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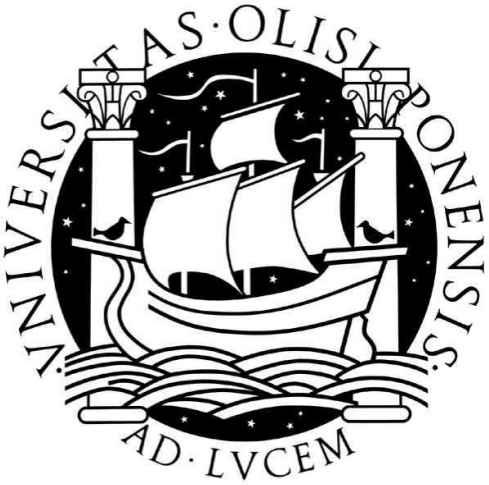

Evaluation of *Paracentrotus lividus* (Lamarck, 1816) exotrophic larvae as live feed for marine decapod crustacean larvae

Tiago Filipe Baptista da Rosa Repolho

Doutoramento em Biologia

Biologia Marinha e Aquacultura

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Tiago Filipe Baptista da Rosa Repolho

Tese orientada pelo Professor Auxiliar Doutor Orlando de Jesus Luís
e Professor Doutor Manuel Yúfera Ginés, especialmente elaborada
para a obtenção do grau de Doutor em Biologia
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NOTA PRÉVIA

A presente dissertação de doutoramento baseia-se num conjunto de trabalhos experimentais, desenvolvidos no Laboratório Marítimo da Guia (Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa), em colaboração com o Instituto de Ciências Marinhas da Andalucia (ICMAN, CSIC), cujo principal objectivo foi o de avaliar o uso de estadios larvares exotróficos do ouriço-do-mar *Paracentrotus lividus* como presas vivas na larvicultura de crustáceos decápodes marinhos. Deste modo, foram testadas como alimento vivo, larvas exotróficas de *P. lividus* (pluteus de 4 braços) com 3 e 5 dias após fertilização. A investigação desta hipótese teve por base estudos anteriores que evidenciam a importância e a abundância sazonal de estadios larvares de ouriço-do-mar no plâncton marinho, assim como trabalhos científicos que comprovam a ingestão destes organismos por estadios larvares de peixes, crustáceos e outros vertebrados e invertebrados marinhos. De igual modo, os resultados do trabalho agora apresentado, revestem-se de importância acrescida no que respeita à sua aplicabilidade na área da echinocultura, assim como noutras áreas de investigação fundamental e/ou aplicada, como sejam a ecotoxicologia aquática e ciências biomédicas, onde os vários estadios de desenvolvimento desta espécie são usados como modelos biológicos. O presente trabalho foi suportado financeiramente através de uma Bolsa de Doutoramento da Fundação para a Ciência e Tecnologia (SFRH/BD/36232/2007) e pelo Programa Plurianual de Financiamento de Unidades de Investigação e Desenvolvimento (CO/LMG). Mais declaro a minha participação integral na elaboração da presente dissertação, desde o seu planeamento e formulação teórica, até à concretização prática e redação dos trabalhos efectuados. No trabalho referente ao capítulo 2.2, declaro a minha participação nos trabalhos de análise bioquímica e revisão crítica dos resultados.

A dissertação aqui apresentada com vista à obtenção do Grau de doutor, encontra-se organizada em 7 capítulos, sendo 1 referente a 2 trabalhos já publicados e 4 capítulos referentes a trabalhos em fase de submissão a revistas internacionais da especialidade com arbitragem científica.

No primeiro capítulo (General Introduction), é apresentada uma breve introdução onde se descreve o estado da arte inerente à investigação realizada e às áreas da ciência com

possível aplicabilidade dos resultados obtidos, assim como os principais objectivos da dissertação.

No segundo capítulo (Broodstock diets for sea urchin larvae production) é analisada a formulação e utilização de dietas inertes, com fontes proteicas de origem vegetal, na alimentação de progenitores de *P. lividus* e seus efeitos ao nível de composição bioquímica (aminoácidos) de óvulos, taxas de fertilização e desenvolvimento larvar. De igual modo, o presente capítulo estuda o melhoramento da qualidade nutricional (ácidos gordos), de óvulos e estadios larvares endotróficos, através da manipulação da composição de dietas inertes de progenitores desta espécie. Este capítulo é composto por dois trabalhos que já se encontram publicados nas revistas internacionais da especialidade, "*Ecotoxicology and Environmental Safety*" e "*Aquaculture Nutrition* ", respectivamente.

O terceiro capítulo (The effect of sperm:egg ratio and egg concentration on egg fertilization, larvae malformations and polyspermy events in the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea)), apresenta os resultados referentes ao efeito que diferentes rácios de esperma:óvulos e concentração de óvulos, aplicados durante a fertilização *in vitro*, podem ter ao nível das taxas de fertilização, malformações larvares e possível incidência de eventos associados a polispermia na espécie *P. lividus.* O presente capítulo encontra-se em fase de submissão à revista "*Development, Growth and Differentiation*".

O quarto capítulo (Culture conditions of the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) exotrophic plutei larvae: Implications to larvae production) avalia determinadas condições bióticas (alimentação, densidades de cultivo e enriquecimento) e abióticas (arejamento e mudança de meio de cultivo) no cultivo larvar da espécie *P. lividus*, por intermédio da análise de parâmetros biométricos, sobrevivência, crescimento e desenvolvimento larvar. Este capítulo encontra-se em fase de submissão à revista "*Aquaculture*".

O quinto capítulo (Potential of the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) exotrophic larvae as live feed for rearing marine decapod crustacean larvae) avalia o potencial de larvas exotróficas de ouriço-do-mar *P. lividus* como alimento vivo na larvicultura de crustáceos decápodes marinhos, face a outra presa viva (*Artemia* spp.). Este capítulo encontra-se em fase de submissão à revista "*Aquaculture Research*".

No sexto capítulo (Can sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) spines be used as a tool for sex identification of adult individuals?) analisou-se a

possibilidade da utilização de uma característica morfológica externa dos espinhos de *P. lividus*, como metodologia não letal para diferenciação de sexos em indivíduos adultos.

Por fim, no sétimo capítulo (Final Considerations), são discutidos, sucintamente, os resultados obtidos e é apresentada uma breve conclusão sobre a potencialidade de larvas exotróficas de *P. lividus* serem usadas como alimento vivo na larvicultura de crustáceos decápodes marinhos. Paralelamente, são indicadas possíveis linhas de trabalho futuro, inerente à temática da dissertação aqui apresentada e áreas de investigação associadas.

Lisboa, 22 de Junho de 2012

_____

(Tiago Filipe Baptista da Rosa Repolho)

AGRADECIMENTOS

Em primeiro lugar gostaria de agradecer ao Professor Orlando de Jesus Luís, pela sua orientação teórica e prática na concretização do trabalho inerente a esta tese de doutoramento, assim como pelo incentivo permanente na elaboração da mesma. De igual modo, gostaria de agradecer ao Doutor Manuel Yúfera Ginés, pelo seu apoio, assim como pelas críticas construtivas na elaboração desta tese.

Em segundo lugar, gostaria de dar uma palavra de agradecimento à instituição Laboratório Marítimo da Guia (Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa), pelas condições logísticas proporcionadas e sem as quais a realização dos trabalhos conducentes a esta tese de doutoramento, não teriam sido possíveis.

Paralelamente, os meus agradecimentos são igualmente endereçados a todos os meus colegas de trabalho, do Laboratório Marítimo da Guia, não só pela sua boa disposição muitas das vezes necessária em alturas mais difíceis, mas também por todo o convívio salutar que tive o prazer de desfrutar durante o período de elaboração do presente trabalho.

Aos meus pais por toda a educação que me deram e proporcionaram, fazendo-me sempre ver e encarar novos desafios como oportunidades.

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ABSTRACT

In the present study, we have evaluated 4-arm exotrophic larvae of sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) as live feed in marine decapod crustacean larviculture, in comparison to *Artemia* spp. naupliar stages, a commonly used live prey in marine hatcheries.

We therefore investigated several key parameters to assess the potential of *P. lividus* plutei as live feed for crustacean larvae, namely: 1) broodstock diet manipulation in order to obtain both quantitative and qualitative year round production of 4-arm echinoplutei, free from environmental and reproductive seasonal constraints; 2) best possible biotic conditions to perform sea urchin *in vitro* fertilization, namely sperm:egg ratios and egg concentrations, and its relation to early stage segmentation and larvae development; 3) mass production possibility in terms of larvae media culture conditions, plutei stocking densities and larvae feed and enrichment; and acceptability as live feed by predator larvae (*Lysmata seticaudata*, *Palaemon elegans*, *Maja brachydactyla*, *Pachygrapsus marmoratus* and *Xantho incisus*). Additionally, we studied the possibility to differentiate *P. lividus* broodstock sex by means of a spine external morphological characteristic.

Under controlled laboratory conditions, percent egg fertilization above 99% and high values for percent normal development (≥85%) were obtained when captive breed *P. lividus* broodstock was fed an inert diet based upon maize and wheat flour mixture. Whole egg total amino acid composition was similar between *P. lividus* fed this inert diet and wild caught organisms. Enrichment potential in terms 4-arm plutei fatty acid profile and lipid content was possible through manipulation of the lipid source chosen for captive *P. lividus* broodstock diet.

Sperm:egg ratios were found to influence percent egg fertilization and segmentation development after 90 minutes post-fertilization, but not plutei total length, post-oral arm length and normal percent larvae development.

Water renewal (every 2 days) during *P. lividus* larviculture was found to be an essential abiotic culture condition to sustain plutei development and survival until 18 days post-fertilization. All plutei larval feeds (live microalgae, inert microdiets, single or mixed provided), supported *P. lividus* larvae growth and survival until 18 days post-fertilization

(DPF), with *D. tertiolecta* being found to be the most appropriate diet in terms of larvae development and survival outcome. When cultured at a initial stocking density of 40 plutei.ml^{-1}, inert microdiets were unable to sustain *P. lividus* plutei survival to 10 DPF. Plutei survival was found to be inversely correlated with increasing culture densities. *Paracentrotus lividus* plutei enrichment with Algamac 3050 flake, was found to cause mass mortality at 5 and 10 DPF.

Paracentrotus lividus exotrophic 4-arm plutei was ingested and preyed by 4 (*Lysmata seticaudata*, *Maja brachydactyla*, *Pachygrapsus marmoratus* and *Xantho incisus*) out of 5 crustacean larvae tested, but were unable to sustain complete larvae development and survival to settlement of *L. seticaudata*, *M. brachydactyla* and *P. marmoratus* species. No apparent advantage of *P. lividus* 4-arm plutei as live feed for marine crustacean decapod larvae was found in comparison to *Artemia* spp..

Finally, the analysed spine morphological characteristic, as a tool for sex differentiation between *P. lividus* adults, does not allow accurate and feasible results.

Keywords: *Paracentrotus lividus***;** exotrophic larvae; live prey; crustacean; larviculture.

