and Gametogenesis in Southern Africa’s Endemic Box Jellyfish *Carybdea branchi* (Cubozoa, Carybdeidae). Afr. J. Mar. Sci. 2019, 41, 83–91.

46. Mayer, A.G. Medusae of the World; Publication no. 109; Carnegie Institution of Washington: Washington, DC, USA, 1910; Volume 3.

47. Migotto, A.E.; Marques, A.C.; Morandini, A.C.; da Silveira, F.L. Checklist of the Cnidaria Medusozoa of Brazil. Biota Neotrop. 2002, 2, 1–31.

48. Morandini, A.C.; Ascher, D.; Stampar, S.N.; Ferreira, J.F.V. Cubozoa e Scyphozoa (Cnidaria: Medusozoa) de Águas Costeiras Do Brasil. Iheringia Ser. Zool. 2005, 95, 281–294.

49. Nogueira, M., Jr.; Haddad, M.A. Macromedusae (Cnidaria) from the Paraná coast, southern Brazil. J. Coast. Res. 2006, 2, 1161–1164.

50. Gershwin, L.A. Comments on Chiropsalmus (Cnidaria: Cubozoa: Chirodropida): A Preliminary Revision of the Chiropsalmidae, with Descriptions of Two New Genera and Two New Species. Zootaxa 2006, 42, 1–42.

51. Nogueira Júnior, M. Article Gelatinous Zooplankton Fauna (Cnidaria, Ctenophora and Thaliacea) from Baía da Babitonga (Southern Brazil). Zootaxa 2012, 3398, 1–21.

52. Guest, W.C. The Occurence of the Jelllyfish *Chiropsalmus quadrumanus* in Matagorda Bay, Texas. Bull. Mar. Sci. 1959, 9, 79–83.

53. Kraeuter, J.N.; Setzler, E.M. The Seasonal Cycle of Scyphozoa and Cubozoa. Bull. Mar. Sci. 1975, 25, 66–74.

54. Phillips, P.J.; Burke, W.D. The Occurrence of Sea Waps (Cubomedusae) in Mississipi Sound and the Northern Gulf of Mexico. Bull. Mar. Sci. 1970, 20, 853–859.

55. Straehler-Pohl, I. Critical Evaluation of Characters for Species Identification in the Cubomedusa Genus *Malo* (Cnidaria, Cubozoa, Carybdeida, Carukiidae). Plankton Benthos Res. 2014, 9, 83–98.

56. Pitt, K.A.; Kingsford, M.J. Reproductive Biology of the Edible Jellyfish *Catostylus mosaicus* (Rhizostomeae). Mar. Biol. 2000, 137, 791–799.

57. Humason, G.L. Animal Tissue Techniques; W.H. Freeman and Company: San Francisco, CA, USA, 1962.

58. Behmer, O.A.; Tolosa, E.M.C.; Freitas Neto, A.G. Manual de Tecnicas Para Histologia Normal e Patologica; Livraria Editora: São Paulo, Brazil, 1976.

59. Bancroft, J.; Stevens, A. Theory and Practice of Histological Techniques; Churchill Livingstone: London, UK, 1982.

60. Pearse, A.G.E. Histochemistry Theoretical and Applied. In Analytical Tehnology; Churchill Livingstone: London, UK, 1985; Volume 2.

61. Junqueira, L.C. Histology Revisited—Technical Improvement Promoted by the Use of Hydrophilic Resin Embedding. Cienc. Cult. 1995, 47, 92–95.

62. Mendoza-Becerril, M.A.; Marian, J.E.A.R.; Migotto, A.E.; Marques, A.C. Exoskeletons of Bougainvilliidae and Other Hydroidolina (Cnidaria, Hydrozoa): Structure and Composition. PeerJ 2017, 5, e2964.

63. Schneider, C.; Rasband, W.; Eliceiri, K.W. NIH Image to ImageJ: 25 Years of Image Analysis. Nat. Methods 2010, 9, 671–675.

64. Marian, J.E.A.R. Spermatophoric Reaction Reappraised: Novel Insights into the Functioning of the Loliginid Spermatophore Based on *Doryteuthis plei* (Mollusca: Cephalopoda). J. Morphol. 2012, 273, 248–278.

65. Eckelbarger, K.J.; Larson, R. Ultrastructure of the Ovary and Oogenesis in the Jellyfish *Linuche unguiculata* and *Stomolophus meleagris*, with a Review of Ovarian Structure in the Scyphozoa. Mar. Biol. 1992, 114, 633–643.

66. Mammone, M.; Bosch-Belmar, M.; Milisenda, G.; Castriota, L.; Sinopoli, M.; Allegra, A.; Falautano, M.; Maggio, T.; Rossi, S.; Piraino, S. Reproductive Cycle and Gonadal Output of the Lessepsian Jellyfish *Cassiopea andromeda* in NW Sicily (Central Mediterranean Sea). PLoS ONE 2023, 18, e0281787.

67. Bengston, K.; Nichols, M.M.; Schnadig, V.; Ellis, M.D. Sudden Death in a Child Following Jellyfish Envenomation by *Chiropsalmus quadrumanus*: Case Report and Autopsy Findings. JAMA J. Am. Med. Assoc. 1991, 266, 1404–1406.

68. Eckelbarger, K.J.; Larson, R.J. Ultrastructural Study of the Ovary of the Sessile Scyphozoan, *Haliclystus octoradiatus* (Cnidaria: Stauromedusae). J. Morphol. 1993, 218, 225–236.

69. Toshino, S.; Miyake, H.; Srinui, K.; Luangoon, N.; Muthuwan, V.; Sawatpeera, S.; Honda, S.; Shibata, H. Development of *Tripedalia binata* Moore, 1988 (Cubozoa: Carybdeida: Tripedaliidae) Collected from the Eastern Gulf of Thailand with Implications for the Phylogeny of the Cubozoa. Hydrobiologia 2017, 792, 37–51.

70. Alexandrova, O.; Schade, M.; Böttger, A.; David, C.N. Oogenesis in *Hydra*: Nurse Cells Transfer Cytoplasm Directly to the Growing Oocyte. Dev. Biol. 2005, 281, 91–101.

71. Honegger, T.G.; Zürrer, D.; Tardent, P. Oogenesis in *Hydra carnea*: A New Model Based on Light and Electron Microscopic Analyses of Oocyte and Nurse Cell Diferentiation. Tissue Cell 1989, 21, 381–393.

72. Toyokawa, M.; Shimizu, A.; Sugimoto, K.; Nishiuchi, K.; Yasuda, T. Seasonal Changes in Oocyte Size and Maturity of the Giant Jellyfish, *Nemopilema nomurai*. Fish. Sci. 2010, 76, 55–62.

73. Iguchi, N.; Lee, H.E.; Yoon, W.D.; Kim, S. Reproduction of the Giant Jellyfish, *Nemopilema nomurai* (Scyphozoa: Rhizostomeae), in 2006–2008 as Peripherally-Transported Populations. Ocean. Sci. J. 2010, 45, 129–138.

74. Jarms, G.; Tiemann, H.; Båmstedt, U. Development and Biology of *Periphylla periphyll*a (Scyphozoa: Coronatae) in a Norwegian Fjord. Mar. Biol. 2002, 141, 647–657.

75. Schiariti, A.Í.; Christiansen, E.; Morandini, A.C.; da silveira, F.L.; Giberto, D.A.; Mianzan, H.W. Reproductive Biology of *Lychnorhiza lucerna* (Cnidaria: Scyphozoa: Rhizostomeae): Individual Traits Related to Sexual Reproduction. Mar. Biol. Res. 2012, 8, 255–264.

76. Hargitt, G.T. Germ Cells of Coelenterates. VI. General Considerations, Discussion, Conclusions. J. Morphol. 1919, 33, 1–59.

77. Jarms, G.; Båmstedt, U.; Tiemann, H.; Martinussen, M.B.; Fosså, J.H.; Høisœter, T. The Holopelagic Life Cycle of the Deep-Sea Medusa *Periphylla periphylla* (Scyphozoa, Coronatae). Sarsia 1999, 84, 55–65.

78. Berrill, N.J. Developmental Analysis of Scyphomedusae. Biol. Rev. 1949, 24, 393–409.

79. Russell, F.S. On the Scyphomedusa *Poralia rufescens* Vanhöffen. J. Mar. Biol. Assoc. UK 1962, 42, 387–390.

80. Lucas, C.H.; Reed, A.J. Gonad Morphology and Gametogenesis in the Deep-Sea Jellyfish *Atolla wyvillei* and *Periphylla periphylla* (Scyphozoa: Coronatae) Collected from Cape Hatteras and the Gulf of Mexico. J. Mar. Biol. Assoc. UK 2010, 90, 1095–1104.

81. Toshino, S.; Miyake, H.; Shibata, H. Development of *Carybdea brevipedalia* Kishinouye, 1891 (Cnidaria: Cubozoa: Carybdeida: Carybdeidae) Collected from Northern Japan. Plankton Benthos Res. 2018, 13, 116–128.

Figures

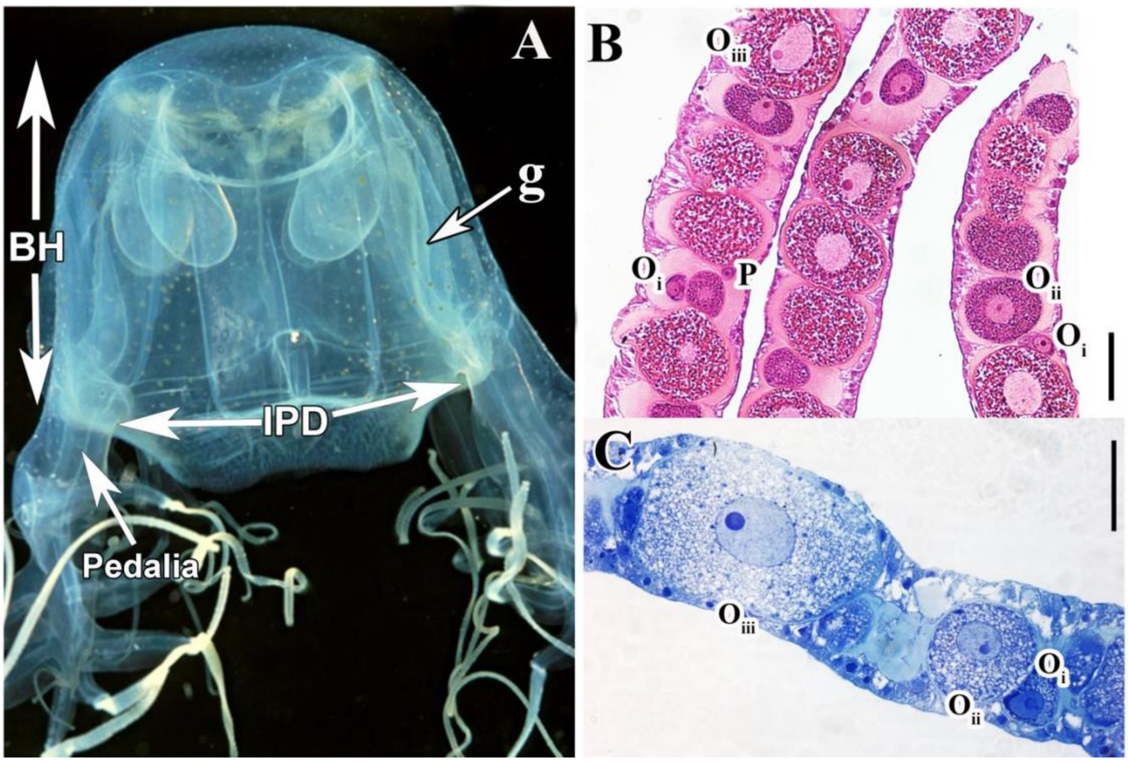

Figure 5. (A). Chiropsalmus quadrumanus live specimen [20]. **(B, C).** Gonadal transversal sections of females of C. quadrumanus from São Sebastião Channel bearing oocytes in the four different stages of development. **(B).** Specimen (CH6, Table 1) stained with toluidine blue (TB). Scale bars 50 µm. BH—bell height; IPD—interpedalial distance; g—gonads; Oiii—late-vitellogenic oocyte; Oii—mid-vitellogenic oocyte; Oi—early-vitellogenic oocyte; P—pre-vitellogenic oocyte.

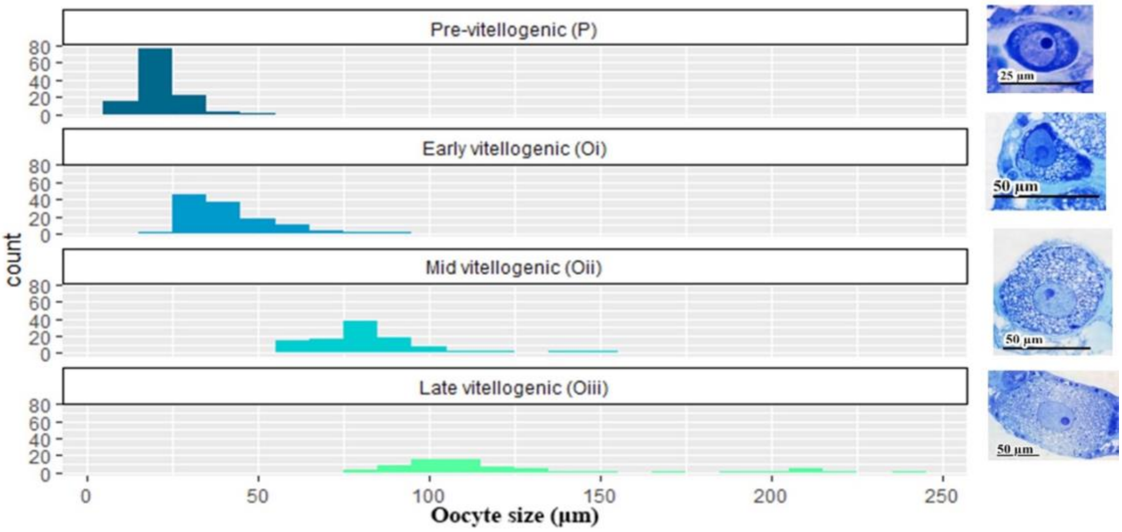

Figure 6. Distribution of oocyte diameter for each of the four stages of development in *C. quadrumanus*. Images on the right reveal morphological variations in oocyte features, including vitellogenic content.

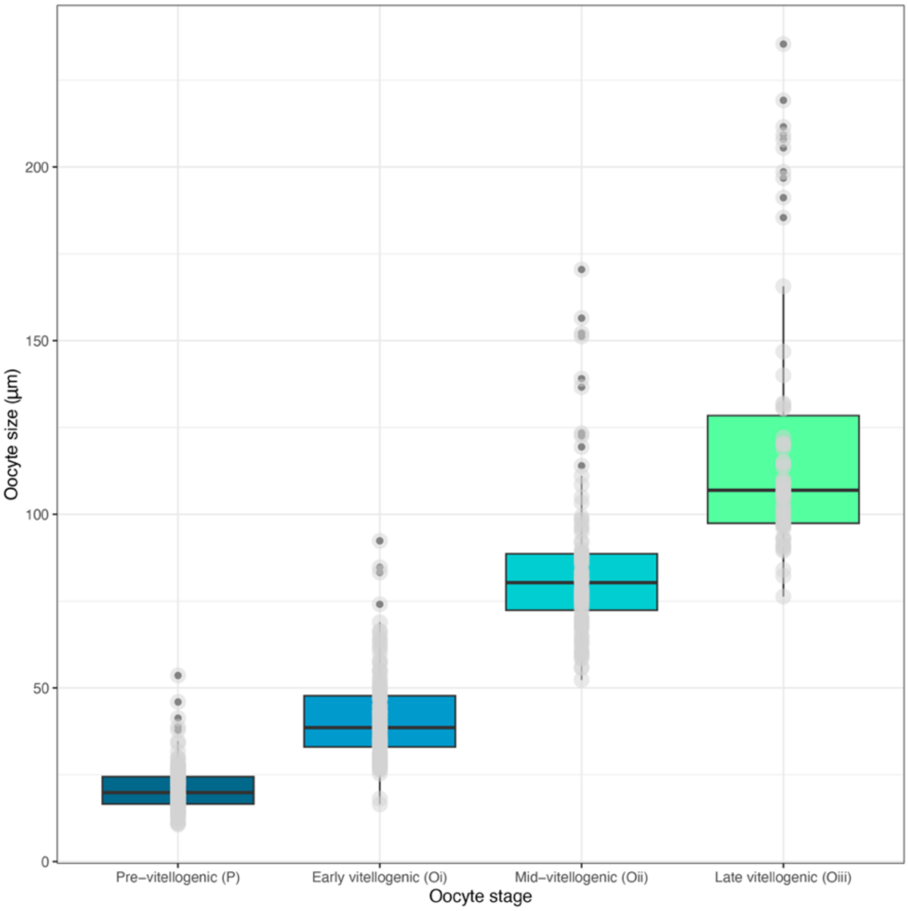

Figure 7. Boxplot representing the four stages of development based on the oocyte diameter of *Chiropsalmus quadrumanus.*

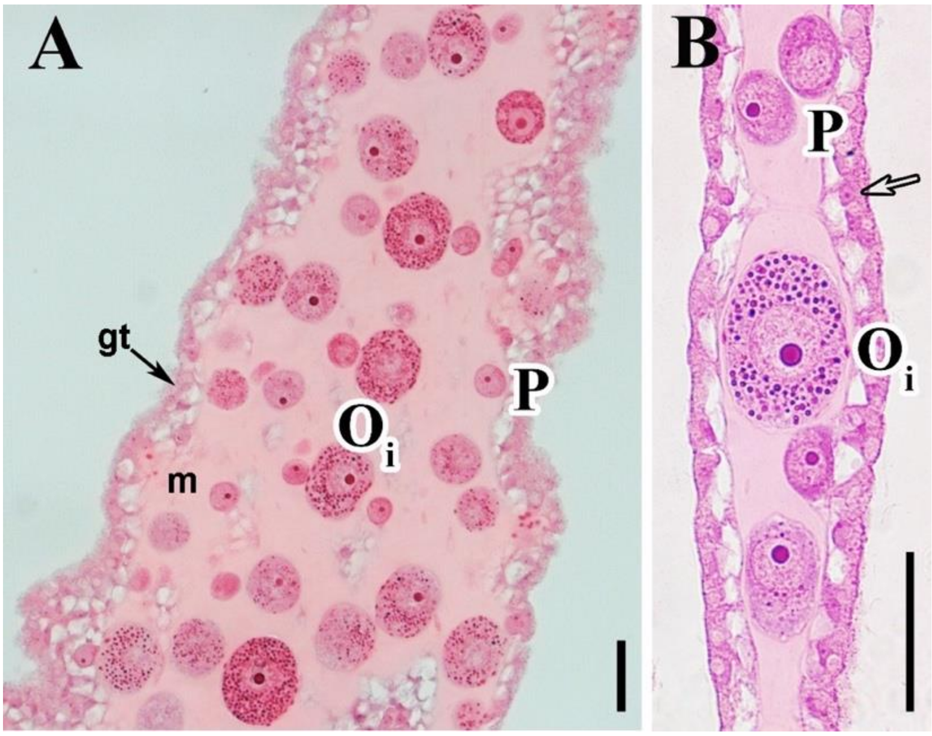

Figure 8. Gonadal section of the most immature studied female of *Chiropsalmus quadrumanus* (from Macaé, Rio de Janeiro). Stained with Gomori trichrome and hematoxylin (GT + H) and HE, respectively **(A, B).** Arrows show the columnar gonadal epithelium with basal vacuoles. Scale bars: 50 µm. gt—gastrodermal epithelium; m—mesoglea; Oi—early-vitellogenic oocyte; P = pre-vitellogenic oocyte.

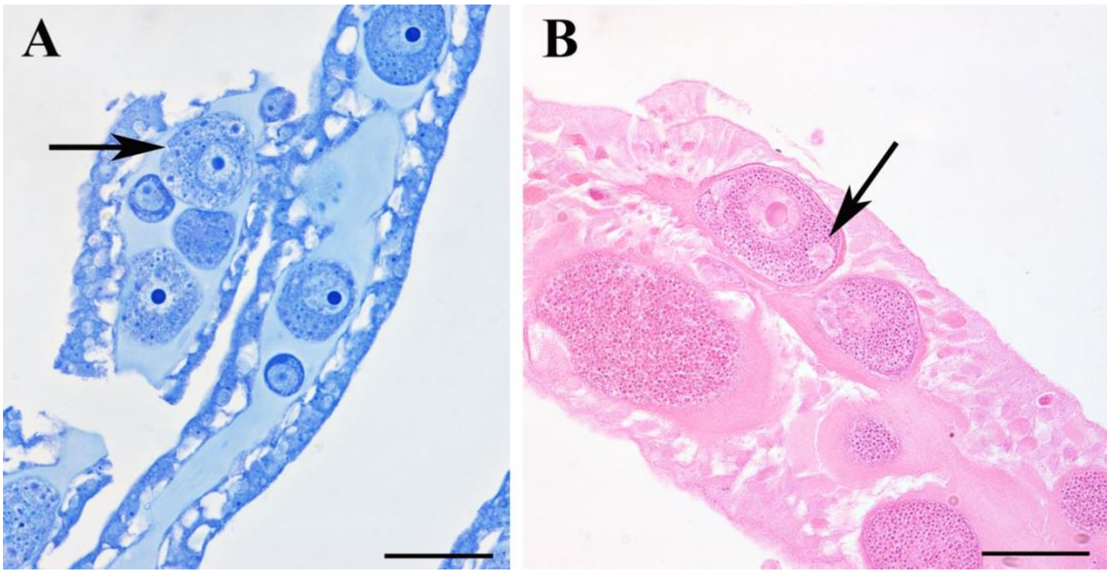

Figure 9. Gonads section of two different specimens of *Chiropsalmus quadrumanus* (from Macaé, Rio de Janeiro) stained with toluidine blue **(A)** and HE **(B)**, respectively. Arrows show inclusions of unknown natures within the oocytes. Scale bars 50 µm.

Tables

Table 1. Material data for *Chiropsalmus quadrumanus* females examined in this study. Abbreviation: MEL: Marine Evolution Lab; MZUSP: Museum of Zoology of the University of São Paulo; RJ: Rio de Janeiro; SP: São Paulo.

Specimen number	**Museum number (MZUSP) and MEL number**	**Bell height (mm)**	**Interpedalial distance (mm)**	**Locality**	**Depth (m)**	**Date (Y/M/D)**	**Collector**
CH2	MEL-CH2	4.5	3.8	São Sebastião Channel (SP)	10	2014/06/10	JGR
CH6	MEL-CH6	6.5	4.7	São Sebastião Channel (SP)	10	2014/08/19	JGR
MA49	MZUSP-1920	6.5	4.5	Macaé (RJ)	5-10	2008/09/13	F.P.L. Marques
MA50	MZUSP-1921	7.5	5	Macaé (RJ)	5-10	2008/09/13	F.P.L. Marques
MA52	MZUSP-1923	6.5	4.4	Macaé (RJ)	5-10	2008/09/13	F.P.L. Marques
MA53	MZUSP-1924	7	4.7	Macaé (RJ)	5-10	2008/09/13	F.P.L. Marques
MA56	MZUSP-1927	7	5	Macaé (RJ)	5-10	2008/09/13	F.P.L. Marques
MA57	MZUSP-1928	5.4	4	Macaé (RJ)	5-10	2008/09/13	F.P.L. Marques
53A	MEL-53A	6.7	10.54	Baía de Santos e São Vicente (SP)	10-40	2010/04	Tiseo, G. R.; Zara, F. J.
54C	MEL-54C	5.6	8	Baía de Santos e São Vicente (SP)	10-40	2010/04	Tiseo, G. R.; Zara, F. J.

Table 2. Measurements of oocyte diameter (maximum, minimum, average, standard deviation) in each stage of gonadal development of *Chiropsalmus quadrumanus*. Blank cells indicate no oocytes of that specific size category was observed in the sample. Oiii = late-vitellogenic oocyte, Oii = mid-vitellogenic oocyte, Oi = early vitellogenic oocyte, P = pre-vitellogenic oocyte.

Specimen number	**Oiii** diameter (µm)				**Oii** diameter (µm)				**Oi** diameter (µm)				**P** diameter (µm)			
	Max	Min	Average	Standard deviation	Max	Min	Average	Standard deviation	Max	Min	Average	Standard deviation	Max	Min	Average	Standard deviation
CH2	140.014	76.292	110.796	22.54687	75.617	52.308	62.1977	6.2079	48.703	16.492	28.484	7.81417	31.68	11.544	20.5662	5.98748
CH6	235.397	146.857	197.781	23.70315	170.486	99.398	132.068	22.297	92.352	49.656	69.34	12.3727	53.582	13.919	28.242	13.5341
MA49					83.02	58.667	74.9411	7.3262	61.273	34.359	46.186	8.19608	26.104	16.271	20.8476	3.6763
MA50					98.593	70.107	85.8052	8.959	58.029	31.074	42.923	8.00621	37.838	14.411	24.7551	7.08516
MA52	131.602	96.736	114.187	10.50545	97.173	65.079	81.6818	11.168	59.958	26.203	38.339	10.4808	27.778	13.889	20.9081	4.47119
MA53	113.525	95.89	103.493	5.407642	87.199	60.336	76.8158	8.5688	41.82	27.397	33.926	4.1092	34.013	13.784	20.6236	5.4142
MA56					119.398	75.18	90.0476	13.532	63.774	26.698	43.078	10.8529	27.68	10.99	20.4653	5.60585
MA57									47.507	33.333	38.761	4.44204	23.292	10.623	16.8126	4.18917
53A	122.144	89.443	101.199	10.10663	91.952	56.063	79.3338	8.9946	57.007	25.298	37.208	10.46	24.828	14.717	19.9075	3.43497
54C	131.966	82.262	99.711	15.20876	88.511	62.639	74.9315	8.3674	61.837	27.402	39.457	9.03138	29.829	14.583	20.7193	5.1459

Table 3. Percentage of oocytes in the four different stages of the oogenesis of *Chiropsalmus quadrumanus*. *Mature females, IPD = Interpedalial distance (mm), Oiii = late-vitellogenic oocyte, Oii = mid-vitellogenic oocyte, Oi = early-vitellogenic oocyte, P = pre-vitellogenic oocyte.

Specimen number	IPD	Oiii %	Oii %	Oi %	P %	Sampling Month
CH2	3.8	6.45	45.16	41.94	6.45	June/2014
CH6*	4.7	37.68116	27.53623	24.63768	10.14493	August/2014
MA49	4.5	0	8.474576	83.89831	7.627119	September/2008
MA50	5	0	38.70968	38.70968	22.58065	September/2008
MA52*	4.4	35.38462	47.69231	12.30769	4.615385	September/2008
MA53*	4.7	26.05042	35.29412	24.36975	14.28571	September/2008
MA56	5	0	62.63736	32.96703	4.395604	September/2008
MA57	4	0	0	85.65022	14.34978	September/2008
53A*	10.54	32.91139	44.3038	14.76793	8.016878	April/2010
54C	8	4.659498	60.9319	28.67384	5.734767	April/2010

Capítulo 3

Novel features and role of sexual reproduction in jellyfishes of the class Cubozoa (Cnidaria: Medusozoa)

Jimena García-Rodríguez[1], Cheryl Lewis Ames[2,3], Amanda Ferreira Cunha[4], Allen G. Collins[5, 6] and Antonio Carlos Marques[1]

[1] Department of Zoology, Institute of Biosciences, University of São Paulo, R. Matão, Tv. 14, 101, 05508-090, São Paulo, Brazil

[2] Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington DC, 20013-7012, USA

[3] Graduate School of Agricultural Science, Faculty of Agriculture, Tohoku University, Sendai, 980-8572, Japan

[4] Departamento de Biologia Animal, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa, Av. P.H. Rolfs, s/n, 36570-900, Viçosa, Brazil

[5] NOAA's National Marine Fisheries Service, Office of Science & Technology

[6] Curator for Medusozoa, Ctenophora, Porifera, National Museum of Natural History, Smithsonian Institution, 10th & Constitution Avenue, Washington, DC 20560 USA

Abstract

Cubozoa, a small class within the subphylum Medusozoa (Cnidaria), is notable for its complex sexual behaviors, setting it apart from other medusozoan classes. The reproductive traits and seasonality of several species are yet to be determined, partly due to the challenges in collecting these animals. This study unveils novel sexual traits by characterizing cubozoan reproductive tissue (gonads) using histological methods. We examined 15 cubozoan species representing males and females of various maturity stages across two orders, seven families and eight genera of Cubozoa. The findings reveal several reproductive attributes unique to some species of this important group within prebilaterian taxa. These include: (1) variation in oocyte shape, such as hexagonal oocytes, characterizing an alternative method of oocyte arrangement; (2) previously unrecognized inclusions within oocytes, suggesting a unique vitellogenesis process in Cnidaria; (3) the development of swollen structures, implying a novel spawning strategy; (4) the occurrence of internal fertilization within the gonad in *Tripedalia cystophora*, indicated by the presence of blastulae; and (5) a diverse repertoire of nematocysts within the gonads, including nematoblasts in non-tripedaliid species, in species with both internal and external fertilization, suggesting the existence of different development pathways for these subcellular organelles. The discovery and characterization of these sexual traits in cubozoan jellyfishes provides crucial baseline data, that can contribute significantly to comparative studies aimed at elucidating the diversity of reproductive strategies in cnidarians.

Keywords: Box-jellyfish, sexual reproduction, oocyte packing, vitellogenesis, oocytes inclusions, nematocyst

Introduction

Jellyfish from the four classes of the subphylum Medusozoa (Hydrozoa, Scyphozoa, Staurozoa, and Cubozoa) (Marques and Collins 2004) often display metagenetic life cycles (Campbell 1974; Collins 2002; Müller and Leitz 2002; Morandini et al. 2016), with sexual reproduction predominantly occurring in the medusa stage, where the gonadal region is located (Werner 1973; Collins 2002; Marques and Collins 2004). Similar to many marine invertebrates, the reproductive and behavioral patterns of Medusozoans are not well-documented due to the challenges in observing sexual behaviors in their natural habitat, and maintaining them alive in laboratory conditions to maturity for the observation of sexual reproduction (Tyler et al. 1982). Anatomical, morphological, and histological studies of gonadal structures in preserved specimens have been instrumental in overcoming these challenges, providing valuable insights into the sexual reproduction of these species (Eckelbarger and Larson 1992; Pitt and Kingsford 2000; Morandini and Silveira 2001). Even so, given the limited number of such studies, many aspects of medusozoan sexual reproduction, and that of cubozoans notwithstanding, remain poorly understood (e.g., Migotto 1998; Garm et al. 2015).

Medusozoans exhibit a wide range of reproductive strategies and life cycles (Fautin 2002; Lewis Ames 2018). Hydrozoans, for example, have undergone several evolutionary modifications, showing various degrees of reduction or even loss of the medusa stage (Govindarajan et al. 2006; Cartwright and Nawrocki 2010). This pattern results in species with fixed gonophores in which the polyps functionally produce the gametes (e.g., *Cordylophora caspia*, Schuchert 2004), or a reduced medusa or medusoid (e.g., *Nemalecium lighti*, Gravier-Bonnet and Migotto 2000), or fully developed free medusa (e.g., *Clytia linearis*, Lindner and Migotto 2002). In Scyphozoa and Cubozoa, different arrangements of gamete packing within reproductive structures have been observed, such as spermatophores and spermatozeugmata, resulting in functionally efficient strategies to transfer the sperm to the female mate (Hofmann and Hadfield 2002; García-Rodríguez et al. 2018; Mammone et al. 2023).

The literature provides limited information regarding the developmental processes involved in gametogenesis within Medusozoa. Notably, gene expression related to vitellogenin was observed in the cubozoan *Alatina alata* during an aggregation event coinciding with ovulation (Lewis Ames et al. 2016). Indeed, two vitellogenic processes (the formation of nutritive yolk for oocytes) are known to be involved with oocyte development (Eckelbarger 2005) – heterosynthesis, in which females produce yolk proteins that are incorporated into oocytes through endocytosis (e.g., in *Aurelia aurita*, Eckelbarger and Larson 1988); and autosynthesis, in which oocytes produce yolk inclusions using their proteosynthetic organelles, such as the Golgi apparatus and rough endoplasmic reticulum (RER) (for discussion in other invertebrate taxa, see Eckelbarger 2005). The widespread occurrence of these vitellogenic processes across animal species suggests that they are primitive mechanisms, employed in various combinations according to the distinct life histories of different metazoans (Eckelbarger and Hodgson 2021).

Oogenesis has been more extensively studied than spermatogenesis in certain medusozoan groups (e.g., Widersten 1965 and Schiariti et al. 2012, both for Scyphozoa). This process involves specialized or accessory cells that contribute to nutrition and/or oocyte maturation across three medusozoan classes. In some Hydrozoans, putative nurse cells, connected to developing oocytes by cytoplasmic bridges, have been identified (Honegger et al. 1989). Particularly in *Hydra*, these nurse cells are phagocytosed by the developing oocyte post-apoptosis (Alexandrova et al. 2005). Conversely, in the scyphozoan orders Semaeostomeae and Rhizostomeae, specialized gastrodermal cells, known as trophocytes, are associated with oocytes to facilitate the uptake of exogenous yolk proteins (Eckelbarger and Larson 1988). In stark contrast, oocytes of holoplanktonic Coronatae scyphozoans do not exhibit a specialized association with gastrodermal cells within the gonads and instead are located freely within the mesoglea (Eckelbarger and Larson 1992, for Linuche unguiculata). In staurozoans, oocytes within ovarian follicles are encircled by follicular cells and, although the function of these cells remains unclear (Eckelbarger and Larson 1993), they may perform similar roles to nurse cells (see Eckelbarger 2005 for examples in other taxa). Interestingly, cubozoans are devoid of accessory cells in oogenesis, and recent studies indicate that their oocytes develop freely within the mesoglea (Mohamed et al. 2019; García-Rodríguez et al. 2023), paralleling the pattern observed in coronates.

This study focuses on the class Cubozoa, particularly the medusae (cubomedusae) within this relatively small clade of Medusozoa, which comprises approximately 50 known species (Collins, 2024). Cubozoans exhibit complex sexual reproductive traits that are not typically observed in other classes. Notably, species within the family Tripedaliidae demonstrate unique behaviors such as courtship and sperm transfer (Stewart 1996; Lewis and Long 2005). Over the past decade, substantial research efforts have revealed detailed histomorphological traits of spermatogenesis in cubomedusae (García-Rodríguez et al. 2018, 2020; Tiseo et al. 2019). These studies have shown that spermatogenesis occurs within sperm follicles in species such as *Carybdea marsupialis*, *Chiropsalmus quadrumanus*, *Morbakka virulenta*, and *Tamoya haplonema* (García-Rodríguez et al. 2020), with sperm subsequently being released into the gastrovascular cavity and externally by the rupture of the gonadal epithelium (García-Rodríguez et al. 2018, 2020; Tiseo et al. 2019). Among the taxa studied so far, two species from distinct families, *Alatina alata* and *Copula sivickisi*, have been identified to engage in internal fertilization, employing differing methods of sperm packaging, namely spermatophore and spermatozeugma, respectively (García-Rodríguez et al. 2018).

A significant characteristic of cubozoans is the potent toxicity exhibited by some species. *Chironex fleckeri*, regarded as the most dangerous jellyfish to humans (Bolte et al. 2021), has been responsible for numerous stings in tropical Australian waters, occasionally resulting in fatalities (Williamson et al. 1984; Lumley et al. 1988). Another species, *Carukia barnesi*, is known to cause Irukandji syndrome, characterized by severe skin pain and systemic symptoms (Fenner and Harrison 2000). The source of this harm is specialized stinging organelles in cnidarians, known as nematocysts, which are secreted by specialized cells called nematocytes. These organelles primarily serve to defense and prey capture (Mariscal 1974), therefore nematocysts are typically concentrated in the medusa tentacles. However, in certain cubozoan species, nematocysts are also found in warts on the exumbrellar surface (Calder and Peters 1975), as well as within the gonads, embryo strands, and subgastric sacs (Garm et al. 2015; García-Rodríguez et al. 2018; Helmark and Garm 2019). The latter observations have led to speculations about their potential roles in protecting spermatozoa during transfer to the female or during the release of the embryo strand (Helmark and Garm 2019). Cubozoans are not unique in this regard, as previous studies on Hydrozoa have reported eggs associated with nematocysts, presumably for protective purposes (Szollosi 1969; Piraino 1992; Migotto 1996; Marques and Calder 2000; Denitto et al. 2007). These nematocysts are believed to originate from maternal tissue, developing from the ectoderm and actively migrating to the oocyte surface (Piraino 1992). The presence and hypothesized function of nematocysts in reproductive processes underscore their complex role in medusa adaptation, survival, and proliferation. Nonetheless, the extent to which nematocysts are incorporated into male and/or female gonads and their functions within these structures, remain unknown due to limited research in this area.

Although studies on cubozoan gametogenesis remains relatively limited, there is a greater body of literature on spermatogenesis (García-Rodríguez et al. 2018, 2020; Tiseo et al. 2019) compared to oogenesis (e.g., Mohamed et al. 2019; García-Rodríguez et al. 2023). This study aims to provide a more balanced understanding of reproductive processes in both sexes of cubomedusae. To this end, we utilized histomorphological data to delve into the gonadal features of both males and females, with a particular focus on oogenesis, as well as on the role of accessory cells and nematocysts in the reproductive structures. We provided new data on sexual characteristics across several species from seven different cubozoan families, thereby enabling an evaluation of the variation in sexual traits within the class. This research offers a detailed examination of oogenesis, gamete packing phenomena, and the presence of nematocysts in reproductive structures. As such, this constitutes one of the most comprehensive overviews of sexual reproduction for the class Cubozoa to date.

Material and Methods

Material samples

Subsamples of mature and immature Cubozoan medusae, preserved in formalin, were obtained from the collections of the National Museum of Natural History (NMNH), Smithsonian Institution, Washington DC, USA, and the Marine Evolution Laboratory (MEL) at the University of São Paulo, Brazil. In this sense, we highlight the significant benefits of utilizing curated museum collections in this kind of scientific research. The sex of the specimens and their maturity were determined by the presence of developed gonads under an Olympus SZX16 dissecting microscope. This study analyzed 35 specimens representing 15 different cubozoan species at various developmental stages (Tables 1-2). The bell height (BH), measured from the apex of the umbrella to the center of the projected margin, and the interpedalial distance (IPD), the distance between alternate pedalia along the bell margin, were recorded for each specimen analyzed (cf. Straehler-Pohl 2014; Mohamed et al. 2019; García-Rodríguez et al. 2023) (Table 1).

Histological analysis

Gonadal tissue samples were carefully dissected from each specimen using forceps under an Olympus SZX16 dissecting microscope. The preparation of specimens from MEL followed a tissue dehydration protocol using an alcohol reverse dilution series, then staining as per García-Rodríguez et al. (2018). For NMNH specimens, a modified version of this protocol was employed, detailed in (Appendices I-II). Additionally, a third protocol was utilized, involving the embedding kit Technovit 7100 Glycol Methacrylate. All samples were serially sectioned into 5 µm cuts using a MT990 microtome and mounted with Entellan™.

Oocyte stages

Four oocyte stages were defined for the classification of female medusa maturity: pre-vitellogenic (P), early-vitellogenic (Oi), mid-vitellogenic (Oii), and late vitellogenic (Oiii). This classification was based on the oocyte diameter and vitellogenic content, drawing upon previous research conducted on two cubozoan species, *Carybdea murrayana* (originally referred to as *Carybdea branchi* in Mohamed et al. 2019) and *Chiropsalmus quadrumanus* (García-Rodríguez et al. 2023).

Results

Order Carybdeida Gegenbaur, 1857

Family Alatinidae Gershwin, 2005

Alatina alata (Reynaud, 1830)

Specimens (n= 4): one mature male sectioned (USNM 54385) with intermittent sets of sperm at mesoglea. No spermatic follicles observed (Fig. 1A). Large umbrellar size (bell height 8 cm, interpedalial distance 5.4 cm, Table 1) indicates that specimen probably already spawned. Simple cuboidal gastrodermal epithelium vacuolated with glandular cells (Fig. 1B). Sections of three additional mature females (USNM 1205447, 1195801 and 1195803) with oocytes in late vitellogenesis filled with lipid droplets, showed previously unreported hexagonal oocytes with nuclei at apical position of cell (Fig. 1C-D).

Family Carukiidae Bentlage, Cartwright, Yanagihara, Lewis, Richards & Collins, 2010

Morbakka virulenta (Kishinouye, 1910)

Specimen (n=1): one mature female dissected with asynchronous oogenesis (USNM 1124251) inferred by the presence of oocytes with different maturity stages (early and mid-vitellogenic oocytes). Inclusions of unknown nature within oocytes (Fig. 2A), similar to those reported as reminiscent in *Chiropsalmus quadrumanus* (García-Rodríguez et al. 2023). Apparent absence of mesoglea within oocytes must be artifactual. Nematocysts at gastrodermal epithelium (Fig. 2B).

Family Carybdeidae Gegenbaur, 1857

Carybdea arborifera Maas, 1897

Specimens (n=3): two females (USNM 22304) with asynchronous oogenesis inferred by presence of oocytes within the mesoglea in early and mid-vitellogenesis with visible nuclei. Gastrodermal epithelium poorly preserved (Fig. 3A-B); one mature male (bell height 3 cm, interpedalial distance 2 cm, Table 1) (USNM 22304) with spermatic follicles preserved likely after spawning (Fig. 3C).

Carybdea brevipedalia Kishinouye, 1891

Specimens (n=3): one mature female (USNM 1154099) with simple columnar gastrodermal epithelium and asynchronous oogenesis, nearly spawning an oocyte in late vitellogenesis (Fig. 4A); one mature female likely preserved during spawning (Fig. 4B); one immature male (USNM 1154103) with spermatic follicles (Fig. 4C).

Carybdea marsupialis (Linnaeus, 1758)

Specimens (n=2): one mature female (MEL CM_1F) with columnar gastrodermal epithelium (Fig. 5A) exhibiting asynchronous oogenesis (Fig. 5B) with oocytes at mesoglea in late-vitellogenesis filled with lipid droplets.

Carybdea murrayana Haeckel, 1880 (as *Carybdea branchi* Gershwin & Giboons, 2009)

Specimens (n=2): one female (USNM 1124255) likely preserved after spawned, with early vitellogenic oocytes close to gastrodermal epithelium and mesoglea free of oocytes (Fig. 6A); one mature male (USNM 1124258) with spermatic follicles filled with sperm (Fig. 6B). Both specimens with nematocysts at gastrodermal epithelium.

Carybdea rastonii Haacke, 1886

Specimens (n=3): two mature females (USNM 88365) with asynchronous oogenesis, showing oocytes in two different stages of maturity, and nematocysts at gastrodermal epithelium (Fig. 7A). Oocytes with lipid droplets covering mesoglea, some in early vitellogenesis. One immature male (USNM 88365) with spermatic follicles and nematocysts at gastrodermal epithelium (Fig. 7B).

Carybdea xaymacana Conant, 1897

Specimens (n=4): one spawning female (USNM 54462) showing some oocytes with inclusions of unknown nature (Fig. 8A); one mature male (USNM 54450) with spermatic follicles. Gonad with swollen areas probably preceding spawning (Fig. 8B-C), as observed in one of these swellings (Fig. 8C). Gonads with simple cuboidal vacuolated epithelium. Sperm released into the gastrovascular cavity by gonadal opening. Samples with some preparation artifacts (white gaps).

***Carybdea* sp.**

Specimen (n=1): one mature female (USNM 1155724) spawning with some hexagonal oocytes (similar to *A. alatina* above), presumably packed within mesoglea (Fig. 9).

Family Tamoyidae Haeckel, 1880

Tamoya haplonema F. Müller, 1859

Specimens (n=2): two females, similar in size (Table 1), with free oocytes at mesoglea. One female (MEL-TH3) with oocytes in early and mid-vitellogenesis exhibiting asynchronous oogenesis (Fig. 10A); second female (MEL-TH2) showing simple vacuolated gastrodermal epithelium, likely preserved after spawning, with fewer oocytes at mesoglea (Fig. 10B-C). Inclusions of unknown nature within oocytes in some sections (as per other taxa above, see *M. virulenta* and *C. xaymacana*) (Fig. 10A, C-D).

Family Tripedaliidae Conant, 1897

Copula sivickisi (Stiasny, 1926)

Specimens (n=1): one mature female (MEL-F1-4(1) with asynchronous oogenesis; oocyte developmental gradient from septum toward apical section (Fig. 11A) (corroborating description by Lewis and Long 2005). Oocytes in early-vitellogenesis proximate to interradial septum, later stage oocytes distant from interradial septum and in expanded gonads (Fig. 11B). Nematocysts and nematoblasts in simple gastrodermal epithelium (Fig. 11C).

Tripedalia cystophora Conant, 1897

Specimens (n=3): one female (USNM 54410) with fertilized eggs (at blastulae stage), and with nematocysts within mesoglea (Fig.12A); one female (USNM 54410) with blastulae inside gastrovascular canal (Fig. 12B); one mature male (USNM 54410) with spermatic follicles full of sperm, with nematocysts and nematoblasts at gastrodermal epithelium (Fig. 12C).

Order Chirodropida Haeckel, 1880

Family Chirodropidae Haeckel, 1880

Chironex fleckeri Southcott, 1956

Specimens (n=2): one immature female (USNM 100353) with inclusions of unknown nature within some oocytes (as per other taxa above, see *M. virulenta, C. xaymacana* and *T. haplonema*) (Fig. 13A), bearing nematocysts and nematoblasts at gastrodermal epithelium (Fig. 13A-B); one immature male (USNM 1155730) with spermatic follicles lacking sperm or nematocysts within gonads (Fig. 13C).

Chironex yamaguchii Lewis & Bentlage, 2009

Specimens (n=3): mature female (USNM 28692) with hexagonal oocytes and asynchronous oogenesis, showing early and late-vitellogenic oocytes (Fig. 14A); mature male (USNM 28694) with sperm in spermatic follicles (Fig. 14B).

Family Chiropsellidae Toshino, Miyake & Shibata, 2015

Chiropsella bronzie Gershwin, 2006

Specimens (n=1): immature female (USNM 1131157) with asynchronous oogenesis, showing oocytes in early and mid-vitellogenesis (Fig. 15). Neither inclusions in oocytes nor nematocysts within gonads were observed.

Discussion

Female gonads and gamete packing

Hexagonal shapes, frequently observed in nature (e.g., honeycombs and crystals), represent an efficient organizational strategy for arranging objects, including cells, while supporting structural and functional integrity (Sugimura and Ishihara 2013). This geometric configuration efficiently maximizes space usage while minimizing the length of connecting edges. Polygonal oocytes within ovaries have been described for hydrozoans (Galea et al. 2012; Galea 2018). Though the adaptive benefit of this configuration for hydrozoans is unknown, such ovarian arrangement, resembling honeycomb packing, has been reported for other invertebrate taxa (e.g., polychaetes, Eckelbarger 2005) involving different oocyte developmental stages, such as in immature gastropods (Shepherd and Laws 1974), and mature nematodes (Rudel et al. 2005). This suggests a beneficial strategy for packing gonads.

In this study, we observed polygonal oocytes are densely packed within the gonads, leaving little visible mesoglea in females of different cubozoan taxa, such as *Alatina alata*, *Carybdea* sp., and *Chironex yamaguchi* (Figs. 1C, 9, 14A, respectively). This represents the first account of honeycomb gamete packing within female gonads in cubomedusae. Previous research has documented similar packing in the spermatic follicles of cubozoan males, particularly in species like *Copula sivickisi*, which exhibit internal fertilization and sperm transfer (García-Rodríguez et al. 2018).

Oocytes with yolk bodies as accessory nuclei cells

Cnidarians are recognized for utilizing their mesoglea as a pathway for nutrient transportation from the gastrovascular cavity to the oocytes, facilitating a heterosynthetic mechanism for yolk formation (Eckelbarger and Hodgson 2021). Our research indicates that cubozoan oocytes undergo vitellogenesis within the mesoglea, paralleling patterns observed in Anthozoa (Eckelbarger et al. 1998; Shikina et al. 2013) and Scyphozoa (Eckelbarger and Larson 1992), and evidently not being comparable with the thin and delicate hydrozoan mesoglea (e.g., Mendoza-Becerril et al. 2017). Interestingly, this process in Cubozoa appears to occur without the participation of supporting or accessory cells, such as trophocytes, which have been reported in at least one scyphozoan species (*Periphylla periphylla*, Tiemann and Jarms 2010). Accessory cells, including nurse cells and follicle cells surrounding the oocytes (Eckelbarger and Larson 1993), have been identified in Hydrozoa and Staurozoa, respectively (Freeman 1987; Honegger et al. 1989; Eckelbarger and Larson 1993), suggesting diverse oocyte-somatic cell relationships across cnidarian clades.

Despite vitellin being a prevalent egg protein in the animal kingdom (Hagedorn and Kunkel 1979; Chen et al. 1997), details about its production by females and incorporation into developing oocytes (vitellogenesis) remain limited to Medusozoa, more specifically to species of Hydrozoa (Kessel 1968; Spracklin 1984) and Scyphozoa (Eckelbarger and Larson 1988; Avian and Sandrini 1991). In Cubozoa (as with Staurozoa), gene expression related to vitellogenesis has been documented in *Alatina alata* during ovulation (Lewis Ames et al. 2016), yet the underlying mechanisms of vitellogenesis are not fully understood. Optical microscopy studies have revealed lipid droplets in the oocytes of *C. quadrumanus* (García-Rodríguez et al. 2023) but the details regarding their origin remains unclear. Our findings highlight the presence of unidentified electron-dense, spherical structures in the yolk of oocytes from five cubozoan species (*Carybdea xaymacana*, *Chironex fleckeri*, *Chiropsalmus quadrumanus*, *Morbakka virulenta*, and *Tamoya haplonema*), matching those recently reported as unknown oocyte inclusions in *C. quadrumanus* (García-Rodríguez et al. 2023). These inclusions, observed during early and mid-stages of vitellogenesis (Figs. 2, 10C-D, 13A) and disappearing when yolk granules become prominent (Fig. 10D), may comprise proteinaceous yolk spheres, lipid droplets, and glycogen particles as reported for other taxa (for details see Eckelbarger and Hodgson 2021).

In other invertebrate taxa, various yolk oocyte inclusions of different origins, referred to as spherical bodies, have been documented (e.g., Schmekel and Fioroni 1974). In the tunicate ovary, follicular and test cells contain prominent Golgi systems, numerous vesicles and cisternae of endoplasmic reticulum, and typical mitochondria (Kessel and Kemp 1962). Yolk globules are formed through the biosynthetic activity of the Golgi and RER in Platyhelminthes (Falleni et al. 2006), while sea urchins exhibit yolk granules or platelets (Armant et al. 1986). Scorpions show nucleolar extrusions and Golgi yolk (Nath 1925); *Lumbricus* has yolk-nucleus formations (Bronte Gatenby and Nath 1926), and insects display accessory nuclei (Jaglarz et al. 2008).

The evidence gathered in this study points to the identification of accessory nuclei as the primary candidate for the spherical bodies observed in the oocytes. Indeed, these inclusions are structurally similar to accessory nuclei by the observation of an outer envelope adjacent to the cytoplasm of the spherical inclusions (Fig. 2). Notably, multiple inclusions were observed within a single oocyte, with some instances showing up to four inclusions (Fig. 2A), suggesting they are individually delimited by a membrane. This supports the notion of an autosynthetic contribution to yolk production in Cubozoa. Autosynthetic yolk production is typically associated with long-lived, iteroparous species (Eckelbarger and Hodgson 2021), such as *C. quadrumanus* (discussed in García-Rodríguez et al. 2023). This process entails extended periods of vitellogenesis and prolonged intervals between breeding episodes (Eckelbarger and Hodgson 2021). Consequently, our findings suggest that cubozoan species exhibiting these unique accessory nuclei may have the capacity for multiple reproductive cycles throughout their lifespan.

Investigating the molecular and cellular signaling mechanisms in vitellogenesis during medusozoan oogenesis could provide fundamental insights into the evolution of metazoan oogenesis and its ecological implications. Employing advanced electron microscopy techniques, particularly cryo-SEM, to examine these putative accessory nuclei in Cubozoa will enhance our understanding of yolk production evolution and its ecological significance, particularly concerning medusa blooms in marine ecosystems.

Spawning

In medusozoan species, two primary mechanisms for gamete release into the gastrovascular cavity have been identified. The first involves release through a gonopore, often accompanied by accessory mucus cells, as observed in *Periphylla periphylla* (Tiemann and Jarms 2010), or via a gametoduct that develops upon gonad maturation, a feature characteristic of Staurozoa (Miranda et al. 2016). The second mechanism is characterized by the rupture of the gonad wall, a phenomenon documented in scyphozoans (Schiariti et al. 2012) and cubomedusae, in which studies noted specific rupture sites along the gonad in species such as *Alatina alata*, *Tamoya* cf. *haplonema* (García-Rodríguez et al. 2018, 2020), and *Copula sivickisi* (as *Carybdea sivickisi* in Lewis and Long 2005).

Our observations revealed that oocytes are expelled through the gastrodermis in taxa that exhibit external fertilization, such as in *Carybdea brevipedalia* (Toshino et al. 2018) (Fig. 4B), and in those with internal fertilization like *A. alata* (Lewis Ames et al. 2016). The female gonad typically forms a blind sac, and our findings suggest that in most cases, mature oocytes are released instinctively through rupture sites at the weakest points of the gastrodermis. This process appears to occur without the involvement of any accessory cells, which aligns with our inability to identify nurse cell candidates despite conducting an extensive histomorphological study of multiple female cubozoans.

We identified distinctive morphological features in the gonads of a male specimen of *Carybdea xaymacana*. Notably, the gonads develop swollen areas that are visually and structurally different from the rupture sites observed in other species examined in this study, such as *Carybdea arborifera* (Fig. 3C). The gonads of *Carybdea xaymacana* are particularly remarkable, exhibiting a unique morphology that resembles “small balls” visible to the naked eye (this study). *Carybdea arborifera* (Fig. 3C) shows a similar method of sperm release, however, no swollen areas were observed along the gonads. We hypothesize that these swellings may be specific to mature males of only a few species. To confirm whether this is an isolated case, additional sectioning of mature *Carybdea xaymacana* is necessary.

In the context of female reproductive biology, an intriguing question pertains to the nature of ovulated material. Our observations indicate that, in some species, nuclei positioned apically within oocyte (Figs. 4A, 4B and Fig. 9) may indicate the female is about to spawn.

Fertilization in Cubozoa

In Cubozoa, fertilization can occur either externally, in the surrounding water (Yamaguchi and Hartwick 1980), or internally, within the gastrovascular cavity (Lewis Ames et al. 2016) or even inside the female gonad (Garm et al. 2015). External fertilization is regarded as a plesiomorphic trait (an ancestral character state) in Medusozoa (Marques and Collins 2004) and typically takes place in the water column. This mode of fertilization has been documented in species such as *Morbakka virulenta* (Toshino et al. 2013), *Chironex fleckeri* (Yamaguchi and Hartwick 1980), and is inferred for *C. quadrumanus* due to the presence of mature oocytes but absence of planulae in the gastrovascular cavity (García-Rodríguez et al. 2023). Conversely, some cubomedusae exhibit internal fertilization, characterized by courtship behaviors and sperm storage, as seen in the family Tripedaliidae (Hartwick 1991; Lewis and Long 2005; Garm et al. 2015; García-Rodríguez et al. 2018).

Regarding internal fertilization, the site of oocyte fertilization varies by species. One scenario involves fertilization within the gonad (e.g., *Copula sivickisi*, Garm et al. 2015) as observed in *Tripedalia cystophora*, where planulae develop inside the gonad (Fig. 12A). In such cases, spermatozoa must penetrate the gonad either through existing or temporarily formed pits, pores, or sinuses, or by being engulfed (phagocytosed) by epithelial cells (Garm et al. 2015). Another scenario is fertilization within the gastrovascular cavity. Although embryos have not been reported in the gastrovascular cavity in histological studies, interactions between sperm and ovum within the gastrovascular cavity of *A. alata* have been documented prior to the release of blastulae into the water (Lewis Ames et al. 2016). This suggests that fertilization may also occur in the gastrovascular cavity.

Nematocyst role within the gonads

The nematocyst, a specialized stinging organelle, is a synapomorphy of the phylum Cnidaria (Arai 1996; Marques and Collins 2004). In this regard, as a universal and fundamental characteristic of the group, nematocysts are expected to interact with various systems of cnidarian biology, including the reproductive system. However, our findings indicates that the occurrence of nematocysts, or their precursors, nematoblasts, does not uniformly manifest within the gonads of both sexes of certain cubozoan species. For instance, female specimens of *Chironex fleckeri* and *Morbakka virulenta* (García-Rodríguez et al. 2020) were found to possess nematocysts within their gonads, whereas their male counterparts did not, despite these species engaging in external fertilization (Table 2). This disparity raises the hypothesis that nematocysts may not be present, or at least not detectable, in the immature males of these species, as evidenced in the case of *C. fleckeri* and *M. virulenta* (García-Rodríguez et al. 2020). However, the limited number of samples examined makes it challenging to assure this as a general characteristic across the species.

An alternative hypothesis concerns the potential function of nematocysts in female gonads. It is conceivable that these organelles serve a protective role for the oocyte (Garm et al. 2015; García-Rodríguez et al. 2018), safeguarding it until fertilization, which occurs in the surrounding water. This hypothesis aligns with the notion that nematocysts, while primarily associated with defense and prey capture, may have evolved additional, specialized roles in cnidarians, particularly in the context of reproduction.

In members of the Tripedaliidae family within the Cubozoa class, nematocysts have been documented in the gonads of both male and female medusae, such as in *Copula sivickisi*. Notably, the specific types of nematocysts differ between male and female gonads. In females, the presence of eurytele nematocysts in the "embryo strand" of *C. sivickisi* is believed to protect the embryos from predators as they are deposited onto the substrate for planulae release (Lewis and Long 2005; Toshino et al. 2014; Garm et al. 2015). García-Rodríguez et al. (2018) further identified that *C. sivickisi* possesses nematocyst nests within the subgastric sacs, from which nematocysts are hypothesized to be incorporated into the embryo strand as it is released by the female, demonstrating a morphologically and phylogenetically concerted function. Conversely, males of *C. sivickisi* are thought to use their isorhiza nematocysts to anchor the spermatophore to the female gonad, aiding in fertilization (Garm et al. 2015). This sexually dimorphic cnidome in cubozoan gonads highlights the specialized adaptations these non-bilaterian metazoans have evolved for successful reproduction.

Conversely, in another well-studied Tripedaliidae species, *Tripedalia cystophora*, the absence of nematocysts in female gonads has been reported (Helmark and Garm 2019). Unlike *C. sivickisi*, *T. cystophora* does not produce an embryo strand; rather, blastulation occurs internally within the female gastrovascular cavity and/or gonads (Stewart 1996; Garm et al. 2015; Helmark and Garm 2019; this study, Fig. 12A-B). Contrary to previous observations, our data from histological sections of *T. cystophora* revealed the presence of eurytele-type nematocysts within the female gonads (Fig. 12A). Heterotrichous euryteles have been reported in almost all species within Tripedaliidae. They are found on the tentacles of polyps and on the exumbrella warts of medusae of *T. binata* (Toshino et al. 2017), tentacles of polyps and medusae of *T. cystophora* (Straehler-Pohl and Jarms 2011), and on tentacles and exumbrellar warts of medusae of *C. sivickisi* (Toshino et al. 2014). Additionally, other nematocysts, including prolate spheroid p-rhopaloids and ovoid isorhizas, have been reported for these species (Straehler-Pohl and Jarms 2011; Toshino et al. 2014, 2017). The presence of euryteles in the female gonads of both *C. sivickisi* (Garm et al. 2015) and *T. cystophora* (this study) suggests that this nematocyst type may be specific to the gonads in female tripedaliid medusae species. The occurrence of nematocysts within the gonads of species producing embryo strand, such as *C. sivickisi*, implies a protective role for the planulae upon their release into the ocean. Our study also included the examination of blastulae sections from the gastrovascular cavity of *T. cystophora* (Fig. 12B). Notably, this observation does not provide a definitive conclusion, as the absence of nematocysts could be due to a preparation artifact that may have caused their detachment from the mucus during sample handling.

Our research has identified the presence of nematocysts within the gastrodermal epithelium of the gonads in several non-Tripedaliidae cubozoan species. This includes members of the family Carybdeida, such as Carybdeidae *Carybdea murrayana* (Fig. 6) and *Carybdea rastonii* (Fig. 7), as well as Carukiidae *Morbakka virulenta* (Fig. 2B), and Chirodropida, such as Chirodropidae *Chironex fleckeri* (Fig. 13A-B). However, in a contrasting observation, a recent study on *Chiropsalmus quadrumanus*, another Chirodropida species but belonging to the family Chiropsalmidae, did not report the presence of gonadal nematocysts despite comprehensive investigations of oogenesis in numerous specimens (García-Rodríguez et al. 2023).

The discovery of nematocysts within the reproductive structures of these medusae highlights the complex and varied roles of these organelles throughout different life stages and anatomical regions of medusozoans. However, the specific function of these nematocysts, particularly in relation to gametogenesis and reproduction, remains a topic for further exploration. While reproductive and protective functions have been identified in species that exchange sperm packages, a form of putative copulation, the presence of nematocysts in species that undergo external fertilization, such as *M. virulenta* and *Chironex fleckeri*, or internal fertilization without sperm package transfer, like *Carybdea murrayana*, calls for more studies focusing on the functional significance of gonadal nematocysts to the reproductive biology of Cubozoans.

The study of nematogenesis, which refers to the development of nematocysts, remains largely unexplored in cubozoans, with notable exceptions in the Tripedaliidae family, particularly *Copula sivickisi*, for which specialized nematocyst nests were described within its subgastric sacs (García-Rodríguez et al. 2018). Our research extends this understanding by documenting nematoblasts, the precursor cells to nematocysts, within the gonads of several cubozoan species. Specifically, nematoblasts were observed in females of *C. sivickisi* (Fig. 11B), males of *Tripedalia cystophora* (Fig. 12C), and females of *Chironex fleckeri* (Fig. 13B).

Investigations into other classes of Medusozoa have revealed distinct structures associated with nematogenesis. For instance, in Hydrozoa, nematogenesis has been identified in specific nests (Yamada et al. 2007), and in Staurozoa, white spots frequently associated with gonads have been reported as sites of nematocyst development (Miranda et al. 2016). The identification of nematoblasts in locations other than the tentacles, such as in subgastric sacs (García-Rodríguez et al. 2018), nematocyst warts (*C. sivickisi*, this study, Fig. 11A), and gonads (*T. cystophora* and *C. fleckeri*, this study), indicates the presence of diverse nematogenic sites within Tripedaliidae. This finding suggests a more complex and varied process of nematocyst development than previously understood. Our study contributes to this understanding by demonstrating the presence of nematoblasts in nematocyst warts in *C. sivickisi* (Fig. 11A) and in the gastrodermal epithelium of *T. cystophora* and *C. fleckeri* (Figs. 12 and 13, respectively), in this last case supporting the hypothesis that gonads may also serve as a site for nematogenesis. Although we did not observe large number of nematoblasts within the gonads that could further support their origination in these reproductive structures, we also did not find evidence to support the scenario of nematocysts being transported as nematoblasts to the gonads, where they would then complete their development. Based on current knowledge, we hypothesize at least four potential sites of nematocyst formation in cubozoans: (1) in the subgastric sacs (e.g., García-Rodríguez et al. 2018); (2) in tentacles (Lewis Ames 2018); (3) in nematocyst warts (this study); and (4) in the gastrodermal epithelium (this study). The identification of these four distinct sites of nematocyst development in cubozoans not only highlights the multifaceted nature of these stinging organelles but also underscores their innovative roles in the biology of these organisms. This expanded view of nematocyst formation and functionality accentuates the complex life history and ecological adaptations of cubozoans, offering a richer perspective on their role in marine ecosystems.

Conclusions

From an invertebrate reproductive biology perspective, the Cubozoa class distinguishes itself from other medusozoans through its diverse and complex reproductive strategies. These strategies underscore the intricate and innovative evolutionary characteristics associated with their sexual reproduction processes and behaviors. Key findings in cubozoans, unique among non-bilaterians, such as: (1) the frequent occurrence of polygonal oocytes with a honeycomb structure; (2) a distinctive vitellogenic process characterized by electron-dense yolk inclusions which we have identified as putative accessory nuclei – these nuclei suggest an autosynthetic approach to yolk production; (3) fertilization inside the gonad have been corroborated due to the presence of planulae; (4) presence of both gonadal nematocysts and nematoblasts indicates the gonads as potential sites of nematogenesis. These features add layers of sexual complexity and set Cubozoa apart within the phylum Medusozoa.

Our comprehensive study, examining the gonadal histomorphologies of 15 cubozoan species, roughly 1/3 of the species diversity of the class, represents the most extensive research to date in this area. It significantly enhances our understanding of cubozoan reproductive strategies, a group that has traditionally been challenging to study. We hope that these findings will encourage further research within the scientific community, particularly with the objective of tracing the ancestral states of these reproductive characteristics from a phylogenetic standpoint (Fig. 16). Such investigations are crucial for shedding light on the unique features that distinguish the Cubozoa group from other classes within the Medusozoa classes.

References

Alexandrova O, Schade M, Böttger A, David CN (2005) Oogenesis in *Hydra*: Nurse cells transfer cytoplasm directly to the growing oocyte. *Developmental Biology*, *281*, 91–101. doi: 10.1016/j.ydbio.2005.02.015

Arai MN (1996) A functional biology of Scyphozoa. Springer Science & Business Media.

Armant R, Carson DD, Decker GL, Welply JK, Lennarz WJ (1986) Characterization of Yolk Platelets isolated from Developing Embryos of *Arbacia punctulata*. *Developmental biology*, *113*(2), 342-355. doi: 10.1016/0012-1606(86)90169-7

Avian M, Sandrini LR (1991) Oocyte development in four species of scyphomedusa in the northern Adriatic Sea. *Hydrobiologia, 216,*189–195. doi: 10.1007/BF00026461

Bolte B, Goldsbury J, Huerlimann R, Jerry D, Kingsford M (2021) Validation of eDNA as a viable method of detection for dangerous cubozoan jellyfish. *Environmental DNA, 3,* 769–779. doi: 10.1002/edn3.181

Bronte Gatenby J, Nath V (1926) The oogenesis of certain Invertebrata, with special reference to *Lumbricus*. *Journal of Cell Science*, *2*(279), 371-389.

Calder DR, Peters EC (1975) Nematocysts of *Chiropsalmus quadrumanus* with comments on the systematic status of the Cubomedusae. *Helgoländer Wissenschaftliche Meeresuntersuchungen, 27,* 364–369. doi: 10.1007/BF01611701

Campbell RD (1974) Cnidaria. Reproduction of Marine invertebrates 1:133–199.

Cartwright P, Nawrocki AM (2010) Character evolution in Hydrozoa (phylum Cnidaria). *Integrative and Comparative Biology*, *50*, 456–472. doi: 10.1093/icb/icq089

Chen JS, Sappington TW, Raikhel AS (1997) Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *Journal of Molecular Evolution*, *44*, 440–451. doi: 10.1007/PL00006164

Collins AG (2002) Phylogeny of Medusozoa and the evolution of cnidarian life cycles. *Journal of Evolutionary Biology*, *15*, 418–432. doi: 10.1046/j.1420-9101.2002.00403.x

Collins AG (2024) World List of Cubozoa. Cubozoa. Accessed through: World Register of Marine Species at: https://www.marinespecies.org/aphia.php?p=taxdetails&id=135219 on 2024-01-27

Denitto F, Miglietta MP, Boero F (2007) Life cycle of *Bougainvillia nana* (Cnidaria: Hydrozoa: Bougainvilliidae) from Italy, including a discussion of *Bougainvillia muscus* in the Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom, 87,* 853–857. doi: 10.1017/S0025315407054574

Eckelbarger KJ (2005) Oogenesis and oocytes. *Hydrobiologia 535,* 179–198. doi: 10.1007/s10750-004-4397-y

Eckelbarger KJ, Hodgson AN (2021) Invertebrate oogenesis–a review and synthesis: comparative ovarian morphology, accessory cell function and the origins of yolk precursors. *Invertebrate Reproduction and Development*, *65*, 71–140. doi: 10.1080/07924259.2021.1927861

Eckelbarger KJ, Larson R (1992) Ultrastructure of the ovary and oogenesis in the jellyfish *Linuche unguiculata* and *Stomolophus meleagris,* with a review of ovarian structure in the Scyphozoa. *Marine Biology*, *114*, 633–643. doi: 10.1007/BF00357260

Eckelbarger KJ, Larson RJ (1993) Ultrastructural study of the ovary of the sessile scyphozoan, *Haliclystus octoradiatus* (Cnidaria: Stauromedusae). *Journal of Morphology*, *218*, 225–236. doi: 10.1002/jmor.1052180210

Eckelbarger KJ, Larson RL (1988) Ovarian morphology and oogenesis in *Aurelia aurita* (Scyphozoa: Semaeostomae): ultrastructural evidence of heterosynthetic yolk formation in a primitive metazoan. *Marine Biology*, *100*, 103–115. doi: 10.1007/BF00392960

Eckelbarger KJ, Tyler PA, Langton RW (1998) Gonadal morphology and gametogenesis in the sea pen *Pennatula aculeata* (Anthozoa: Pennatulacea) from the Gulf of Maine. *Marine Biology*, *132*, 677-690. doi: 10.1007/s002270050432

Falleni A, Lucchesi P, Ghezzani C, Silveira M, Gremigni V (2006) Ultrastructural and cytochemical aspects of the female gonad of *Geoplana burmeisteri* (Platyhelminthes, Tricladida, Terricola). *Journal of Morphology*, *267*, 318–332. doi: 10.1002/jmor.10405

Fautin DG (2002) Reproduction of Cnidaria. *Canadian Journal of Zoology*, *80*, 1735–1754. doi: 10.1139/z02-133

Fenner PJ, Harrison SL (2000) Irukandji and *Chironex fleckeri* jellyfish envenomation in tropical Australia. *Wilderness and Environmental Medicine*, *11*, 233–240.