RESUMO

Os estudos efectuados e incluídos nesta tese de doutoramento, resultam da formulação de uma hipótese que se baseou em evidências cientificamente comprovadas e que pretendeu a avaliar a utilização de larvas exotróficas do equinoderme *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata: Echinoidea) como presas vivas na larvicultura de crustáceos decápodes marinhos. No seguimento de evidências científicas baseadas em estudos ao nível de comunidades planctónicas marinhas, que demonstram a importância de estadios larvares de ouriços-do-mar na sua composição e biomassa, acrescido de resultados laboratoriais que comprovam a sua predação por estadios larvares de crustáceos marinhos, pretendeu-se inferir o potencial de larvas exotróficas de *P. lividus* como presa vivas face a outras espécie zooplanctónica normalmente usada como alimento vivo (*Artemia* spp.).

Neste contexto, efectuou-se um conjunto de estudos experimentais com o objectivo de verificar a aplicabilidade da hipótese sugerida. Deste modo escolheu-se esta espécie uma vez que se trata do ouriço-do-mar de maior abundância na zona rochosa intertidal e subtidal da costa Portuguesa. Em segundo lugar, informação cientifica disponível permite a manutenção desta espécie em cativeiro, com custos operacionais baixos. De igual modo sendo possível a manutenção anual de organismos adultos maturos em cativeiro, independentemente da época reprodutiva no meio natural, torna possível a obtenção em cativeiro de grandes quantidades de gâmetas e consequentemente de larvas. A escolha de estadios larvares exotróficos prendeu-se por um lado com o facto da sua biometria ser semelhante a naúplios recém-eclodidos e metanáuplios (24h) de *Artemia* spp., assim como à hipótese da sua qualidade nutricional poder ser melhorada por intermédio de alimentação exógena. Finalmente e dada a sua fraca capacidade natatória, estes estadios podem constituir uma presa com maior capacidade de captura por parte de potenciais predadores.

De modo a avaliar a potencialidade destas larvas como presas vivas na larvicultura marinha, procedeu-se a elaboração de um conjunto de trabalhos experimentais, com vista à verificação da hipótese apresentada. Em primeiro lugar foi analisado o efeito da alimentação (dietas naturais e artificiais) de adultos desta espécie em cativeiro ao nível do índice gonadossomático, conteúdo proteico de óvulos (aminoácidos), taxas de fertilização, biometria e desenvolvimento larvar normal. Foi efectuada uma comparação com ouriços-do-

mar selvagens, recolhidos durante a época reprodutiva. Obtiveram-se valores similares de desenvolvimento larvar normal entre organismos selvagens e adultos alimentados com uma das dietas inertes usadas. Como principal resultado ficou provada a possibilidade de obtenção de taxas de desenvolvimento larvar normal elevadas, quando adultos de *P. lividus*, mantidos em cativeiro, eram alimentados com uma dieta inerte apropriada, cuja fonte proteica é baseada numa mistura de farinha de milho e trigo.

De seguida inferiu-se a qualidade nutricional (conteúdo lipídico e composição em ácidos gordos) de óvulos e larvas endotróficas de *P. lividus* mantidos em cativeiro e alimentados com dietas naturais (grãos de milho, macrolgas e mistura) e inertes. Procedeu-se posteriormente à manipulação lipídica de dietas inertes artificiais, pela incorporação de óleos ricos em ácidos gordos poliinsaturados. Como principal resultado, verificou-se a incorporação de ácidos gordos poliinsaturados em óvulos e larvas endotróficas resultantes de adultos cuja alimentação tinha sido efectuada com dietas artificiais elaboradas com fontes lípidicas com uma maior proporção de lípidos insaturados.

Posteriormente foram investigados quais os efeitos que diferentes racios de espermatozóides:óvulos e concentração de óvulos, aplicados na fertilização *in vitro* desta espécie, poderiam ter ao nível de taxas de fertilização, desenvolvimento embrionário, biometria e desenvolvimento larvar resultante. Pretendeu-se deste modo inferir se existia alguma relação directa entre a hipótese formulada e os resultados obtidos, para os parâmetros analisados. Verificou-se que com o aumento de rácios de espermatozóides:óvulos testados, ocorre um aumento nas percentagens de taxas de fertilização, embriões fertilizados (não desenvolvidos) e embriões segmentados (4 células), contrariamente ao decréscimo observado para embriões em estadio de segmentação de 2 células. De igual modo, verificou-se um decréscimo nos valores obtidos de desenvolvimento larvar normal e tamanho de braços pós-orais, com o aumento na concentração de óvulos testadas, o que possivelmente poderá estar relacionado com o aumento de densidades de cultivo larvares. Como principal resultado, racios de espermatozóides:óvulos superiores a 300:1, aparentemente influenciam negativamente a segmentação embrionária normal de *P. lividus*, mas não a morfogénese de echinopluteus de 4 braços, 72 horas após fertilização.

Seguidamente, foram avaliadas e testadas várias condições de cultivo larvar de *P. lividus*, tendo como objectivo a aferição da possibilidade da sua larvicultura em massa. Foram analisados diversos parâmetros (arejamento, mudança de meio de cultivo, dietas, densidades de cultivo e enriquecimento). Como principal resultado, verificou-se a obtenção de taxas de sobrevivência superiores a 77.0 %, para larvas exotróficas até 6 dias após

fertilização, independentemente das densidades de cultivo testadas. De igual modo, taxas de sobrevivência larvar superiores a 52.9 %, aos 18 dias após fertilização, só foram obtidas quando pluteus foram alimentados com *D. tertiolecta*, a uma densidade de cultivo inicial de 1.5 pluteus.mL^{-1} e sob determinadas condições de cultivo (com arejamento e mudança de meio de cultivo a cada 2 dias). Adicionalmente, verificou-se no que respeita às dietas testadas, que a microalga *D. tertiolecta*, foi a alimentação que melhores resultados proporcionou, quer em taxas de sobrevivência larvar, quer na percentagem final de pluteus no estadio de 8 braços, após 18 dias de larvicultura, face a dietas microencapsuladas. Adicionalmente verificou-se a possibilidade de efectuar o enriquecimento de pluteus aos 5 e 10 dias após fertilização, por intermédio da utilização de um produto comercial (Algamac 3050 flake). Como principal resultado, verificou-se a obtenção de taxas de mortalidade elevadas em larvas exotróficas de *P. lividus* (5 e 10 dias após fertilização) após a tentativa de enriquecimento com Algamac 3050 Flake, por um período de 12h. De igual modo, verificou-se a possibilidade de produção em massa de pluteus (40 pluteus.mL^{-1}), até 5 e 10 dias após fertilização, por intermédio da utilização da microalga *D. tertiolecta* e microdietas artificiais (Frippak 1#CAR e Lanzy spirulina+) como fonte de alimentação larvar, tendo-se verificado valores de sobrevivência larvar baixos (15,1 % e 11,9 %) aos 5 dias após fertilização e 100% de mortalidade aos 10 dias após fertilização, respectivamente, nas experiências em que as dietas artificiais foram usadas como alimento para as larvas exotróficas.

Posteriormente, foram efectuadas uma série de experiências com vista ao estudo do potencial da utilização de larvas exotróficas de *P. lividus* (4 braços), como alimento vivo na larvicultura de crustáceos decápodes marinhos. Deste modo, foram verificadas em primeiro lugar, taxas de ingestão (24 horas) de 5 potenciais predadores (*Lysmata seticaudata*, *Palaemon elegans*, *Maja brachydactyla*, *Pachygrapsus marmoratus* e *Xantho incisus*) face a oito presas vivas (ovócitos; gástrulas; pluteus com 4 braços, com 5 e 10 dias após fertilização; pluteus com 6 seis braços, com 12 dias após fertilização; Pluteus com 8 braços, 18 dias após fertilização; *Artemia* spp. recém-eclodida; *Artemia* spp. 24horas após eclosão). Verificou-se para as espécies de predadores testadas, que unicamente *Lysmata seticaudata*, *Maja brachydactyla*, *Pachygrapsus marmoratus* e *Xantho incisus*, consumiam estadios larvares de *P. lividus*. *Lysmata seticaudata* foi a espécie de predador que ingeriu todas as presas vivas de entre os predadores testados, tendo mesmo sido a única onde se constatou a ingestão de pluteus com 6 e 8 braços. Posteriormente, foi avaliada o desenvolvimento e sobrevivências larvares das espécies *Lysmata seticaudata*, *Maja brachydactyla* e *Pachygrapsus marmoratus* alimentadas com larvas exotróficas de *P. lividus* (3 e 5 dias após

fertilização) *vs. Artemia* spp. ao longo do cultivo larvar destes crustáceos. Como resultado principal, verificou-se que as larvas exotróficas de *P. lividus* permitiram a sobrevivência e desenvolvimento larvar de *L. seticaudata* até 28 dias após eclosão e estadio IV; *M. brachydactyla* até 12 dias após eclosão e estadio II; e *P. marmoratus* até 9 dias após eclosão, não se tendo observado para esta última espécie, o desenvolvimento larvar além do estadio de zoea I. De igual modo, verificou-se que os equinopluteus exotróficos usados como presas vivas, não permitiram a obtenção de resultados superiores em termos de desenvolvimento e taxas de sobrevivência larvar dos predadores testados, face a *Artemia* spp..

Por fim foi analisada a possibilidade da utilização de uma determinada característica morfológica externa dos espinhos de *P. lividus*, com o objectivo de a testar como método não letal na identificação do sexo de organismos adultos desta espécies. Como principal resultado, verificou-se a não validade desta característica como potencial método de identificação sexual entre machos e fêmeas de *P. lividus*.

Em conclusão, verificou-se que os estadios larvares exotróficos de *P. lividus*, testados como potenciais presas vivas na larvicultura de espécies de crustáceos decápodes marinhos, não apresentam nenhuma vantagem adicional como alimento vivo alternativo ou mesmo complementar face a *Artemia* spp., no que respeita à obtenção de taxas de sobrevivência superiores ao longo do desenvolvimento larvar. Paralelamente e apesar dos resultados obtidos, este estudo contribui para o aperfeiçoamento, desenvolvimento e aplicação de técnicas e/ou métodos em determinadas áreas da investigação, como é exemplo a ecotoxicologia marinha e estuarina, onde gâmetas e estadios de desenvolvimento embrionário e larvar desta espécie, são utilizados como biomodelos.

Palavras-chave: *Paracentrotus lividus*; larvas exotróficas; presas vivas; crustáceos; larviculture.