Freeman G (1987) The role of oocyte maturation in the ontogeny of the fertilization site in the hydrozoan *Hydractinia echinata*. *Roux's Archives of Developmental Biology*, *196*, 83–92. doi: 10.1007/BF00402029

Galea HR (2018) A swimming medusoid gonophore in the life cycle of *Ventromma halecioides* (Alder, 1859) (Hydrozoa: Leptothecata: Kirchenpaueriidae). *Revue Suisse de Zoologie, 125,* 137–147. doi: 10.5281/zenodo.l196038

Galea HR, Ferry R, Bertot JM (2012) Medusoids in the life cycle of *Dentitheca dendritica* (Nutting, 1900) and *Nemalecium gracile* sp. nov. (Cnidaria: Hydrozoa). *Zootaxa* 43–54. doi: 10.11646/zootaxa.3527.1.3

García-Rodríguez J, Lewis Ames C, Marian JEAR, Marques AC (2018) Gonadal histology of box jellyfish (Cnidaria: Cubozoa) reveals variation between internal fertilizing species *Alatina alata* (Alatinidae) and *Copula sivickisi* (Tripedaliidae). *Journal of Morphology, 279*, 841–856. doi: 10.1002/jmor.20815

García-Rodríguez J, Ames CL, Marian JEAR, Marques AC (2020) Histomorphological comparison of testes in species of box jellyfish (Cnidaria; Cubozoa): does morphology differ with mode of reproduction and fertilization? *Organisms Diversity & Evolution*, *20,* 25–36. doi: 10.1007/s13127-019-00427-x

García-Rodríguez J, Ames CL, Jaimes-Becerra A, Tiseo GR, Morandini AC, Cunha AF, Marques AC (2023) Histological Investigation of the Female Gonads of *Chiropsalmus quadrumanus* (Cubozoa: Cnidaria) Suggests Iteroparous Reproduction. *Diversity, 15*, 816. doi: 10.3390/d15070816

Garm A, Lebouvier M, Tolunay D (2015) Mating in the box jellyfish *Copula sivickisi* -Novel function of cnidocytes. *Journal of Morphology, 276,* 1055–1064. doi: 10.1002/jmor.20395

Govindarajan AF, Boero F, Halanych KM (2006) Phylogenetic analysis with multiple markers indicates repeated loss of the adult medusa stage in Campanulariidae (Hydrozoa, Cnidaria). *Molecular Phylogenetics and Evolution*, *38*(3), 820–834. doi: 10.1016/j.ympev.2005.11.012

Gravier-Bonnet N, Migotto AE (2000) Gonangium development and medusoid of *Nemalecium lighti* (Hargitt, 1924) (Cnidaria: Hydrozoa, Haliciidae). *Scientia Marina, 64,* 207–213. doi: 10.3989/scimar.2000.64s1207

Hagedorn HH, Kunkel JG (1979) Vitellogenin and vitellin in insects. *Annual review of entomology*, *24*, 475–505. doi: 10.1146/annurev.en.24.010179.002355

Hartwick RF (1991) Observations on the anatomy, behaviour, reproduction and life cycle of the cubozoan *Carybdea sivickisi*. *Hydrobiologia 216–217*, 171–179. doi: 10.1007/BF00026459

Helmark S, Garm A (2019) Gonadal cnidocytes in the cubozoan *Tripedalia cystophora* Conant, 1897 (Cnidaria: Cubozoa). *Journal of Morphology*, *280*, 1530–1536. doi: 10.1002/jmor.21046

Hofmann DK, Hadfield MG (2002) Hermaphroditism, gonochorism, and asexual reproduction in *Cassiopea* sp.— an immigrant in the islands of Hawaii. *Reproduction and Development*, *41*, 215–221. doi: 10.1080/07924259.2002.9652754

Honegger TG, Zürrer D, Tardent P (1989) Oogenesis in *Hydra carnea*: a new model based on light and electron microscopic analyses of oocyte and nurse cell diferentiation. *Tissue and Cell, 2*(3), 381–393. doi: 10.1016/0040-8166(89)90052-9

Jaglarz MK, Kloc M, Bilinski SM (2008) Accessory nuclei in insect oogenesis: In search of the function of enigmatic organelles. *International Journal of Developmental Biology, 52*, 179–185. doi: 10.1387/ijdb.072303mj

Kessel RG (1968) Electron Microscope Studies on Developing Oocytes of a Coelenterate Medusa with Special Reference to Vitellogenesis. *Journal of Morphology*, *126*(2), 211–247. doi: 10.1002/jmor.1051260205

Kessel RG, Kemp NE (1962) An Electron Microscope Study on the Oocyte, Test Cells and Follicular Envelope of the Tunicate, *Molgula manhattensis*. *Journal of Ultrastructure Research, 6*, 57–76. doi: 10.1016/S0022-5320(62)90061-8

Lewis Ames C (2018) Medusa: A Review of an Ancient Cnidarian Body Form. *In:* In: Kloc, M., Kubiak, J. (eds) Marine Organisms as Model Systems in Biology and Medicine. Results and Problems in Cell Differentiation, vol 65. Springer, Cham. doi: 10.1007/978-3-319-92486-1_7

Lewis Ames C, Ryan JF, Bely AE, Cartwright P, Collins AG (2016) A new transcriptome and transcriptome profiling of adult and larval tissue in the box jellyfish *Alatina alata*: An emerging model for studying venom, vision and sex. *BMC Genomics, 17*, 1–25. doi: 10.1186/s12864-016-2944-3

Lewis C, Long TAF (2005) Courtship and reproduction in *Carybdea sivickisi* (Cnidaria: Cubozoa). *Marine Biology, 147*, 477–483. doi: 10.1007/s00227-005-1602-0

Lindner A, Migotto AE (2002) The life cycle of *Clytia linearis* and *Clytia noliformis*: Metagenic campanulariids (Cnidaria: Hydrozoa) with contrasting polyp and medusa stages. *Journal of the Marine Biological Association of the United Kingdom, 82,* 541–553. doi: 10.1017/S0025315402005866

Lumley J, Williamson JA, Fenner PJ, Burnett JW, Colquhoun DM (1988) Fatal envenomation by *Chironex fleckeri*, the north Australian box jellyfish: The continuing search for lethal mechanisms. *Medical Journal of Australia, 148,* 527–534. doi: 10.5694/j.1326-5377.1988.tb99466.x

Mammone M, Bosch-Belmar M, Milisenda G, Castriota L, Sinopoli M, Allegra A, Falautano M, Maggio T, Rossi S, Piraino S (2023) Reproductive cycle and gonadal output of the Lessepsian jellyfish *Cassiopea andromeda* in NW Sicily (Central Mediterranean Sea). *PLoS One*, *18,* e0281787. doi: 10.1371/journal.pone.0281787

Mariscal RN (1974) Nematocyst. In: Coelenterate biology: Reviews and new perspectives. Muscatine L, Lenhoff HM, editors. pp 129–178

Marques AC, Calder DR (2000) *Eudendrium bathyalis*, a new species of hydroid (Hydrozoa: Anthomedusae: Eudendriidae) from Bermuda. *Proceedings-Biological Society of Washington*, *113*(1), 124-128.

Marques AC, Collins AG (2004) Cladistic analysis of Medusozoa and cnidarian evolution. *Invertebrate Biology, 123,* 23–42. doi: 10.1111/j.1744-7410.2004.tb00139.x

Mendoza-Becerril MA, Marian JEAR, Migotto AE, Marques AC (2017) Exoskeletons of Bougainvilliidae and other Hydroidolina (Cnidaria, Hydrozoa): structure and composition. *PeerJ, 5*, e2964. doi: 10.7717/peerj.2964

Migotto AE (1996) Benthic shallow-water hydroids (Cnidaria, Hydrozoa) of the coast of Sao Sebastiao, Brazil, including a checklist of Brazilian hydroids. *Zoologische Verhandelingen, 306*, 3–125.

Migotto AE (1998) The life cycle of *Sertularia marginata* Kirchenpauer, 1864 (Cnidaria: Hydrozoa): A medusoid-producing sertulariid. *Journal of Natural History*, *32*, 1–12. doi: 10.1080/00222939800770011

Miranda LS, Collins AG, Hirano YM, Mills CE, Marques AC (2016) Comparative internal anatomy of Staurozoa (Cnidaria), with functional and evolutionary inferences. *PeerJ, 4*, e2594. doi: 10.7717/peerj.2594

Mohamed R, Skrypzeck H, Gibbons MJ (2019) Describing gonad development and gametogenesis in southern Africa's endemic box jellyfish *Carybdea branchi* (Cubozoa, Carybdeidae). *African Journal of Marine Science*, *41*, 83–91. doi: 10.2989/1814232X.2019.1575282

Morandini AC, Silveira FL Da (2001) Sexual reproduction of *Nausithoe aurea* (Scyphozoa, Coronatae). Gametogenesis, egg release, embryonic development, and gastrulation. *Scientia Marina, 65*, 139–149. doi: 10.3989/scimar.2001.65n2139

Morandini AC, Schiariti A, Stampar SN, Maronna MM, Straehler-Pohl I, Marques AC (2016) Succession of generations is still the general paradigm for scyphozoan life cycles. *Bull Mar Sci, 92(3),* 343–351. doi: 10.5343/bms.2016.1018

Müller WA, Leitz T (2002) Metamorphosis in the Cnidaria. *Canadian Journal of Zoology,* 80, 1755–1771. doi: 10.1139/z02-130

Nath V (1925) Cell inclusions in the oogenesis of scorpions. *Proceedings of the Royal Society of London Series B, 98*, 44–58. doi: 10.1098/rspb.1925.0022

Piraino S (1992) The «stinging» egg of *Clavopsella michaeli* (Berrill) (Hydrozoa, Cnidaria). *Bolletino di zoologia, 59*, 251–256. doi: 10.1080/11250009209386678

Pitt K a., Kingsford MJ (2000) Reproductive biology of the edible jellyfish *Catostylus mosaicus* (Rhizostomeae). *Marine Biology, 137*, 791–799. doi: 10.1007/s002270000399

Rudel D, Riebesell M, Sommer RJ (2005) Gonadogenesis in *Pristionchus pacificus* and organ evolution: Development, adult morphology and cell-cell interactions in the hermaphrodite gonad. *Developmental Biology*, *277*, 200–221. doi: 10.1016/j.ydbio.2004.09.021

Schiariti A, Christiansen E, Morandini AC, da silveira FL, Giberto DA, Mianzan HW (2012) Reproductive biology of *Lychnorhiza lucerna* (Cnidaria: Scyphozoa: Rhizostomeae): Individual traits related to sexual reproduction. *Marine Biology Research, 8,* 255–264. doi: 10.1080/17451000.2011.616897

Schmekel L, Fioroni P (1974) The Ultrastructure of the Yolk Nucleus during Early Cleavage of *Nassarius reticulatus* L. (Gastropoda, Prosobranchia). *Cell Tissue Res, 153*, 79–88. doi: 10.1007/BF00225447

Schuchert P (2004) Revision of the European athecate hydroids and their medusae (Hydrozoa, Cnidaria): Families Oceanidae and Pachycordylidae. *Revue Suisse de Zoologie, 111*, 315–369. doi: 10.5962/bhl.part.80242

Shepherd SA, Laws HM (1974) Studies on southern Australian abalone (Genus *Haliotis*) II: Reproduction of five species. *Marine and Freshwater Research*, *25*, 49–62. doi: 10.1071/MF9740049

Shikina S, Chen CJ, Chung YJ, Shao ZF, Liou JY, Tseng HP, Lee YH, Chang CF (2013) Yolk formation in a stony coral *Euphyllia ancora* (Cnidaria, Anthozoa): Insight into the evolution of vitellogenesis in nonbilaterian animals. *Endocrinology, 154*, 3447–3459. doi: 10.1210/en.2013-1086

Spracklin BW (1984) Oogenesis in *Tubularia larnyx* and *Tubularia indivisa* (Hydrozoa, Athecata). University of New Hampshire

Stewart S (1996) Field behavior of *Tripedalia cystophora* (class Cubozoa). *Marine and Freshwater Behaviour and Physiology, 27,* 175–188. doi: 10.1080/10236249609378963

Straehler-Pohl I (2014) Critical evaluation of characters for species identification in the cubomedusa genus *Malo* (Cnidaria, Cubozoa, Carybdeida, Carukiidae). *Plankton and Benthos Research*, *9*(2), 83–98. doi: 10.3800/pbr.9.83

Straehler-Pohl I, Jarms G (2011) Morphology and life cycle of *Carybdea morandinii*, sp. nov. (Cnidaria), a cubozoan with zooxanthellae and peculiar polyp anatomy. *Zootaxa, 56,* 36–56. doi: 10.11646/zootaxa.2755.1.2

Sugimura K, Ishihara S (2013) The mechanical anisotropy in a tissue promotes ordering in hexagonal cell packing. *Development, 140*(19), 4091–4101. doi: 10.1242/dev.094060

Szollosi D (1969) Unique envelope of a jellyfish ovum: The armed egg. *Science*, *163*(3867), 586-587. doi: 10.1126/science.163.3867.586

Tiemann H, Jarms G (2010) Organ-like gonads, complex oocyte formation, and long-term spawning in *Periphylla periphylla* (Cnidaria, Scyphozoa, Coronatae). *Marine Biology, 157,* 527–535. doi: 10.1007/s00227-009-1338-3

Tiseo GR, García-Rodríguez J, Zara FJ, Ames CL, Marques AC, Morandini AC (2019) Spermatogenesis and gonadal cycle in male *Tamoya haplonema* and *Chiropsalmus quadrumanus* (Cnidaria, Cubozoa). *Zoologischer Anzeiger, 279*, 59–67. doi: 10.1016/j.jcz.2018.12.008

Toshino S, Hiroshi M, Ohtsuka S, Okuizumi K, Adachi A, Hamatsu Y, Urata M, Nakaguchi K, Yamaguchi S (2013) Development and polyp formation of the giant box jelly fish *Morbakka virulenta* (Kishinouye, 1910) (Cnidaria: Cubozoa) collected from the Seto Inland Sea, western Japan. *Plankton and Benthos Research, 8*, 1–8. doi: 10.3800/pbr.8.1

Toshino S, Miyake H, Iwanaga S (2014) Development of *Copula sivickisi* (Stiasny, 1926) (Cnidaria: Cubozoa: Carybdeidae: Tripedaliidae) collected from the Ryukyu Archipelago, southern Japan. *Plankton and Benthos Research, 9*, 32–41. doi: 10.3800/pbr.9.32

Toshino S, Miyake H, Srinui K, Luangoon N, Muthuwan V, Sawatpeera S, Honda S, Shibata H (2017) Development of *Tripedalia binata* Moore, 1988 (Cubozoa: Carybdeida: Tripedaliidae) collected from the eastern Gulf of Thailand with implications for the phylogeny of the Cubozoa. *Hydrobiologia, 792*, 37–51. doi: 10.1007/s10750-016-3022-1

Toshino S, Miyake H, Shibata H (2018) Development of *Carybdea brevipedalia* Kishinouye, 1891 (Cnidaria: Cubozoa: Carybdeida: Carybdeidae) collected from northern Japan. *Plankton and Benthos Research, 13*, 116–128. doi: 10.3800/pbr.13.116

Werner B (1973) New Investigations on systematics and evolution of the class Scyphozoa and the phylum Cnidaria. *Publications of the Seto Marine Biological Laboratory, 20*, 35–61.

Widersten B (1965) Genital organs and fertilization in some Scyphozoa. *Zoologiska Bidrag fran Uppsala 37,* 45–58. doi: 10.1016/0011-7471(66)90698-x

Williamson JA, Le Ray LE, Wohlfahrt M, Fenner PJ (1984) Acute management of serious envenomation by box-jellyfish (*Chironex fleckeri*). *Medical journal of Australia*, *141*(12-13), 851–853. doi: 10.5694/j.1326-5377.1984.tb132969.x

Yamada S, Morimoto H, Fujisawa T, Sugahara K (2007) Glycosaminoglycans in *Hydra magnipapillata* (Hydrozoa, Cnidaria): Demonstration of chondroitin in the developing nematocyst, the sting organelle, and structural characterization of glycosaminoglycans. *Glycobiology, 17,* 886–894. doi: 10.1093/glycob/cwm051

Yamaguchi M, Hartwick R (1980) Early life history of the sea wasp *Chironex fleckeri* (class Cubozoa). *Development and cellular biology of coelenterates,* 11–16.

Figures

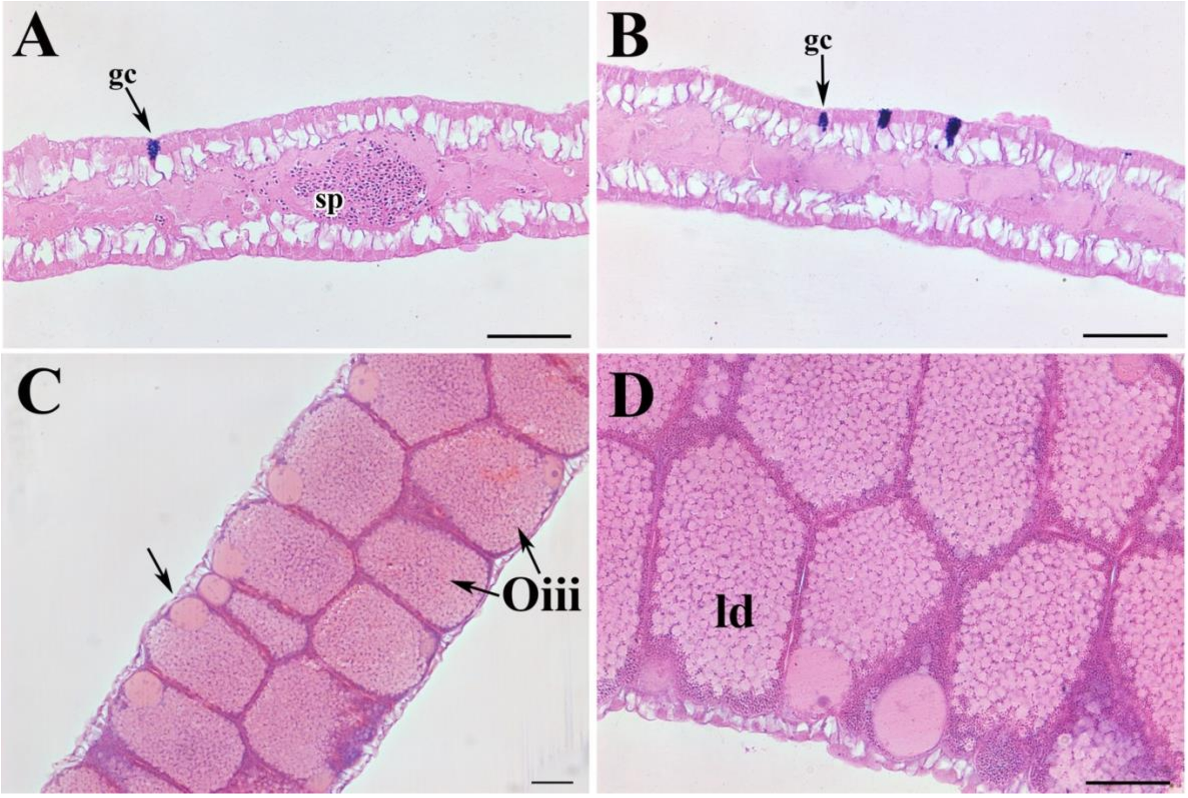

Figure 1. *Alatina alata* gonad sections. **A-B.** Mature male with intermittent distribution of sperm within the mesoglea and epithelial cells interspersed with occasional gland cells in the gastrodermal vacuolated epithelium. **C-D.** Mature females (USNM 1195801) characterized by hexagonal oocytes and the nuclei (arrow) positioned apically. Oocytes filled with lipid droplets (ld). Hematoxylin-Eosin staining. gc = glandular cell, Oiii = late-vitellogenic coocyte, sp = sperm. Scale bars 50 µm.

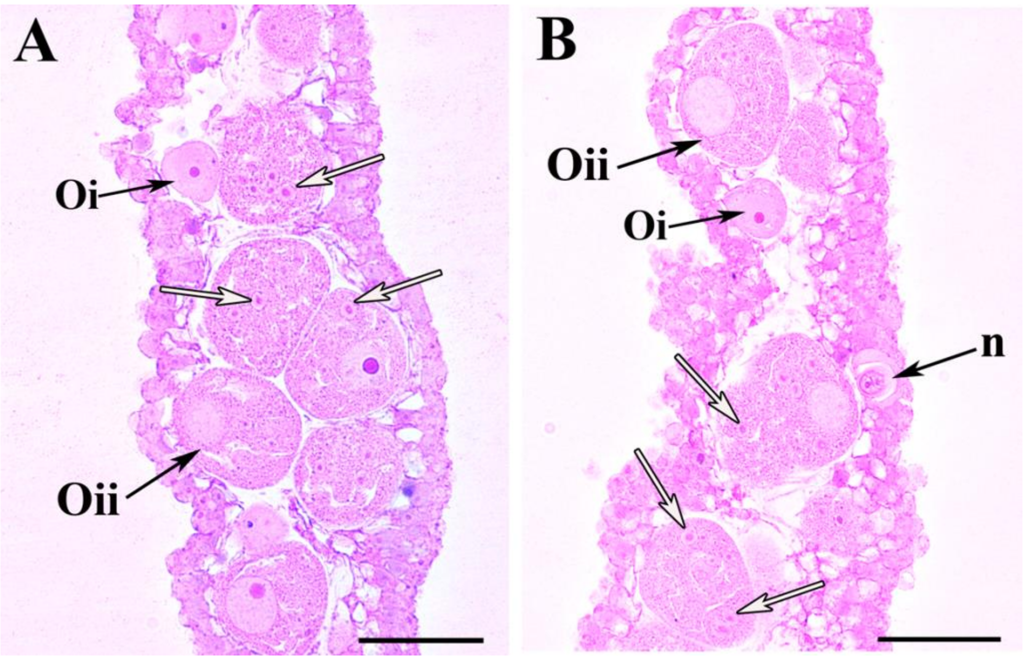

Figure 2. *Morbakka virulenta* female gonad sections. **A.** Inclusions of unknown nature (white arrows) within oocytes. **B.** Nematocyst located within the gastrodermal epithelium. Hematoxylin-Eosin staining. n = nematocyst, Oi = early-vitellogenic oocyte, Oii = mid-vitellogenic oocyte. Scale bars 50 µm.

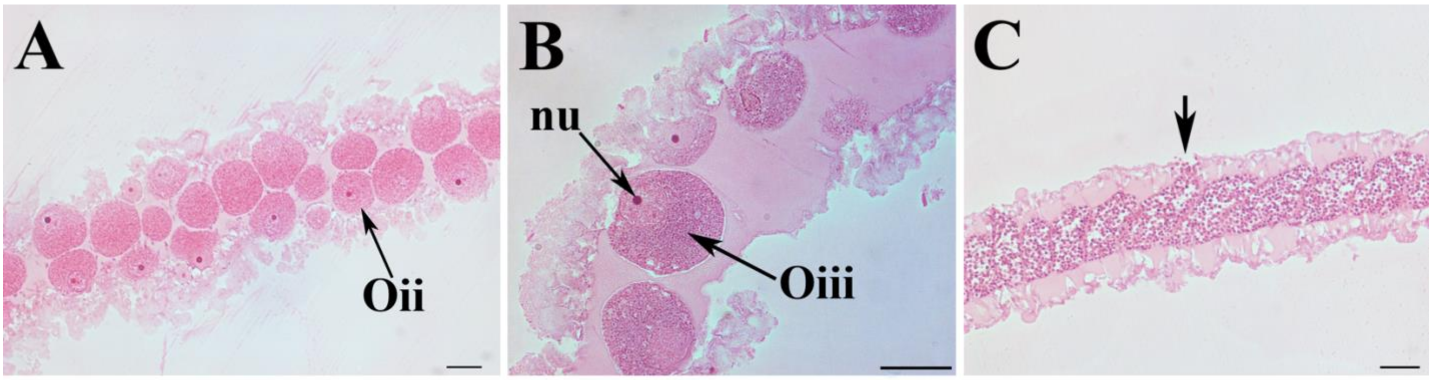

Figure 3. *Carybdea arborifera* gonad sections. **A-B.** Mature female with gastrodermal epithelium poorly preserved. **C.** Mature male with rupture of the gonads (arrow). nu = nuclei, Oii = mid-vitellogenic oocyte, Oiii = late-vitellogenic oocyte, Hematoxylin-Eosin staining. Scale bars 50 µm.

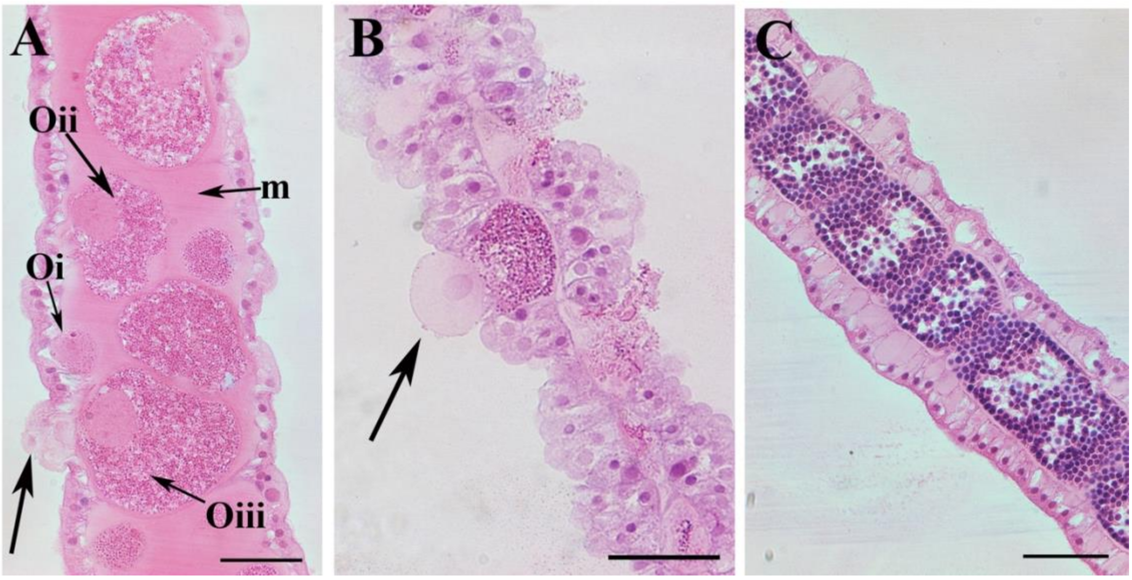

Figure 4. *Carybdea brevipedalia* gonad sections. **A.** Female gonad with asynchronous oogenesis with oocytes freely within the mesoglea (m). Single arrow indicates the oocyte about to be spawn. **B.** Female gonad during spawning (arrow). **C.** Immature male gonad with spermatic follicles. Hematoxylin-Eosin staining. gs = gastrodermal epithelium, Oi = early-vitellogenic oocyte, Oii = mid-vitellogenic oocyte, Oiii = late-vitellogenic oocyte. Scale bars 50 µm.

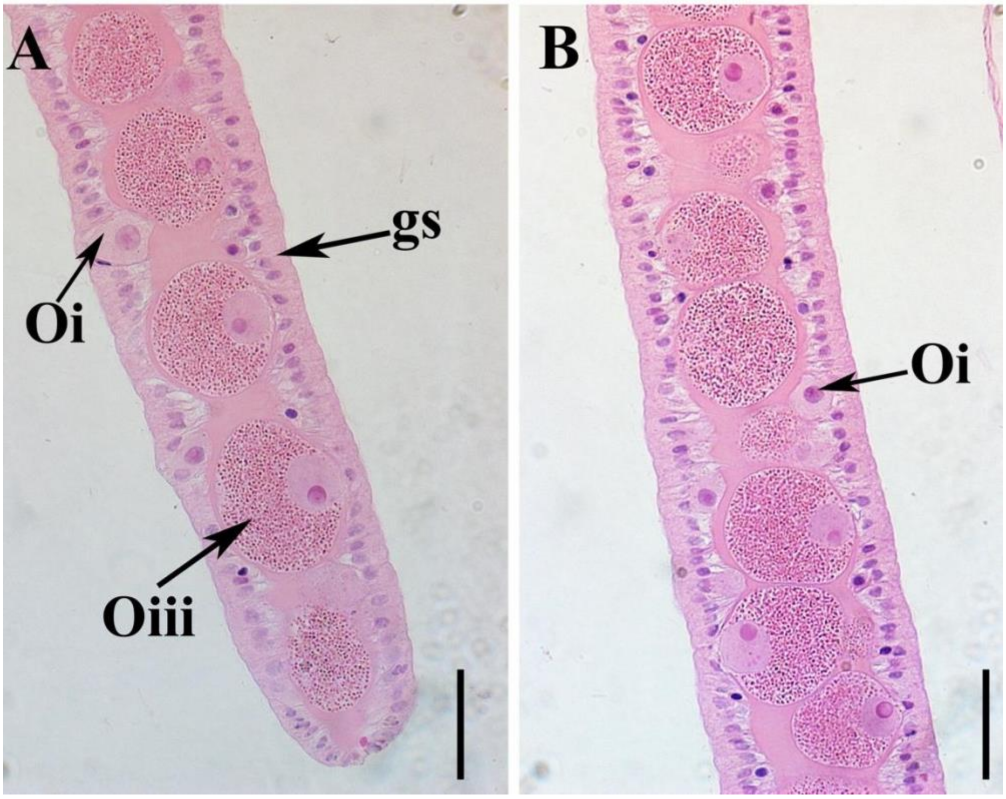

Figure 5. *Carybdea marsupialis* female gonad sections. **A.** Apical section of the gonad. **B.** Middle section of the gonad. Hematoxylin-Eosin staining. gs = gastrodermal epithelium, Oi = early-vitellogenic oocyte, Oiii = late-vitellogenic oocyte. Scale bars 50 µm.

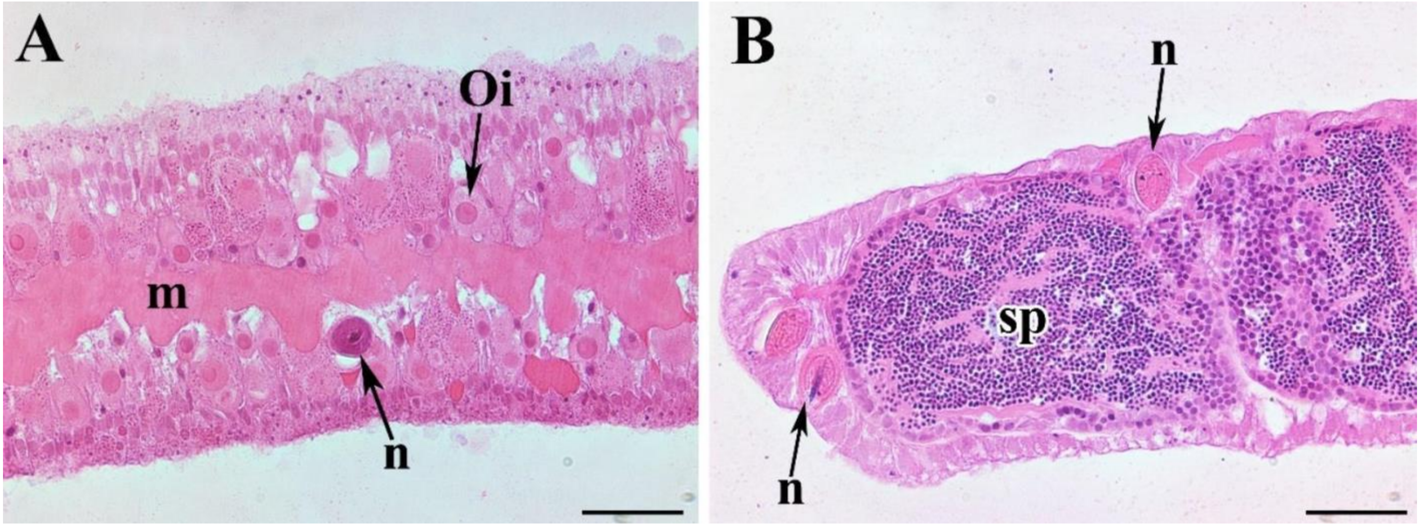

Figure 6. *Carybdea murrayana* gonad sections. **A**. Spawned female gonad with early-vitellogenic oocytes (Oi) within the gastrodermal epithelium. **B.** Male gonad with ripe sperm in spermatic follicles. Hematoxylin-Eosin staining. m = mesoglea, n = nematocyst, sp = sperm. Scale bars 50 µm.

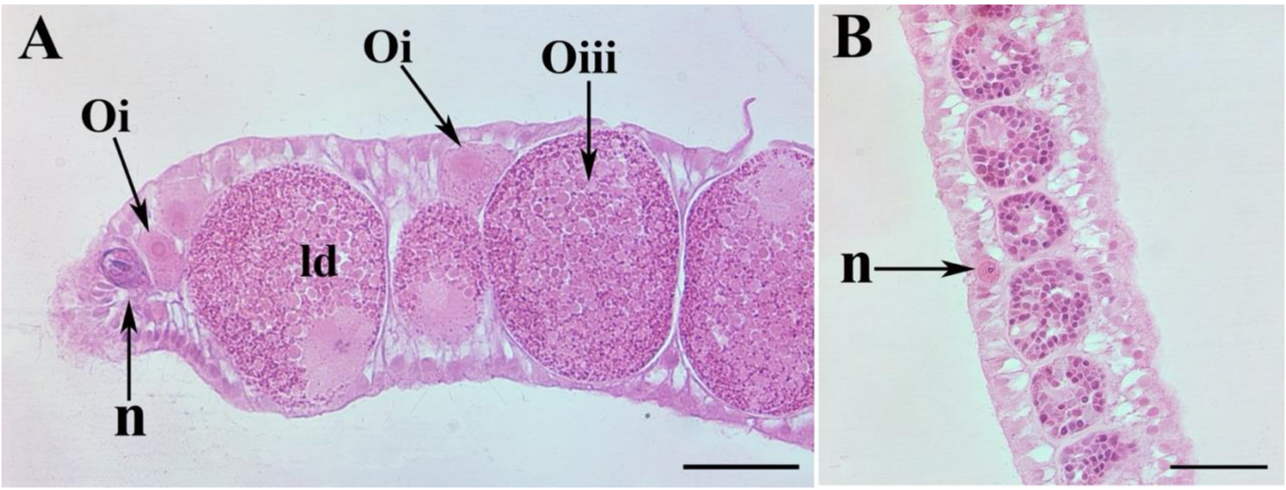

Figure 7. *Carybdea rastonii* gonad sections*.* **A.** Mature female (USNM 88365) with oocytes in late vitellogenesis (Oiii) containing lipid droplets. Nematocyst on the apex of the gonad**. B.** Immature male with nematocyst in gastrodermal epithelium. Hematoxylin-Eosin staining. ld = lipid droplets, n = nematocyst, Oi = early-vitellogenic oocyte. Scale bars 50 µm.

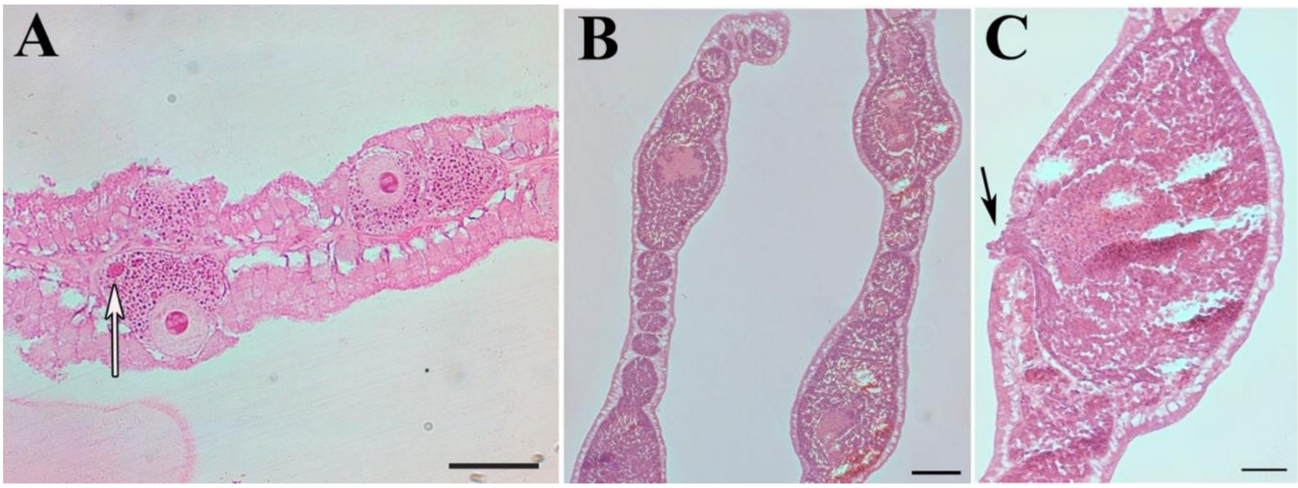

Figure 8. *Carybdea xaymacana* gonads section. **A.** Female gonad with inclusions of unknown nature in some oocytes (white arrow). Scale bar 50 µm. **B-C.** Male gonads swollen, presumably for spawning (black arrow). Image C shows some artifacts (White gaps) probably due to improper embedding of the resin. Hematoxylin-Eosin staining. Scale bars 50 µm.

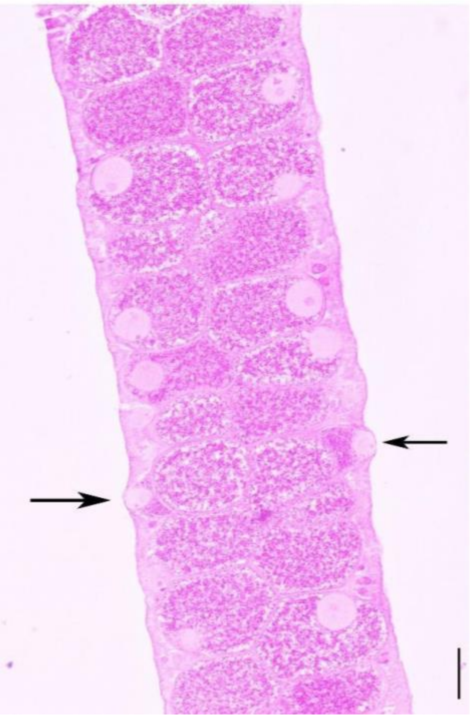

Figure 9. *Carybdea* sp. gonad sections. Mature female with hexagonal oocytes, spawning (arrows). Hematoxylin-Eosin staining. Scale bars 50 µm.