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Fisheries regulation and aquaculture of a particular species should be based upon knowledge over its biology and ecology. Over the last decades, increasing market demand for sea urchins, with special emphasis to its gonads, generally termed as “roe”, have led to the depletion and over-exploitation of natural stocks of these organisms (Lawrence *et al.*, 2001), with severe associated ecological consequences within coastal ecosystems biological structure and dynamics. Due to a general reduction in sea urchin fisheries landings and growing market demand for its roe, interest and subsequent development of commercial echinoculture as steadily increased over the years. Despite recent advances, echinoculture is still based upon the collection of wild adult organisms and subsequent captive fattening with artificial feeds, until its gonads reach desired market size and quality. As a consequence, commercial echinoculture development has contributed also with deleterious effects over natural populations once fisheries pressure over these organisms has not been eased but steadily increased instead. Additionally, since most of inert processed diets used to feed sea urchins, are based upon fish meal, the pressure over wild fish stocks has also increased, leading to a medium to long term unsustainable situation, ecologically speaking. In order to overcome this situation, the development of a sustainable echinoculture has received increased attention by the research community, through the study and optimization of generally termed “key areas” such as, sea urchin larviculture and nutrition.

It is well known that the main cost associated with the captive production of aquatic organisms is feed. From hatchery production of early life stages till grow-out of juvenile and adult individuals, it is imperative that feed quality as well as quantity is properly provided so survival and growth of targeted species as well as production feasibility is not compromised. In this context, research performed in the development of artificial inert diets, applied through out the production stages of commercial important species, has positively contributed for the possibility to complement or partial/complete replacement of live prey usage in early life stages production and wild origin feeds in juveniles or adult aquaculture. Thus, artificial feeds represent a reliable and cost-effective alternative to live feed (hatchery production) and raw feeds (grow-out), presenting several advantages such as: The possibility to customize their nutritional and proximal composition in terms of nutritional

requirements of targeted species and development stages; their ready availability and long-term storage; the ability to incorporate in its composition specific components target for specific needs such as disease resistance (probiotics and immunostimulants) and the reduction of accidental pathogens incorporation through feed delivery. But despite developments in microdiets formulation (D'Abramo, 2002, 2003; Holme *et al.*, 2009), further research in terms of microfeeds water stability and nutrient leaching, as well as palatability, digestibility and supply methodology are needed, to overcome existing problems in their applicability as complement, alternative or co-feeding larval feeds.

Brachyuran and caridean marine crustaceans are species of commercial interest, whose natural stock declines due to human seafood supply, live bait or ornamental trade demand have prompt their aquaculture development. Like most marine invertebrates, their successful larviculture is dependent upon several factors like maternal energy and nutritional provisioning, exogenous feeding, qualitative and quantitative nutrition, biotic and abiotic culture conditions. In this context, micro-encapsulated/bounded feeds have increasingly been used in marine crustacean larviculture (Kumlu and Jones, 1995; D'Abramo, 2003; Holme *et al.*, 2006; Andrés *et al.*, 2011). Total replacement of live feeds by artificial diets has been accomplished, for penaeid larvae (Jones, 1998) but in some other species such as the case of phyllosoma and puerulus culture, survival has only been achieved by using *Artemia* spp. and *Mytilus galloprovincialis* gonads as larval feeds (Takeuchi and Murakami, 2007).

In this context, *Artemia* spp. still remains as one of the most generally used live preys in crustacean larviculture, mainly due to the year-round easiness of mass-culture at high densities, as well as the possibility to bioencapsulate with desired nutrients, making them available to target species. However, alternative/complementary lives preys should be evaluated, in order to avoid or even diminish the dependence of hatchery larvae production upon this crustacean. Furthermore, new live feeds could offer the possibility in the development of new species aquaculture, still dealing with high early life stages mortality.

Sea urchin embryos and larvae are considered as the most sensitive and complex development stages of these marine invertebrates. Larval development and survival are strongly dependent upon maternal energy and nutritional provisioning (George *et al.*, 2001), larval nutritional status (George *et al.*, 1990; Bertram and Strathmann, 1998; Meidel *et al.,* 1999) and environmental conditions (Bressan *et al.*, 1995; Sewell and Young, 1999; Denny *et al.*, 2002; Carballeira *et al.*, 2011). Under captive conditions, larvae development of several sea urchin species, has been thoroughly studied namely *Arbacia punctulata*, *Lytechinus pictus*

and *Echinometra niathaei*, (Hinegardner, 1969) *Asthenosoma ijimai* (Amemiya and Tsuchiya, 1979), *Sterechinus neumayeri* (Bosch *et al.*, 1987), *Strongylocentrotus droebachiensis* (Bertram and Strathmann, 1998; Meidel *et al.*, 1999), *Psammechinus miliaris* (Kelly *et al.*, 2000), *Lytechinus variegatus* (Hinegardner, 1969; McEdward and Herrera, 1999; George *et al.*, 2004; Buitrago *et al.*, 2005; Miloslavich *et al.*, 2007), *Tripneustes ventricosus* (Wolcott and Messing, 2005), *Strongylocentrotus purpuratus* (Hinegardner, 1969; Meyer *et al.*, 2007; Smith *et al.*, 2008), *Cassidulus mitis* (Contins and Ventura, 2011), including *Paracentrotus lividus* (Fenaux *et al.*, 1985b; Gosselin and Jangoux, 1998; Vaïtilingon *et al.*, 2001; Liu *et al.*, 2007; Carboni *et al.*, 2012). Despite the majority of sea urchin larviculture research aims to improve larval survival till metamorphosis, some authors have already emphasized the hypothesis of early life planktonic stages of sea urchins as live feeds in marine aquaculture for *P. lividus* (Luís *et al.*, 2005) and for *Lytechinus variegatus* (Hubbard *et al.*, 2003), but only (Gago *et al.*, 2010) have evaluated mass-produced endotrophic stages as life feeds for fish larviculture.

When assessing the potential of a zooplanktonic species, as live prey for marine larviculture, certain eligibility criteria have to be addressed, like nutritional composition, size biometry, mass production feasibility and acceptance by targeted predators. Likewise, development and optimization of mass-culture conditions and techniques (water quality, temperature, culture densities and apparatus), diets (natural or artificially processed) and feeding regimes are of critical importance, if high survival and culture success is to be obtained, whatever the final aim of the proposed research.

In this context, the aim of our study was to evaluate the potential of the sea urchin *P. lividus* exotrophic larvae (4, 6 and 8-arm plutei) as live feed for marine decapods crustacean larviculture. Our working hypothesis was based upon findings from both ecological as well as laboratory studies. Firstly, several authors have emphasized echinoplutei importance within marine zooplankton composition and biomass (Fransz *et al.,* 1984; Lindley *et al.,* 1995; López *et al.,* 1998). Secondly, experimental data, demonstrates that sea urchin plutei are preyed by zooplanktonic predators, including fish and crustacean species (Rumrill *et al.* 1985; Pennington *et al.* 1986; Allen, 2008; Gago *et al.*, 2010). Thirdly, simple spawning induction techniques, easy broodstock maintenance under captive conditions and large year-round spawnings, are some characteristics, which sustained the hypothesis of *P. lividus* potential as live prey for marine larviculture (Luís *et al.*, 2005). Finally, the easiness of mass-producing 4-arm exotrophic larvae and size similarity (between 450-500 μm and 580-650

µm in length, for 3 and 5 days post-fertilization 4-arm plutei) in comparison to *Artemia* spp. nauplii (420-475 µm) and 24h metanauplii (600-650 µm) (Narciso, 2000).

Our study focused on the sea urchin *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata: Echinoidea), the dominant sea urchin species along the Portuguese subtidal rocky shores. This species has a wide distribution within rocky marine environments (Lozano *et al*., 1995), extending from the Northeast Atlantic Ocean until the Mediterranean Sea (Spirlet *et al*., 2001). It plays a key ecological role, namely by acting in algae population control, as well as nutrient recycling, within its larval, juvenile and adult life stages (Rassoulzadegan and Fenaux, 1979; Fernandez, 1997).

Overexploitation of *P. lividus* natural stocks (Fernandez, 1997; Barnes and Crook, 2001; Pais *et al.*, 2007; Symonds *et al.*, 2007) associated to increasing demand and commercial value of its roe, led to increasing study of this species echinoculture over the last 2 decades (Frantzis and Grémare, 1992; Basuyaux and Blin, 1998; Grosjean *et al.*, 1998; Spirlet *et al*., 1998; Fernandez and Boudouresque, 2000; Spirlet *et al.*, 2000; Spirlet *et al.*, 2001; Shpigel *et al.*, 2004; Shpigel *et al.*, 2005; Shpigel et al., 2006; Cook and Kelly, 2007a, 2007b; Liu *et al.*, 2007; Symonds *et al.*, 2007; Pantazis, 2009; Carboni *et al.*, 2012).

The ability to obtain gametes in large quantities, external fertilization, easy manipulation and possibility to observe their *in vitro* development, as well as availability from research institutions or wild caught and easy maintenance under captive conditions at low financial cost, are some of the factors that also contributed to this species importance as biological model, both for fundamental or applied research.

Paracentotus lividus gametes, fertilization and embryo/larval development have been applied in cellular, molecular and development mechanisms studies underlying biological processes (Roccheri *et al.*, 1997; Dubois and Ameye, 2001), as well as within other research areas such as aquatic toxicology (Ozretic *et al.*, 1998; His *et al.*, 1999; Fernández and Beiras, 2001; Beiras, 2002; Pesando *et al.*, 2003; Ghirardini and Novelli, 2001; Ghirardini *et al.*, 2005; Soualili *et al.*, 2008; Caeiro *et al.*, 2009). Thus considerable effort has been applied in the development of culture methodology, behavior studying, feeding and nutritional requirements of *P. lividus* larvae (Rassoulzadegan and Fenaux, 1979; Bressan *et al.*, 1995; Gosselin and Jangoux, 1998; Vaïtilingon *et al.*, 2001; Liu *et al.*, 2007; Pétinay et al., 2009; Carboni *et al.*, 2012), although further research is needed in order to optimize certain aspects of larval nutrition, like feed acceptance (Liu *et al.*, 2007).

The present study aims directly to evaluate the potential of sea urchin exotrophic larvae as live preys for marine decapods crustacean larvae. The criteria to be tested in this

hypothesis, namely year-round gamete production, larvae nutritional improvement, mass-production conditions and live prey acceptability by crustacean larvae, are tested and findings are discussed. Likewise, the results obtained in the present study positively contribute to other research areas, i.e. embryology, aquatic and pharmaceuticals toxicology assessment, where sea urchin gamete and larvae development are used as biological models.