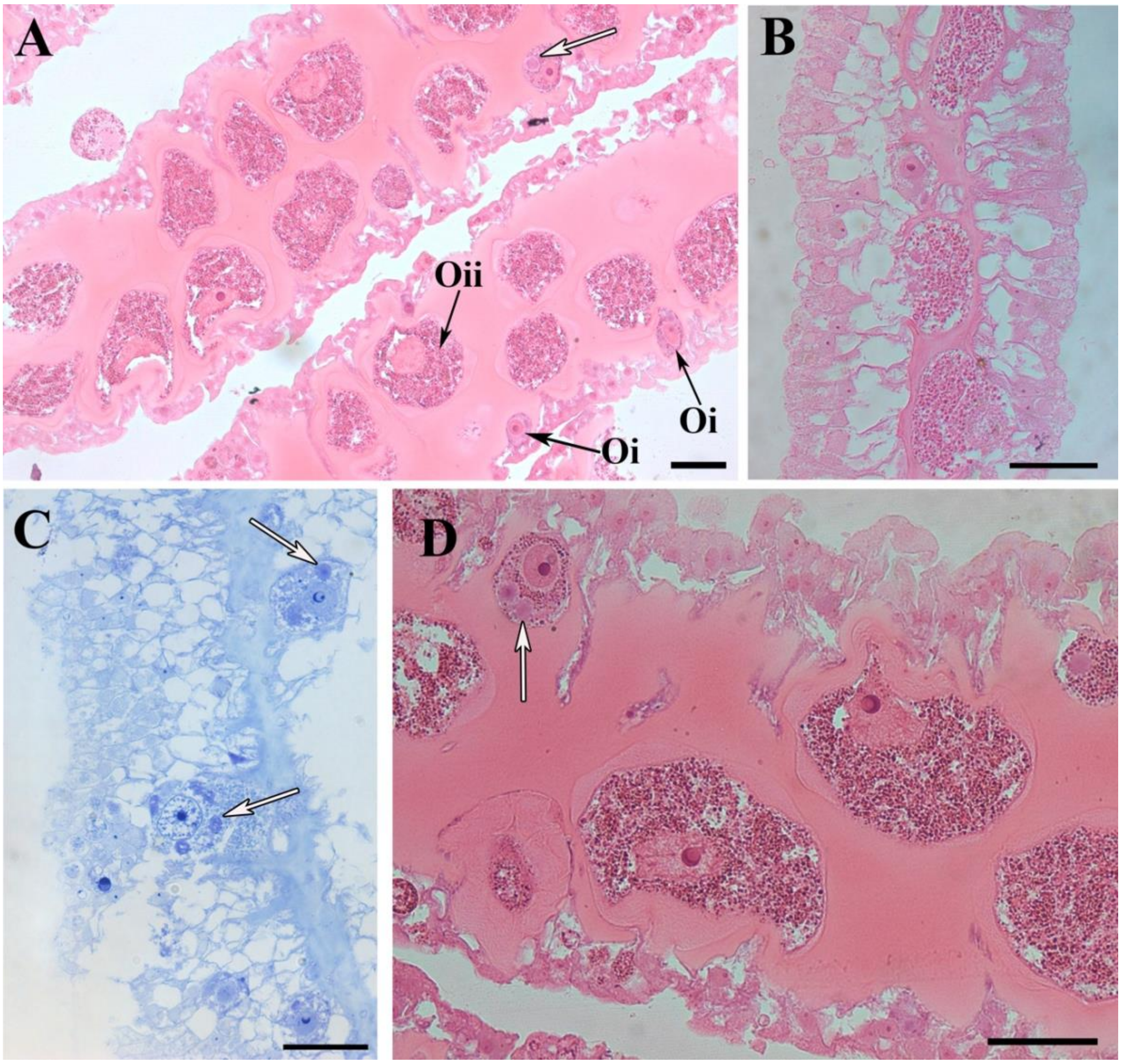

Figure 10. *Tamoya haplonema* female gonad sections. **A.D.** Female with asynchronous oogenesis**. B.C.** Female already spawned. Hematoxylin-Eosin staining (A-B, D) and toluidine blue (C). Unknown inclusions (white arrows) within the oocytes. Oi = early-vitellogenic oocyte, Oii = mid-vitellogenic oocyte. Scale bars 50 µm.

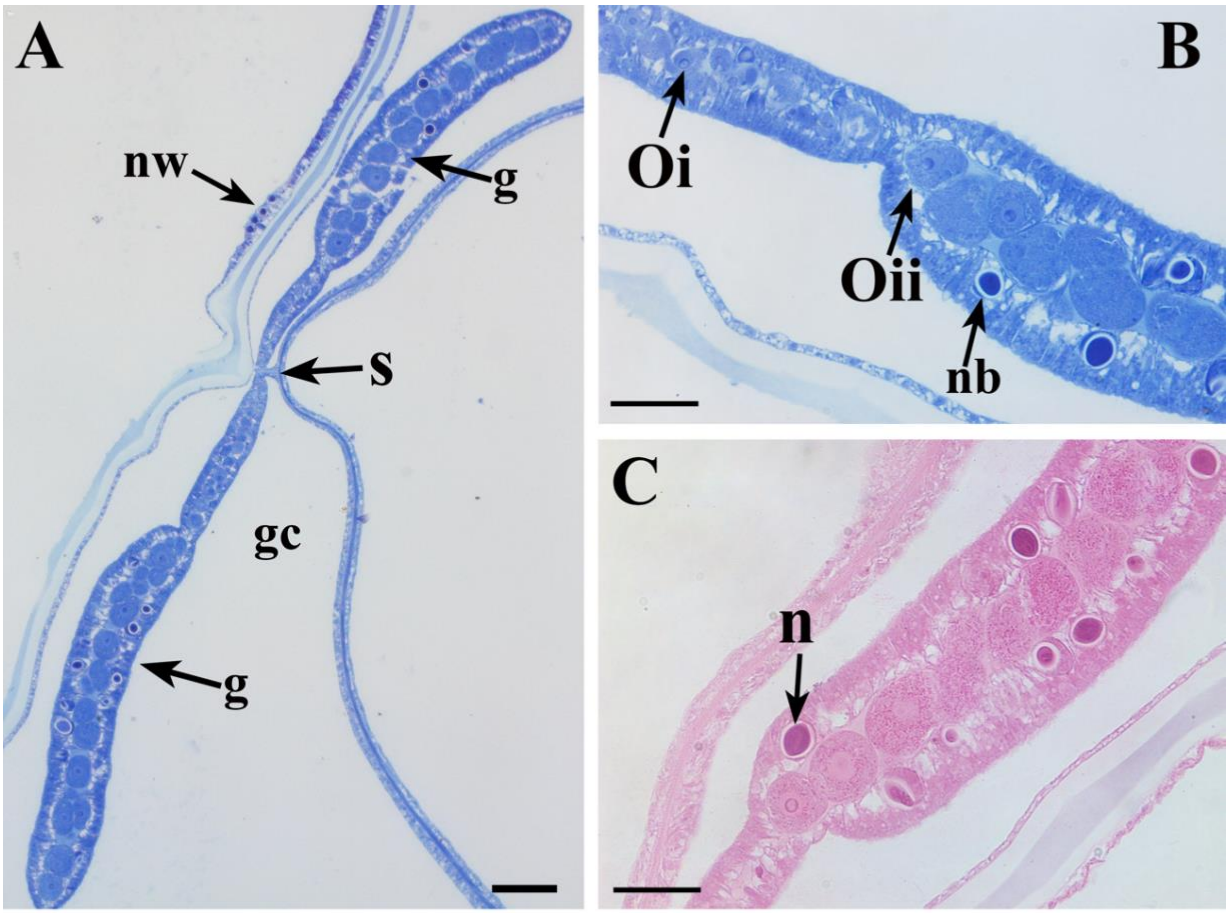

Figure 11. *Copula sivickisi* female gonad sections. **A.** Hemigonads divided by the septum (s). **B-C.** Thin section with oocytes in early-vitellogenesis and wide section with oocytes in mid-vitellogenesis and nematocysts within the gastrodermal epithelium. g = gonad, gc = gastrovascular cavity, n = nematocyst, nb = nematoblasts, nw = nematocyst wart, Oi = early vitellogenic oocyte, Oii = mid-vitellogenic oocyte. Toluidine blue staining (A-B) and hematoxylin-eosin (C). Scale bars 50 µm.

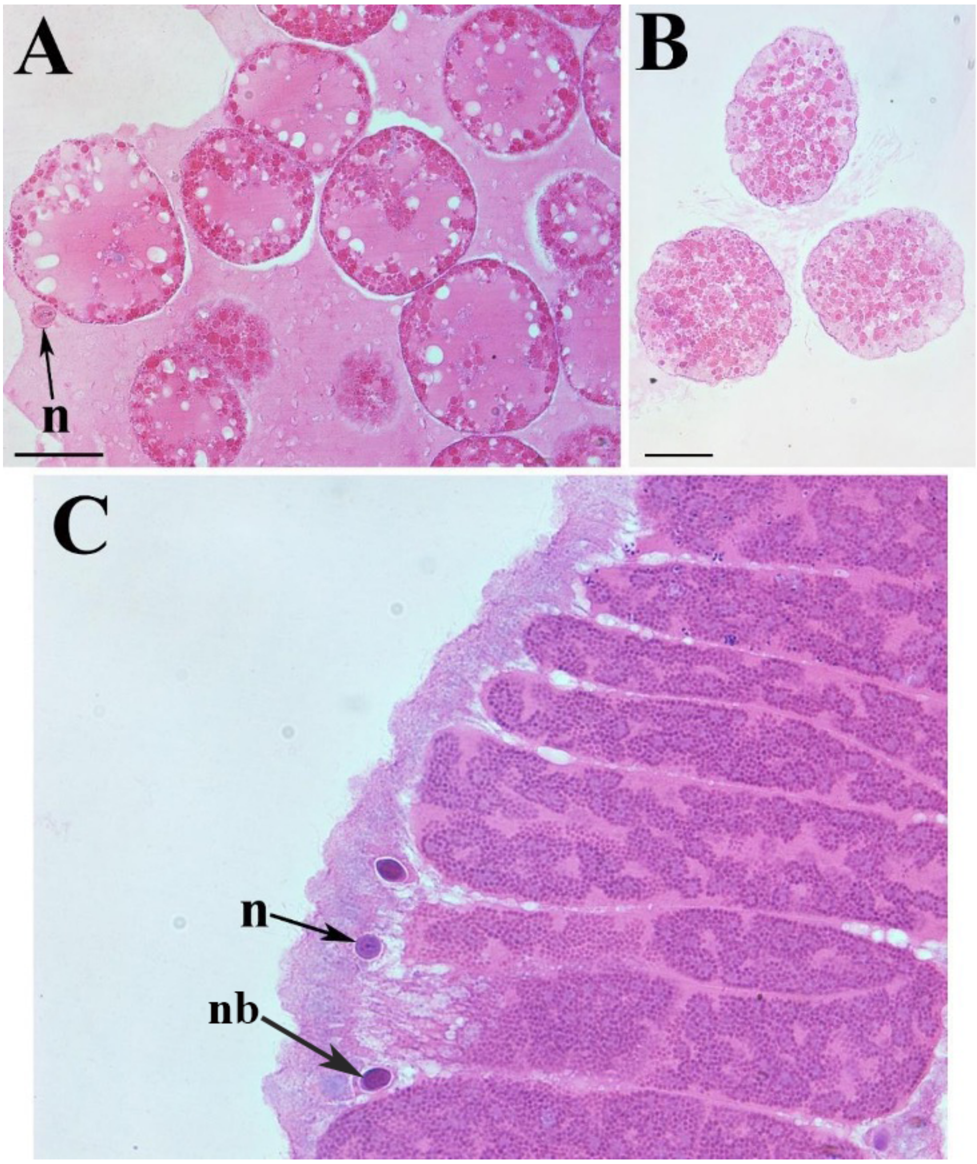

Figure 12. *Tripedalia cystophora* gonad sections. **A.** Female bearing fertilized oocytes (blastulae) and nematocyst within the gonad. **B.** Blastulae obtained from the gastrovascular cavity. **C.** Mature male with nematocysts and ripe sperm in spermatic follicles. Hematoxylin-Eosin staining. n = nematocyst, nb = nematoblast. Scale bars 50 µm.

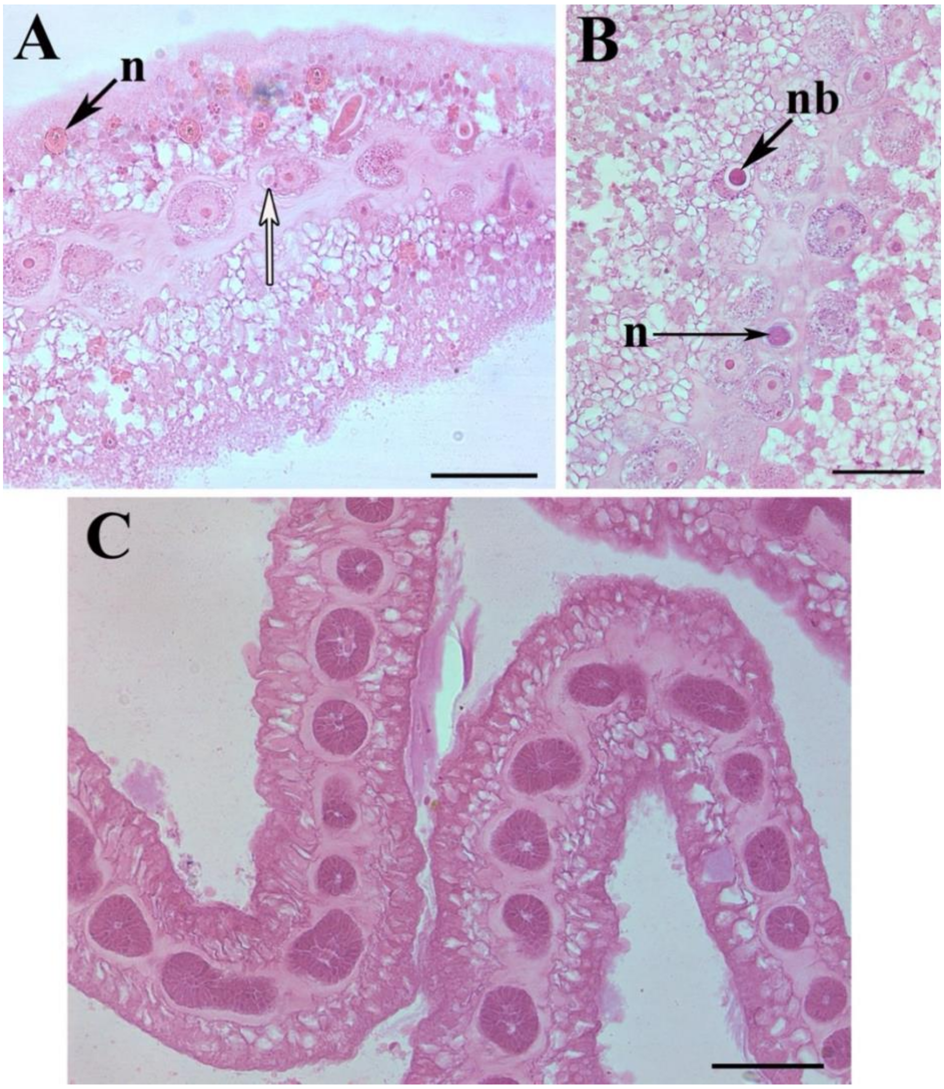

Figure 13. *Chironex fleckeri* gonad sections. **A-B.** Female gonad bearing nematocyst, nematoblast, and oocyte inclusions (white arrow). **C.** Immature male with spermatic follicles. Hematoxylin-Eosin staining. n = nematocyst, nb = nematoblast. Scale bars 50 µm.

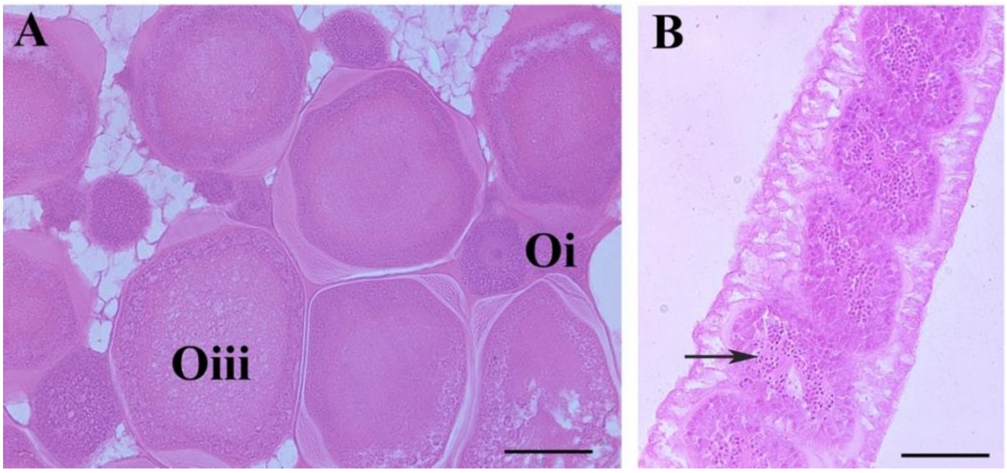

Figure 14. *Chironex yamaguchii* gonad sections. **A.** Female packing oocytes (polygonal) in different stages of development. **B.** Mature male with spermatic follicles full of sperm (arrow). Hematoxylin-Eosin staining. Oi = early-vitellogenic oocyte, Oiii = late-vitellogenic oocyte. Scale bars 50 µm.

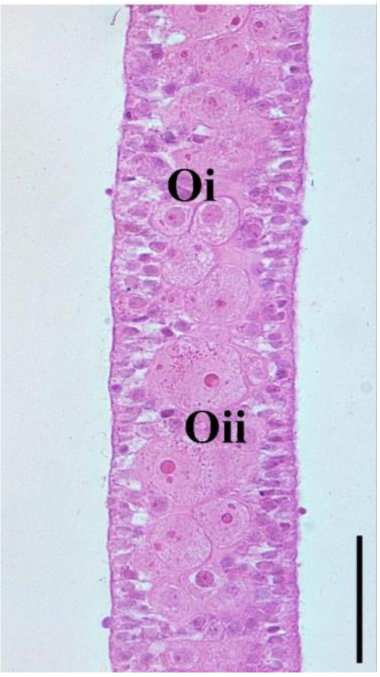

Figure 15. *Chiropsella bronzie* female gonad with oocytes in early and mid-vitellogenesis. Hematoxylin-Eosin staining. Oi = early-vitellogenic oocyte, Oii = mid-vitellogenic oocyte. Scale bars 50 µm.

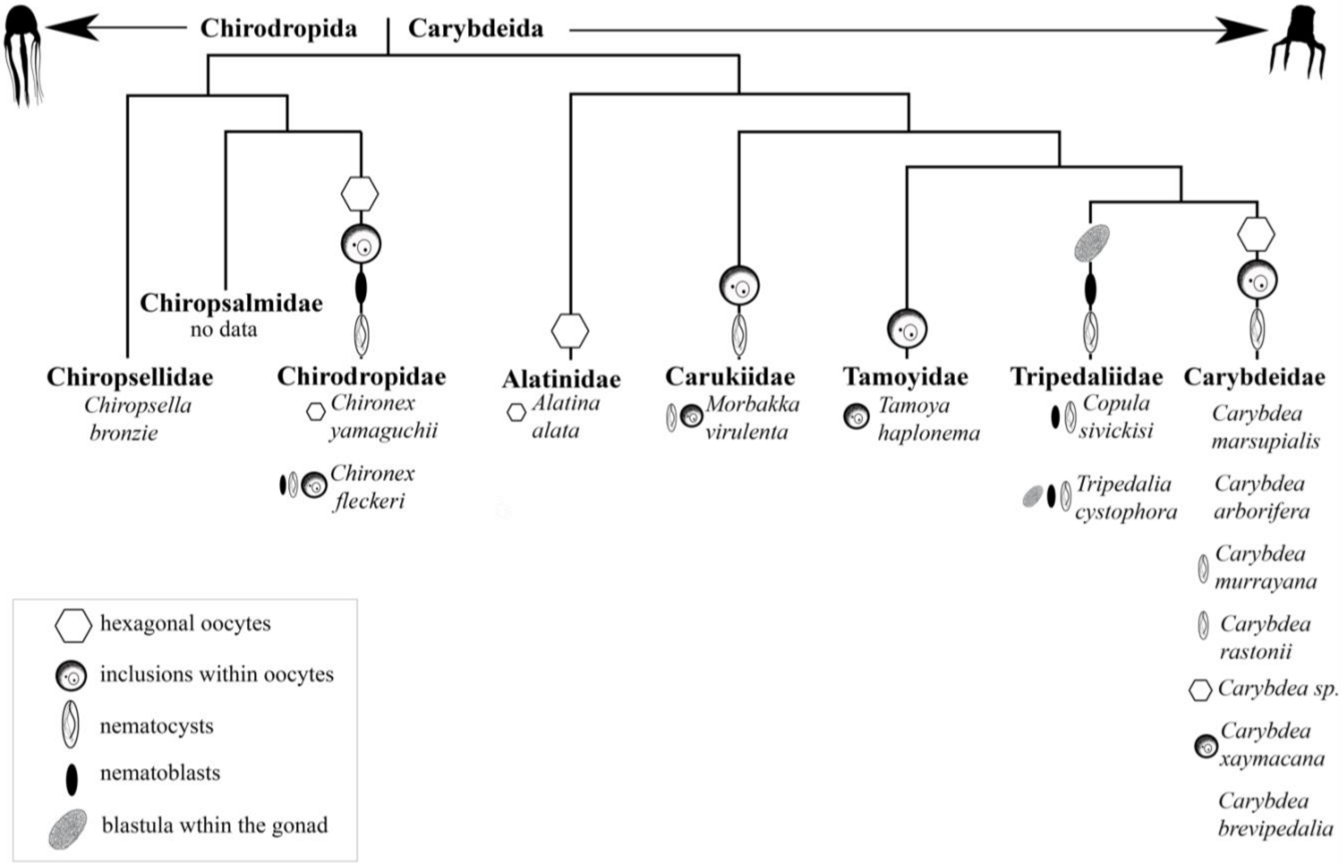

Figure 16. Reproductive characters analyzed in this study (hexagonal oocytes, inclusions within oocytes, nematocyst and nematoblasts within the gonads, blastula within the gonad) plotted onto the phylogeny published by Bastian et al. (2010). The pattern does not represent a character optimization.

Tables

Table 1. List of Cubozoa species sampled from the invertebrate zoology collections of the National Museum of Natural History (Smithsonian Institution, Washington DC) and MEL (Marine Evolution Lab, University of Sao Paulo, Brazil). BH = bell height, IPD = interpedalial distance.

Species	USNM numbers / MEL codes	Locality	BH (cm)	IPD (cm)	Collection date	Sex
Alatina alata	USNM 54385	Antigua Island, Antigua and Barbuda	8	5.4	04/Apr/1956	male
	USNM 1205447	Kralendijk, Bonaire, The Netherlands	7.7	5.8	24/Jun/2011	female
	USNM 1195801	Kralendijk, Bonaire, The Netherlands	7.4	6.1	24/Jun/2011	female
	USNM 1195803	Kralendijk, Bonaire, The Netherlands	7.7	5.9	24/Jun/2011	female
Carybdea arborifera	USNM 22304	Honolulu, Oahu Island, USA	2.4	2.1	08/May/2002	female
	USNM 22304	Honolulu, Oahu Island, USA	3	2	08/May/2002	male
	USNM 22304	Honolulu, Oahu Island, USA	2.1	1.5	08/May/2002	female
Carybdea murrayana (as Carybdea branchi)	USNM 1124255	Cape Town, South Africa	4	2.8	Apr/2008	female
	USNM 1124258	Cape Town, South Africa	4.4	3.4	Apr/2008	male
Carybdea brevipedalia	USNM 1154099	Hiroshima, Japan	2.5	1.9	20/Dec/2006	female
	USNM 1154103	Hiroshima, Japan	1.6	1	20/Dec/2006	male
	MEL C.brevi1	Iwate, Japan	1.5	1.3	No data	female
	MEL C.brevi2	Iwate, Japan	2.3	1.4	No data	female
	MEL C.brevi3	Iwate, Japan	2.5	1.4	No data	male
Carybdea rastonii	USNM 88365	Marino, Australia	1.8	0.9	29/Mar/1962	female
	USNM 88365	Marino, Australia	1.5	1	29/Mar/1962	female
	USNM 88365	Marino, Australia	1.7	1	29/Mar/1962	male
Carybdea marsupialis	USNM 54378	Tunisia	4.7	3	21/Jan/1973	female
	MEL CM_1F	Mediterranean Sea, Spain	2	1.1	No data	female
Carybdea xaymacana	USNM 54462	La Parguera, Puerto Rico	2.8	2.4	May/1975	female
	USNM 54462	La Parguera, Puerto Rico	1.9	1.4	May/1975	female
	USNM 54464	Puerto Cabello, Venezuela	2.8	1.5	29/set/1965	female
	USNM 54450	La Parguera, Puerto Rico	3.2	1.6	24/Feb/1974	male
Tamoya haplonema	MEL- TH2	Ubatuba, Brazil	11.5	5	01/Aug/2012	female
	MEL- TH3	Ubatuba, Brazil	12	4.5	01/Aug/2012	female
Tripedalia cystophora	USNM 54410	La Parguera, Puerto Rico	1	0.5	11/Aug/1973	female
	USNM 54410	La Parguera, Puerto Rico	1.8	0.5	11/Aug/1973	female
	USNM 54410	La Parguera, Puerto Rico	0.9	0.6	11/Aug/1973	male

Copula sivickisi	MEL- F1-4(1)	Shirahama, Japan	0.5	0.45	Aug/2006	female
Chironex fleckeri	USNM 100353	Fannie Bay Beach, Darwin, Australia	No data	No data	05/Jan/1999	female
	USNM 1155730	Mackay Island, Queensland, Australia	7	3.5	18/Dez/2008	male
Chironex yamaguchii	USNM 28694	Bolinao Bay, Luzon Island, The Philippines	9 (ca)	5 (ca)	10/May/2009	male
	USNM 28692	Malcochin Harbor, Linapacan Island, The Philippines	9 (ca)	4 (ca)	19/Dez/2008	female
	USNM 28696	Linapacan Strait, Linapacan Island, The Philippines	No data	No data	18/Dez/2008	female
Chiropsella bronzie	USNM 1131157	Newell Beach, Queensland, Australia	3	2	No data	female
Carybdea sp.	USNM 1155724	Goleta, California, USA	2.4	1.5	No data	female
Morbakka virulenta	USNM 1124251	Hiroshima, Japan	No data	No data	20/Dez/2006	female

Table 2. Summary of morphological and reproductive information obtained from gonadal samples of Cubozoa species analyzed in this study. NA = no data available.

Order	**Family**	**Species**	**Total specimens analyzed**	**Male**	**Female**	**Nematocyst within the gonads**		**Fertilization**	**Reference**	**Notes**
						male	**female**			
Carybdeida	*Alatinidae*	*Alatina alata*	4	1	3	Not observed		internal	Arneson and Cutress 1976 (as *Carybdea alata*); and this study	Glandular cells on gastrodermal epithelium. Hexagonal oocytes
	Carybdeidae	*Carybdea arborifera*	4	1	2	Not observed		NA	This study	Spawning male
		Carybdea murrayana (as *Carybdea branchi)*	2	1	1	yes	yes	NA	This study	Apical nematocysts within male gonads
		Carybdea brevipedalia	3	1	2	Not observed		external	Toshino et al. 2018; and this study	Asynchronous oogenesis. Spawning females.
		Carybdea rastonii	3	1	2	yes	yes	Internal?	Santhanam 2020; and this study	Asynchronous oogenesis. Nematocysts at gastrodermal epithelium.
		Carybdea marsupialis	2		2	Not observed		internal	Corbelli et al. 2003; and this study	Asynchronous oogenesis
		Carybdea xaymacana	4	1	3	Not observed		internal	Studebaker 1972 (as *Carybdea marsupialis*); and this study	Swellings to release sperm and inclusions of unknown nature in some oocytes.
		Carybdea *sp.*	1		1	Not observed		NA	This study	Some hexagonal oocytes. Spawning female.
	Carukiidae	*Morbakka virulenta*	1		1	Not observed	yes	external	Toshino et al. 2013; and this study	Inclusions of unknown nature within the oocyte
	Tamoyidae	*Tamoya haplonema*	2		2	Not observed		NA	This study	Inclusions of unknown nature within some oocytes
	Tripedaliidae	*Tripedalia cystophora*	3	1	π2	yes	yes	internal	Stewart 1996; and this study	Blastulae within the gonads. Nematoblasts present
		Copula sivickisi	1		1	yes	yes	internal	This study	Gonad widens in distalmost regions from the septum. Nematocysts and nematoblasts within the gonads

Chirodropida	Chirodropidae	*Chironex fleckeri*	2	1	1	Not observed	yes	external	Yamaguchi and Hartwick 1980; and this study	Nematoblasts within the gonads. Inclusions of unknown nature witin some oocytes
		Chironex yamaguchii	3	1	2			NA	This study	Oocyte packing
	Chiropsellidae	*Chiropsella bronzie*	1		1			external	Yamaguchi and Hartwick 1980; and this study	Asynchronous oogenesis

Appendix

Appendix I

Histological protocol for cubozoan jellyfishes (histoR).

Material fixed in formalin 4%.

1. Distilled water (3 x 30 min)

2. Alcohol dehydration series

- Alcohol 30 % (30 min)
- Alcohol 50 % (30 min)
- Alcohol 70 % (30 min) – we can stop here
- Alcohol 80 % (30 min)
- Alcohol 95 % (30 min)
- Alcohol 100 % (3 x 60 min)

3. Historesin infiltration

- resin + alcohol (1:1): infiltration solution 1: 1 (overnight in fridge)
- Replaced with an infiltration solution (resin) pure. Vacuum chamber for 3 days. Then put it in the fridge for 3 more days.
- Hardener inclusion - mix of 5 ml of pure resin and 0.35 ml of hardener (approximate measurement for 10 molds)

4. Orientation of material in resin molds (allow to harden for one day)

5. Mounting on wooden blocks using epoxy adhesive

6. Silica storage minimum one day before section.

Appendix II

Staining method Hematoxylin-Eosin (HE) in 5 µm section

- Mayer's hematoxylin (20 min at 37°C)
- Running tap water (2 min) – dry and analyze slides under a microscope to verify staining intensity
- Eosin (4 min at 37º C)
- Distilled water (2 min x 3)
- Differentiate in 70% alcohol (to remove excess Eosin)
- Wash in distilled water
- Drying on a slide warmer (37º C)
- Final assembly of slides with mounting medium (Entellan) and coverslip.

Before mounting the slides, dry in the oven at 60 °C (15 min)

Discussão Geral e Conclusões

Discussão Geral

Esta tese investigou a reprodução sexual em espécies de Medusozoa, tomando por base os dados de 193 espécies. Dentre estas, os dados de 172 foram obtidos em revisões bibliográficas e de 21 foram gerados por meio de pesquisas conduzidas no Marine Evolution Laboratory (MEL- USP) e no National Museum of Natural History, Smithsonian Institution (NMHN). Os resultados contribuem significativamente para o entendimento das estratégias reprodutivas e as caraterísticas da reprodução sexual nessa classe cosmopolita e diversa.

A histologia como ferramenta para inferir padrões reprodutivos

A importância da histologia para o estudo de animais marinhos é fundamental para avançar nosso entendimento sobre sua biologia e evolução. Por meio da análise histológica, acessamos informações detalhadas sobre a estrutura e função dos tecidos e estruturas desses animais, como demonstrado nos **Capítulos 1 a 3**. Essa abordagem permitiu inferir sobre processos evolutivos, incluindo a descrição da oogênese nos **Capítulos 2 e 3**, além de padrões morfológicos específicos, como o tamanho dos ovos (**Capítulo 1**) e a descrição de nematocistos (**Capítulo 3**).

Um dos principais desafios ao estudar animais marinhos reside na dificuldade de realizar experimentos em laboratório, devido às exigências específicas de seu habitat, como temperatura, salinidade, pH e fotoperíodo. Adicionalmente, a observação direta desses animais em seus ambientes naturais muitas vezes é inviável devido a fatores como profundidade do oceano, localização remota ou seu comportamento elusivo (e.g., espécies de profundidade; Jarms et al. 1999; Morandini 2003; Herring & Widder 2004). Nesse contexto, a histologia emerge como uma abordagem valiosa, oferecendo a possibilidade de investigar características internas dos organismos sem a necessidade de manipulação direta, acessando coleções de materiais de biológicos.

A adoção da histologia na literatura científica tem permitido descrições detalhadas, algumas já antigas, que continuam sendo referenciadas atualmente (e.g., Widersten 1965; Chapman 1974, 1978). Essa técnica também facilita a inferências de processos evolutivos, como adaptações ao ambiente (por exemplo, o aumento do tamanho dos ovos em espécies de águas mais profundas, García-Rodríguez et al. 2023, **Capítulo 1**), a identificação de padrões morfológicos (Miranda et al. 2016, 2017; García-Rodríguez et al. 2018, 2023a, **Capítulo 2**) e das relações filogenéticas entre espécies (e.g., García-Rodríguez et al. 2020, 2023b; **Capítulo 1** e **Capítulo 3**).

Análises de caráteres sexuais para inferir comportamentos ecológicos

Os atributos (= *traits*) sexuais, incluindo estruturas como gônadas e comportamentos de corte para a transferência de esperma (Lewis & Long 2005), são fundamentais para a reprodução e frequentemente refletem adaptações ao ambiente e ao modo de vida de uma espécie (García-Rodríguez et al. 2023a, b, **Capítulos 1 e 2**). Contudo, o conhecimento sobre a reprodução sexual em Medusozoa tem sido limitado, representando um desafio significativo nesse campo de estudo.

A análise dos caracteres sexuais serve também para inferir comportamentos ecológicos, tornando-se uma abordagem valiosa para uma compreensão mais ampla da ecologia e biologia evolutiva em Medusozoa. Neste estudo, a análise do tamanho dos ovócitos (García-Rodríguez et al. 2023b, **Capítulo 1**) mostrou-se uma ferramenta determinante para inferir padrões reprodutivos ecológicos (Thorson 1950; Laptikhovsky 2006), como já se sabia para outras espécies de invertebrados (e.g., moluscos e equinodermos, Grant & Tyler 1983). Igualmente, a observação de estágios variados de desenvolvimento de ovócitos nas gônadas (vitelogênese tardia, mediana e precoce; Schiariti et al. 2012; Mohamed et al. 2019; García-Rodríguez et al. 2023a) nos permitiu definir a ocorrência de um comportamento iteróparo (i.e., mais de um ciclo reprodutivo durante a vida) nas espécies com oogênese assíncrona (García-Rodríguez et al. 2023a, **Capítulo 2, Capítulo 3**). Por fim, a integração dos atributos sexuais com inferências em ecologia e biologia evolutiva permite uma compreensão mais qualificada e completa das complexas interações entre os organismos e seus ambientes.

Desafios na comparação e padrões reprodutivos da classe Medusozoa

Sabendo-se que Cnidaria é uma linhagem chave na compreensão da divergência precoce dos grupos de metazoários, os padrões de características depreendidos no estudo desse grupo podem representar características precursoras e expressas em outras linhagens ao longo da evolução animal (Galliot & Schmid, 2002). Porém, a comparação e compreensão de padrões de expressão dentre as espécies de Medusozoa representou um dos maiores desafios deste estudo (García-Rodríguez et al. 2023b, **Capítulo 1**). Em parte, isso ocorreu porque Medusozoa se destaca por uma notável diversidade de ciclos de vida, incluindo medusas planctônicas livre-natantes, medusas reduzidas que podem ou não ser liberadas, e até mesmo a completa ausência da fase de medusa, na qual a reprodução sexual é restrita a gonóforos que permanecem fixos nos pólipos bentônicos (Marques & Collins, 2004; Cartwright & Nawrocki, 2010). Essa diversidade multiplica-se em diferentes estratégias reprodutivas, levando a distintas capacidades de dispersão, padrões biogeográficos e níveis de conectividade populacional (Gibbons et al. 2009; Cunha et al. 2016). Além disso, foi constatado que variações nas características morfológicas e de desenvolvimento das gônadas e gonóforos refletem padrões macroevolutivos relevantes para o grupo (e.g., Widersten, 1965; Morandini & Silveira 2001; Bormaud

& Gravier-Bonnet, 2004; Schuchert & Brinckmann-Voss 2006; Schiariti et al. 2012). Diante de toda essa diversidade, a comparação entre diferentes espécies de Medusozoa revelou padrões interessantes, como a conservação do tamanho dos ovos, apesar da variedade de estratégias reprodutivas e tamanhos individuais observados dentro do grupo. A análise do tamanho dos ovos destacou-se como um atributo conservado (García-Rodríguez et al. 2023b, **Capítulo 1**) oferecendo *insights* valiosos para compreender a evolução e a biologia reprodutiva desses organismos tão distintos.

Conclusões

Os resultados apresentados nesta tese permitem destacar as seguintes conclusões principais:

1. Este é o primeiro estudo comparativo abordando o tamanho dos ovos em Medusozoa, demostrando que o tamanho do ovo nesse grupo apresenta um forte sinal filogenético, o que permitem compreender porque Medusozoa, com suas muitas estratégias reprodutivas diferentes (medusa, medusoide, gonóforo fixo) e tamanhos corpóreos diversos, tende a ter ovos de dimensões semelhantes. De fato, o tamanho do ovo é consideravelmente conservado, sendo a classe de tamanhos intermediários a condição ancestral do grupo.
2. O tamanho do ovo em Medusozoa é influenciado por outros atributos reprodutivos, como o número de ovos, em que ovos maiores estão frequentemente associados a espécies com poucos ovos (1–15); e a características ambientais, como profundidade e temperatura.
3. Além das caraterísticas morfológicas e de desenvolvimento únicas compreendidas em uma estauromedusa, Staurozoa é também a classe mais peculiar dentre os Medusozoa no que tange às características reprodutivas, com os menores ovos do subfilo, provavelmente associados às suas plânulas bentônicas rastejantes diminutas.
4. A presença de diferentes estágios de maturidade dos ovócitos em um indivíduo maduro é uma caraterística ocorrente em espécies com oogênese assíncrona, como demonstrado para *Chiropsalmus quadrumanus* (Cubozoa), o que sugere mais de um ciclo reprodutivo na vida do animal (iteroparidade).
5. Novos padrões foram desvelados para a reprodução sexual em Cubozoa, como a produção autosintética de vitelo, e a forma hexagonal nos ovócitos como arranjo de organização ocorrente na gônada de algumas espécies.
6. As gônadas de Cubozoa foram identificadas como locais de nematogênese em algumas espécies, evidenciado pela presença de nematoblastos, adicionando camadas de complexidade na reprodução do grupo.