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CHAPTER 2

Broodstock diets for sea urchin larvae production

CHAPTER 2.1

Broodstock diet effect on sea urchin Paracentrotus lividus (Lamarck, 1816) endotrophic larvae development: Potential for their year-round usei n environmental toxicology assessment

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Broodstock diet effect on sea urchin *Paracentrotus lividus* (Lamarck, 1816) endotrophic larvae development: Potential for their year-round use in environmental toxicology assessment

Tiago Filipe Baptista da Rosa Repolho [a,*], Maria Helena Costa [b], Orlando de Jesus Luís [a], João André Evaristo de Matos Gago [a]

[a] Centro de Oceanografia, Laboratório Marítimo da Guia, Faculdade de Ciências da Universidade de Lisboa, Avenida Nossa Senhora do Cabo 939, 2750-354 Cascais, Portugal
[b] IMAR-Instituto do Mar, Departamento de Ciências e Engenharia do Ambiente, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus da Caparica, 2829-516, Caparica, Portugal

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ABSTRACT

The effect of captive broodstock diet on fertilization and endotrophic larvae development of the sea urchin *Paracentrotus lividus* was assessed. Maize grain and five inert pelleted diets were tested, during a three-month experimental period. Maize flour, wheat flour, soybean flour, maize/wheat flour (MWF) and maize/soybean flour mixes were used as vegetal sources for inert feed. Gonad index, percent egg fertilization and larvae malformation occurrence were compared with the results obtained from wild sea urchins (W). Whole egg total amino acid composition was concomitantly analyzed as a tool to explain eventual endotrophic larvae malformations caused by lack of specific nutrients. For all treatment groups (wild and captive), percent egg fertilization values above 96% were always observed, fulfilling the requisites (70–90%) necessary to conduct environmental monitoring bioassays, according to USEPA (2002). Similar values for normal percent larval development were only obtained from *P. lividus* broodstock subjected to an inert feeding diet based on a maize/wheat flour mix ($85.0 \pm 1.45\%$), in comparison to wild *P. lividus* ($82.5 \pm 1.75\%$). Likewise, no statistical differences on resultant whole egg total amino acid composition were observed between *P. lividus* fed MWF and wild treatments. Moreover, statistical differences between MWF and all the other captive feeding treatments were found for six out of the seventeen amino acids analyzed. This study demonstrates the possibility to obtain high values for *P. lividus* endotrophic larvae percent normal development based on broodstock held in captivity as long as an appropriate inert diet is provided.

1. Introduction

Environmental pollution affects biological communities in aquatic habitats at different organizational levels (Vaschenko and Zhadan, 2003). Physicochemical monitoring is insufficient to predict the effects of pollutants at the ecosystem level. Therefore, bioassays increasingly are used to determine the contamination fraction that pose a potential biological risk (Kobayashi and Okamura, 2004). In this context, sea urchin embryos and larvae have been widely used in environmental toxicity assessments (e.g. Garman et al., 1997; Vaschencko et al., 1999; Woodworth et al., 1999; Au et al., 2001a, 2001b; Böttger and McClintock, 2002; Pagano et al., 2002; Yaroslavtseva and Sergeeva, 2002; Bielmyer et al., 2005; Bellas et al., 2008; Geffard et al., 2009; Caeiro et al., 2009).

Organizations like the United States Environmental Protection Agency (USEPA) developed standardized toxicity evaluation methodologies using *Arbacia punctulata* (USEPA, 2002) and *Strongylocentrotus purpuratus* (USEPA, 1995) embryos and larvae. Other scientific institutions such as the International Council for the Exploration of the Sea (ICES) have recommended the use of sea urchin embryos as bioassay testing models for environmental monitoring programs and toxic effect determination (ICES, 2003).

Embryos and larvae of the sea urchin, *P. lividus* (Lamarck, 1816), have also been widely used in environmental monitoring and assessment (e.g. Ozretic and Ozretic, 1985; Ozretic et al., 1998; His et al., 1999; Marin et al., 2000; Fernández and Beiras, 2001; Radenac et al., 2001; Beiras, 2002; Beiras et al., 2003a, Pesando et al., 2003;

Abbreviations: MF, maize flour; WF, wheat flour; SF, soya flour; WMF, wheat and maize flour mix; MSF, maize and soya flour mix; W, wild; PSU, practical salinity unit; GI, gonadic index; ppm, parts per million; atm, atmosphere; Cys, Cysteine; Asx, asparagine and aspartic acid complex; Glx, glutamine and glutamic acid complex; Ser, Serine; Gly, Glycine; His, Histidine; Arg, Arginine; Thr, Threonine; Ala, Alanine; Pro, Proline; Tyr, Tyrosine; Val, Valine; Met, Methionine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Lys, Lysine

* Corresponding author. Fax: +351 214869720.
E-mail address: biotiago@yahoo.com (T. Repolho).

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Ghirardini et al., 2005; Bellas et al., 2008; Geffard et al., 2009; Caeiro et al., 2009). However, all previous studies using *P. lividus* embryos and larvae were limited to the season when this species is naturally mature. Year-round availability of embryos and larvae for environmental bioassay testing requires maintenance of broodstock under controlled conditions. Artificial feeds are increasingly favored over natural diets in sea urchin husbandry because of predictable availability, reduction of pathogenic contamination, durability, and the possibility of altering/customizing the biochemical composition (George et al., 2000). Although Luís et al. (2005) achieved year-round spawning with captive *P. lividus*, they attributed the high percent of larvae malformation to deficiencies in nutrients of the tested raw diets. Although culture conditions for some echinoderm species, including *P. lividus*, are well known, little or no attention has been given to their reproductive biochemistry (Fernandez, 1997; Garcia et al., 2000). Maturation stage, as well as gamete size and quality, can be affected by diet. Embryonic and larval development and viability are strongly dependent on nutritive reserve accumulation during oocyte development (Brooks and Wessel, 2003). Likewise, survival and percent larval development are determined directly and indirectly by gonad and larval nutritional status (Meidel et al., 1999). To promote high quality gamete production, nutritionally balanced artificial diets must be evaluated. It is imperative that these diets do not bias percent egg fertilization and normal larval development because these attributes are used as quantitative and qualitative measures in environmental monitoring studies. The development of balanced diets that promote gamete quality, as well as normal larval and juvenile development, is essential to maintain the reliability of using sea urchin fertilization and development in environmental bio-monitoring studies (Warnau et al., 1995; Fichet et al., 1998; Fernández and Beiras, 2001; Radenac et al., 2001; Beiras et al., 2003a, 2003b;Pesando et al., 2003). In this study we evaluated the effects of six artificial diets on *P. lividus* broodstock by comparing gonadic index, whole egg total amino acid composition, percent egg fertilization and 4-armed pluteus malformation. The whole egg total amino acid composition was determined to detect possible deficiencies and potentially explain larval development results. All these parameters were compared with levels of wild broodstock during the reproductive season. The main goal was to develop a dry pellet-based feed that would yield similar values to a natural diet for *P. lividus*. The success of this diet would allow percent egg fertilization and percent normal larval development to be used year-round in bioassay testing.

2. Material and methods

2.1. Collection of adult sea urchins from natural habitat

P. lividus were collected from the wild in February, during out-of-season gametogenesis, (Gago et al., 2003) at Cabo Raso, located westwards from Lisbon metropolitan area (38°42′30″N/9°29′11″W). Absence of water pollution sources and sea urchin population abundance were key factors for the selection of this collection site. According to the values registered on site, a previous salinity and temperature adjustment on the culture system was performed, to avoid osmotic and thermal shock on collected sea urchins. *P. lividus* were collected and transported in thermal cases, completely immersed and with continuous aeration. After collection, sea urchins were randomly allocated to culture tanks.

2.2. Culture system

Maintenance of *P. lividus* under captive conditions was performed in a recirculating aquaculture system (RAS), equipped with mechanical (Glass Wool), physical (Fernando Ribeiro, Protein skimmer) and biological (Fernando Ribeiro, Ouriço®) filtration, UV disinfection (De Bary Aquaristik, AN25), automatic thermoregulation and water level control probes. Water circulation was ensured through magnetic pumps (Iwaki, MD-40 RX). System aeration was performed through an air compressor (Hiblow, 220). Twelve black polyethylene right circular cone tanks (0.03 m^3 in volume and 1.38 m^2 of total submerged area, per tank) were set, with continuous aeration and water renewal (1.2 L min^{-1}). The entire culture system was set on an environmental controlled room. Culture parameters were set taking into account sea urchin culture conditions in captivity described in available references (Table 1). The temperature of 18 °C and photoperiod of 14:10 (L:D) were selected in order to mirror the prevailing conditions of mid-spring, when most *P. lividus* are mature in the field (Gago et al., 2003). During the entire experimental period, constant monitoring of abiotic culture parameters was performed daily and a 10% seawater change on a weekly basis.

Table 1
Culture parameters for *P. lividus* broodstock feeding experiments.

Culture parameters	Values	References
Salinity	35 ± 1 PSU	Radenac et al. (2001)
Temperature	18 ± 0.5 °C	Radenac et al. (2001), Luís et al. (2005)
Photoperiod	L:D, 14 h/10 h	Luís et al. (2005)
Illuminance	800 lux	Luís et al. (2005)
Ammonia, nitrite	< 0.05 ppm	Basuyaux and Mathieu (1999)
Nitrate	< 50 ppm	Basuyaux and Mathieu (1999)
Dissolved O_2	> 8 ppm	–
pH	8.2 ± 0.1	–
Flow rate	1.2 L min^{-1}	–
KH	> 89 ppm	–

On each culture tank, eighteen adult individuals (mean test diameter = 4.82 ± 0.02 cm, mean test height = 1.95 ± 0.03 cm and total mean wet weight = 63.0 ± 0.69 g) were placed at a density of thirteen individuals m^{-2}. Additionally, twenty-two *P. lividus* were stocked in a similar culture tank coupled to the water reservoir of the RAS, for posterior inanition parameter analysis. Acclimatization of individuals to experimental conditions was performed during a six-week time period (February–March), without any feed supply.

2.3. Feed production

Six inert dry diets were selected for testing: whole maize grain Zea mays (M) and five pelleted feeds with vegetable based protein source from maize flour (MF), wheat flour (WF), soya flour (SF), wheat and maize flour mix (MWF), and maize and soya flour mix (MSF). Proximal composition of each flour type was estimated, as well as the proximal composition of each manufactured pelleted feed, according to Crampton and Harris (1969) and through technical datasheets of average nutritional composition. Maize oil was used as surplus lipid source. Binding agent percentage was 4% weight of the dry feed (2% sodium alginate, Sigma Aldrich, ref. A728/180947 and 2% sodium hexametaphosfate, José M. Vaz Pereira, code 946). An additional vitamin mixture (Lab Antral S.A., Plurivit vet) was included on each pelleted feed. All feed ingredients were weighted using a ± 0.001 g precision balance (Sartorius GMBH, 1518-B-MP8-1), mixed and homogenized together using a home kitchen stand mixer (Phillips, HR2970A). Afterwards 1 L aqueous solution containing seawater (35 PSU), as solvent, and the binding agent was preheated on a thermal plate (Corning, PC-351) for 5 min at 100 °C and then added to the ingredient mix. Pellets were manufactured by forcing each dough feed mix through the meat mincer of the stand mixer. Resulting pellets were placed on a forced draft air oven (Memmert, UL50) at 35 °C, during a 72 h time period and cut to final cylindrical size (diameter ≈ 0.8 cm, length ≈ 1 cm). Pellets, thus obtained, were tested concerning consistency under seawater-submerged conditions. All pellets were stored in the refrigerator at 5 °C. The proximal composition range of all dry pellets used on *P. lividus* broodstock nutrition assays is described in Table 2.

Total amino acid composition from maize grain and maize, wheat and soya flowers was analyzed prior to feed elaboration and according to the protocol used in the present study to determine whole egg total amino acid composition (as detailed in Section 2.9.).