Referências

Bourmaud C, Gravier-Bonnet N (2004) Medusoid release and spawning of *Macrorynchia philippina* Kirchenpauer, 1872 (Cnidaria, Hydrozoa, Aglaopheniidae). *Hydrobiologia*, *530*(1-3), 365-372. doi: 10.1007/s10750-004-2665-5

Chapman D (1974) Cnidarian histology. *In:* Coelenterate biology. Reviews and new perspectives, United Kin. Academic Press, pp 2–36

Chapman DM (1978) Microanatomy of the cubopolyp, *Tripedalia cystophora* (Class Cubozoa). *Helgoländer Wissenschaftliche Meeresuntersuchungen, 31,*128–168. doi: 10.1007/BF02296994

Cunha AF, Maronna MM, Marques AC (2016) Variability on microevolutionary and macroevolutionary scales: a review on patterns of morphological variation in Cnidaria Medusozoa. *Organisms Diversity & Evolution*, *16*, 431-442. doi: 10.1007/s13127-016-0276-4

García-Rodríguez J, Lewis Ames C, Marian JEAR, Marques AC (2018) Gonadal histology of box jellyfish (Cnidaria: Cubozoa) reveals variation between internal fertilizing species *Alatina alata* (Alatinidae) and *Copula sivickisi* (Tripedaliidae). *Journal of Morphology, 279,* 841–856. doi: 10.1002/jmor.20815

García-Rodríguez J, Ames CL, Marian JEAR, Marques AC (2020) Histomorphological comparison of testes in species of box jellyfish (Cnidaria; Cubozoa): does morphology differ with mode of reproduction and fertilization? *Organisms Diversity & Evolution*, *20,* 25–36. doi: 10.1007/s13127-019-00427-x

García-Rodríguez J, Ames CL, Jaimes-Becerra A, Tiseo GR, Morandini AC, Cunha AF, Marques AC (2023a) Histological Investigation of the Female Gonads of *Chiropsalmus quadrumanus* (Cubozoa: Cnidaria) Suggests Iteroparous Reproduction. *Diversity, 15*(7), 816. doi: 10.3390/d15070816

García-Rodríguez J, Cunha AF, Morales-Guerrero A, González-Chaves A, Camacho A, Miranda LS, Serrano FC, Jaimes-Becerra A, Marques AC (2023b) Reproductive and environmental traits explain the variation in egg size among Medusozoa (Cnidaria). *Proceedings of the Royal Society B: Biological Sciences, 290,* 20230543. doi: 10.1098/rspb.2023.0543

Gibbons MJ, Janson LA, Ismail A, Samaai T (2010) Life cycle strategy, species richness and distribution in marine Hydrozoa (Cnidaria: Medusozoa). *Journal of Biogeography*, *37*(3), 441-448. doi:10.1111/j.1365-2699.2009.02226.x

Grant A, Tyler PA (1983) The analysis of data in studies of invertebrate reproduction. II. The analysis of oocyte size/frequency data, and comparison of different types of data. *International Journal of Invertebrate Reproduction, 6,* 271–283. doi: 10.1080/01651269.1983.10510053

Herring PJ, Widder EA (2004) Bioluminescence of deep-sea coronate medusae (Cnidaria: Scyphozoa). *Marine Biology, 146*, 39–51. doi: 10.1007/s00227-004-1430-7

Jarms G, Båmstedt U, Tiemann H, Martinussen MB, Fosså JH, Høisœter T (1999) The holopelagic life cycle of the deep-sea medusa *Periphylla periphylla* (Scyphozoa, Coronatae). *Sarsia, 84,* 55–65. doi: 10.1080/00364827.1999.10420451

Laptikhovsky V (2006) Latitudinal and bathymetric trends in egg size variation: A new look at Thorson's and Rass's rules. *Marine Ecology, 27,* 7–14. doi: 10.1111/j.1439-0485.2006.00077.x

Lewis C, Long TAF (2005) Courtship and reproduction in *Carybdea sivickisi* (Cnidaria: Cubozoa). *Marine Biology, 147,*477–483. doi: 10.1007/s00227-005-1602-0

Marques AC, Collins AG (2004) Cladistic analysis of Medusozoa and cnidarian evolution. *Invertebrate Biology*, *123*, 23–42. doi: 10.1111/j.1744-7410.2004.tb00139.x

Miranda LS, Collins AG, Hirano YM, Mills CE, Marques AC (2016) Comparative internal anatomy of Staurozoa (Cnidaria), with functional and evolutionary inferences. *PeerJ, 4,* e2594. doi: 10.7717/peerj.2594

Miranda LS, García-Rodríguez J, Collins AG, Morandini AC, Marques AC (2017) Evolution of the claustrum in Cnidaria: comparative anatomy reveals that it is exclusive to some species of Staurozoa and absent in Cubozoa. *Organisms Diversity & Evolution, 17,* 753–766. doi: 10.1007/s13127-017-0342-6

Mohamed R, Skrypzeck H, Gibbons MJ (2019) Describing gonad development and gametogenesis in southern Africa's endemic box jellyfish *Carybdea branchi* (Cubozoa, Carybdeidae). *African Journal of Marine Science*, *41*(1), 83–91. doi: 10.2989/1814232X.2019.1575282

Morandini AC, Silveira FL (2001) Sexual reproduction of *Nausithoe aurea* (Scyphozoa, Coronatae). Gametogenesis, egg release, embryonic development, and gastrulation. *Scientia Marina*, *65*(2), 139-149. doi: 10.3989/scimar.2001.65n2139

Morandini AC (2003) Deep-Sea medusae (Cnidaria: Cubozoa, Hydrozoa and Scyphozoa) from the coast of Bahia (western South Atlantic, Brazil). *Mitt Hamb Zool Mus Inst, 100,* 13–25.

Cartwright P, Nawrocki AM (2010) Character Evolution in Hydrozoa (phylum Cnidaria). *Integrative and Comparative Biology*, *50*(3), 456–472. doi:10.1093/icb/icq089

Schiariti A, Christiansen E, Morandini AC, da silveira FL, Giberto DA, Mianzan HW (2012) Reproductive biology of *Lychnorhiza lucerna* (Cnidaria: Scyphozoa: Rhizostomeae): Individual traits related to sexual reproduction. *Marine Biology Research, 8,* 255–264. doi: 10.1080/17451000.2011.616897

Schuchert P, Brinckmann-Voss A (2006) The true and false gonophores of *Coryne epizoica* and its shell colonizing mechanisms. *Italian Journal of Zoology*, *73*(4), 319-324. doi: org/10.1080/11250000600881340

Thorson G (1950) Reproductive and larval ecology of marine bottom invertebrates. *Biological Reviews 25,* 1–45. doi: 10.1111/j.1469-185X.1950.tb00585.x

Widersten B (1965) Genital organs and fertilization in some Scyphozoa. *Zoologiska Bidrag fran Uppsala, 37,* 45–58. doi: 10.1016/0011-7471(66)90698-x

Resumo

Estudos descritivos e comparativos sobre a reprodução sexuada em Medusozoa, um grupo diversificado de Cnidaria, são escassos, o que restringe a compreensão de sua evolução, especialmente em relação a padrões históricos e funcionais. Esse estudo buscou elucidar, por meio de dados da literatura e de dados próprios, aspectos histomorfológicos da reprodução em quatro classes de Medusozoa — Hydrozoa, Cubozoa, Scyphozoa e Staurozoa — explorando suas variadas estratégias reprodutivas. Nossos objetivos foram: (1) investigar a variação do tamanho dos ovócitos em espécies de Medusozoa por meio de uma abordagem comparativa; (2) inferir padrões reprodutivos da cubomedusa *Chiropsalmus quadrumanus* a partir de descrições histomorfológicas das gônadas e do desenvolvimento dos ovócitos; (3) elucidar padrões reprodutivos e a oogênese em Cubozoa, expandindo a base taxonômica e empregando dados histomorfológicos para explorar características reprodutivas inéditas. Este estudo revelou aspectos únicos e complexos da reprodução em animais não-bilaterais. Entre os principais resultados, destacam-se o pioneirismo em demonstrar que o tamanho do ovo é uma característica conservada e com significativo sinal filogenético em Medusozoa, influenciado tanto por fatores ambientais, como a profundidade e temperatura, quanto por aspectos da história de vida, como o número de ovos produzidos. Também propusemos a primeira escala de maturação de ovócitos baseada em dados histomorfológicos para Chirodropida, e inferimos a iteroparidade (reprodução contínua) e fertilização externa em *Chiropsalmus quadrumanus*, com base na análise das gônadas e ovócitos. Adicionalmente, descrevemos características inéditas associadas às estruturas reprodutivas de Cubozoa, incluindo a presença de ovócitos hexagonais, fertilização interna da gônada, síntese autossintética de vitelo a partir de possíveis núcleos acessórios, e a ocorrência de nematocistos e nematoblastos nas gônadas, sugerindo o tecido gonadal (gastroderme) como possível sítio de nematogênese. Essa investigação enriqueceu e qualificou o conhecimento sobre a diversidade e complexidade dos Medusozoa, especialmente no que concerne à reprodução sexual e seus atributos reprodutivos. As novas evidências trazidas por esse estudo adicionam complexidade à compreensão das características reprodutivas e da história de vida dos Cubozoa, ampliando a perspectiva sobre sua ecologia e evolução no ambiente marinho.

Abstract

Descriptive and comparative studies on sexual reproduction in Medusozoa, a diverse group of Cnidaria, are scarce, limiting the understanding of their evolution, especially regarding historical and functional patterns. This study sought to elucidate, through literature data and original data, histomorphological aspects of reproduction in four classes of Medusozoa — Hydrozoa, Cubozoa, Scyphozoa, and Staurozoa — exploring their varied reproductive strategies. Our objectives were: (1) to investigate the variation in oocyte size in Medusozoa species through a comparative approach; (2) to infer reproductive patterns of the box jellyfish *Chiropsalmus quadrumanus* from histomorphological descriptions of the gonads and oocyte development; (3) to elucidate reproductive patterns and oogenesis in Cubozoa, expanding the taxonomical base and using histomorphological data to explore novel reproductive characteristics. This study revealed unique and complex aspects of reproduction in non-bilateral animals. Among the main results, it was pioneering in demonstrating that egg size is a conserved characteristic with a significant phylogenetic signal in Medusozoa, influenced by both environmental factors, such as depth and temperature, and life history aspects, such as the number of eggs produced. We also proposed the first oocyte maturation scale based on histomorphological data for Chirodropida, and inferred iteroparity (continuous reproduction) and external fertilization in *Chiropsalmus quadrumanus*, based on the analysis of gonads and oocytes. Additionally, we described novel characteristics associated with the reproductive structures of Cubozoa, including the presence of hexagonal oocytes, internal fertilization of the gonad, autosynthetic synthesis of yolk from possible accessory nuclei, and the occurrence of nematocysts and nematoblasts within the gonads, suggesting the gonadal tissue (gastroderm) as a possible site of nematogenesis. This investigation enriched and qualified knowledge about the diversity and complexity of Medusozoa, especially concerning sexual reproduction and its reproductive attributes. The new evidence brought by this study adds complexity to the understanding of reproductive characteristics and life history of Cubozoa, broadening the perspective on their ecology and evolution in the marine environment.

ANEXOS I e II

PROCEEDINGS B

royalsocietypublishing.org/journal/rspb

Research

Cite this article: García-Rodríguez J, Cunha AF, Morales-Guerrero A, González-Chaves A, Camacho A, Miranda LS, Serrano FC, Jaimes-Becerra A, Marques AC. 2023 Reproductive and environmental traits explain the variation in egg size among Medusozoa (Cnidaria). *Proc. R. Soc. B* **290**: 20230543. https://doi.org/10.1098/rspb.2023.0543

Received: 6 March 2023
Accepted: 30 June 2023

Subject Category:
Evolution

Subject Areas:
cellular biology, evolution, ecology

Keywords:
oocyte, reproduction, medusa, phylogenetic signal, depth, temperature

Author for correspondence:
Jimena García-Rodríguez
e-mail: jimena.gar88@gmail.com

Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.6730050.

THE ROYAL SOCIETY
PUBLISHING

Reproductive and environmental traits explain the variation in egg size among Medusozoa (Cnidaria)

Jimena García-Rodríguez[1], Amanda Ferreira Cunha[1,3], Adriana Morales-Guerrero[1], Adrian González-Chaves[2], Agustín Camacho[4], Lucília Souza Miranda[5], Filipe C. Serrano[2], Adrian Jaimes-Becerra[1,6] and Antonio Carlos Marques[1]

[1]Department of Zoology, Institute of Biosciences and [2]Department of Ecology, Institute of Biosciences, University of São Paulo, R. Matão, Tv. 14, 101, 05508-090 São Paulo, Brazil
[3]Departamento de Biologia Animal, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa, Av. P.H. Rolfs, s/n, 36570-900 Viçosa, Brazil
[4]Departamento de Ecología Evolutiva, Estación Biológica de Doñana, CSIC, Av. Américo Vespucio s/n, 41092 Sevilla, Spain
[5]Department of Zoology, Institute of Biological Sciences, Federal University of Minas Gerais, Av. Presidente Antônio Carlos 6627, 31270-901 Belo Horizonte, Brazil
[6]Department of Ecology, Evolution and Behavior, Alexander Silberman Institute of Life Sciences, Faculty of Science, Hebrew University of Jerusalem, 9190401 Jerusalem, Israel

Medusozoa (Cnidaria) are characterized by diverse life cycles, with different semaphoronts (medusa, medusoid, fixed gonophore, polyp) representing the sexual phase and carrying the gametes. Although egg size is often considered a proxy to understand reproductive and developmental traits of medusozoans, understanding of the processes influencing egg size variation in the group under an evolutionary context is still limited. We carried out a comprehensive review of the variation of egg size in Medusozoa to test whether this variation is related to biological/sexual or environmental traits. Egg size presents a strong phylogenetic signal ($\lambda = 0.79$, $K = 0.67$), explaining why closely related species with different reproductive strategies and different individual sizes have similar egg sizes. However, variation in egg size is influenced by the number of eggs, depth and temperature, with larger eggs frequently present in species with few eggs (1–15), in deep-sea species and in cold-water species. Conversely, the production of small eggs among cold-water species of Staurozoa might be associated with the development of a small benthic larvae in this group. Our study reinforces that egg sizes respond to reproductive and environmental traits, although egg size is highly conserved within medusa classes.

1. Introduction

Interspecific comparison of sexual traits helps to understand the diversity and evolution of reproductive patterns, for example, why species living in shallow and deep waters have different reproductive performances [1,2]. Some frequently studied sexual traits are gonadal morphology [3], fecundity (e.g. number of eggs) [4], gametogenesis [5], reproductive cycles (e.g. phenology) [6] and sexual strategies (e.g. predominance of asexual or sexual reproduction) [7]. Egg size has raised interest because it is considered a proxy to infer characteristics of the reproductive cycle of marine species [8]. Variation in egg size has been found to predict variation in larval and female size [9,10], developmental mode (lecithotrophic larval species have larger eggs in many taxa) [11], reproductive cycle (e.g. deep-sea echinoderms show egg size variation depending on their reproductive biology) [6] and fertilization success (e.g. larger eggs have a higher chance of fertilization in marine invertebrates with broadcast-spawning) [12]. Egg size was also demonstrated to respond to environmental conditions

[13,14], with larger eggs present in species living at low salinity (e.g. in estuarine amphipods [15]), in deep-sea habitats (e.g. in deep-sea fishes) [13], in low temperatures (with lipid-rich yolk stocks) [14] and in food-rich environments (e.g. in echinoderms) [16]. Similarly, a correlation between egg size and a given environmental factor may also depend on the species and latitude [14,17].

Sexual reproduction in Cnidaria (including Anthozoa, Endocnidozoa and Medusozoa [18]) is highly diverse [19]. Among the major cnidarian lineages, Medusozoa (a subphylum including the classes Hydrozoa, Scyphozoa, Cubozoa and Staurozoa) is particularly interesting because of its unique metagenetic life cycle [20]. The life cycle includes a swimming larva or planula, a sedentary polyp, and a free-living medusa [20], and the two main stages (polyp and medusa) may undertake sexual and asexual reproduction [21,22]. The medusa, when present, is the sexual adult phase carrying the gonads and gametes [22,23]. The development of the medusae is particularly plastic in the class Hydrozoa, where it may show several degrees of morphological reduction, even among species of the same genus [24]. Some species have medusoids (i.e. a short-lived reduced medusae [25,26]) that may remain attached to the gonotheca in a structure named a meconidium [27]. Other species have the medusa stage reduced to a fixed gonophore or sporosac [28], which is attached to the hydroid and considered to be the most reduced state of a medusa [29].

Medusozoan gonads are usually the focus in studies concerning sexual reproduction [30], and have been considered either as the regions where gametes are formed (therefore not true 'gonads', or not even an organ [31,32]), or true reproductive individualized organs with meiotic cells, sperm, and vitellogenic oocyte stages [33]. Gonadal development, location, and shape are also distinctive characters among medusozoans [34]. Scyphozoa, Staurozoa and Cubozoa are predominantly gonochoristic with gonads developing from the endodermal tissue of the bell [31,35,36]. Meanwhile Hydrozoa have gonads of ectodermic origin developing either in the region of the radial canals (e.g. in the leptothecate *Mitrocoma cellularia* [37]), around the manubrium (e.g. in the 'anthoathecate' *Sarsia lovenii* [38]) or spadix (in fixed gonophores such as in the 'anthoathecate' *Eudendrium* [39]). Gametes (egg and sperm) can be liberated directly into the water for external fertilization (broadcasting as in *Nemalecium lighti* [40]), which is the presumed ancestral state in marine invertebrates [41], or only the sperm is liberated and fertilizes the eggs inside the gastrovascular cavity (e.g. *Alatina alata* [42]), sometimes including brooding (e.g. *Eudendrium* [39]).

Some topics of the sexual reproduction in Medusozoa have been relatively well studied, such as gametogenesis [43] and fertilization [44]. However, there is little information concerning egg morphology and nutrient content in Medusozoa [45]. Egg size data are also scarce, and comparative studies focusing on its variation within the group have not yet been published, despite the importance and high variability of this reproductive trait among Medusozoa. For instance, the contribution of historical processes and environmental pressures to Medusozoa reproductive patterns is limited, with no estimates of the relative influence of phylogeny and environment in reproductive trait variation.

In this study we use comparative phylogenetic methods to understand the evolution of egg size in Medusozoa, considering the phylogenetic relationships within Medusozoa, and the relative contributions of reproductive (viz. egg number, individual size, reproductive strategies [polyp, medusa, medusoid], fertilization mode [internal, external]) and environmental (viz. depth range, water temperature) traits.

2. Material and methods

(a) Oocyte and egg

We highlight that the technical literature generally uses egg (or ovum, pl. ova) and oocyte (or ovocyte) as synonymous, referring to stages of differentiation of the female gametes from the primary oocyte during the oogenesis [46]. The egg is derived from the maturation of the ootid that resulted from the meiosis II of the secondary oocyte. Ovum is considered the mature female gamete after meiosis is completed, whereas the secondary oocyte is an immature egg cell produced by the meiosis I of the primary oocyte [47,48]. Therefore, strictly, the terms egg and oocyte are not synonymous. However, the use of the term egg to refer to a late vitellogenic oocyte stage (O_{iii}) is widespread, and we adopt the term herein. The largest oocyte in the gonad is considered to represent the late vitellogenic stage [49] and is therefore the one usually measured to obtain the egg size.

(b) Reproductive and ecological traits

Information on egg size was obtained for a total of 187 species of Medusozoa (136 Hydrozoa, 8 Staurozoa, 32 Scyphozoa and 11 Cubozoa), for which the taxonomy was standardized according to the World Register of Marine Species (WoRMS, 2021). Most of the species had their measurement surveyed in the literature, either by collecting data directly as provided in the paper or by measuring the eggs from photos or drawings available in species descriptions using ImageJ [50] (electronic supplementary material, table S1). We complemented the data by measuring eggs from histological preparations of gonads of hydrozoans (*Liriope tetraphylla*, *Orthopyxis crenata*, *Turritopsis nutricula*) and cubozoans (*Alatina alata*, *Carybdea marsupialis*, *Chiropsalmus quadrumanus*, *Copula sivickisi*, *Tamoya haplonema*) (electronic supplemental methods). When measuring eggs both from literature illustrations and histological preparations, we selected the largest eggs, considered to be in late vitellogenic stage (O_{iii}). For histological preparations, we measured up to 20 O_{iii} and used the average size value in the analyses. When egg size data available from the literature were given in ranges ($n = 90$), we used the average size value for subsequent analyses.

Information of reproductive traits was obtained for each species based on a literature review. We considered the following traits: (1) fertilization mode (external or internal), (2) number of eggs (1–15, 16–50, 51–100 and ≥101), (3) reproductive strategy (medusa, medusoid, fixed gonophore and polyp), and (4) individual size (see supplemental methods for further details). We also obtained information on the following environmental traits: (1) the mean sea surface temperature data for each species ($n = 185$; electronic supplementary material, table S1), and (2) the maximum depth of occurrence ($n = 187$; electronic supplemental material, methods).

(c) Phylogenetic signal

In order to evaluate if the evolution of egg size in Medusozoa is constrained by phylogeny, we tested for phylogenetic signal (statistical dependence among species' trait values due to their phylogenetic relatedness [51]) of egg size with Blomberg's K and Pagel's lambda (λ) [52], with the 'phylosig' function in the package 'phytools' in R [52]. Both use Brownian motion (BM) evolutionary process in their implementation but differ in their approach. Blomberg's K compares the variance of a trait with the variance from a BM model. Values of K can take higher than 1 representing more phylogenetic signal than expected (more variance between clades) and K lower than 1 representing less phylogenetic signal than expected (more variance within clades) [51]. Pagel's λ, on the other hand, illustrates the transformation of the phylogeny that fits a BM model so when λ equals 1, the phylogeny can explain changes in traits, indicating a high phylogenetic signal and when λ equals 0 the trait is evolving independently of the phylogeny [51]. We therefore use high indices (λ close to 1 and p-values significative) to assess that closely related species are similar in egg size [53]. In addition, we tested the phylogenetic signal for environmental traits which turned out significant in the analysis of model selection and phylogenetic ANCOVA. This was done in order to test the pattern of phylogenetic niche conservatism.

(d) Ancestral state reconstruction

To investigate the evolutionary history of egg size, egg number, temperature and depth we reconstructed ancestral states of these variables using the tree topology of 134 species (see supplemental methods for further details). Briefly, a composite phylogenetic tree including sequences of mitochondrial (16S and COI) and nuclear genes (18S and 28S) of 134 species was constructed based on the available published molecular phylogenies [54–59], and species for which we did not have egg size data were later removed from the tree. Bayesian estimates of divergence times were obtained using BEAST2 [53].

The ancestral state of egg size and temperature (continuous variable) was estimated using the 'fastAnc' function in the R package 'phytools' [53]. The model of evolution (Brownian motion, Ornstein–Uhlenbeck or early-burst) was selected using the R package 'geiger' [60] based on AIC scores. The ancestral states were mapped on the phylogeny using the 'contMap' function in R package 'phytools' [53]. We used maximum likelihood to compare models of traits evolution: Brownian motion (BM), Ornstein–Uhlenbeck (OU) and early burst (EB) models of trait evolution [61]. The ancestral state of egg number, and depth (categorical traits) were inferred by using stochastic character mapping implemented in R [62]. Transitions from one state to another can occur at different rates and in different directions in the phylogeny. For that, we first chose the best-fitting model of evolution for the reconstruction of this categorical trait among equal-rates (ER), symmetrical (SYM) and all-rates-different (ARD), identified by the lowest AICc score, calculated with the 'ace' function in the package 'APE' in R [63]. The uncertainty on the value of the trait at each ancestral node was described with the 'make.simmap' function (in 'phytools') after 1000 generations, resulting in a posterior probability distribution of character histories. The posterior distribution of character state history along each branch was summarized by using the 'describe.simmap' function [53].

(e) Model selection

We used PGLS to investigate the influence of phylogeny, reproductive (number of eggs, individual size, reproductive strategies), and ecological (temperature and depth) traits' on egg size. For this analysis, phylogenetic distances among species were obtained from our phylogenetic hypothesis based on molecular data (n = 134; see electronic supplementary material, methods), the same one used for the ancestral state reconstructions.

We assumed a Gaussian error distribution for the log transformed egg size variable, which seemed appropriated given the high number of species with data (134). Using the full model (constructing with all the variables), we compared different covariance structures corresponding to alternative models of evolution based on the lowest AIC value [64]. The tested covariance structures were BM, Martins (equivalent to OU model), Grafen (incorporates a calculation of branch lengths based on number of descendants), Blomberg (assumes that traits evolve under a BM model which rates either accelerate or slow through time) and Pagel (a modification of a BM model that incorporates phylogenetic signal). Between the plausible models we tested the multicollinearity using the 'vif' function in the R package 'car' [65].

We use the 'vif' function to remove the variable (greater than 3) to obtain our full model without multicollinearity. Then, we used the function 'model.sel' to identify which factors led to the lowest AIC. After building the full model considering additive effects among the predictive variables, we tested all possible combinations (between the variables number of eggs, individual size, reproductive strategies, class and temperature) using 'dredge' function from 'MuMIn' packages [66]. All models with delta AIC lower than 2 were considered equally plausible (see electronic supplementary material, table S3).

Model validation was performed with residual analyses (standardized residuals versus fitted values and Q-Q plots) (see the script in electronic supplementary material).

Finally, we tested the association of egg number and both reproductive strategy and fertilization mode fitting a phylogenetic generalized linear squares model (PGLS) [67]. Since evolutionary correlation of discrete characters is prone to reporting spurious significant associations [68], we transformed 'egg number' from a categorical to a continuous variable by using the mean number of each interval. Furthermore, has visual inspection of data is likely better to interpret these results [68,69], we mapped these traits using boxplots (see electronic supplementary material, figures S1 and S2, and script).

(f) Phylogenetic ANCOVA

In addition to model selection and in order to test the hypothesis that egg number, individual size, reproductive strategy, depth, or temperature affects egg size in Medusozoa, we implement an ANCOVA model using phylogenetic generalized least squares (PGLS). This was done because we have a combination of continuous and discrete traits as independent variables [70].

3. Results

(a) Phylogenetic signal

There is strong phylogenetic signal related to egg size across Medusozoa (Pagel's $\lambda = 0.79$, $p < 0.001$; $K = 0.67$, $p < 0.001$), considering that related species have similar egg sizes. The λ and K values (0.79 and 0.67 respectively) (close to 1) correspond to similar egg sizes between close taxa (figure 1), even though $K < 1$ shows that there is also moderate intra-clade variance. Likewise, it was also found that there is phylogenetic signal for temperature (Pagel's $\lambda = 0.80$, $p < 0.001$ and $K = 0.60$, $p < 0.001$) and depth ($p < 0.05$—rejects no signal model). This means that related species in the phylogeny have similar temperature values and depth ranges.

(b) Ancestral reconstructions of egg size and related reproductive/environmental traits

Reconstruction of the ancestral state of egg size in Medusozoa shows that intermediate egg sizes are plesiomorphic and highly conserved within the group (figure 1; best-fit was OU model, AIC = 1750.46). The smallest eggs occur in Staurozoa (18–72 µm), intermediate in Cubozoa and Hydrozoa, and some of the largest in Scyphozoa (figure 1).

A higher number of eggs ($n \geq 101$) is the ancestral condition in Medusozoa, conserved in Staurozoa, Scyphozoa, and Cubozoa (figure 2; best-fit was symmetrical model,

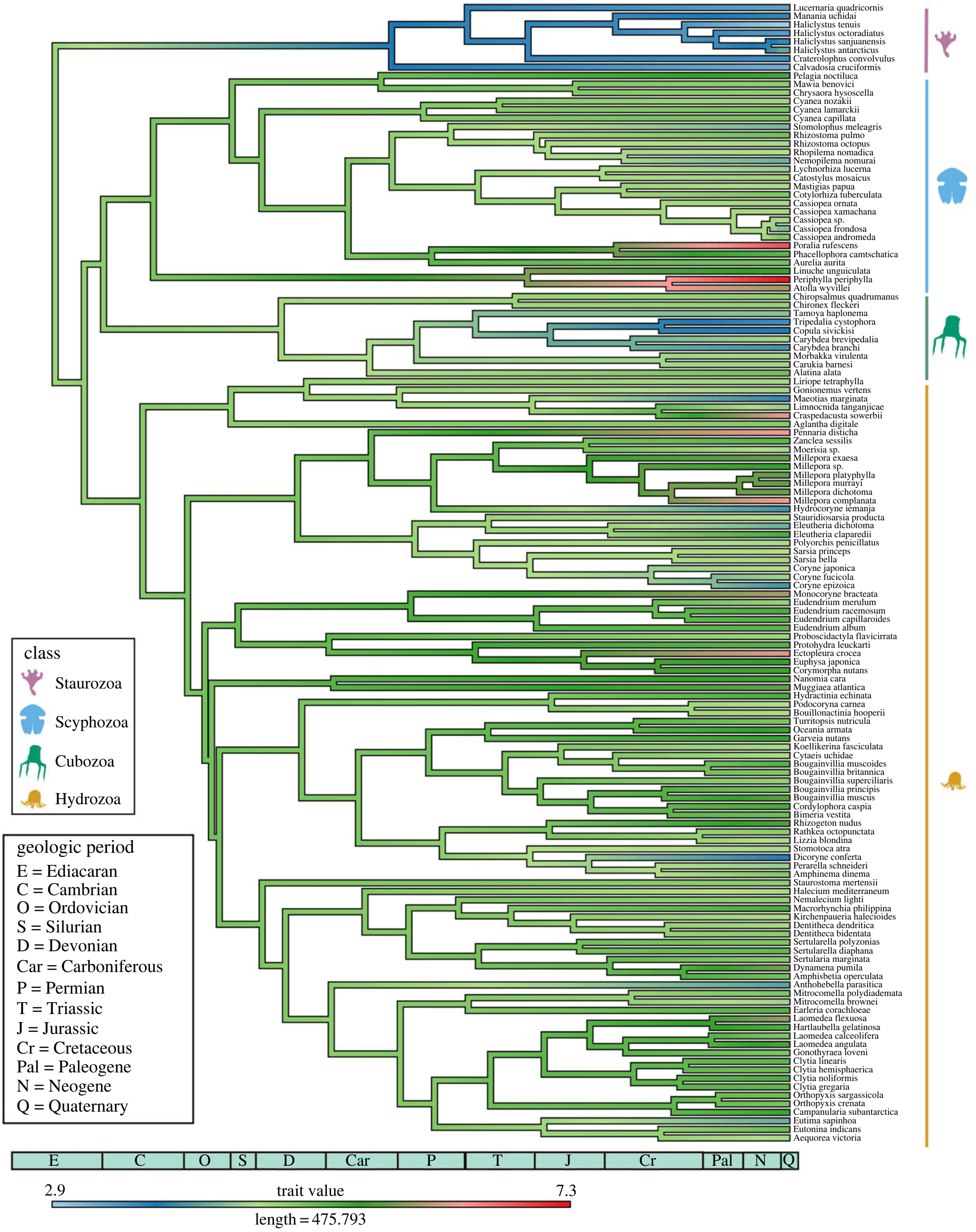

Figure 1. Reconstruction of ancestral egg size (log) in Medusozoa for the 134 species analysed in this study. Colour on branches indicates egg size along the topology. Trait value = min and max egg size (log) (2.9–7.3).

AIC = 135.43) ($n = 103$). The ancestral condition changed to fewer eggs ($n = 1$–15) in the origin of Hydroidolina. However, an intermediate number of eggs ($n = 16$–50 and 51–100) has evolved multiple times in less inclusive groups of Hydroidolina, such as Siphonophorae (e.g. *Muggiaea atlantica*, *Nanomia cara*), Capitata (e.g. *Coryne fucicola*, *Coryne epizoica*) and Macrocolonia (e.g. *Dentitheca dendritica*, *Sertularella polyzonias*). In addition, a reversal to a higher number of eggs ($n \geq 101$) originated independently in Eirenida (e.g. *Aequorea victoria*), Campanulinida (e.g. *Earleria corachloeae*), Hebellidae (e.g. *Anthohebella parasitica*) and Bougainvilliidae (*Bougainvillia superciliaris*).

Reconstruction of the ancestral state of temperature (figure 3; best-fit was OU model, AIC = 885.96) shows that the Medusozoa ancestor (≈ 680 millions of years) probably lived in temperate waters (≈ 14°C). There was an increase in the average temperature (Devonian/Carboniferous period) occupied by the species throughout the evolution of Cubozoa and Scyphozoa (Kolpophorae) in tropical waters, as well as Hydrozoa, with some decreases to cold

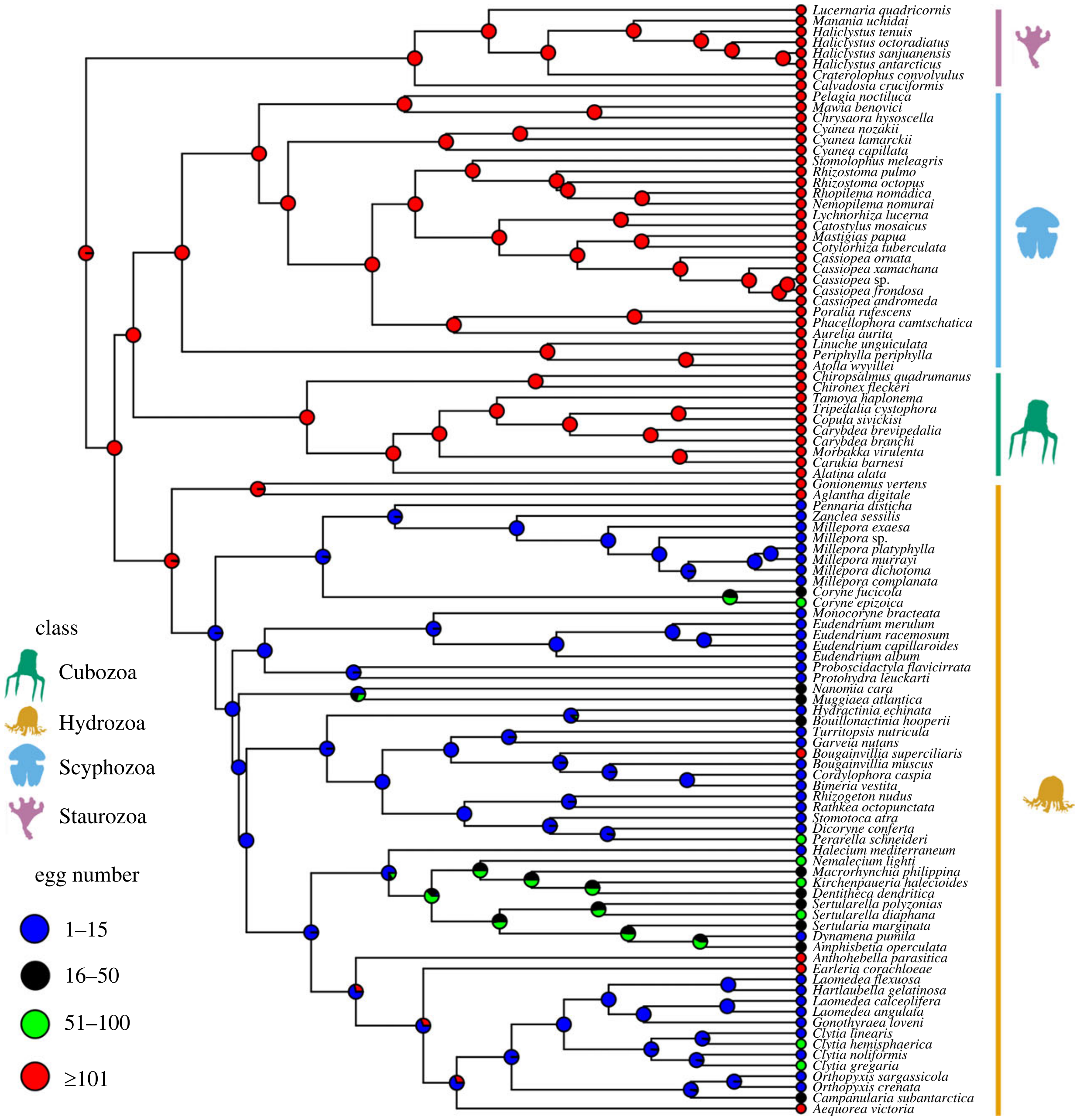

Figure 2. Inferred reconstruction of the number of eggs (1–15, 16–50, 51–100, ≥ 101) within Medusozoa, for 103 species included in the analysis. Colours inside the pie charts at the internal nodes represent the posterior probability of alternative categories of egg number.

temperatures in several clades (Campanulariida, Haleciida, Bougainvillia, Aplanulata). It is important to note that Staurozoa and a few Scyphozoa species currently occurring in cold waters present contrasting egg sizes (small eggs in Staurozoa and large eggs in deep sea Scyphozoa species, compare figure 1 and figure 3).

The ancestral state of the depth (best-fit was ER model, AIC = 118.8) shows that Medusozoa inhabited shallow waters (0–50 m), independently appearing in only a few species in deep waters (greater than 101 m) and intermediate waters (51–100 m) (electronic supplementary material, figure S3).

(c) Reproductive and environmental predictors of egg size

The evolutionary model based on AIC was for Grafen correlation (electronic supplementary material, table S2). Prior to model selection, multicollinearity was tested from the first model with six predictors (egg number, reproductive strategy, individual size, depth, and temperature). All predictors lower than 3, are showed on the final model. The best 5 models resulted under dredge function were compared using AIC (electronic supplementary material, table S3) and the best-fit model show that egg number, temperature and depth had an effect on egg size (AIC = 166.1, SE = 0.68, d.f. = 103) (figure 4 and electronic supplementary material, tables S3 and S4). The phylogenetic ANCOVA analyses show the same significant variables explaining the variation in egg size (depth, $p = 0.003$; egg number, $p = 0.037$; temperature, $p = 0.045$) as the results obtained with model selection.

Results from the phylogenetic generalized estimating equations analyses revealed a positive and significant correlation between the number of eggs and the 'medusa' reproductive strategy of Medusozoa (medusa: 1797.76, $t = 16.03$, $p < 0.001$) while not being significant for the other reproductive strategies (gonophore: 15.85, $t = 0.08$, $p = 0.93$;

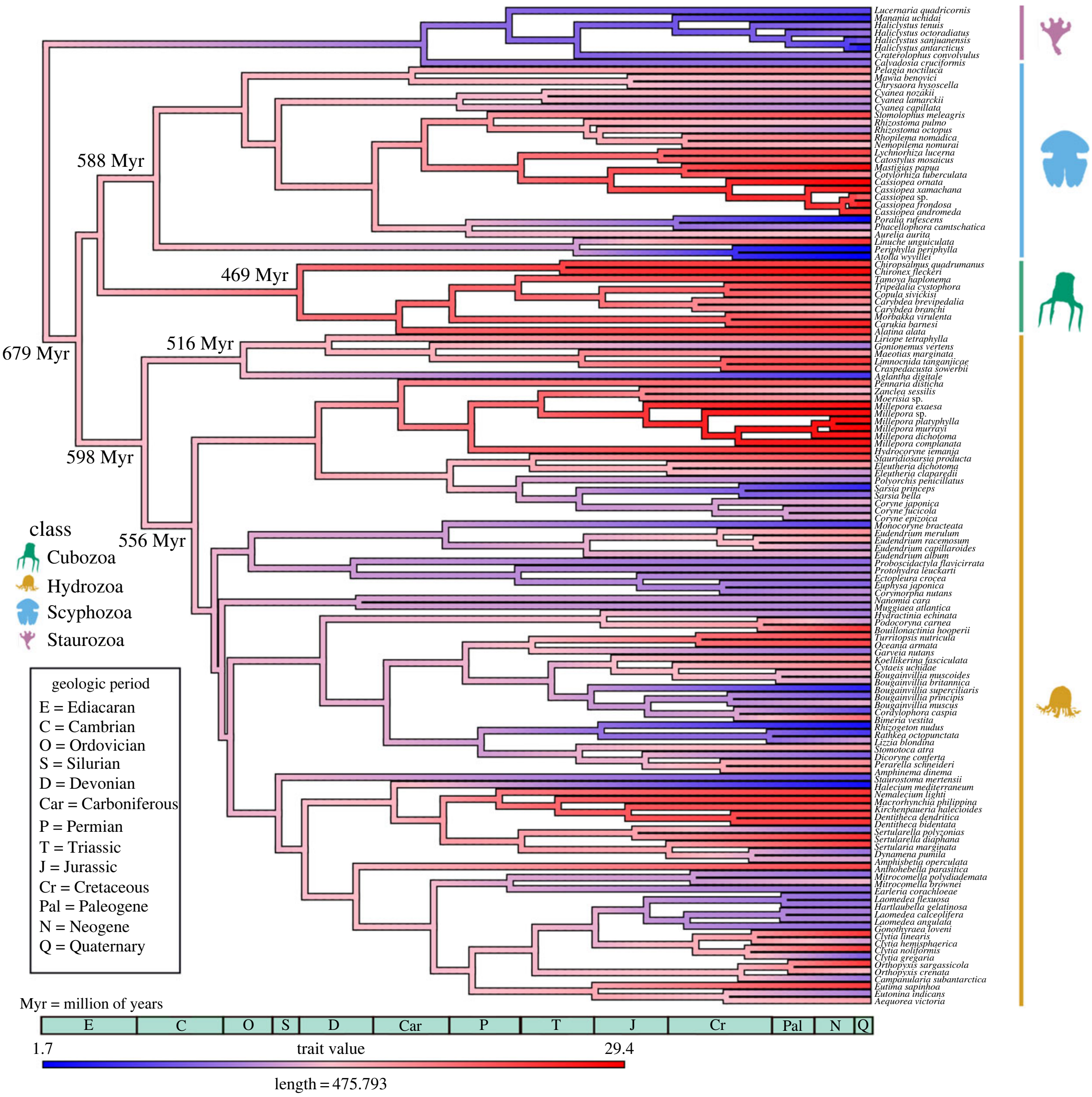

Figure 3. Ancestral reconstruction with time (in millions of years corresponding with geological periods) for temperature in Medusozoa. This graph assumes that the thermal niche of each lineage has not changed across the years. Colour figures: yellow = Hydrozoa, green = Cubozoa, blue = Scyphozoa, pink = Staurozoa. Trait value = min and max temperature (1.7–29.4°C).

medusoid: 242.44, $t = 0.91$, $p = 0.37$; polyp: 7.12, $t = 0.008$, $p = 0.99$) (electronic supplementary material, figure S1). Likewise, a significant correlation with the number of eggs was found for external fertilization (1626.54, $t = 8.71$, $p > 0.001$) but not for internal fertilization (–393.09, $t = -1.39$, $p = 0.174$) (electronic supplementary material, figure S2).

4. Discussion

(a) Evolution of egg size and its relationship with reproductive traits

Although small egg sizes are presumably a basal condition for animals [71], our analyses support that intermediate egg size is the ancestral condition in Medusozoa, being conserved along most of its phylogenetic history (figure 1). Among Medusozoa classes, however, Staurozoa and the cubozoan Tripedaliidae differ by having a relatively early transition (Carboniferous and Jurassic, respectively) to smaller eggs, while transitions to larger eggs in the scyphozoan Coronatae occurred since the Cretaceous (figure 1). Both genetic and environmental factors may explain egg size variation in marine invertebrates at different evolutionary levels, from individuals to populations, or even in species and more inclusive taxonomic groups [72]. Several medusozoan lineages have independent evolutionary shifts in egg size, but overall variation in egg size is correlated to the number of eggs related to different reproductive strategies (electronic supplementary material, figure S1). This suggests that egg size may be modulated by life-history tradeoffs and/or selective pressures unique to the evolutionary history of each taxa. Thus, for instance, species from different classes (e.g. Hydrozoa and Scyphozoa) living in similar habitats (e.g. deep sea species) are prone to have similar egg size.

Several models for the evolution of egg size in marine invertebrates have been suggested, often involving tradeoffs between egg size and egg number (e.g. [73]). For instance, larger eggs require a lower concentration of sperm for fertilization, and are related to reduced developmental time and

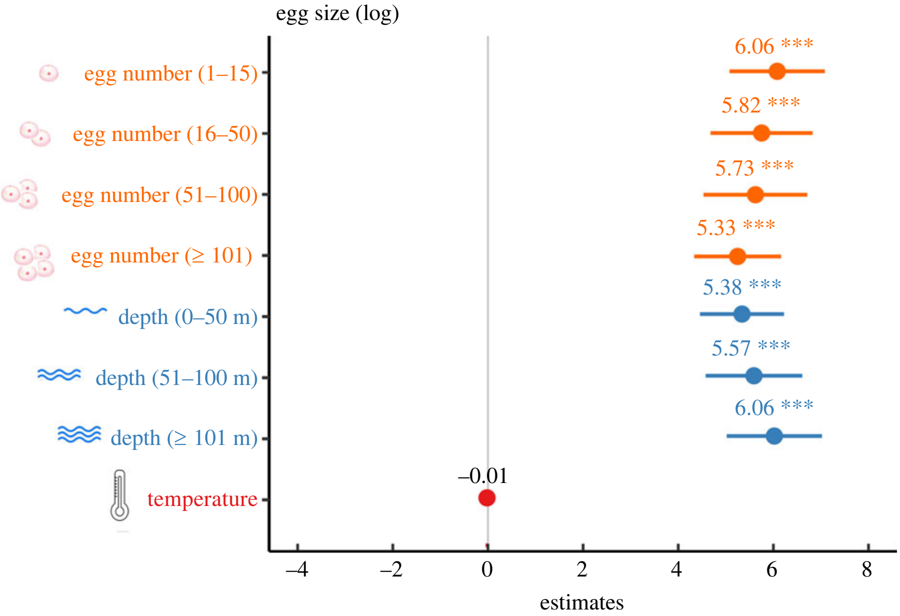

Figure 4. Forest plot of estimates of the best model with 95% confidence intervals. Variables that estimate overlap the line of null effect are not statistically significant while asterisks highlight significant variables (***$p > 0.001$). Values on the right side of the grey line represent positive estimates while values on the left side of the line (red) represent negative estimates.

offspring mortality, but have the disadvantage of an increase in resources allocated to each offspring, leading to a reduction in fecundity (or number of eggs) [12,73]. In Medusozoa, a higher number of eggs ($n \geq 101$) is the ancestral condition for the group (figure 2; see also [54]). In addition, a higher number of eggs is characteristic of species that show small to intermediate egg sizes (figure 4). A clear shift to a smaller number of eggs was observed in the appearance of Hydroidolina (Hydrozoa), which also presents a higher frequency of occurrence of fixed gonophores (electronic supplementary material, table S1). Indeed, our results show that species with medusa produce more eggs ($n \geq 101$) (electronic supplementary material, figure S1) and also show a tendency to have smaller eggs than species with fixed gonophores ($n = 1$–15) (e.g. *Eudendrium bentart*, *Eudendrium klausi*, *Laomedea flexuosa*, *Synthecium flabellum*; figure 4; electronic supplementary material, table S1).

The relationship between egg size, egg number and reproductive strategy in Hydrozoa was proposed by Teissier [71], who suggested that the reduction of the medusa in some lineages of Hydrozoa could explain the fewer and larger eggs in species with fixed gonophores. This explanation was subsequently corroborated in differences concerning egg number and egg size among hydrozoans with different reproductive strategies (e.g. [26,74]). For instance, most species with a fixed gonophore strategy (in which eggs are produced by the polyp stage) are colonial hydroids that, as modular organisms, compartmentalize the functions in different modules [75] (polymorphs) [76,77] (see supplemental methods). This compartmentalization, allow the resources to be shared among the modules, with growth and reproduction often proceeding simultaneously [77,78]. Differently, the medusa, as a unique individual, has to balance growth and reproduction [79]. In modular colonies, however the eggs are produced in small numerous modules called gonozoids, which are distributed along the colony [78,80] and may also contribute to the existence of larger eggs. In fact, brooding in medusozoans is often associated with modular colonies, which present smaller and more sparsely distributed masses of eggs when compared to medusae. This is consistent with the view that the cost of brooding is associated with oxygen provision [81], as smaller and more spaced embryos facilitate oxygen supply (e.g. [82]), and in the case of modular colonies, could favour the occurrence of brooding [83].These different patterns would have consequences from basic physiology (e.g. energetic demand of the different reproductive system and trade off with other systems) to species interaction (e.g. competition for substrate or prey) and biogeography (e.g. advective events and range distribution).

Among species with a fixed gonophore (within Hydrozoa), the relationship of brooders producing fewer and larger eggs, as well as larger planulae, than non-brooders have also been suggested elsewhere [26]. Our results statistically corroborate and extend part of this hypothesis, showing that egg number could be related to fertilization mode in Medusozoa (electronic supplementary material, figure S2). Indeed, our study corroborates that species with extrernal fertilization more often produces a higher number of eggs [84], while we found no support for internal fertilization being associated with the production of fewer eggs, as suggested before [85]. This, however, may reflect insufficient data on egg size of species with internal fertilization, and highlights the need for additional studies on reproductive patterns in Medusozoa, especially considering that several species are thought to present internal fertilization, even though most inferences are based on indirect evidence [86,87]. In a scenario in which external fertilization is usually considered as a primitive character [41], Medusozoa presents a high diversity and complexity of reproductive traits when compared to other basal Metazoa, and, given the few studies, a high potential for the discovery of additional complex reproductive traits. For instance, it includes viviparous species, as *Crossota millsae* [34], as well as elaborate sexual behaviors, such as the courtship and sperm transfer via spermatophore in *Copula sivickisi* [88].

(b) Egg size and environmental conditions in Medusozoa

Environmental conditions are often correlated with the evolution of morphological characters (e.g. different egg morphologies depending on temperature or salinity in copepods [89]) and with reproductive traits in marine invertebrates [90]. Several environmental variables are suggested to trigger variation in the morphology of Medusozoa as a result of phenotypic plasticity [91]. For instance, species reared under laboratory conditions, such as *Millepora complanata* [92] and *Pennaria disticha* [93], produced larger eggs (both 600 µm) than wild collected specimens, suggesting that a greater investment of energy in reproduction under controlled environment with higher food supply increments the female gamete size [94].

Temperature plays an important role in reproduction, triggering mass reproductive events based on synchronized gametogenetic cycles [95], and affecting egg number [96] and egg size [97]. Many marine taxa (e.g. amphipods, echinoids, fishes) generally produce larger eggs in colder temperatures (e.g. [98]). Within Medusozoa, large eggs are known to be related to direct development, in which the developing medusa benefits from the high amount of energy stored in the yolk of these eggs [99–101]. Direct development mainly occurs in species adapted to colder temperatures in deep-sea species [3]. Indeed, we found that the largest eggs among medusozoans are in deep-sea species, *Atolla wyvillei* (403 µm), and *Periphylla periphylla* (1480 µm), that also present direct development [102] and are associated with lower temperatures [14].

Besides, temperature increases in ectothermic animals, leading to increased energetic costs of development which could explain for the smaller egg sizes [103]. However, the relationship between temperature and egg size may also reflect adaptive strategies shaped throughout the evolutionary history of the group [14,104], which could be the case of Staurozoa that mainly occurs in intertidal cold waters [105] and has small eggs.

According to the results obtained from the phylogenetic signal for temperature and depth, we recognize the possible existence of a pattern of phylogenetic niche conservatism (PNC) that may arise from multiple process including phylogenetic constraint where a common selective environment is shared in related species [106].

(c) Staurozoa, the class with smaller eggs

Staurozoa showed an early shift to smaller eggs in the ancestral state reconstruction, coinciding with their colonization of colder waters [105] (figure 1). In Cnidaria, the planula stage follows egg fertilization and embryonic development. The planula and egg in Medusozoa are similar in size, as described for eggs and larvae of other organisms (e.g. echinoderms [107]). It is possible that the conspicuous difference between the egg size of Staurozoa and other medusozoans is related to the staurozoan unique planula [108]. The diminutive eggs of Staurozoa (18–72 µm recorded in this study) are followed by diminutive (e.g. 100 µm long and 20 µm wide in *Haliclystus* sp.), non-swimming (benthic) planulae, with a constant number of 16 endodermal cells [109] and apparently able to feed when attached [110] (although staurozoan eggs were reported with yolk substances [108,109,111]). Moreover, these small planulae have limited movement ability, crawling on the substrate (i.e. vermiform movements of elongation and retraction) [108,109]. Indeed, staurozoan planulae may settle in groups, living side-by-side, apparently maximizing the efficiency in prey capture [112,113] and maybe in defence, and promoting genetically homogeneous populations [112]. Finally, their small eggs and planulae dimensions and habit would also be associated with lower metabolic demands, as suggested for other marine invertebrates [114].

5. Conclusion

This is the first comparative study addressing egg size in Medusozoa and we show the importance of evaluating this trait and its relationship with historical, sexual, and ecological traits. Egg size in Medusozoa has a strong phylogenetic signal, explaining why this group with many different reproductive strategies and different individual sizes tend to have eggs of similar dimensions. Indeed, egg size is considerably conserved, with intermediate egg sizes as the ancestral condition in the group. Egg size is also related to sexual traits, such as the number of eggs, with larger eggs frequently associated with species with few eggs (1–15); and to environmental traits as depth and temperature. On the other hand, Staurozoa has small eggs probably associated with their small benthic crawling planulae, which therefore would have presumably lower metabolic demands. After this study we can conclude that variation in egg size in Medusozoa is explained by biological/sexual and environmental factors, stressing the role of past ecological and genetic processes in Medusozoa evolution. Further information on deep sea species, as well as data on fertilization mode and egg size of more species of Medusozoa, shall contribute to provide a broader understanding of the evolutionary patterns of sexual reproduction in this group, as well as in other marine taxa.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material [115].

Authors' contributions. J.G.-R.: conceptualization, data curation, formal analysis, investigation, project administration, resources, software, supervision, validation, visualization, writing—original draft, writing—review and editing; A.F.C.: conceptualization, supervision, validation, writing—review and editing; A.M.-G.: data curation, formal analysis, investigation, methodology, resources, software, validation, visualization, writing—review and editing; A.G.-C.: data curation, formal analysis, methodology, resources, software, validation, writing—review and editing; A.C.G.: data curation, formal analysis, methodology, software, supervision, validation, writing—review and editing; L.S.M.: validation, writing—review and editing; F.C.S.: formal analysis, methodology, software, writing—review and editing; A.J.-B.: data curation, formal analysis, investigation, methodology, resources, software, validation, visualization, writing—review and editing; A.C.M.: supervision, validation, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. The authors have no competing of interests.

Funding. This study was supported by São Paulo Research Foundation (FAPESP) (grants 2011/50242-5 to A.C.M., 2017/00641-7 to A.F.C., 2017/00312-3 to A.M.-G., 2017/19411-1 to A.G.-C., 2019/01370-2 and 2021/09739-5 to J.G.-R.), CNPq (grants 140235/2018-3 to J.G.-R., 316095/2021-4 to A.C.M.), CAPES (grant 23650751852 to A.J.-B.).

Acknowledgements. We are grateful to Prof. Alvaro Migotto, Prof. André Morandini and Dr Marina O. Fernandez for suggestions in response to a previous version of the manuscript. We thank Dr Edgar Gamero-Mora for providing pictures to measure egg size of *Cassiopeia* spp, and Prof. José Eduardo Marian for helping with the histological procedures. We also thank M. Enio Matos and Mr Phillip Lenktaitis for their support in the histology lab. We thank the four anonymous reviewers who improved the manuscript.

References

1. Shlesinger T, Loya Y. 2019 Sexual reproduction of Scleractinian corals in mesophotic coral ecosystems vs. shallow reefs. In *Mesophotic coral ecosystems* (eds Y Loya, KA Puglise, TCL Bridge), pp. 653–666. Berlin, Germany: Springer.
2. Waller RG. 2006 Deep-water Scleractinia (Cnidaria: Anthozoa): current knowledge of reproductive processes. In Cold-water corals and ecosystems (eds A Freiwald, JM Roberts), pp. 691–700. Berlin, Germany: Springer.
3. Lucas CH, Reed AJ. 2010 Gonad morphology and gametogenesis in the deep-sea jellyfish *Atolla wyvillei* and *Periphylla periphylla* (Scyphozoa: Coronatae) collected from Cape Hatteras and the Gulf of Mexico. *J. Mar. Biol. Assoc. U. K.* **90**, 1095–1104. (doi:10.1017/S0025315409000824)
4. Costa EFS, Dias JF, Murua H. 2015 Reproductive strategy and fecundity of the keystone species *Paralonchurus brasiliensis* (Teleostei, Sciaenidae): an image processing techniques application. *Environ. Biol. Fishes* **98**, 2093–2108. (doi:10.1007/s10641-015-0432-2)
5. Waller RG, Tyler PA, Gage JD. 2005 Sexual reproduction in three hermaphroditic deep-sea *Caryophyllia species* (Anthozoa: Scleractinia) from the NE Atlantic Ocean. *Coral Reefs* **24**, 594–602. (doi:10.1007/s00338-005-0031-3)
6. Gage JD, Tyler PA. 1991 Reproduction, recruitment and growth of deep-sea organisms. In *Deep-sea biology: a natural history of organisms at the deep-sea floor*, pp. 301–336. Cambridge, UK: Cambridge University Press.
7. Fischer EA, Petersen CW. 1987 The evolution of sexual patterns in the seabasses. *Bioscience* **37**, 482–489. (doi:10.2307/1310420)
8. Grant A, Tyler PA. 1983 The analysis of data in studies of invertebrate reproduction. II. The analysis of oocyte size/frequency data, and comparison of different types of data. *Int. J. Invertebr. Reprod.* **6**, 271–283. (doi:10.1080/01651269.1983.10510053)
9. Johnston TA, Leggett WC. 2002 Maternal and environmental gradients in the egg size of an iteroparous fish. *Ecol. Soc. Am.* **83**, 1777–1791.
10. McEdward LR. 1986 Comparative morphometrics of echinoderm larvae. I. Some relationships between egg size and initial larval form in echinoids. *J. Exp. Mar. Biol. Ecol.* **96**, 251–265. (doi:10.1016/0022-0981(86)90206-6)
11. Thorson G. 1950 Reproductive and larval ecology of marine bottom invertebrates. *Biol. Rev.* **25**, 1–45. (doi:10.1111/j.1469-185X.1950.tb00585.x)
12. Levitan DR. 2006 The relationship between egg size and fertilization success in broadcast-spawning marine invertebrates. *Integr. Comp. Biol.* **46**, 298–311. (doi:10.1093/icb/icj025)
13. Marshall NB. 1953 Egg size in Artic, Antartic and deep-sea fishes. *Evolution***7**, 328–341. (doi:10.1111/j.1558-5646.1953.tb00096.x)
14. Laptikhovsky V. 2006 Latitudinal and bathymetric trends in egg size variation: A new look at Thorson's and Rass's rules. *Mar. Ecol.* **27**, 7–14. (doi:10.1111/j.1439-0485.2006.00077.x)
15. Steele DH, Steele VJ. 1991 Effects of salinity on the survival, growth rate, and reproductive output of *Gammarus lawrencianus* (Crustacea, Amphipoda). *Mar. Ecol. Prog. Ser.* **78**, 49–56. (doi:10.3354/meps078049)
16. George SB. 1996 Echinoderm egg and larval quality as a function of adult nutritional state. *Oceanolog. Acta* **19**, 297–308.
17. Fleming IA, Gross MR. 1990 Latitudinal clines: A trade-off between egg number and size in Pacific salmon. *Ecol. Soc. Am.* **71**, 1–11. (doi:10.2307/20167213)
18. Kayal E, Bentlage B, Sabrina Pankey M, Ohdera AH, Medina M, Plachetzki DC, Collins AG, Ryan JF. 2018 Phylogenomics provides a robust topology of the major cnidarian lineages and insights on the origins of key organismal traits. *BMC Evol. Biol.* **18**. (doi:10.1186/s12862-018-1142-0)
19. Fautin DG. 2002 Reproduction of Cnidaria. *Can. J. Zool.* **80**, 1735–1754. (doi:10.1139/z02-133)
20. Müller WA, Leitz T. 2002 Metamorphosis in the Cnidaria. *Can. J. Zool.* **80**, 1755–1771. (doi:10.1139/z02-130)
21. Morandini AC, Schiariti A, Stampar SN, Maronna MM, Straehler-Pohl I, Marques AC. 2016 Succession of generations is still the general paradigm for scyphozoan life cycles. *Bull. Mar. Sci.* **92**, 343–351. (doi:10.5343/bms.2016.1018)
22. Collins AG. 2002 Phylogeny of medusozoa and the evolution of cnidarian life cycles. *J. Evol. Biol.* **15**, 418–432. (doi:10.1046/j.1420-9101.2002.00403.x)
23. Werner B. 1973 New Investigations on systematics and evolution of the class Scyphozoa and the phylum Cnidaria. *Publ. Seto Mar. Biol. Lab.* **20**, 35–61. (doi:10.5134/175791)
24. Boero F, Sarà M. 1987 Motile sexual stages and evolution of Leptomedusae (Cnidaria). *Bolletino di zoologia* **54**, 131–139. (doi:10.1080/11250008709355572)
25. Cornelius P. 1992 Medusa loss in leptolid Hydrozoa (Cnidaria), hydroid rafting, and abbreviated life-cycles among their remote-island faunae: an interim review. *Sci. Mar.* **56**, 245–261.
26. Migotto A. 1998 The life cycle of Sertularia marginata Kirchenpauer, 1864 (Cnidaria: Hydrozoa): a medusoid-producing sertulariid. *J. Nat. Hist.* **32**, 1–12. (doi:10.1080/00222939800770011)
27. Cornelius PFS. 1995 *North-west European thecate hydroids and their medusae, part 2: Sertulariidae to Campanulariidae*. London, UK: Field Studies Council.
28. Allman GJ. 1983 Report on the present state of our knowledge of the reproductive system in the Hydroida. *Report of the Thirty-third Meeting of the British Association for the Advancement of Science.*
29. Teissier G. 1926 Notes critiques sur la morphologie des gonophores chez les hydraires. *Archives de Zoologic Expdrimentale, notes et reveu* **65**, 75–86.
30. Eckelbarger KJ, Larson RJ. 1993 Ultrastructural study of the ovary of the sessile scyphozoan, *Haliclystus octoradiatus* (Cnidaria: Stauromedusae). *J. Morphol.* **218**, 225–236. (doi:10.1002/jmor.1052180210)
31. Campbell RD. 1974 Cnidaria. *Reprod. Mar. Invertebr.* **1**, 133–199.
32. Eckelbarger KJ, Hodgson AN. 2021 Invertebrate oogenesis–a review and synthesis: comparative ovarian morphology, accessory cell function and the origins of yolk precursors. *Invertebr. Reprod. Dev.* **65**, 71–140. (doi:10.1080/07924259.2021.1927861)
33. Schiariti A, Kawahara M, Uye S, Mianzan HW. 2008 Life cycle of the jellyfish Lychnorhiza lucerna (Scyphozoa: Rhizostomeae). *Mar. Biol.* **156**, 1–12. (doi:10.1007/s00227-008-1050-8)
34. Thuesen E v. 2003 *Crossota millsae* (Cnidaria: Trachymedusae: Rhopalonematidae), a new species of viviparous hydromedusa from the deep sea off California and Hawaii. *Zootaxa* **309**, 1. (doi:10.11646/zootaxa.309.1.1)
35. Widersten B. 1965 Genital organs and fertilization some Scyphozoa. *Zoological Institute, Uppsala* **37**, 45–58. (doi:10.1016/0011-7471(66)90698-x)
36. Hyman LH. 1940 Metazoa of the tissue grade of construction: the radiate phylum Cnidaria. In *The invertebrates: Protozoa through Ctenophora.* New York, NY: McGraw-Hill.
37. Arai MN, Brinckmann-Voss A. 1980 Hydromedusae of British Columbia and Puget Sound. *Can. Bull. Fish. Aquat. Sci.* **204**, 1–192.
38. Edwards C. 1978 The hydroids and medusae *Sarsia occulta* sp. nov., *Sarsia tubulosa* and *Sarsia loveni*. *J. Mar. Biol. Assoc. U. K.* **58**, 291–311. (doi:10.1017/S0025315400027995)
39. Marques A, Peña CA, Vervoort W. 2000 Mediterranean species of Eudendrium Ehrenberg, 1834 (Hydrozoa, Anthomedusae, Eudendriidae) with the description of a new species. *J Zool.* **252**, 197–213. (doi:10.1111/j.1469-7998.2000.tb00615.x)
40. Gravier-Bonnet N, Migotto AE. 2000 Gonangium development and medusoid of *Nemalecium lighti* (Hargitt, 1924) (Cnidaria: Hydrozoa, Haliciidae). *Sci Mar* **64**, 207–213. (doi:10.3989/scimar.2000.64s1207)
41. Subramoniam T. 2018 Mode of reproduction: Invertebrate animals. In *Encyclopedia of reproduction* (ed. MK Skinner), pp. 32–40. New York, NY: Elsevier.
42. Lewis C, Bentlage B, Yanagihara A, Gillan W, van Blerk J, Keil DP, Bely AE, Collins AG. 2013 Redescription of *Alatina alata* (Reynaud, 1830) (Cnidaria: Cubozoa) from Bonaire, Dutch Caribbean.

Zootaxa **3737**, 473–487. (doi:10.11646/zootaxa.3737.4.8)

43. Morandini AC, da Silveira FL. 2001 Sexual reproduction of *Nausithoe aurea* (Scyphozoa, Coronatae): gametogenesis, egg release, embryonic development, and gastrulation. *Sci Mar* **65**, 139–149. (doi:10.3989/scimar.2001.65n2139)
44. Freeman G, Miller RL. 1982 Hydrozoan eggs can only be fertilized at the site of polar body formation. *Dev. Biol.* **94**, 142–152. (doi:10.1016/0012-1606(82)90077-X)
45. Beckwith CJ. 1914 The genesis of the plasma-structure in the egg of *Hydractinia echinata*. *J. Morphol.* **25**, 189–251. (doi:10.1002/jmor.1050250202)
46. Gilbert SF. 2000 Oogenesis. In *Developmental biology* (ed. SF Gilbert), pp. 453–454. Sunderland, MA: Sinauer Associates.
47. Sagata N. 1996 Meiotic metaphase arrest in animal oocytes: its mechanisms and biological significance. *Trends Cell Biol.* **6**, 22–28. (doi:10.1016/0962-8924(96)81034-8)
48. Taieb F, Thibier C, Jessus C. 1997 On cyclins, oocytes, and eggs. *Mol. Reprod. Dev.* **48**, 397–411. (doi:10.1002/(SICI)1098-2795(199711)48:3<397::AID-MRD14>3.0.CO;2-T)
49. Schiariti A, Christiansen E, Morandini AC, da Silveira FL, Giberto DA, Mianzan HW. 2012 Reproductive biology of Lychnorhiza lucerna (Cnidaria: Scyphozoa: Rhizostomeae): Individual traits related to sexual reproduction. *Marine Biology Research* **8**, 255–264. (doi:10.1080/17451000.2011.616897)
50. Schneider CA, Rasband WS, Eliceiri KW. 2012 NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods.* **9**, 671–675. (doi:10.1038/nmeth.2089)
51. Münkemüller T, Lavergne S, Bzeznik B, Dray S, Jombart T, Schiffers K, Thuiller W. 2012 How to measure and test phylogenetic signal. *Methods Ecol Evol* **3**, 743–756. (doi:10.1111/j.2041-210X.2012.00196.x)
52. Blomberg SP, Garland T, Ives AR. 2003 Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* **57**, 717–745. (doi:10.1111/j.0014-3820.2003.tb00285.x)
53. Revell LJ. 2012 Phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* **3**, 217–223. (doi:10.1111/j.2041-210X.2011.00169.x)
54. Collins A, Schuchert P, Marques A, Jankowski T, Medina M, Schierwater B. 2006 Medusozoan phylogeny and character evolution clarified by new large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models. *Syst. Biol.* **55**, 97–115. (doi:10.1080/10635150500433615)
55. Miranda LS, Hirano YM, Mills CE, Falconer A, Fenwick D, Marques AC, Collins AG. 2016 Systematics of stalked jellyfishes (Cnidaria: Staurozoa). *PeerJ* **4**, e1951. (doi:10.7717/peerj.1951)
56. Collins AG. 2009 Recent insights into cnidarian phylogeny. *Smithson Contrib Mar Sci* **38**, 139–149. (doi:10.5479/si.1943667X.0)
57. Cartwright P, Evans NM, Dunn CW, Marques AC, Miglietta MP, Schuchert P, Collins AG. 2008 Phylogenetics of Hydroidolina (Hydrozoa: Cnidaria). *J. Mar. Biol. Assoc. U. K.* **88**, 1663–1672. (doi:10.1017/S0025315408002257)
58. Maronna MM, Miranda TP, Peña Cantero ÁL, Barbeitos MS, Marques AC. 2016 Towards a phylogenetic classification of Leptothecata (Cnidaria, Hydrozoa). *Sci. Rep.* **6**, 18075. (doi:10.1038/srep18075)
59. Kayal E, Bentlage B, Cartwright P, Yanagihara AA, Lindsay DJ, Hopcroft RR, Collins AG. 2015 Phylogenetic analysis of higher-level relationships within Hydroidolina (Cnidaria: Hydrozoa) using mitochondrial genome data and insight into their mitochondrial transcription. *PeerJ* **3**, e1403. (doi:10.7717/peerj.1403)
60. Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W. 2008 GEIGER: Investigating evolutionary radiations. *Bioinformatics* **24**, 129–131. (doi:10.1093/bioinformatics/btm538)
61. Cooper N, Jetz W, Freckleton RP. 2010 Phylogenetic comparative approaches for studying niche conservatism. *J. Evol. Biol.* **23**, 2529–2539. (doi:10.1111/j.1420-9101.2010.02144.x)
62. Huelsenbeck JP, Nielsen R, Bollback JP. 2003 Stochastic mapping of morphological characters. *Syst. Biol.* **52**, 131–158. (doi:10.1080/10635150390192780)
63. Paradis E, Claude J, Strimmer K. 2004 APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**, 289–290. (doi:10.1093/bioinformatics/btg412)
64. Grafen A. 1989 The phylogenetic regression. *Phil. Trans. R. Soc. Lond. B* **326**, 119–157. (doi:10.1098/rstb.1989.0106)
65. Kassambara A. 2018 Multicollinearity essentials and VIF in R. See http://www.sthda.com/english/articles/39-regression-model-diagnostics/160-multicollinearity-essentials-and-vif-in-r.
66. Barton K. 2020 MuMIn: multi-model inference. R package version 1.43.17. See https://cran.r-project.org/web/packages/MuMIn/MuMIn.pdf.
67. Paradis E, Claude J. 2002 Analysis of comparative data using generalized estimating equations. *J. Theor. Biol.* **218**, 175–185. (doi:10.1006/jtbi.2002.3066)
68. Maddison WPM, Fitzjohn RG. 2015 The unsolved challenge to phylogenetic correlation tests for categorical characters. *Syst. Biol.* **64**, 127–136. (doi:10.1093/sysbio/syu070)
69. Rabosky DL, Goldberg EE. 2015 Model inadequacy and mistaken inferences of trait-dependent speciation. *Syst. Biol.* **64**, 340–355. (doi:10.1093/sysbio/syu131)
70. Smaers JB, Rohlf FJ. 2016 Testing species' deviation from allometric predictions using the phylogenetic regression. *Evolution* **70**, 1145–1149. (doi:10.1111/evo.12910)
71. Teissier G. 1922 Observation des médusoides libres et des planulas de *Sertularia operculata* L. *Bull. Soc. Zool. Fr.* **47**, 357–361.
72. Lessios HA. 1990 Adaptation and phylogeny as determinants of egg size in echinoderms from the two sides of the isthmus of Panama. *Am. Nat.* **135**. (doi:10.1086/285028)
73. Podolsky RD, Strathmann RR. 1996 Evolution of egg size in free-spawners: consequences of the fertilization-fecundity trade-off. *Am. Nat.* **148**, 160–173. (doi:10.1086/285916)
74. Bourmaud C, Gravier-bonnet N. 2005 Non-released cryptomedusoid in the life cycle of *Antennella* sp. (Cnidaria, Hydrozoa, Halopterididae) from La Réunion, Indian Ocean. *Invertebr. Reprod. Dev.* **48**, 55–62. (doi:10.1080/07924259.2005.9652170)
75. Rosen BR. 1986 Modular growth and form of corals: a matter of metamers? *Phil. Trans. R. Soc. Lond. B* **313**, 115–142. (doi:10.1098/rstb.1986.0029)
76. Mackie GO. 1986 From aggregates to integrates: physiological aspects of modularity in colonial animals. *Phil. Trans. R. Soc. Lond. B* **313**, 175–196. (doi:10.1098/rstb.1986.0032)
77. Harvell CD. 1994 The evolution of polymorphism in colonial invertebrates and social insects. *Q Rev. Biol.* **69**, 155–185. (doi:10.1086/418538)
78. Hughes RN. 1989 Modular colonies. In *A functional biology of clonal animals*, pp. 231–275. London, UK: Chapman and Hall.
79. Gershwin L. 2005 Taxonomy and phylogeny of Australian Cubozoa. Thesis, James Cook University, Townsville, Australia.
80. Millard NAH. 1975 Monograph on the Hydroida of Southern Africa. *Annals of the South African Museum* **68**, 1–513.
81. Fernández M, Calderón R, Cifuentes M, Pappalardo P. 2006 Brooding behaviour and cost of brooding in small body size brachyuran crabs. *Mar. Ecol. Prog. Ser.* **309**, 213–220. (doi:10.3354/meps309213)
82. Lee CE, Strathmann RR. 1998 Scaling of gelatinous clutches: Effects of siblings' competition for oxygen on clutch size and parental investment per offspring. *Am. Nat.* **151**, 293–310. (doi:10.1086/286120)
83. Strathmann RR. 2020 The association of coloniality with parental care of embryos. *J Exp Zool.* **336**, 221–230.
84. Szmant-Froelich A, Yevich P, Pilson MEQ. 1980 Gametogenesis and early development of the temperate coral *Astrangia danae* (Anthozoa: Scleractinia). *Biol. Bull.* **158**, 257–269. (doi:10.2307/1540935)
85. Fadlallah YH, Pearse JS. 1982 Sexual reproduction in solitary corals: overlapping oogenic and brooding cycles, and benthic planulas in *Balanophyllia elegans*. *Mar. Biol.* **71**.
86. Toshino S, Hiroshi M, Ohtsuka S, Okuizumi K, Adachi A, Hamatsu Y, Urata M, Nakaguchi K, Yamaguchi S. 2013 Development and polyp formation of the giant box jelly fish *Morbakka virulenta* (Kishinouye, 1910) (Cnidaria: Cubozoa) collected from the Seto Inland Sea, western Japan. *Plankton Benthos Res. Benthos Res.* **8**, 1–8. (doi:10.3800/pbr.8.1)
87. García-Rodríguez J, Ames CL, Marian JEAR, Marques AC. 2020 Histomorphological comparison of testes in species of box jellyfish (Cnidaria; Cubozoa): does morphology differ with mode of reproduction and fertilization? *Org Divers Evol* **20**, 25–36. (doi:10.1007/s13127-019-00427-x)
88. Lewis C, Long TAF. 2005 Courtship and reproduction in *Carybdea sivickisi* (Cnidaria: Cubozoa). *Mar. Biol.* **147**, 477–483. (doi:10.1007/s00227-005-1602-0)