2.4. Captive broodstock feeding

After acclimatization period, nutritional assays were performed during a twelve-week period (April–June). Each tested diet was presented to *P. lividus* randomly stocked in two out of twelve culture tanks. Manual feeding was performed three times a week, supplying each individual with one pellet and visually assuring its transport from the aboral to the oral region. Eventual uneaten pellets and all produced excreta were daily removed by siphoning.

2.5. Broodstock biometry and gonadic index (GI)

P. lividus test height, test diameter and total wet weight were registered for all captive sea urchins at the beginning (after inanition period) and at the end of the twelve week feeding captive period. For gonadic index (GI) determination, three sampling times were used as follows: Initially collected from natural habitat ($n = 22$); after six-week inanition period ($n = 22$) and at the end of the feeding captive

Table 2
Proximal composition (%) of all dry pellets used on feeding treatments of *P. lividus* broodstock.

Dry pellets composition	Centesimal composition range
Fiber	[2.70; 2.73]
Minerals	[5.82; 6.22]
Total protein	[8.72; 8.81]
Total lipids	[4.15; 4.18]
Carbohydrates	[73.2; 73.9]
Binding agent	[3.64; 3.96]
Vitamin premix	[0.91; 0.99]

period (twelve sea urchins per each tested diet, i.e., six from each replicate tank). Coinciding with the end of the captive feeding, already during the gametogenesis season, sea urchins ($n=12$) from the wild were collected for GI determination. A ruler (± 0.1 cm) and a compass were used for test height and diameter determination. Total and gonad wet weights were registered through a ± 0.01 g precision balance (Kern & Sohn GmbH, Kern 470). Gonadic index, expressed as percentage, was calculated according to the formula:

$GI = (\text{Gonad wet weight}/\text{total wet weight}) \times 100$

2.6. Spawning induction and egg collection

Spawning induction was performed through a peristomial injection of 0.5 mL KCl, 0.5 M (Leahy, 1986; Triquet et al., 1998; Manzo, 2004), on the remaining twenty-four sea urchins (twelve from each replicate tank) of each feeding treatment. Likewise, at the end of the feeding period (during the gametogenesis season), a sample of twenty-four sea urchins freshly collected from the wild were induced to spawn for posterior comparison with captive *P. lividus* broodstock. Prior to spawning induction, sea urchins were rinsed with seawater, for debris removal. After injection and for gamete collection, sea urchins were placed in 100 mL glass beakers, with the aboral region facing downwards in contact with seawater, for a time period not exceeding 30 min (Leahy, 1986). During this experimental phase, sea urchins were kept moist at all times. Gametes were collected in previously filtered (0.35 μm) and autoclaved seawater. Sperm and egg concentrations were estimated for each individual, through a counting chamber (Marienfeld, Neubaeur improved) observations, under 100× optic microscopy (Leica, ATC 2000). Sperm mobility was also checked under microscopy observation.

For each feeding treatment, eggs sampled from three female spawnings were taken and filtered through a 60 μm nylon mesh. Excess seawater was absorbed through filter paper. Samples were stored in 1.5 mL eppendorf tubes and freezed (−80 °C) for posterior whole egg total amino acid analysis. All the other egg samples corresponding to spawning events were used for *in vitro* fertilization and larval development assays.

2.7. In vitro fertilization

For each *in vitro* fertilization assay, a single male sperm and the three single female egg spawning samples derived from broodstock of the same feeding treatment were used at a 1:3000 egg/sperm ratio (Ozretic et al., 1998), on a 600 mL glass beaker. Similar procedure was used for specimens from the wild, as the control group. After 15 min, a 30 mL sample, from each *in vitro* fertilization replicate was taken and preserved in neutralized formaldehyde (4%), for posterior analysis. Percent egg fertilization was checked under a 100× stereomicroscope (Leica, MZ125) observations, using Sedgewick Rafter chambers (KC, Denmark). Presence and absence of fertilization membranes were counted by image analysis (Leica, TWAIN module for DFC 480) for every 1000 eggs.

2.8. Larval development assays

Whenever percent egg fertilization was above 96%, larval development assays were performed in 2 L plastic beakers. For each fertilization assay, a sample corresponding to a concentration of 1000 embryos L^{-1} was added to each larval development culture beaker. Larval culture was performed during 72 h, in autoclaved and filtered seawater (0.35 μm). Physicochemical parameters were higher than 8 ppm (dissolved oxygen), 35 ± 1 PSU (Salinity), 18 ± 0.5 °C (temperature), L:D 14 h/10 h (photoperiod); 800 lux (illuminance). Water circulation within the culture medium was achieved by light bubble aeration. After larval development assays, a 30 mL volume sample was taken from each replicate of each treatment and preserved in 4% neutralized formaldehyde. A random observation of 30 larvae was performed for each replicate. Normal vs. abnormal larvae development was observed through 100× optical microscopy (Leica, model ATC 2000) on 1 mL Sedgewick Rafter chambers (KC—Denmark). *P. lividus* 4-armed pluteus larvae were considered abnormal when, at least, one of the following characteristics were observed: asynchronous development; arm absence; presence of deformed arms; and morphological asymmetry over the longitudinal axis. Larvae without any of the above mentioned characteristics were classified as normal (Fig. 1). The total length for observed normal larvae was measured by means of a micrometric eye lens and expressed as the distance (μm) between the apical and the posterior lateral arm extremities.

2.9. Whole egg total amino acid composition

Three egg samples obtained from three independent female spawnings for each nutritional treatment plus three egg samples from wild caught sea urchins, comprising twenty-one samples in total, were analyzed for whole egg total amino acid composition. Samples were lyophilized (Edwards, model PIRANI 10) for 72 h, at −40 °C and 2.5 atm pressure (Elnor Motors, model TYPE 35 °C), on eppendorf tubes. Samples were piston minced afterwards and store at 5 °C temperature, prior to analysis. Four replicates of each sample were weighted in glass tubes, containing norleucine internal control. Two replicates were submitted to performic acid oxidation, followed by acid hydrolysis, under vacuum conditions, with HCl 6 N/Phenol 1%, during 24 h, at 110 °C, for cysteine quantification. Remaining replicates ($n=2$) were submitted to acid hydrolysis, under vacuum conditions with HCl 6 N/Phenol 1%, during 24 h, at 110 °C, for the remaining amino acid quantification. Samples were hydrolyzed in inert atmosphere, obtained through vacuum/nitrogen alternate cycles, in a PicoTag workstation (Waters). After hydrolysis, samples were neutralized and derivated with phenylisothiocyanate (PITC). Chromatographic separation (PTH-AA) was performed through HPLC (Alliance, model 2695) using a Waters Nova-Pak® C18 column (60 Å, 4 μm, 3.9 × 15 mm dimension) at 38 °C temperature, with a 254 nm detection (Waters, PDA 2996). Software Empower (Waters) was used for result analysis. Amino acid final concentration was expressed as percentage (%) of the total amino acid mass:

$\text{Amino acid mass percentage} = (\text{mAA} \times 100)/\text{tAA}$

whereas:

- mAA = individual amino acid mass
- tAA = Total amino acid mass for each analyzed sample

During acid hydrolysis, asparagine and aspartic acid, as well as glutamine and glutamic acid, were converted into the same chemical species and accounted as Asx and Glx, respectively. Tryptophan was not quantified since it is destroyed during acid hydrolysis.

2.10. Statistical analysis

Prior to statistical analysis, percentage data (gonadic index, percent egg fertilization and percent normal larvae development) was normalized by means or arcsen transformation. Levene test ($\alpha > 0.05$) was used for data variance homogeneity testing. For all the analyzed variables, since no statistical differences were found between replicate tanks for the same diet, data were pooled. ANOVA analysis was applied to data whereas data normality and variance homogeneity were observed. Tukey test was applied for multiple comparison analysis. Non-parametric analysis (Kruskal–Wallis) was applied, in the first instance, where data normality and/or variance homogeneity were/was not observed, as well as Tukey and Dunn testing, for multiple comparison analysis among different treatments. Statistical analysis was performed using SigmaStat software (Systat Software Inc., Windows version 3.10). For egg amino acid composition, three egg samples were randomly picked from all the samples obtained in the two replicates of each captive feeding treatment.

3. Results

3.1. Biometry and gonadic index (GI)

Mean test height, test diameter and total wet weight for all captive *P. lividus* at the beginning and at the end of the twelve week feeding captive period are shown in Table 3.

An overall increase on total wet weight, test diameter and height was observed for every feeding treatment. Significant differences (*T*-student, $P < 0.05$) were found between the mean values at the beginning and at the end of each experimental trial, among the three variables analyzed.

Mean GI values corresponding to sea urchins initially collected from the wild (initial) and after inanition period (inanition), as well as after the feeding captive period (M: maize grain; MF: maize