89. Castro-Longoria E, Williams JA. 1999 The production of subitaneous and diapause eggs: a reproductive strategy for *Acartia bifilosa* (Copepoda: Calanoida) in Southampton Water, UK. *J Plankton Res* **21**, 65–84. (doi:10.1093/plankt/21.1.65)
90. Stimson JS. 1978 Mode and timing of reproduction in some common hermatypic corals of Hawaii and Enewetak. *Mar. Biol.* **48**, 173–184. (doi:10.1007/BF00395017)
91. Cunha AF, Maronna MM, Marques AC. 2016 Variability on microevolutionary and macroevolutionary scales: a review on patterns of morphological variation in Cnidaria Medusozoa. *Org Divers Evol.* **16**, 431–442. (doi:10.1007/s13127-016-0276-4)
92. Lewis JB. 1991 The ampullae and medusae of the calcareous hydrozoan *Millepora complanata*. *Hydrobiologia* **216–217**, 165–169. (doi:10.1007/BF00026458)
93. Genzano GN, Kubota S. 2003 Synchronous mass release of mature medusae from the Hydroid *Halocordyle disticha* (Hydrozoa, Halocordylidae) and experimental induction of different timing by light changes. *Publ. Seto Mar. Biol. Lab.* **39**, 221–228. (doi:10.5134/176311)
94. Chester CM. 1996 The effect of adult nutrition on the reproduction and development of the estuarine nudibranch, *Tenellia adspersa* (Nordmann, 1845). *J. Exp. Mar. Biol. Ecol.* **198**, 113–130. (doi:10.1016/0022-0981(95)00197-2)
95. Keith SA *et al.* 2016 Coral mass spawning predicted by rapid seasonal rise in ocean temperature. *Proc. R. Soc. B* **283**, 20160011. (doi:10.1098/rspb.2016.0011)
96. Galea HR, Ferry R, Bertot JM. 2012 Medusoids in the life cycle of *Dentitheca dendritica* (Nutting, 1900) and *Nemalecium gracile* sp. nov. (Cnidaria: Hydrozoa). *Zootaxa* **3527**, 43–54. (doi:10.11646/zootaxa.3527.1.3)
97. Pitt KA, Kingsford MJ. 2000 Reproductive biology of the edible jellyfish *Catostylus mosaicus* (Rhizostomeae). *Mar. Biol.* **137**, 791–799. (doi:10.1007/s002270000399)
98. Skadsheim A. 1989 Regional variation in amphipod life history: effects of temperature and salinity on breeding. *J. Exp. Mar. Biol. Ecol.* **127**, 25–42. (doi:10.1016/0022-0981(89)90207-4)
99. Hargitt GT. 1919 Germ cells of coelenterates. VI. General considerations, discussion, conclusions. *J. Morphol.* **33**, 1–59. (doi:10.1002/jmor.1050330102)
100. Berrill NJ. 1949 Developmental analysis of Scyphomedusae. *Biol. Rev.* **24**, 393–409. (doi:10.1111/j.1469-185X.1949.tb00581.x)
101. Larson RJ. 1986 Pelagic scyphomedusae (Scyphozoa: Coronatae and Semaeostomeae) of the Southern Ocean. In *Biology of the antarctic seas XVI* (ed. LS Kornicker), pp. 59–165. Washington, DC: American Geophysical Union.
102. Jarms G, Båmstedt U, Tiemann H, Martinussen MB, Fosså JH, Høisœter T. 1999 The holopelagic life cycle of the deep-sea medusa *Periphylla periphylla* (Scyphozoa, Coronatae). *Sarsia* **84**, 55–65. (doi:10.1080/00364827.1999.10420451)
103. Parry GD. 1983 The influence of the cost of growth on ectotherm metabolism. *J. Theor. Biol.* **101**, 453–477. (doi:10.1016/0022-5193(83)90150-9)
104. Lardies MA, Medina MH, Correa JA. 2008 Intraspecific biogeographic pattern breakage in the snapping shrimp *Betaeus emarginatus* caused by coastal copper mine tailings. *Mar. Ecol. Prog. Ser.* **358**, 203–210. (doi:10.3354/meps07347)
105. Miranda LS, Mills CE, Hirano YM, Collins AG, Marques AC. 2018 A review of the global diversity and natural history of stalked jellyfishes (Cnidaria, Staurozoa). *Mar. Biodivers.* **48**, 1695–1714. (doi:10.1007/s12526-017-0721-4)
106. Losos JB. 2011 Seeing the forest for the trees: The limitations of phylogenies in comparative biology. *Am. Nat.* **177**, 709–727. (doi:10.1086/660020)
107. Rahman MA, Uehara T, Rahman M. 2002 Effects of egg size on fertilization, fecundity and offspring performance: a comparative study between two sibling species of tropcial sea urchins (Genus Echinometra). *Pak. J. Biol. Sci.* **5**, 114–121. (doi:10.3923/pjbs.2002.114.121)
108. Mayorova TD, Osadchenko B, Kraus Y. 2020 How to build a larval body with less than a hundred cells? Insights from the early development of a stalked jellyfish (Staurozoa, Cnidaria). *Org Divers Evol* **20**, 681–699. (doi:10.1007/s13127-020-00459-8)
109. Otto JJ. 1976 Early development and planula movement in Haliclystus (Scyphozoa: Stauromedusae). In *Coelenterate ecology and behavior* (ed. GO Mackie), pp. 319–329. Berlin, Germany: Springer.
110. Jarms G, Tiemann H. 1996 On a new hydropolyp without tentaeles, Microhydrula limopsicola n. sp., epibiotic on bivalve shells from the Antarctic. *Sci Mar* **60**, 109–115.
111. Hanaoka K-I. 1934 Notes on the Early Development of a Stalked Medusa. *Proc. Imp. Acad.* **10**, 117–120. (doi:10.2183/pjab1912.10.117)
112. Miranda LS, Collins AG, Marques AC. 2010 Molecules clarify a cnidarian life cycle: the 'hydrozoan' *Microhydrula limopsicola* is an early life stage of the staurozoan *Haliclystus antarcticus*. *PLoS ONE* **5**, 1–9. (doi:10.1371/journal.pone.0010182)
113. Wietrzykowski W. 1912 Recherches sur le développement des Lucernaires. *Archives de zoologie expérimentale et générale* **10**, 1–95.
114. McEdward LR, Morgan KH. 2001 Interspecific relationships between egg size and the level of parental investment per offspring in echinoderms. *Biological Bulletin* **200**, 33–50. (doi:10.2307/1543083)
115. García-Rodríguez J, Cunha AF, Morales-Guerrero A, González-Chaves A, Camacho A, Miranda LS, Serrano FC, Jaimes-Becerra A, Marques AC. 2023 Reproductive and environmental traits explain the variation in egg size among Medusozoa (Cnidaria). Figshare. (doi:10.6084/m9.figshare.c.6730050)

diversity

MDPI

Article

Histological Investigation of the Female Gonads of *Chiropsalmus quadrumanus* (Cubozoa: Cnidaria) Suggests Iteroparous Reproduction

Jimena García-Rodríguez [1,*], Cheryl Lewis Ames [2,3,*], Adrian Jaimes-Becerra [1,4], Gisele Rodrigues Tiseo [1], André Carrara Morandini [1,5], Amanda Ferreira Cunha [6] and Antonio Carlos Marques [1]

[1] Department of Zoology, Institute of Biosciences, University of São Paulo, R. Matão, Tv. 14, 101, São Paulo 05508-090, Brazil; ajjb80@gmail.com (A.J.-B.); giseletiseo@gmail.com (G.R.T.); acmorand@ib.usp.br (A.C.M.); marques@ib.usp.br (A.C.M.)
[2] Graduate School of Agricultural Science, Faculty of Agriculture, Tohoku University, Sendai 980-8572, Japan
[3] Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20013-7012, USA
[4] Department of Ecology, Evolution and Behavior, Alexander Silberman Institute of Life Sciences, Faculty of Science, Hebrew University of Jerusalem, Jerusalem 9190401, Israel
[5] Center for Marine Biology, University of São Paulo, Manoel Hypólito do Rego, São Sebastião 11612-109, Brazil
[6] Department of Zoology, Institute of Biological Sciences, Federal University of Minas Gerais, Av. 17 Presidente Antônio Carlos 6627, Belo Horizonte 31270-901, Brazil; amanda.cunha@ufv.br
* Correspondence: jimena.gar88@gmail.com (J.G.-R.); ames.cheryl.lynn.a1@tohoku.ac.jp (C.L.A.)

Abstract: The box jellyfish *Chiropsalmus quadrumanus* (Chirodropida: Cubozoa: Cnidaria) is common in warm waters. Although it is assumed that external fertilization is a characteristic of Chirodropida, the life history of *C. quadrumanus* is not yet known since its reproductive behavior has never been described, nor has the polyp has been found in nature. As a result, in the absence of documentation of reproductive behavior, we sought to test the hypothesis of external fertilization through a histological analysis of the female gonads. Herein, we analyze ten females collected in São Paulo and Rio de Janeiro (Brazil) and describe the gonadal organization and pattern of oocyte development. The discovery of four distinct stages of oocyte differentiation augments the scant existing reports of the structural and functional maturation of sex cells in Cubozoa species. Furthermore, the gonads of mature females comprise both mature (average diameter of 122 µm) and immature oocytes, suggesting that *C. quadrumanus* is iteroparous and exhibits multiple reproductive cycles during its life. Medusa bell size was not found to correlate with maturity state as even small females possessed a high percentage of oocytes in late vitellogenesis, suggesting that sexual maturation occurs rapidly in *C. quadrumanus* females.

Keywords: oocyte; medusa; box jellyfish; fertilization; oogenesis; differentiation gradient; seasonality

Citation: García-Rodríguez, J.; Ames, C.L.; Jaimes-Becerra, A.; Tiseo, G.R.; Morandini, A.C.; Cunha, A.F.; Marques, A.C. Histological Investigation of the Female Gonads of *Chiropsalmus quadrumanus* (Cubozoa: Cnidaria) Suggests Iteroparous Reproduction. *Diversity* **2023**, *15*, 816. https://doi.org/10.3390/d15070816

Academic Editors: Angelo Poliseno and Luc Legal

Received: 5 May 2023
Revised: 20 June 2023
Accepted: 23 June 2023
Published: 28 June 2023

1. Introduction

Reproductive studies in Cubozoa (box jellyfish) are hampered because reports of their jellyfish (cubomedusae) or medusoid form often refer to sightings of a single individual [1] or a limited (seasonal) reproductive period [2,3], and many species are notoriously venomous to humans, making cubomedusae challenging to collect [4]. Cubozoans, like many medusozoans (jellyfishes), have complex metagenetic life cycles, metamorphosing from a sessile polyp stage (asexual larval stage) into the characteristic, free-swimming medusa (sexual adult stage) [5–7]. Asexual polypoid reproduction is favorable under certain environmental conditions because it allows for a rapid increase in the number of clonal medusozoan individuals; this is also the case for other bi- or multiphasic marine invertebrates [8,9]. Meanwhile, in marine invertebrates, sexual reproduction, which generates genetic novelties via mixing genotypes, is triggered by specific environmental and genetic factors [8], many of which are undetermined. Unfortunately, female gonadal maturation (oogenesis), reproductive strategies and dynamics, and fertilization modes are particularly understudied in cubozoans [10]. This lack of knowledge limits actions to monitor cubozoan populations with the aim of managing the influence of cubomedusae on tourism and recreation while at the same time considering biodiversity conservation and public safety.

The accumulated knowledge on the sexual reproduction of cubomedusae is derived from seminal lab studies on five species of Carybdeida (*Alatina alata* (Reynaud, 1830) [3,11,12]; *Carybdea marsupialis* (Linnaeus, 1758) [13]; *Copula sivickisi* (Stiasny, 1926) [12,14–17]; *Morbakka virulenta* (Kishinouyea, 1910) [18]; *Tamoya haplonema* F. Müller, 1859 [19,20]) and two species of Chirodropida (*Chironex fleckeri* Southcott, 1956 [21,22], and *Chiropsalmus quadrumanus* (F. Müller, 1859) [19]). The sexual behaviors of a few species have been described from the field (e.g., [3,23,24]) or from tank observations (e.g., [15,25]), proving the difficulty of performing in situ observations as cubomedusae are active swimmers [26,27] with long tentacles and the ability to avoid obstacles [27] using their complex eyes [28,29]. Accordingly, only *Tripedalia cystophora* Conant, 1897, a mild stinger and the second smallest cubomedusan species, has been reared to sexual maturity in vitro (cf. [30]). Therefore, mechanisms of gametogenesis and reproductive behaviors in this small class are often inferred via methods of histomorphology rather than relying exclusively on in situ observations.

The mode of fertilization in cubomedusae can be external or internal depending on the species, but external fertilization is considered plesiomorphic in marine invertebrates (cf. [31]). External fertilization is considered typical for Chirodropida based on scant reports for just two species, *C. fleckeri* [21] and *C. quadrumanus* [19]. In contrast, internal fertilization is considered a synapomorphy of Carybdeida [2] and is well documented for two species of Tripedaliidae (*C. sivickisi* and *T. cystophora*) that exhibit complex reproductive behaviors involving spermatophore transfer [14–16,23,32,33]. However, reports of external fertilization for *M. virulenta* (Carukiidae) [18] cast doubts on the universality of internal fertilization in Carybdeida. Regarding periodicity, most cubomedusae are considered seasonal spawners based on the few reports of reproductive events witnessed in the field (e.g., *C. marsupialis* [34]; *C. fleckeri* [35]; *C. sivickisi* [15]). However, the scattered knowledge about carybdeid species makes it difficult to establish either semelparity or iteroparity as a universal pattern at the level of class or even order. For example, *A. alata* medusae reproduce during monthly spermcasting aggregations in which the entire gonad tissue ruptures and the reproductive cells are released [36]; it may be unique among cubozoans as a semelparous species (for references on iteroparous cubomedusae, see [19,20]).

In many gonochoristic marine organisms, sexual dimorphism can be exhibited in the form of marked phenotypic differences between males and females (e.g., in body size, color, and shape), but a disproportionate focus on a handful of “model” species has led to a skewed representation of the underlying mechanisms of the evolution of different modes of reproduction (reviewed by [8]). In cubozoans, although all species are gonochoristic, only species of Tripedaliidae exhibit sexual dimorphism with respect to gonadal shape, development, and/or color (e.g., *C. sivickisi* [15–17,37,38] and *T cystophora* [23,32,37]). During gametogenesis, male and female cubomedusae develop their gonads (defined as the “area where gametes are formed” by [39], p. 142) from endodermal tissue in the bell [39–43]. In most cubomedusae, subtle phenotypic variation between males and females typically occurs at the level of reproductive tissues and in germ cell morphology [19,37,44]. However, reports of sexually dimorphic gonadal morphologies and patterns of gametogenesis are limited to cubomedusae that exhibit internal fertilization and exhibit sperm “packet” transfer [12], suggesting the potential for gonad morphology to infer reproductive modes in Cubozoa.

Recent histomorphological studies have elucidated spermatogenesis in several species of cubomedusae [19,20], but studies concerning oogenesis are limited to *C. marsupialis* [13] and *C. sivickisi* (as *Carybdea sivickisi* [15,17]). Most recently, oogenesis was reported in *Carybdea murrayana* Haeckel, 1880 (as *Carybdea branchi* [45]) from southern African waters, and a "maturation scale" for female sex cells was established for the first time in the class. During the process of vitellogenesis, oocytes accumulate yolk protein granules and subsequently increase in diameter. Oocyte maturation patterns corresponded to significant egg size differences documented during the oogenesis of a single cubozoan species, *viz. C. murrayana* [45]. Nevertheless, scarce information on egg size and its relation to cubomedusae maturity precludes the ability to establish a baseline of sexual maturation related to reproductive season for the 50 estimated species.

Herein, we report on the hitherto obscure reproductive strategy of the chirodropid species *C. quadrumanus* (Chirodropida, Chiropsalmidae), a relatively common species in the western tropical Atlantic from Brazil to the USA [46–50]. *Chiropsalmus quadrumanus* is represented by conspicuous cubomedusae (with a bell height of 10 cm and a width of 12 cm on average) which are poorly studied; for instance, a mere three sightings have been reported in the literature in the past decade [19,20,51]. This species' mode of fertilization was previously suggested to be external [19,20], but no evidence supports this claim as the life cycle and cubopolyp location in nature remain undetermined. Herein, we carry out a histolomorphological analysis to elucidate an oocyte structural maturation "scale" in a chirodropid species for the first time while aiming to infer the sexual reproductive strategy and contemplate previous reports speculating on the fertilization mode of this species. We also refute the hypothesis that sexual maturity in all cubomedusae can be inferred accurately by bell size [15,16], as our outcomes for *C. quadrumanus* fail to align with previous findings on carybdeid species.

2. Materials and Methods

2.1. Material Samples

Medusae of *C. quadrumanus* have been collected in the field at typical marine ecosystem salinities (20–30‰) and at shallow depths ranging from 5 m to 10 m. In the northern hemisphere, specimens have been found during the summer (May–August) [52,53] and sometimes in the fall (September) (e.g., Matagorda Bay, Texas [54]). Conversely, in the southern hemisphere (e.g., Brazil), specimens have been collected during the winter, in the dry season (July–August) [20], and sometimes during spring (March–April) [19].

In this study, we analyzed 10 females (Table 1) collected via trawling from a depth of 5 m to 40 m during the dry season (April to September) in 2008, 2010, and 2014. The specimens were collected from the São Sebastião Channel (São Paulo State, n = 2), Santos Bay and São Vicente (São Paulo State, n = 2), and Macaé (Rio de Janeiro State, n = 6) (Table 1). The medusae were fixed in 10% formaldehyde solution in seawater. The bell height (BH, from the apex of the umbrella to the margin) and interpedalial distance (IPD, distance along the bell margin between alternate pedalia) were measured for all specimens (cf. [45,55]) (Figure 1A) (Table 1). All necessary approvals for the sampling of specimens were obtained (sampling permit 16802 SISBIO/ICMBIO—Instituto Chico Mendes de Conservação da Biodiversidade). The specimens from Macaé were deposited in the collection of the Museum of Zoology (MZ), and the remaining specimens were deposited in the Marine Evolution Lab (MEL), both belonging to the University of São Paulo (Table 1).

Table 1. Material data for *Chiropsalmus quadrumanus* females examined in this study. Abbreviation: MEL: Marine Evolution Lab; MZUSP: Museum of Zoology of the University of São Paulo; RJ: Rio de Janeiro; SP: São Paulo.

Specimen Number	Museum Number (MZUSP) and MEL Number	Bell Height (mm)	Interpedalial Distance (mm)	Locality	Depth (m)	Date (D/M/Y)	Collector
CH2	MEL-CH2	4.5	3.8	São Sebastião Channel (SP)	10	10 June 2014	JGR
CH6	MEL-CH6	6.5	4.7	São Sebastião Channel (SP)	10	19 August 2014	JGR

Table 1. *Cont.*

Specimen Number	Museum Number (MZUSP) and MEL Number	Bell Height (mm)	Interpedalial Distance (mm)	Locality	Depth (m)	Date (D/M/Y)	Collector
MA49	MZUSP-1920	6.5	4.5	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA50	MZUSP-1921	7.5	5	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA52	MZUSP-1923	6.5	4.4	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA53	MZUSP-1924	7	4.7	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA56	MZUSP-1927	7	5	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA57	MZUSP-1928	5.4	4	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
53A	MEL-53A	6.7	10.54	Baía de Santos e São Vicente (SP)	10–40	April 2010	Tiseo, G. R.; Zara, F. J.
54C	MEL-54C	5.6	8	Baía de Santos e São Vicente (SP)	10–40	April 2010	Tiseo, G. R.; Zara, F. J.

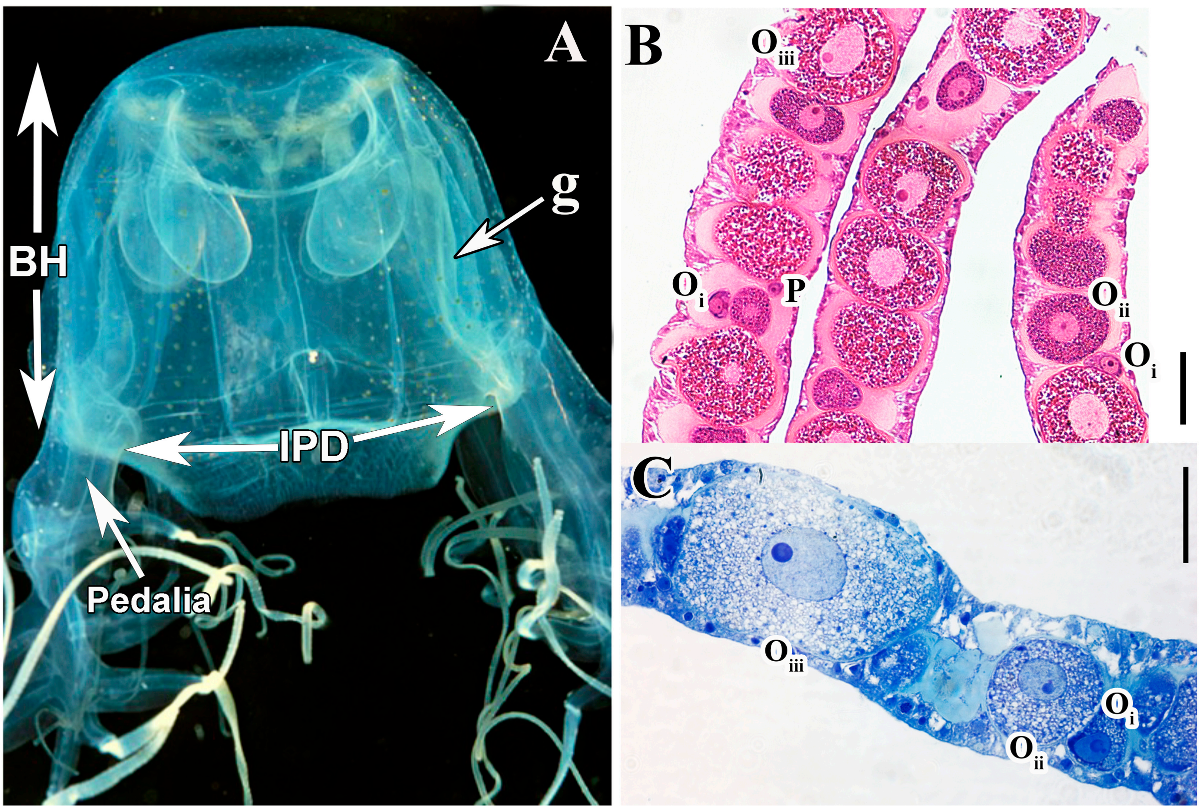

Figure 1. (**A**). *Chiropsalmus quadrumanus* live specimen [20]. (**B**,**C**). Gonadal transversal sections of females of *C. quadrumanus* from São Sebastião Channel bearing oocytes in the four different stages of development. (**B**). Specimen (CH6, Table 1) stained with hematoxylin–eosin (HE). (**C**). Specimen (CH2, Table 1) stained with toluidine blue (TB). Scale bars 50 µm. BH—bell height; IPD—interpedalial distance; g—gonads; Oiii—late-vitellogenic oocyte; Oii—mid-vitellogenic oocyte; Oi—early-vitellogenic oocyte; P—pre-vitellogenic oocyte.

2.2. Maturity of Specimens

Chiropsalmus quadrumanus has eight hemi-gonads arranged in four pairs, with each pair located at interradial septa [50]. Mature female medusae were distinguished based on the microscopic observation of oocytes, characterized by white spheres in the female gonads. Sexual maturity was inferred by the presence of more than 15% of gonadal oocytes in the late vitellogenic stage (O_{iii}) (cf. [45,56]). We propose a maturity scale for the different oocyte developmental states observed in our samples, following the scale proposed for *Carybdea murrayana* (as *Carybdea branchi* [45]), based on oocyte diameter and quantity of yolk.

2.3. Histological Analysis

Several fragments over the entire length of each gonad were dissected individually using forceps. The samples were fixed with formalin 4%, dehydrated in an ethanol series, and embedded in glycol methacrylate using the Leica Historesin Embedding Kit (Leica Microsystems Nussloch GmbH, Germany), following the manufacturer's protocol. The embedded material was cut into 5 µm transversal sections with a Leica RM2255 microtome, with ca. 12 sections set per slide (ca. 60 µm of gonad tissue); each gonad subsample was accommodated on 6–8 slides (i.e., ca. 480 µm of gonadal tissue). The slides were stained with hematoxylin–eosin (HE), toluidine blue (TB) and Gomori's trichrome (GT) (according to [57–62]) and cover-slipped using Entellan. The slides were subsequently analyzed using a Zeiss Axio Imager.M2 microscope, images enhanced with Adobe Photoshop CC 2017 version 24.0.1, and the oocytes were measured using the software ImageJ 1.53s [63].

This study is the first reported attempt to document oogenesis in jellyfish using a glycol methacrylate (historesin) method. This methodology has been successfully used in previous studies on other taxonomic groups [62,64] to obtain high-quality results.

2.4. Statistical Analysis

Several slides per specimen (ca. one third) were selected to measure the oocytes with a 4x objective lens on a compound microscope, and 12 oocytes (per tissue sample) were measured for each of the four established oogenesis stages. The maximum and minimum oocyte diameters of each specimen were averaged, and the standard deviations were calculated (Table 2). Average diameters for each stage were compared using the R package dplyr (v.1.0.7, function "summarise") to discriminate between the different maturation stages based on size, and the results were visualized using R package ggplot2 (v.3.3.3). One slide per specimen was selected to calculate the percentage of oocytes present at each stage of oogenesis. Furthermore, evidence for a correlation between cubomedusa size (interpedalial distance) and maturity (percentage of oocyte stage) was tested using the Spearman correlation method with the R package ggpubr (v.0.4.0, function "ggscatter"). The code is available as Supplementary Material.

Table 2. Measurements of oocyte diameter (maximum, minimum, average, and standard deviation) in each stage of gonadal development of *Chiropsalmus quadrumanus*. Blank cells indicate no oocytes of that specific size category were observed in the sample. Oiii—late-vitellogenic oocyte; Oii—mid-vitellogenic oocyte; Oi—early vitellogenic oocyte; P—pre-vitellogenic oocyte.

Specimen Number	**Oiii Diameter (µm)**				**Oii Diameter (µm)**				**Oi Diameter (µm)**				**P Diameter (µm)**			
	Max	**Min**	**Average**	**Standard Deviation**	**Max**	**Min**	**Average**	**Standard Deviation**	**Max**	**Min**	**Average**	**Standard Deviation**	**Max**	**Min**	**Average**	**Standard Deviation**
CH2	140.014	76.292	110.796	22.54687	75.617	52.308	62.1977	6.2079	48.703	16.492	28.484	7.81417	31.68	11.544	20.5662	5.98748
CH6	235.397	146.857	197.781	23.70315	170.486	99.398	132.068	22.297	92.352	49.656	69.34	12.3727	53.582	13.919	28.242	13.5341
MA49					83.02	58.667	74.9411	7.3262	61.273	34.359	46.186	8.19608	26.104	16.271	20.8476	3.6763
MA50					98.593	70.107	85.8052	8.959	58.029	31.074	42.923	8.00621	37.838	14.411	24.7551	7.08516
MA52	131.602	96.736	114.187	10.50545	97.173	65.079	81.6818	11.168	59.958	26.203	38.339	10.4808	27.778	13.889	20.9081	4.47119
MA53	113.525	95.89	103.493	5.407642	87.199	60.336	76.8158	8.5688	41.82	27.397	33.926	4.1092	34.013	13.784	20.6236	5.4142
MA56					119.398	75.18	90.0476	13.532	63.774	26.698	43.078	10.8529	27.68	10.99	20.4653	5.60585
MA57									47.507	33.333	38.761	4.44204	23.292	10.623	16.8126	4.18917
53A	122.144	89.443	101.199	10.10663	91.952	56.063	79.3338	8.9946	57.007	25.298	37.208	10.46	24.828	14.717	19.9075	3.43497
54C	131.966	82.262	99.711	15.20876	88.511	62.639	74.9315	8.3674	61.837	27.402	39.457	9.03138	29.829	14.583	20.7193	5.1459

3. Results

In *Chiropsalmus quadrumanus*, oogenesis presented a pattern of increased size and yolk density during the process of sexual maturation. Though late-vitellogenic oocytes were large (on average, 122 μm in diameter), all gonads were intact with no sign of ovulation underway in the cubomedusae examined.

Four different oocyte states (Figures 2 and 3) are defined for *C. quadrumanus* females according to their diameter and vitellogenic content, following the scale previously established by [45]: (1) pre-vitellogenic oocytes (p), round-shaped, average diameter of 21 ± 6.88 μm, without vitellogenic content and basophil cytoplasm; (2) early-vitellogenic oocytes (O_i) with some yolk granules, average diameter of 42 ± 13.5 μm; (3) mid-vitellogenic oocytes (O_{ii}), average diameter of 84 ± 21.6 μm; (4) late-vitellogenic oocytes (O_{iii}), rich in yolk granules, average diameter of 122 ± 39.1 μm. The average oocyte size differs significantly among the stages ($\chi^2 = 355.82$; df = 3; $p < 2.2 \times 10^{-16}$), although their ranges overlap (Figure 2).

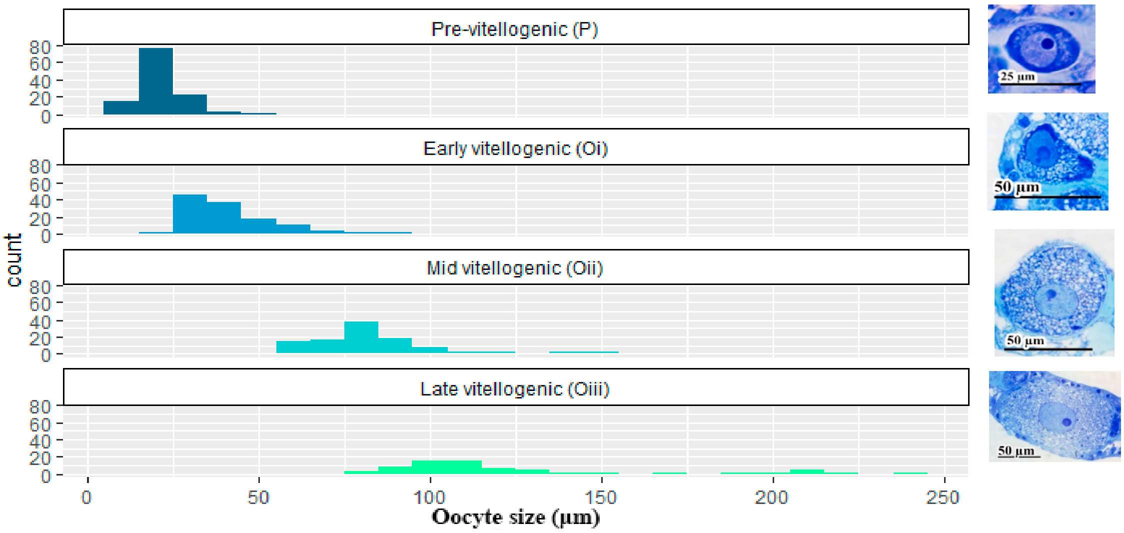

Figure 2. Distribution of oocyte diameter for each of the four stages of development in *C. quadrumanus*. Images on the right reveal morphological variations in oocyte features, including vitellogenic content.

Oogenesis in *C. quadrumanus* was found to be asynchronous, and oocytes of all four stages of development (p—O_{iii}) were observed in almost all specimens analyzed (Figure 1B,C and Table 2). All the specimens possessed pre-vitellogenic oocytes, but late-vitellogenic oocytes (O_{iii}) were not present in four specimens from Macaé, suggesting that they were immature despite their relatively large bells (IPD 4–5 mm). Conversely, a small specimen (IPD 3.8 mm) from the São Sebastião Channel had late-vitellogenic oocytes (O_{iii}), suggesting a non-linear relationship between sexual maturity and bell size. Although all females examined possessed gonads, the maximum oocyte diameter observed for each of the four stages varied between specimens collected at different geographic localities, with maximum diameters of 235 μm for the São Sebastião Channel and 113 μm for Macaé, despite the similarity of the bell sizes (IPD 4.7 mm) (Table 2).

Female gonads are composed of an epithelial bilayer corresponding to the external gastrodermal epithelium and an internal gonadal layer within the mesoglea composed of oocytes (Figure 4A). Epithelial gonadal cells are columnar with basal vacuoles (Figure 4B). While there is no developmental gradient seen along the length of the gonad, oocytes increase in size due to the accumulation of yolk (vitellogenesis) during maturation (Figures 1 and 2). Histological cross-sections revealed that late-vitellogenic oocytes (O_{iii}) were found across the entire length of the gonad within the gonadal epithelium (Figure 1C). Neither trophocytes (specialized nutritive gastrodermal cells) nor nurse cells were found in contact with the oocytes and gastrodermal epithelium (Figures 1 and 4), suggesting that mature oocytes develop freely in the mesoglea (in direct contact with the gastrodermis) (cf. [65]). However, we also report here that some specimens did show inclusions of unknown natures within the oocytes (Figure 5).

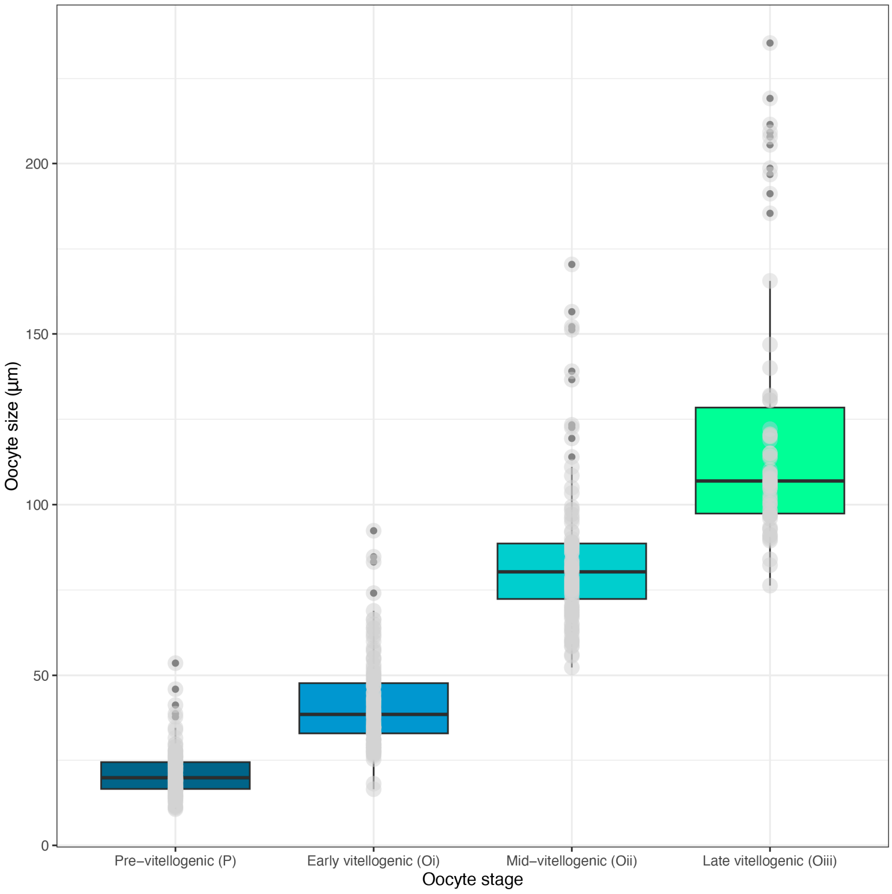

Figure 3. Boxplot representing the four stages of development based on the oocyte diameter of *Chiropsalmus quadrumanus*.

We defined mature females by the presence of gonads with >15% of oocytes in the late-vitellogenic stage [45]. Accordingly, there were four females with this pattern; therefore, they were considered sexually mature (Table 3). Although the ten female specimens had IPD values $\geq$ 4.4 mm, not all were found to be mature. Two specimens from Macaé (IPDs = 4.5 and 5 mm) had no late-vitellogenic oocytes. One specimen from Macaé (MA57, IPD = 4 mm) had pre-vitellogenic (14%) and early-vitellogenic (86%) oocytes, representing the most immature female studied herein (Figure 4). The Spearman correlation test indicates that the bell size of *C. quadrumanus* is not correlated with the proportion of late-vitellogenic oocytes present (r = 0.019; *p* = 0.96).

Table 3. Percentage of oocytes in the four different stages of the oogenesis of *Chiropsalmus quadrumanus*. * Mature females; IPD—interpedalial distance (mm); Oiii—late-vitellogenic oocyte; Oii—mid-vitellogenic oocyte; Oi—early-vitellogenic oocyte; P—pre-vitellogenic oocyte.

Specimen number	IPD	Oiii%	Oii%	Oi%	P%	Sampling Month
CH2	3.8	6.45	45.16	41.94	6.45	June/2014
CH6 *	4.7	37.68116	27.53623	24.63768	10.14493	August/2014
MA49	4.5	0	8.474576	83.89831	7.627119	September/2008

Table 3. *Cont.*

Specimen number	IPD	Oiii%	Oii%	Oi%	P%	Sampling Month
MA50	5	0	38.70968	38.70968	22.58065	September/2008
MA52 *	4.4	35.38462	47.69231	12.30769	4.615385	September/2008
MA53 *	4.7	26.05042	35.29412	24.36975	14.28571	September/2008
MA56	5	0	62.63736	32.96703	4.395604	September/2008
MA57	4	0	0	85.65022	14.34978	September/2008
53A *	10.54	32.91139	44.3038	14.76793	8.016878	April/2010
54C	8	4.659498	60.9319	28.67384	5.734767	April/2010

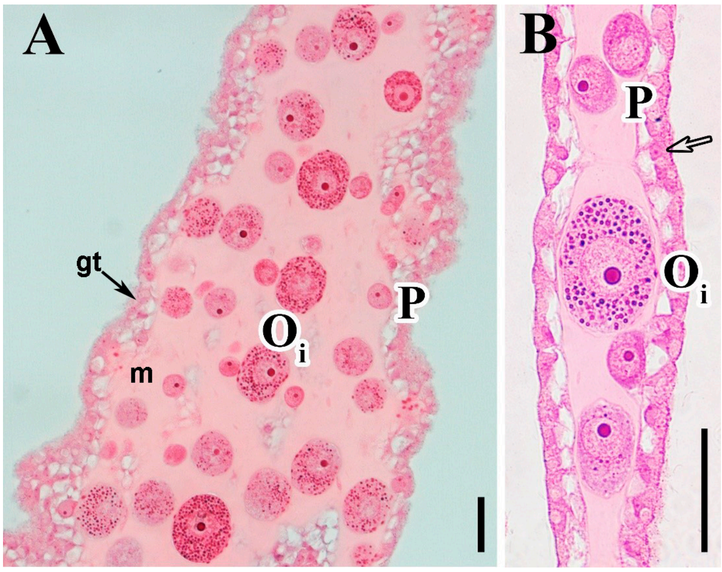

Figure 4. Gonadal section of the most immature studied female of *Chiropsalmus quadrumanus* (from Macaé, Rio de Janeiro). Stained with Gomori trichrome and hematoxylin (GT + H) and HE, respectively (**A**,**B**). Arrows show the columnar gonadal epithelium with basal vacuoles. Scale bars: 50 µm. gt—gastrodermal epithelium; m—mesoglea; Oi—early-vitellogenic oocyte; P = pre-vitellogenic oocyte.

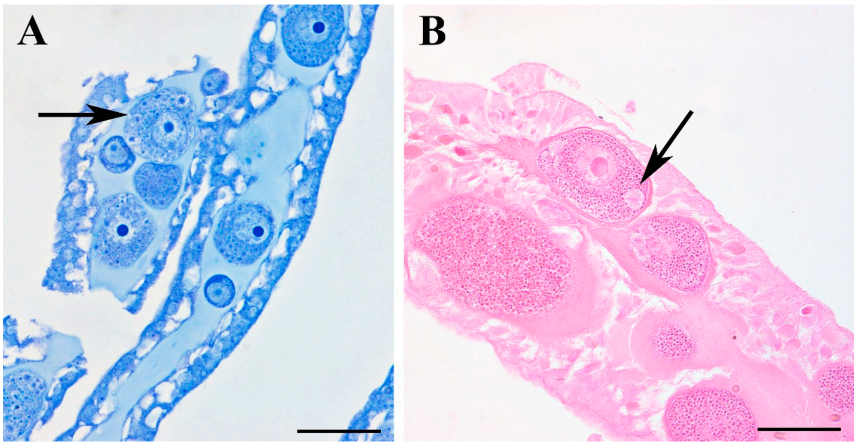

Figure 5. Gonads section of two different specimens of *Chiropsalmus quadrumanus* (from Macaé, Rio de Janeiro) stained with toluidine blue (**A**) and HE (**B**), respectively. Arrows show inclusions of unknown natures within the oocytes. Scale bars 50 µm.

4. Discussion

Patterns of female sex cell maturation (oogenesis) and the reproductive strategy of the cubomedusa *C. quadrumanus* are presented for the first time based on our histological approach. Females have four different stages of oocyte development, as determined via a comparative analysis of vitellogenic content, which was present in most specimens examined (Figures 2 and 3). The presence of pre-vitellogenic oocytes even in mature female gonads is interpreted as an indication of asynchronous oogenesis in which a mature female develops immature oocytes in order to reproduce more than once in her lifetime. This pattern diverges from the pattern reported for *C. murrayana* in which only late-vitellogenic oocytes were observed in mature females, suggesting a single spawning event occurring upon cubomedusae maturity [45]. Several reports of oogenesis in non-cubozoan jellyfishes (e.g., *Cassiopea andromeda,* cf. [66]) have mentioned trophocytes associated with mature oocytes during ovulation; the absence of these specialized nutritive cells in *C. quadrumanus* corroborates findings reported by [45] for the cubomedsusa *C. murayana*. The absence of embryos or planulae within the gastrovascular cavity in females, taken together with knowledge that sperm are released via the rupture of the follicle wall in males of this species [19], strongly indicates that *C. quadrumanus* exhibits external fertilization as a reproductive strategy.

4.1. Seasonality

Chiropsalmus quadrumanus is a shallow-water species inhabiting the Atlantic coast of the Americas. Its seasonality is marked by the presence of mature medusae from April to September in the Southern Hemisphere (e.g., [19,20] and this study). To date, the most sexually mature female (>37% of O_{iii}) was trawled in São Paulo in August (Tables 2 and 3), suggesting that the reproductive season occurs in the dry season in the Southern Hemisphere. Occurrence data for *C. quadrumanus* for the Northern Hemisphere recorded “blooms” in Georgia in July of 1971 [52], a “predominance” in the Mississippi Sound in August of 1968 [53], and floating dead specimens in the fall (September–November) after a Texas rainy season [54]. However, a broader understanding of the distribution and seasonality of this species requires phylogenetic studies to determine whether specimens in Brazil, the Gulf of Mexico, and North Carolina [46] belong to the same species or if a complex of cryptic species exists instead (cf. [1] for a similar western Atlantic cubomedusae). Elucidating the taxonomy of the species will provide a better understanding beyond seasonality, for example, in determining possible differences in relation to its venom, an important public safety area given the case of the death of a child in the Gulf of Mexico associated with *C. quadrumanus* [67].

The female cubomedusae studied herein presented oocytes at different stages of development, but none of the females was undergoing ovulation. The presence of pre-vitellogenic oocytes in all specimens (4–22%, Tables 2 and 3) supports the existence of continuous oogenesis, making it likely that *C. quadrumanus* cubomedusae are iteroparous (shedding their gametes more than once during their lifetime), a pattern observed in a few cubomedusae (e.g., [68]) and the tripedaliid cubozoans [15]. Although the histological data are robust, in situ data on spawning females are needed to corroborate our findings of a maturation scale and its persistence within the population in order to fully validate *C. quadrumanus* as a truly iteroparous species. Large and predictable spermcasting aggregations of the semelparous *A. alata* demonstrated that the leaf-like hemi-gonads visible on radial septa break, becoming a thin line after spawning, and stranded medusae along the rocks on the beach have varying degrees of gonadal rupture (from partial to almost complete) (more details in [3]). However, as is the case for most cubozoan species due to a dearth of histological data on cubomedusa gonad maturation, it remains difficult to corroborate observations on cubomedusae sexual reproduction in situ.

4.2. Fertilization Mode and Oocyte Nutrition

The challenges inherent in rearing cubomedusae for observing sexual reproduction in vitro hamper the experimental study of the modes of fertilization in this class. Even without visual confirmation of sexual reproduction, the presence of fertilized eggs or planulae in the gastric cavity of collected females has served as evidence of internal fertilization in some medusozoan species [15,69]. Additionally, external fertilization in Chirodropida is inferred for *C. fleckeri* [21] and *C. quadrumanus* based on observations of sperm release via the rupture of the follicle wall [19]. Our data corroborate the external fertilization theory for *C. quadrumanus* due to the absence of sperm or embryos within the female gastrovascular cavity, but as no samples had signs of insufficient spawning, gonadal maturity may have precluded the females from signaling males of an ensuing spawning event (for a review of such genetic signals, see [25]).

The presence of inclusions of unknown nature in some oocytes (Figure 5) may indicate that the yolk is supplied to the oocytes by phagocytic bodies, a mode of vitellogenesis previously reported in at least one medusozoan (viz., *Hydra*, [70,71]). However, we avoid further speculation on their origin or function prior to conducting further investigations.

4.3. Individual Size and Maturity

Egg size can indicate the degree of sexual maturity [72–74], defined as the period in which a female is able to reproduce sexually [75]. The average oocyte diameter in *C. quadrumanus* (122 µm) is considerably larger than that reported for the cubomedusa *C. murrayana* (55 µm). This difference may be related to general medusa size, since the bell height of a mature *C. quadrumanus* specimen (>4.4 cm) is greater than that of a mature *C. murrayana* (3.68 cm, as *C. branchi* in [45]). Aside from indicating sexual maturity, egg size is often considered a proxy for understanding the reproductive and developmental traits of medusozoans, as large eggs are related to direct development [76–78] and are found in some deep-sea scyphomedusae [79].

Sexual maturity in medusozoans has previously been connected with bell size [15,80], but these studies have the caveat of defining the sexual maturity of a female cubomedusae simply through the presence of visible oocytes [3], during the act of ovulation [25], or stating that the gonads are full of "mature eggs" [81]. However, our maturation scale and statistical analysis thereof demonstrate that bell size is not necessarily correlated with gonadal maturity in *C. quadrumanus*. A possible explanation for the lack of correlation between bell size and sexual maturity could be that small individuals with high percentages of late-vitellogenic oocytes (e.g., specimen MA52, Table 3) might have undergone more rapid development under different environmental parameters such as temperature, food availability, or breeding period, even within the same population (cf. [3,56,80] for other medusozoan examples). Future in situ studies documenting the reproductive behavior of cubomedusae that are carried out in conjunction with histological studies of the gonads should further elucidate and complement the patterns revealed in this study.

5. Conclusions

In this study, we presented a female oocyte maturation scale based on histological data for the cubozoan *Chiropsalmus quadrumanus*, making it the first of its kind presented for a chirodropid. We demonstrated the existence of four distinct stages of oogenesis, congruent with an iteroparous mode of sexual reproduction with external fertilization. This framework shall serve as a standard for future investigations into sexual maturation and reproductive strategies in cubomedusae. Although large and unpredictable aggregations or "blooms" of *C. quadrumanus* have not been recorded on the Brazilian coast, it is important to be vigilant for potential events related to the emergence of these cubomedusae at the surface to understand the environmental conditions that favor both the sexual and asexual proliferation of this species. Thus, year-round local surveys along the Brazilian coast are needed to fully elucidate the reproductive season of *C. quadrumanus* and corroborate our hypotheses of its iteroparity. Furthermore, additional specimen collections directed at molecular phylogenetic analyses will be important to validate whether the widely distributed *C. quadrumanus* lineage (Gulf of Mexico, North Carolina, Brazil) correspond, in fact, to the same species examined in this work. We expect our findings will serve as a baseline for understanding the structural and functional maturation of female sex cells and the evolution of sexual reproductive strategies within species of the order Chirodropida.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d15070816/s1.

Author Contributions: A.C.M. (André Carrara Morandini) and A.C.M. (Antonio Carlos Marques): Conceptualization, Writing—review & editing. All authors contributed to the study conception and design. J.G.-R. and A.J.-B. conceived the ideas; Material preparation, data collection, and analysis were performed by J.G.-R., A.J.-B. and G.R.T. The first draft of the manuscript was written by J.G.-R. and C.L.A., and all authors reviewed and commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the São Paulo Research Foundation (FAPESP) (grants 2011/50242-5 to A.C.M. (Antonio Carlos Marques), 2017/12970-5 to G.R.T., 2019/01370-2 and 2021/09739-5 to J.G.R.), CNPq (grants 140235/2018-3 to J.G.R., 481399/2007-0 and 309440/2019-0 to A.C.M. (André Carrara Morandini), 316095/2021-4 to A.C.M. (Antonio Carlos Marques)), and CAPES (grant 23650751852 to A.J.B.) and FAPERJ (grant E-26/171.150/2006 to A.C.M. (André Carrara Morandini)).

Data Availability Statement: Our manuscript has data included in the electronic Supplementary Material (DOI 10.5281/zenodo.7901541).

Acknowledgments: We thank R. Mohamed (University of the Western Cape, Cape Town, South Africa) for sharing oocyte data from his study with *Carybdea murrayana* (as *Carybdea branchi*) and Fernando Zara (Univiversidade Estadual Paulista, Brazil) for assistance with field samples. This manuscript was improved due to thorough review and kind feedback by external reviewers.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Collins, A.G.; Bentlage, B.; Gillan, W.; Lynn, T.H.; Morandini, A.C.; Marques, A.C. Naming the Bonaire Banded Box Jelly, *Tamoya Ohboya*, n. sp. (Cnidaria: Cubozoa: Carybdeida: Tamoyidae). *Zootaxa* **2011**, *68*, 53–68. [CrossRef]
2. Bentlage, B.; Cartwright, P.; Yanagihara, A.A.; Lewis, C.; Richards, G.S.; Collins, A.G. Evolution of Box Jellyfish (Cnidaria: Cubozoa), a Group of Highly Toxic Invertebrates. *Proc. R. Soc. B Biol. Sci.* **2010**, *277*, 493–501. [CrossRef] [PubMed]
3. Lewis, C.; Bentlage, B.; Yanagihara, A.; Gillan, W.; van Blerk, J.; Keil, D.P.; Bely, A.E.; Collins, A.G. Redescription of *Alatina alata* (Reynaud, 1830) (Cnidaria: Cubozoa) from Bonaire, Dutch Caribbean. *Zootaxa* **2013**, *3737*, 473–487. [CrossRef]
4. Baxter, E.H.; Marr, A.G.M. Sea Wasp (*Chironex Fleckeri*) Venom: Lethal, Haemolytic and Dermonecrotic Properties. *Toxicon* **1969**, *7*, 195–210. [CrossRef]
5. Werner, B.; Cutress, C.E.; Studebaker, J.P. Life Cycle of *Tripedalia cystophora*, Conant (Cubomedusae). *Nature* **1971**, *232*, 655–657. [CrossRef]
6. Ceh, J.; Gonzalez, J.; Pacheco, A.S.; Riascos, J.M. The Elusive Life Cycle of Scyphozoan Jellyfish—Metagenesis Revisited. *Sci. Rep.* **2015**, *5*, 12037. [CrossRef]
7. Morandini, A.C.; Schiariti, A.; Stampar, S.N.; Maronna, M.M.; Straehler-Pohl, I.; Marques, A.C. Succession of Generations Is Still the General Paradigm for Scyphozoan Life Cycles. *Bull. Mar. Sci.* **2016**, *92*, 343–351. [CrossRef]
8. Picard, M.A.L.; Vicoso, B.; Bertrand, S.; Escriva, H. Diversity of Modes of Reproduction and Sex Determination Systems in Invertebrates, and the Putative Contribution of Genetic Conflic. *Genes* **2021**, *12*, 1136. [CrossRef]
9. Siebert, S.; Juliano, C.E. Sex, Polyps, and Medusae: Determination and Maintenance of Sex in Cnidarians. *Mol. Reprod. Dev.* **2017**, *84*, 105–119. [CrossRef]
10. Eckelbarger, K.J.; Hodgson, A.N. Invertebrate Oogenesis—A Review and Synthesis: Comparative Ovarian Morphology, Accessory Cell Function and the Origins of Yolk Precursors. *Invertebr. Reprod. Dev.* **2021**, *65*, 71–140. [CrossRef]
11. Arneson, A.C.; Cutress, C.E. Life History of *Carybdea alata* Reynaud, 1830 (Cubomedusae). In *Coelenterate Ecology and Behavior*; Mackie, G.O., Ed.; Springer: New York, NY, USA, 1976; pp. 227–236.
12. García-Rodríguez, J.; Lewis Ames, C.; Marian, J.E.A.R.; Marques, A.C. Gonadal Histology of Box Jellyfish (Cnidaria: Cubozoa) Reveals Variation between Internal Fertilizing Species *Alatina alata* (Alatinidae) and *Copula sivickisi* (Tripedaliidae). *J Morphol* **2018**, *279*, 841–856. [CrossRef]
13. Avian, M.; Bonivento, P.; Montanari, G.; Rinaldi, A.; Rottini Sandrini, L. *Carybdea marsupialis* L. (Cubozoa), Istologia Delle Gonadi. *Biol. Mar. Suppl. Notiz. SIBM* **1993**, *1*, 75–78.

14. Hartwick, R.F. Observations on the Anatomy, Behaviour, Reproduction and Life Cycle of the Cubozoan *Carybdea sivickisi*. *Hydrobiologia* **1991**, *216–217*, 171–179. [CrossRef]
15. Lewis, C.; Long, T.A.F. Courtship and Reproduction in *Carybdea sivickisi* (Cnidaria: Cubozoa). *Mar. Biol.* **2005**, *147*, 477–483. [CrossRef]
16. Lewis, C.; Kubota, S.; Migotto, A.E.; Collins, A.G. Sexually Dimorphic Cubomedusa *Carybdea sivickisi* in Seto, Wakayama, Japan. *Publ. Seto Mar. Biol. Lab.* **2008**, *40*, 1–8. Available online: https://repository.si.edu/handle/10088/6250 (accessed on 22 June 2023).
17. Garm, A.; Lebouvier, M.; Tolunay, D. Mating in the Box Jellyfish *Copula sivickisi*—Novel Function of Cnidocytes. *J. Morphol.* **2015**, *276*, 1055–1064. [CrossRef]
18. Toshino, S.; Hiroshi, M.; Ohtsuka, S.; Okuizumi, K.; Adachi, A.; Hamatsu, Y.; Urata, M.; Nakaguchi, K.; Yamaguchi, S. Development and Polyp Formation of the Giant Box Jelly Fish *Morbakka virulenta* (Kishinouye, 1910) (Cnidaria: Cubozoa) Collected from the Seto Inland Sea, Western Japan. *Plankton Benthos Res. Benthos Res.* **2013**, *8*, 1–8. [CrossRef]
19. Tiseo, G.R.; García-Rodríguez, J.; Zara, F.J.; Ames, C.L.; Marques, A.C.; Morandini, A.C. Spermatogenesis and Gonadal Cycle in Male *Tamoya haplonema* and *Chiropsalmus quadrumanus* (Cnidaria, Cubozoa). *Zool. Anz.* **2019**, *279*, 59–67. [CrossRef]
20. García-Rodríguez, J.; Ames, C.L.; Marian, J.E.A.R.; Marques, A.C. Histomorphological Comparison of Testes in Species of Box Jellyfish (Cnidaria; Cubozoa): Does Morphology Differ with Mode of Reproduction and Fertilization? *Org. Divers. Evol.* **2020**, *20*, 25–36. [CrossRef]
21. Yamaguchi, M.; Hartwick, R. Early Life History of the Sea Wasp *Chironex fleckeri* (Class Cubozoa). In *Development and Cellular Biology of Coelenterates*; Elsevier: Amsterdam, The Netherlands, 1980; pp. 11–16.
22. Hartwick, R.F. Distributional Ecology and Behaviour of the Early Life Stages of the Box-Jellyfish *Chironex fleckeri*. *Hydrobiologia* **1991**, *216–217*, 181–188. [CrossRef]
23. Stewart, S. Field Behavior of *Tripedalia cystophora* (Class Cubozoa). *Mar Freshw. Behav. Physiol.* **1996**, *27*, 175–188. [CrossRef]
24. Carrette, T.; Straehler-Pohl, I.; Seymour, J. Early Life History of *Alatina* Cf. *moseri* Populations from Australia and Hawaii with Implications for Taxonomy (Cubozoa: Carybdeida, Alatinidae). *PLoS ONE* **2014**, *9*, e84377. [CrossRef]
25. Lewis Ames, C.; Ryan, J.F.; Bely, A.E.; Cartwright, P.; Collins, A.G. A New Transcriptome and Transcriptome Profiling of Adult and Larval Tissue in the Box Jellyfish *Alatina alata*: An Emerging Model for Studying Venom, Vision and Sex. *BMC Genom.* **2016**, *17*, 650. [CrossRef]
26. Hamner, W.M.; Jones, M.S.; Hamner, P.P. Swimming, Feeding, Circulation and Vision in the Australian Box Jellyfish, *Chironex fleckeri* (Cnidaria: Cubozoa). *Mar. Freshw. Res.* **1995**, *46*, 985–990. [CrossRef]
27. Garm, A.; O'Connor, M.; Parkefelt, L.; Nilsson, D.E. Visually Guided Obstacle Avoidance in the Box Jellyfish *Tripedalia cystophora* and *Chiropsella bronzie*. *J. Exp. Biol.* **2007**, *210*, 3616–3623. [CrossRef]
28. Berger, E.W. *Physiology and Histology of the Cubomedusae, including Dr. F. S. Conant's Notes on the Physiology*; Memoirs from the Biological Laboratory of the Johns Hopkins University; The Johns Hopkins Press: Baltimore, MD, USA, 1900; Volume 4, 84p.
29. Coates, M.M. Visual Ecology and Functional Morphology of Cubozoa (Cnidaria). *Integr. Comp. Biol.* **2003**, *43*, 542–548. [CrossRef]
30. Werner, B. Killermedusen und Ihr Liebesspiel. *Umschau* **1976**, *76*, 80–81.
31. Subramoniam, T. Mode of Reproduction: Invertebrate Animals. In *Encyclopedia of Reproduction*; Elsevier: Amsterdam, the Netherlands, 2018; pp. 32–40. ISBN 9780128151457.
32. Werner, B. Spermatozeugmen und Paarungsverhalten Bei *Tripedalia cystophora* (Cubomedusae). *Mar. Biol.* **1973**, *18*, 212–217. [CrossRef]
33. Helmark, S.; Garm, A. Gonadal Cnidocytes in the Cubozoan *Tripedalia cystophora* Conant, 1897 (Cnidaria: Cubozoa). *J. Morphol.* **2019**, *280*, 1530–1536. [CrossRef] [PubMed]
34. Bordehore, C.; Fuentes, V.L.; Atienza, D.; Barberá, C.; Fernandez-Jover, D.; Roig, M.; Acevedo-Dudley, M.J.; Canepa, A.J.; Gili, J.M. Detection of an Unusual Presence of the Cubozoan *Carybdea marsupialis* at Shallow Beaches Located near Denia, Spain (South-Western Mediterranean). *Mar. Biodivers. Rec.* **2011**, *4*, E69. [CrossRef]
35. Keesing, J.K.; Strzelecki, J.; Stowar, M.; Wakeford, M.; Miller, K.J.; Gershwin, L.A.; Liu, D. Abundant Box Jellyfish, *Chironex* sp. (Cnidaria: Cubozoa: Chirodropidae), Discovered at Depths of over 50 m on Western Australian Coastal Reefs. *Sci. Rep.* **2016**, *6*, 22290. [CrossRef]
36. Lawley, J.W.; Ames, C.L.; Bentlage, B.; Yanagihara, A.; Goodwill, R.; Kayal, E.; Hurwitz, K.; Collins, A.G. Box Jellyfish *Alatina alata* Has a Circumtropical Distribution. *Biol. Bull.* **2016**, *231*, 152–169. [CrossRef]
37. Straehler-Pohl, I.; Garm, A.; Morandini, A.C. Sexual Dimorphism in Tripedaliidae (Conant 1897) (Cnidaria, Cubozoa, Carybdeida). *Zootaxa* **2014**, *3785*, 533–549. [CrossRef] [PubMed]
38. Toshino, S.; Miyake, H.; Iwanaga, S. Development of *Copula sivickisi* (Stiasny, 1926) (Cnidaria: Cubozoa: Carybdeidae: Tripedaliidae) Collected from the Ryukyu Archipelago, Southern Japan. *Plankton Benthos Res.* **2014**, *9*, 32–41. [CrossRef]
39. Campbell, R.D. Cnidaria. *Reprod. Mar. Invertebr.* **1974**, *1*, 133–199.
40. Hyman, L.H. Metazoa of the Tissue Grade of Construction—The Radiate Phyla—Phylum Cnidaria. In *The Invertebrates: Protozoa through Ctenophora*; McGraw-Hill: New York, NY, USA; London, UK, 1940; Volume 1.
41. Widersten, B. Genital Organs and Fertilization in Some Scyphozoa. *Zool. Bidr. Fran Upps.* **1965**, *37*, 45–58. [CrossRef]
42. Miller, R.L. Cnidaria. *Reprod. Biol. Invertebr.* **1983**, *2*, 23–73.
43. Marques, A.C.; Collins, A.G. Cladistic Analysis of Medusozoa and Cnidarian Evolution. *Invertebr. Biol.* **2004**, *123*, 23–42. [CrossRef]

44. Southcott, R.V. Studies on Australian Cubomedusae, Including a New Genus and Species Apparently Harmful to Man. *Mar. Freshw. Res.* **1955**, *7*, 254–280. [CrossRef]
45. Mohamed, R.; Skrypzeck, H.; Gibbons, M.J. Describing Gonad Development and Gametogenesis in Southern Africa's Endemic Box Jellyfish *Carybdea branchi* (Cubozoa, Carybdeidae). *Afr. J. Mar. Sci.* **2019**, *41*, 83–91. [CrossRef]
46. Mayer, A.G. *Medusae of the World*; Publication no. 109; Carnegie Institution of Washington: Washington, DC, USA, 1910; Volume 3.
47. Migotto, A.E.; Marques, A.C.; Morandini, A.C.; da Silveira, F.L. Checklist of the Cnidaria Medusozoa of Brazil. *Biota Neotrop.* **2002**, *2*, 1–31. [CrossRef]
48. Morandini, A.C.; Ascher, D.; Stampar, S.N.; Ferreira, J.F.V. Cubozoa e Scyphozoa (Cnidaria: Medusozoa) de Águas Costeiras Do Brasil. *Iheringia Ser. Zool.* **2005**, *95*, 281–294. [CrossRef]
49. Nogueira, M., Jr.; Haddad, M.A. Macromedusae (Cnidaria) from the Paraná coast, southern Brazil. *J. Coast. Res.* **2006**, *2*, 1161–1164.
50. Gershwin, L.A. Comments on Chiropsalmus (Cnidaria: Cubozoa: Chirodropida): A Preliminary Revision of the Chiropsalmidae, with Descriptions of Two New Genera and Two New Species. *Zootaxa* **2006**, *42*, 1–42. [CrossRef]
51. Nogueira Júnior, M. Article Gelatinous Zooplankton Fauna (Cnidaria, Ctenophora and Thaliacea) from Baía da Babitonga (Southern Brazil). *Zootaxa* **2012**, *3398*, 1–21.
52. Guest, W.C. The Occurence of the Jelllyfish *Chiropsalmus quadrumanus* in Matagorda Bay, Texas. *Bull. Mar. Sci.* **1959**, *9*, 79–83.
53. Kraeuter, J.N.; Setzler, E.M. The Seasonal Cycle of Scyphozoa and Cubozoa. *Bull. Mar. Sci.* **1975**, *25*, 66–74.
54. Phillips, P.J.; Burke, W.D. The Occurrence of Sea Waps (Cubomedusae) in Mississipi Sound and the Northern Gulf of Mexico. *Bull. Mar. Sci.* **1970**, *20*, 853–859.
55. Straehler-Pohl, I. Critical Evaluation of Characters for Species Identification in the Cubomedusa Genus *Malo* (Cnidaria, Cubozoa, Carybdeida, Carukiidae). *Plankton Benthos Res.* **2014**, *9*, 83–98. [CrossRef]
56. Pitt, K.A.; Kingsford, M.J. Reproductive Biology of the Edible Jellyfish *Catostylus mosaicus* (Rhizostomeae). *Mar. Biol.* **2000**, *137*, 791–799. [CrossRef]
57. Humason, G.L. *Animal Tissue Techniques*; W.H. Freeman and Company: San Francisco, CA, USA, 1962.
58. Behmer, O.A.; Tolosa, E.M.C.; Freitas Neto, A.G. *Manual de Tecnicas Para Histologia Normal e Patologica*; Livraria Editora: São Paulo, Brazil, 1976.
59. Bancroft, J.; Stevens, A. *Theory and Practice of Histological Techniques*; Churchill Livingstone: London, UK, 1982.
60. Pearse, A.G.E. Histochemistry Theoretical and Applied. In *Analytical Tehnology*; Churchill Livingstone: London, UK, 1985; Volume 2.
61. Junqueira, L.C. Histology Revisited—Technical Improvement Promoted by the Use of Hydrophilic Resin Embedding. *Cienc. Cult.* **1995**, *47*, 92–95.
62. Mendoza-Becerril, M.A.; Marian, J.E.A.R.; Migotto, A.E.; Marques, A.C. Exoskeletons of Bougainvilliidae and Other Hydroidolina (Cnidaria, Hydrozoa): Structure and Composition. *PeerJ* **2017**, *5*, e2964. [CrossRef]
63. Schneider, C.; Rasband, W.; Eliceiri, K.W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* **2010**, *9*, 671–675. [CrossRef] [PubMed]
64. Marian, J.E.A.R. Spermatophoric Reaction Reappraised: Novel Insights into the Functioning of the Loliginid Spermatophore Based on Doryteuthis Plei (Mollusca: Cephalopoda). *J. Morphol.* **2012**, *273*, 248–278. [CrossRef]
65. Eckelbarger, K.J.; Larson, R. Ultrastructure of the Ovary and Oogenesis in the Jellyfish *Linuche unguiculata* and *Stomolophus meleagris*, with a Review of Ovarian Structure in the Scyphozoa. *Mar. Biol.* **1992**, *114*, 633–643. [CrossRef]
66. Mammone, M.; Bosch-Belmar, M.; Milisenda, G.; Castriota, L.; Sinopoli, M.; Allegra, A.; Falautano, M.; Maggio, T.; Rossi, S.; Piraino, S. Reproductive Cycle and Gonadal Output of the Lessepsian Jellyfish *Cassiopea andromeda* in NW Sicily (Central Mediterranean Sea). *PLoS ONE* **2023**, *18*, e0281787. [CrossRef] [PubMed]
67. Bengston, K.; Nichols, M.M.; Schnadig, V.; Ellis, M.D. Sudden Death in a Child Following Jellyfish Envenomation by *Chiropsalmus quadrumanus*: Case Report and Autopsy Findings. *JAMA J. Am. Med. Assoc.* **1991**, *266*, 1404–1406. [CrossRef]
68. Eckelbarger, K.J.; Larson, R.J. Ultrastructural Study of the Ovary of the Sessile Scyphozoan, *Haliclystus octoradiatus* (Cnidaria: Stauromedusae). *J. Morphol.* **1993**, *218*, 225–236. [CrossRef] [PubMed]
69. Toshino, S.; Miyake, H.; Srinui, K.; Luangoon, N.; Muthuwan, V.; Sawatpeera, S.; Honda, S.; Shibata, H. Development of *Tripedalia binata* Moore, 1988 (Cubozoa: Carybdeida: Tripedaliidae) Collected from the Eastern Gulf of Thailand with Implications for the Phylogeny of the Cubozoa. *Hydrobiologia* **2017**, *792*, 37–51. [CrossRef]
70. Alexandrova, O.; Schade, M.; Böttger, A.; David, C.N. Oogenesis in *Hydra*: Nurse Cells Transfer Cytoplasm Directly to the Growing Oocyte. *Dev. Biol.* **2005**, *281*, 91–101. [CrossRef]
71. Honegger, T.G.; Zürrer, D.; Tardent, P. Oogenesis in *Hydra carnea*: A New Model Based on Light and Electron Microscopic Analyses of Oocyte and Nurse Cell Diferentiation. *Tissue Cell* **1989**, *21*, 381–393. [CrossRef]
72. Toyokawa, M.; Shimizu, A.; Sugimoto, K.; Nishiuchi, K.; Yasuda, T. Seasonal Changes in Oocyte Size and Maturity of the Giant Jellyfish, *Nemopilema nomurai*. *Fish. Sci.* **2010**, *76*, 55–62. [CrossRef]
73. Iguchi, N.; Lee, H.E.; Yoon, W.D.; Kim, S. Reproduction of the Giant Jellyfish, *Nemopilema Nomurai* (Scyphozoa: Rhizostomeae), in 2006–2008 as Peripherally-Transported Populations. *Ocean. Sci. J.* **2010**, *45*, 129–138. [CrossRef]
74. Jarms, G.; Tiemann, H.; Båmstedt, U. Development and Biology of *Periphylla Periphylla* (Scyphozoa: Coronatae) in a Norwegian Fjord. *Mar. Biol.* **2002**, *141*, 647–657. [CrossRef]

75. Schiariti, A.Í.; Christiansen, E.; Morandini, A.C.; da silveira, F.L.; Giberto, D.A.; Mianzan, H.W. Reproductive Biology of *Lychnorhiza lucerna* (Cnidaria: Scyphozoa: Rhizostomeae): Individual Traits Related to Sexual Reproduction. *Mar. Biol. Res.* **2012**, *8*, 255–264. [CrossRef]
76. Hargitt, G.T. Germ Cells of Coelenterates. VI. General Considerations, Discussion, Conclusions. *J. Morphol.* **1919**, *33*, 1–59. [CrossRef]
77. Jarms, G.; Båmstedt, U.; Tiemann, H.; Martinussen, M.B.; Fosså, J.H.; Høisœter, T. The Holopelagic Life Cycle of the Deep-Sea Medusa *Periphylla periphylla* (Scyphozoa, Coronatae). *Sarsia* **1999**, *84*, 55–65. [CrossRef]
78. Berrill, N.J. Developmental Analysis of Scyphomedusae. *Biol. Rev.* **1949**, *24*, 393–409. [CrossRef]
79. Russell, F.S. On the Scyphomedusa Poralia Rufescens Vanhöffen. *J. Mar. Biol. Assoc. UK* **1962**, *42*, 387–390. [CrossRef]
80. Lucas, C.H.; Reed, A.J. Gonad Morphology and Gametogenesis in the Deep-Sea Jellyfish *Atolla wyvillei* and *Periphylla periphylla* (Scyphozoa: Coronatae) Collected from Cape Hatteras and the Gulf of Mexico. *J. Mar. Biol. Assoc. UK* **2010**, *90*, 1095–1104. [CrossRef]
81. Toshino, S.; Miyake, H.; Shibata, H. Development of *Carybdea brevipedalia* Kishinouye, 1891 (Cnidaria: Cubozoa: Carybdeida: Carybdeidae) Collected from Northern Japan. *Plankton Benthos Res.* **2018**, *13*, 116–128. [CrossRef]