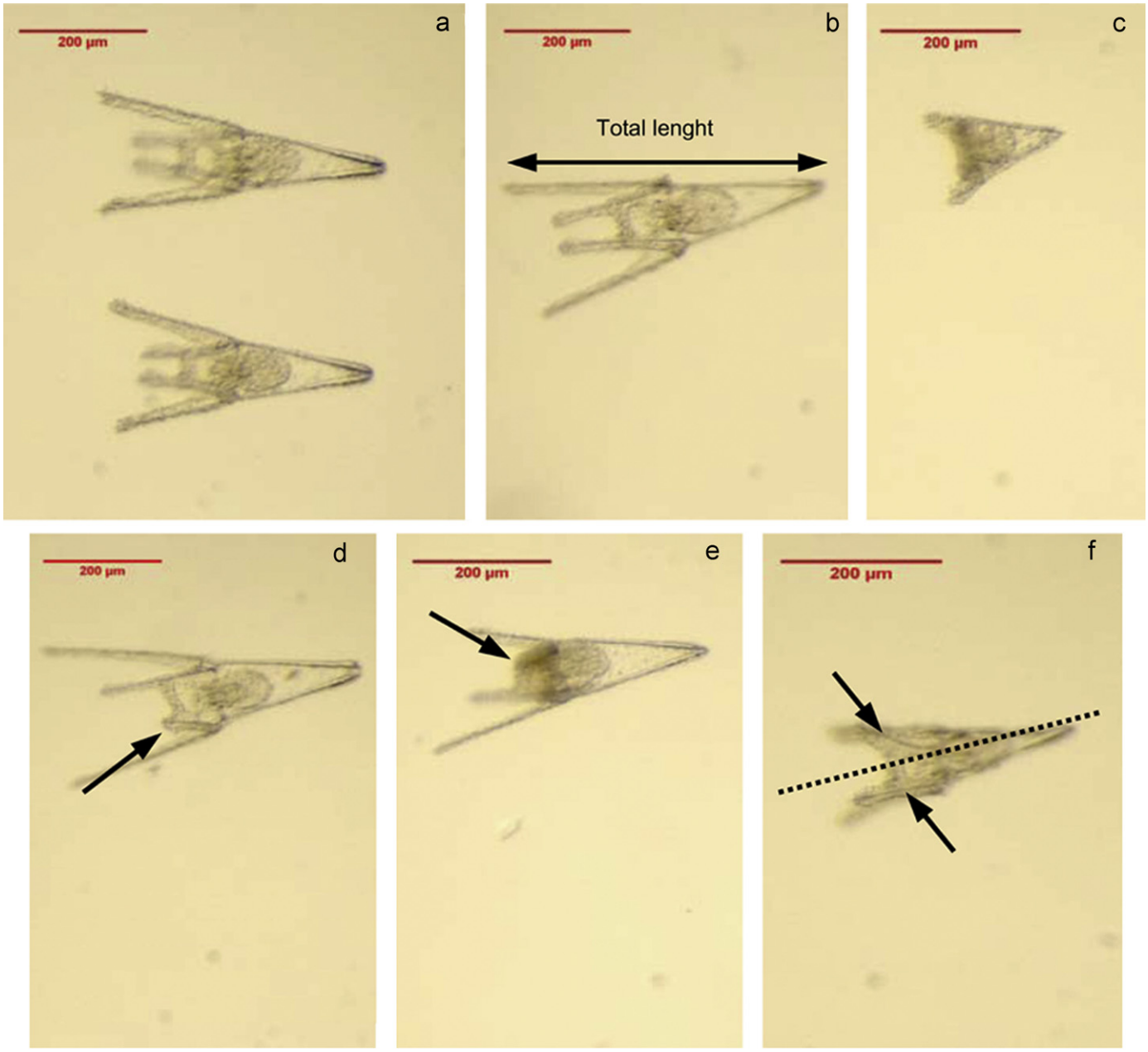

Fig. 1. Pluteus larvae (4-armed) with normal (a,b) and abnormal (c–f) development. (c–e) Absence or presence of deformed arms. (f) Morphological asymmetry over the longitudinal axis.

Table 3
P. lividus mean values for total wet weight (g), test diameter (cm), test height (cm) and associated standard errors at the beginning (after inanition period) and at the end of the twelve-week feeding captive time period. *n* is the number of sampled sea urchins. M: maize grain; MF: maize flour; WF: wheat flour; SF: soya flour; MWF: wheat and maize flour mix; MSF: maize and soya flour mix; W: wild; T: treatments.

T	*n* (initial/end)	Total wet weight ± standard error (initial/end)	Test diameter ± standard error (initial/end)	Test height ± standard error (initial/end)
M	36/36	**59.5** ± 1.67/**61.7** ± 1.65	**4.74** ± 0.06/**4.96** ± 0.06	**1.93** ± 0.02/**2.12** ± 0.03
MF	36/36	**61.3** ± 1.41/**63.4** ± 1.47	**4.76** ± 0.04/**4.95** ± 0.04	**1.97** ± 0.02/**2.05** ± 0.03
WF	36/36	**59.3** ± 1.48/**60.8** ± 1.43	**4.72** ± 0.04/**4.89** ± 0.04	**1.88** ± 0.03/**2.00** ± 0.02
SF	36/36	**70.7** ± 1.43/**73.0** ± 1.36	**5.03** ± 0.04/**5.12** ± 0.04	**2.04** ± 0.02/**2.06** ± 0.03
MWF	36/36	**68.2** ± 1.44/**69.3** ± 1.52	**5.02** ± 0.04/**5.08** ± 0.03	**2.02** ± 0.03/**2.05** ± 0.03
MSF	36/33	**59.2** ± 1.71/**62.1** ± 1.77	**4.67** ± 0.04/**4.85** ± 0.05	**1.86** ± 0.03/**1.99** ± 0.03

flour; WF: wheat flour; SF: soya flour; MWF: wheat and maize flour mix; MSF: maize and soya flour mix) and sea urchins collected from the wild (W) after experimental assays are shown in Fig. 2.

A significant decrease ($P < 0.01$) can be observed between GI values of initial (8.52 ± 0.26%) and inanition (6.49 ± 0.40%) treatments. A significant GI increment ($P < 0.001$) can be observed

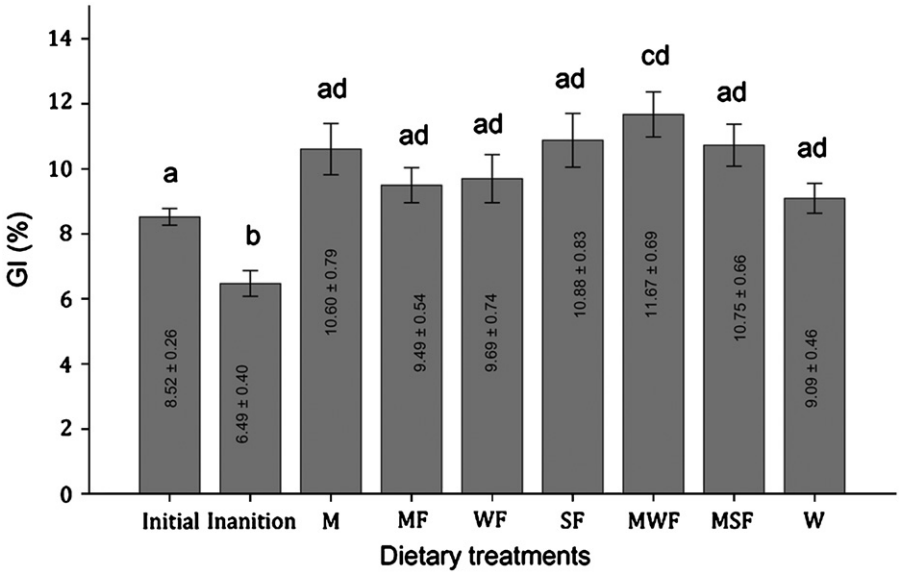

Fig. 2. Mean GI values (%) ± standard error of *P. lividus* broodstock: initially collected from natural habitat ($n=22$), after six-week inanition period ($n=22$) and at the end of the twelve-week feeding captive time period ($n=12$ for each tested diet). M: maize grain; MF: maize flour; WF: wheat flour; SF: soya flour; MWF: wheat and maize flour mix; MSF: maize and soya flour mix; W: wild ($n=12$ *P. lividus* collected from natural habitat coinciding with the end of the captive feeding time period). Different letters placed at the top of the bars indicate statistical differences (one way ANOVA, Tukey test, $P<0.05$) between diet treatments.

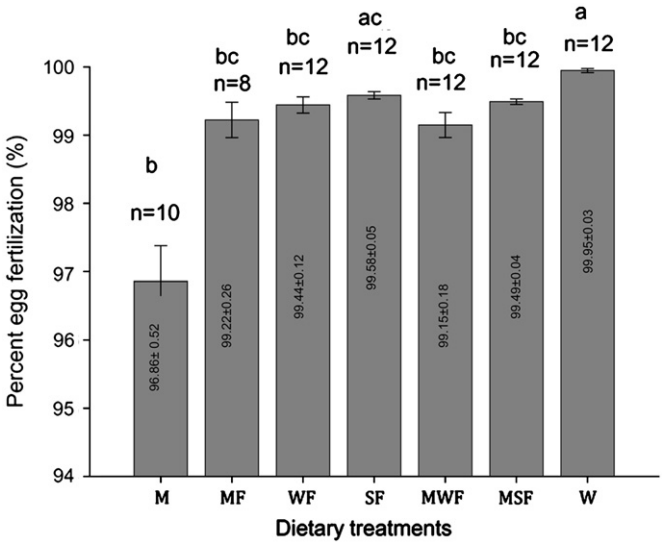

Fig. 3. Mean values for percent egg fertilization (%) ± standard error of *P. lividus*, for feeding treatments and wild caught. M: maize grain; MF: maize flour; WF: wheat flour; SF: soya flour; MWF: wheat and maize flour mix; MSF: maize and soya flour mix; W: wild. Different letters placed at the top of the bars indicate statistical differences (Kruskal–Wallis, Dunn, $P<0.05$) between diet treatments.

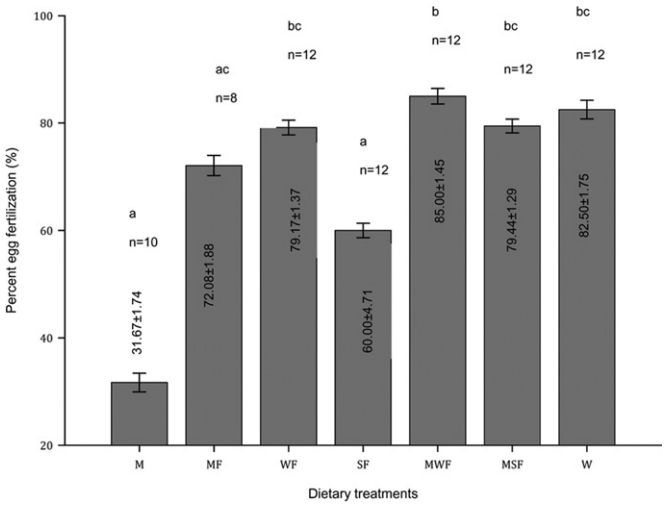

Fig. 4. Mean values for percent normal larval development (%) ± standard error of *P. lividus* for feeding and wild caught sea urchins. M: maize grain; MF: maize flour; WF: wheat flour; SF: soya flour; MWF: wheat and maize flour mix; MSF: maize and soya flour mix; W: wild. Different letters placed at the top of the bars indicate significant differences (Kruskal–Wallis, Dunn, $P<0.05$) between diet treatments.

after inanition period vs. the remaining captive feeding treatments (M, MF, WF, SF, MWF and MSF). Also significant differences ($P<0.001$) were observed between initial and MWF, as well as between inanition and wild caught sea urchins ($P<0.01$).

3.2. Percent egg fertilization

Results of mean values for percent egg fertilization are shown in Fig. 3.

Significant differences ($P<0.05$) of percent egg fertilization values were found between: M vs. SF and W; W vs. MF, WF, MWF and MSF treatments.

3.3. Normal percent larval development

Mean values for normal percent larval development, observed on larvae derived from feeding captive and wild broodstock, are shown in Fig. 4.

Significant differences ($P<0.05$) were observed between: MWF, MSF, WF, W vs. M and SF; MWF vs. MF treatments.

3.4. Normal larval average total length

Data concerning medium total length of normal larvae from feeding captive and wild caught sea urchins are shown in Fig. 5.

Significant differences ($P < 0.05$), between all treatments, were observed, with the exception for MWF vs. MSF and MF vs. W.

3.5. Whole egg total amino acid composition

Mass percentage mean values of each amino acid found in *P. lividus* eggs are shown in Table 4.

Statistical analysis results concerning amino acid mass percentage from eggs derived from feeding captive and wild caught sea urchins are shown in Table 5.

Significant differences ($P < 0.05$) between treatments were observed: MWF and remaining treatments, with the exception for W dietary treatment; W vs. WF and MSF, whereas proline and Pro/Asx complex are concerned.

4. Discussion

4.1. Gonadic index

Sea urchin gonads function as both reproductive organs and nutritive reserve storage (Walker, 1982). During winter, wild *P. lividus* gonads from Cabo Raso (Portugal) are in the phase of reserve accumulation in the form of nutritive phagocytes prior to gamete differentiation and spawning that occur during spring and summer (Gago et al., 2003).

Comparing GI values of wild collected sea urchins with captive held after inanition, a significant decrease was obtained. This decline must be related with the utilization of the gonad nutrients and energy, by starved sea urchins, to assure metabolic vital functions.

Based on GI and histological analysis, Spirlet et al. (2000) referred two months of starvation period, as the time period required for captive *P. lividus* population to be at the same reproductive status. The six-week inanition period used in the present study for *P. lividus* was intended to obtain captive *P. lividus* in the same reproductive status prior to use them in nutrition trials. However the GI values obtained do not allow major conclusions about the reproductive synchronization of experimental sea urchins. On the other hand, considering all treatments, including wild, significant increases were obtained on GI, between inanition and feeding treatments at the end of the trials. This result indicates that all the feed provided was well accepted and was utilized for both somatic and gonadal growth.

When comparing captive and wild sea urchins, GI values were always higher for captive sea urchins, irrespective of the feeding treatment. This result may be related with the quality and availability of nutrients provided by artificial diets, when compared with the natural habitat feed sources. According to Fernandez and

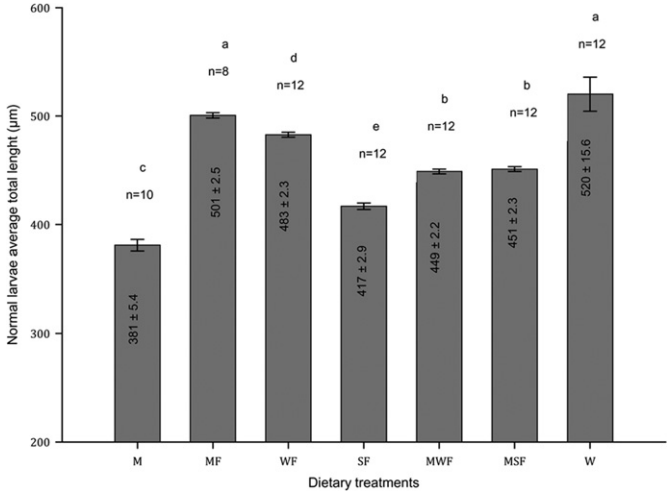

Fig. 5. Average total length of normal larvae (µm) ± standard error of *P. lividus* for feeding and wild caught sea urchins. M: maize grain; MF: maize flour; WF: wheat flour; SF: soya flour; MWF: wheat and maize flour mix; MSF: maize and soya flour mix; W: wild. Different letters placed at the top of the bars indicate statistical differences (Kruskal–Wallis, Dunn, $P < 0.05$) between diet treatments.

Table 5
Statistical analysis results concerning amino acid mass percentage for eggs derived from captive and wild caught sea urchins of *P. lividus* broodstock. Significant differences ($P < 0.05$) between treatments is shown through the presentation of amino acid abbreviators within the table. Non-significant statistical differences are denoted by M: maize grain; MF: maize flour; WF: wheat flour; SF: soya flour; WMF: wheat and maize flour mix; MSF: maize and soya flour mix; W: wild.

	M	MF	WF	SF	MWF	MSF	*W*
M	–	–	–	–	Leu, Ile, Val, Met	–	–
MF	–	–	–	–	Leu, Ile	–	–
WF	–	–	–	–	Asx, Pro	–	Pro
SF	–	–	–	–	Val	–	–
MWF	–	–	–	–	–	Leu, Ile, Met, Asx, Pro	–
MSF	–	–	–	–	–	–	Asx, Pro
W	–	–	–	–	–	–	–

Table 4
Mass percentage mean values of each amino acid (%) ± standard error for eggs derived from captive and wild caught *P. lividus* ($n = 3$). Cys, Cysteine; Asx, asparagine and aspartic acid complex; Glx, glutamine and glutamic acid complex; Ser, Serine; Gly, Glycine; His, Histidine; Arg, Arginine; Thr, Threonine; Ala, Alanine; Pro, Proline; Tyr, Tyrosine; Val, valine; Met, Methionine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Lys, Lysine; M: maize grain; MF: maize flour; WF: wheat flour; SF: soya flour; WMF: wheat and maize flour mix; MSF: maize and soya flour mix; W: wild.

	M	MF	WF	SF	MWF	MSF	W
Cys	**2.0** ± 0.03	**2.1** ± 0.02	**2.1** ± 0.07	**2.2** ± 0.36	**1.9** ± 0.05	**1.9** ± 0.05	**1.7** ± 0.13
Asx	**8.4** ± 0.34	**8.5** ± 0.32	**8.9** ± 0.39	**6.1** ± 0.72	**5.6** ± 0.95	**9.3** ± 0.18	**5.9** ± 1.06
Glx	**11.0** ± 0.24	**11.2** ± 0.36	**10.6** ± 0.18	**10.4** ± 0.27	**9.8** ± 0.49	**11.0** ± 0.03	**9.9** ± 0.81
Ser	**5.6** ± 0.06	**5.6** ± 0.26	**5.6** ± 0.07	**5.4** ± 0.36	**5.3** ± 0.14	**5.6** ± 0.12	**5.3** ± 0.08
Gly	**21.7** ± 0.33	**21.1** ± 0.71	**20.7** ± 0.56	**23.1** ± 0.72	**22.1** ± 0.70	**22.1** ± 1.17	**22.9** ± 0.28
His	**2.4** ± 0.01	**2.3** ± 0.02	**2.4** ± 0.01	**2.3** ± 0.06	**2.4** ± 0.07	**2.3** ± 0.03	**2.3** ± 0.07
Arg	**5.5** ± 0.13	**5.5** ± 0.08	**5.7** ± 0.06	**5.6** ± 0.02	**5.8** ± 0.13	**5.6** ± 0.10	**5.6** ± 0.09
Thr	**5.1** ± 0.03	**5.0** ± 0.06	**5.0** ± 0.08	**5.1** ± 0.01	**5.3** ± 0.18	**5.1** ± 0.04	**5.3** ± 0.09
Ala	**4.8** ± 0.05	**5.0** ± 0.28	**4.9** ± 0.06	**4.6** ± 0.32	**4.9** ± 0.26	**4.8** ± 0.18	**4.5** ± 0.09
Pro	**3.8** ± 0.02	**3.7** ± 0.05	**3.7** ± 0.03	**3.9** ± 0.11	**4.1** ± 0.01	**3.7** ± 0.01	**4.0** ± 0.08
Tyr	**3.4** ± 0.26	**3.5** ± 0.15	**3.6** ± 0.05	**3.2** ± 0.06	**3.5** ± 0.06	**3.5** ± 0.15	**3.6** ± 0.09
Val	**5.1** ± 0.05	**5.2** ± 0.05	**5.3** ± 0.01	**5.1** ± 0.04	**5.5** ± 0.12	**5.2** ± 0.06	**5.4** ± 0.10
Met	**2.4** ± 0.04	**2.4** ± 0.02	**2.5** ± 0.06	**2.5** ± 0.03	**2.6** ± 0.02	**2.4** ± 0.03	**2.5** ± 0.04
Ile	**4.1** ± 0.08	**4.2** ± 0.10	**4.3** ± 0.05	**4.4** ± 0.04	**4.7** ± 0.12	**4.1** ± 0.11	**4.6** ± 0.19
Leu	**6.2** ± 0.09	**6.2** ± 0.13	**6.4** ± 0.10	**6.6** ± 0.14	**7.0** ± 0.19	**6.2** ± 0.09	**6.9** ± 0.28
Phe	**3.2** ± 0.12	**3.1** ± 0.12	**3.4** ± 0.10	**3.4** ± 0.10	**3.6** ± 0.12	**3.1** ± 0.06	**3.5** ± 0.22
Lys	**5.5** ± 0.19	**5.5** ± 0.21	**5.8** ± 0.24	**6.4** ± 0.70	**5.9** ± 0.20	**5.3** ± 0.18	**6.1** ± 0.52

Boudouresque (2000) marine macrophytes biochemistry may vary along the year.

In other studies with captive *P. lividus* (e.g. Fernandez and Boudouresque, 2000, Shpigel et al., 2005) higher GI values, compared to the ones determined in the present study, were obtained. These differences may be related with different factors and/or experimental procedures employed, such as diet formulation, feeding frequency and trial duration period.

4.2. Percent egg fertilization

Percent egg fertilization ranged from $96.96 \pm 0.52\%$ (maize grain treatment) to $99.95 \pm 0.03\%$ (wild treatment). Significant differences occurred between W dietary treatment and all the other feeding treatments. The lowest percent egg fertilization obtained with M diet suggests that oocytes were not all fully mature at the time of spawning induction, and therefore immature gametes could have been released.

Higher percent egg fertilization obtained for wild compared with captive *P. lividus* may be due to sea urchin population reproductive intraspecific variability as well as with experimental factors such as differences in abiotic factors between wild and captivity. However, despite those differences, whatever the captive feeding treatment considered, the percent egg fertilization obtained was always higher than the values (70–90%) required by the USEPA (2002) in regard to bioassays validation and are also concordant with percent egg fertilization values obtained in other studies that used wild (His et al., 1999; Radenac et al., 2001; Beiras et al., 2003a) or captive sea urchins (George et al., 2000).

4.3. Larval development and whole egg total amino acid composition

Few studies related the reproductive biology of *P. lividus* with dietary biochemistry and are generally restricted to the biochemical composition analysis of certain organs such as gonads (Fernandez, 1997, 1998; Garcia et al., 2000; Schlosser et al., 2005). Moreover, little or no attention has been given to *P. lividus* egg biochemical composition and its implications on larval development of this species.

George et al. (2000) obtained similar values for *Lytechinus variegatus* normal larval development whenever broodstock was fed an artificial diet in captivity or when broodstock was caught in natural environments. Contrarily, the results found in this study indicate higher normal larval development for wild *P. lividus* ($82.5 \pm 1.75\%$), except when compared with captive *P. lividus* that were fed the MWF feeding treatment ($85.0 \pm 1.45\%$).

Considering the average length of normal larvae, significant differences were found among all feeding treatments, except when comparing MWF with MSF and MF with W dietary treatment. Highest normal larval length (520 ± 15.6 μm) was obtained with W dietary treatment, while the lowest values were found for larvae derived from broodstock fed the maize grains feeding treatment (381 ± 5.4 μm).

Considering the amino acid biochemical analysis performed in this study, the weight percentage values for some amino acids had statistical differences between MWF with all the other feeding treatments, except for eggs obtained from wild *P. lividus*. For the amino acids analyzed that had statistical differences between treatments, the values obtained for MWF were those closer to the results for W dietary treatment. Additionally, for the amino acids Met, Leu, Ile, Pro and Val, the values obtained in eggs from the MWF treatment were higher than both the eggs from the other captive feeding treatments and the eggs from wild sea urchins (W). Taking into account these differences obtained in egg amino acid composition among treatments, they might indicate that these amino acids, which presented significant differences, can influence *P. lividus* normal larval development.

Glycine was the amino acid most abundant in *P. lividus* eggs obtained from broodstock fed all the tested diets (23.1–21.1%). This same situation was already found by Liyana-Pathirana et al. (2002) for *S. droebachiensis*.

Considering the feeding treatments, whole maize diet presents several advantages like its low cost, annual availability, stability in water and storage compared to other artificial or natural diets such as macroalgae (Basuyaux and Blin, 1998). However, maize is devoid of vitamins B12 and C as well as on the amino acids tryptophan and cysteine (Crampton and Harris, 1969), but the absence of tryptophan in maize is not concordant with OECD (2002). Similarly, according to Crampton and Harris (1969), wheat and soybean are devoid of amino acids such as alanine, aspartic acid, glutamic acid, proline and serine. Contrarily, according to OECD (2003), not only the wheat has all the amino acids mentioned above, as well as is referred for soybean deficiencies in the amino acids asparagine, alanine, aspartic acid, glutamic acid, glycine, glutamine, proline, serine, tyrosine and tryptophan. Despite this information on divergence among authors and institutions, the above referred absence of certain amino acids was not found in the present study (except for tryptophan that was not determined) in the diets based on maize grain, maize, wheat and soybean flour, namely: cysteine for maize, alanine, aspartic acid, glutamic acid, proline and serine for wheat flour and asparagine, alanine, aspartic acid, glutamic acid, glycine, glutamine, proline, serine and tyrosine for soybean flour.

The use of marine macrophytes as captive *P. lividus* diet is problematical due to their temporal and spatial inherent biochemical composition variabilities (Fernandez and Boudouresque, 2000). Therefore, differences observed in the normal larval development and *P. lividus* egg total amino acid composition among captive feeding treatments and the ones obtained from wild sea urchins may be related to differences in the quality, biochemical content and availability of the feed provided.

The low tryptophan concentration both in maize grain and maize flour might explain the differences observed in normal larval development between the feeding treatments based on these diets compared with those that used mixed protein sources of plant origin like the MSF and MWF feeding treatments.

The vitamin and mineral enrichments used in the experimental diets formulation may likewise explain the existence of significant differences between larval development among the maize grain (M) feeding treatment and all the others, particularly when considering the differences among the corn grain and MF treatments. For instance, ascorbic acid plays an important physiological function and influences both growth and development in invertebrates (Cahu et al., 2003).

On the other hand, the values obtained for normal larval development with the SF feeding treatment may be related to the presence of trypsin inhibitors in soybean meal, which interfere in protein digestion and individual growth (OECD, 2001). Similarly, the phytoestrogens present in soybean (OECD, 2001), which may be implicated in the maturation and reproductive capacity of *P. lividus* broodstock, could explain the poor values obtained for normal larval development and larval total length with the SF and MSF feeding treatments.

Results of spawnings and subsequent larval development reported on the present paper were obtained for both broodstock (captive and wild) only during the gametogenesis season for the convenience of comparison. However, it seems possible to extend the production of normal embryos and endotrophic larvae to year round availability (including out-of-season gametogenesis) as shown by Luís et al. (2005) when inert feeds are provided to captive sea urchin broodstock. As long as an appropriate mixture of cereal flours is selected for the manufacture of artificial diets, sea

urchin percent egg fertilization and endotrophic larvae malformations can be reduced to normal wild percents.

5. Conclusions

Considering the results obtained in the present study, it may be concluded that under controlled laboratory conditions, a high percent egg fertilization ($>99\%$) and a low percent larval malformation ($\leq 15\%$) can be achieved when *P. lividus* broodstock kept in captivity is fed with a prepared inert diet based on maize and wheat flour mixture. Therefore the quantitative and qualitative year round production of gametes is possible, thus further allowing the use of embryos and endotrophic larvae as models in ecotoxicological assessment but free from environmental and seasonal constraints.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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CHAPTER 2.2

Fatty acid nutritional quality of sea urchin *Paracentrotus lividus* (Lamarck 1816) eggs and endotrophic larvae: relevance for feeding of marine larval fish

João Gago, Orlando Luís and Tiago Repolho (2009)

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Survival and growth of selected marine fish larvae first fed with eggs and endotrophic larvae of the sea urchin *Paracentrotus lividus*

João Gago[1], Tiago Martins[1], Orlando J Luís[1] & Pedro Pousão-Ferreira[2]

[1]Laboratório Marítimo da Guia, Centro de Oceanografia, Faculdade de Ciências, Universidade de Lisboa, Cascais, Portugal
[2]Instituto Nacional dos Recursos Biológicos I.P. (INRB I.P. – IPIMAR),Olhão, Portugal

Correspondence: J Gago, Laboratório Marítimo da Guia, Centro de Oceanografia, Faculdade de Ciências, Avenida Nossa Senhora do Cabo 939, 2750-354 Cascais, Portugal. E-mail: joaolontra@hotmail.com

Abstract

Two sets of experiments were carried out to evaluate the potential of eggs and endotrophic larvae of captive *Paracentrotus lividus* as alternative live prey for marine fish larvae first feeding. The first consisted in rearing sparids, *Diplodus sargus* and *Sparus aurata*, larvae until 15 days after hatching in a recirculation system. Compared with the commonly used live prey – rotifer *Brachionus* spp. – general lower values of survival and growth were obtained when fish larvae were fed with the alternative live prey. Among these, eggs showed to be the preferred feeding. Broodstock feed showed to play a fundamental role on prey quality and consequent fish larvae survival. In the second set of experiments, the 24-h ingestions of the first feeding larvae in static water were determined for five currently cultured fish larvae species. Except for larger and more predatory *Dicentrarchus labrax* larvae, there was a trend for higher *P. lividus* egg ingestion, followed by pre-plutei and prisms. Prey size, colour and movement affected food selection by fish larvae. It is concluded that, in spite of the alternative live prey being readily consumed by all tested fish larvae, they cannot however presently compete with rotifers in marine fish larvae first feeding.

Keywords: *Paracentrotus lividus*, eggs, endotrophic larvae, marine fish larvae, survival, growth, ingestion rates

Introduction

It is not yet clear which specific organism(s) are preyed upon by a marine fish larva when they start exogenous feeding, although it is found that larvae of marine invertebrates and other small zooplankters are generally exploited (Hunter 1981). In this context, several ecological studies have demonstrated the importance of echinoplutei in the composition and biomass of zooplankton communities (e.g. Rassoulzadegan & Fenaux 1979; Fransz, Miquel & Gonzalez 1984; Lindley, Gamble & Hunt 1995; López, Turon, Montero, Palacín, Duarte & Tarjuelo 1998). Moreover, McEdward and Miner (2001), based on field and laboratory studies, suggested that predation must be the major cause of echinoid larval mortality. Therefore, these facts suggest echinoid larval consumption by marine fish larvae. Consequently, it appears that if echinoplutei are natural prey, they can also be used in marine larviculture as live feed. In this perspective, the potential use of early developmental stages of *Lytechinus variegatus* as larval food was already suggested by Hubbard, Wolcott and Baca (2003), and the same utilization was preconized by Luis, Delgado and Gago (2005) and Gago, Repolho and Luis (2009) for *Paracentrotus lividus* eggs and endotrophic larvae. The body size of these larval stages suggests that this organism could be appropriate to feed early larvae of marine fish.

Several criteria were already analysed to determine the suitability of *P. lividus* eggs and endotrophic larvae as a mass-produced source of zooplankton to feed marine fish larvae. Besides having a short period of embryonic development (Fenaux, Cellario & Etienne 1985), Luis *et al.* (2005) reported consistent large year-round spawnings of captive *P. lividus* and Gago *et al.* (2009) enhanced their eggs and endotrophic larvae nutritional quality with essential fatty acids (EFA)

for fish larvae. But to fully determine the potential of these alternative live preys in larviculture, it is necessary to evaluate the ingestion, survival and growth performance outcomes of marine fish larvae reared with these live feeds. Therefore, several chronological experiments were performed in this study to analyse these parameters for the first-feeding period of marine fish larvae. Several fish larvae species had to be selected because laboratory sea urchin larvae mortality changes not only with larval size or age but also with the type of predator (Rumrill 1990; Allen 2008). Primarily, gilthead seabream (*Sparus aurata* L.) and white seabream (*Diplodus sargus* L.) larvae were reared until 15 days after hatching (DAH) with different live preys. These two species were chosen because *S. aurata* continues to be a majorly produced species in southern Europe and *D. sargus* is considered to be a promising new species with a high market price and demand (Pousão-Ferreira, Dores, Morais & Narciso 2001; Ozorio, Valente, Pousão-Ferreira & Oliva-Teles 2006). Additionally, in order to clarify more precisely general prey acceptability, 24-h ingestion rates were also evaluated for *S. aurata*, *D. sargus* and other commercial important marine fish larvae (*Diplodus vulgaris* G.S.H., *Solea senegalensis* K. and *Dicentrarchus labrax* L.) fed on *P. lividus* eggs and endotrophic larvae. These last ingestion experiments also contribute to discuss the *P. lividus* eggs and endotrophic larvae potential as live feed for marine fish larviculture on a more enlarged basis.

Material and methods

P. lividus broodstock rearing

Sea urchin collection occurred in September from 2007 to 2009 period, on the central west coast of Portugal near Cascais (Lisbon). After collection, sea urchins were transported to the 'Guia Marine Laboratory' and placed in a rearing system, as described by Luis *et al.* (2005) with the same physical–chemical conditions, for at least a 5-month period. This period is considered to be far enough to complete *P. lividus* gametogenesis (Fernandez, Dombrowski & Caltagirone 1996; Luis *et al.* 2005). After this broodstock conditioning period, fully matured sea urchins were transported in aerated seawater-filled containers to the Aquaculture Research Station of IPIMAR (300 km away), in Olhão, South of Portugal, where fish larvae experiments occurred.

For the first fish larvae experiment (Experiment 1 – sea urchin prey from broodstock on raw feed), sea urchins were fed twice a week with commercial whole yellow grains of maize *Zea mays*. This raw diet was chosen because grains of maize are a practical diet (Basuyaux & Blin 1998) and lead to consistent large year-round spawnings (Luis *et al.* 2005).

For all the other fish larvae experiments, sea urchins were fed with a prepared diet that had cod liver oil as the main lipid source, as described by Gago *et al.* (2009). This prepared diet was chosen because the resultant *P. lividus* eggs and endotrophic larvae are enriched with EFA for marine fish larvae (Gago *et al.* 2009).

Production of *P. lividus* eggs and endotrophic larvae

Whenever sea urchins eggs and endotrophic larvae were needed to feed fish larvae, randomly selected *P. lividus* broodstock was induced to spawn by injecting 1 mL KCl 0.5 M through the peristomial membrane. Each sea urchin was then placed for 30 min in an individual plastic beaker filled with 2 L of aerated filtered sea water. Sperm or egg release was evaluated macroscopically. To obtain endotrophic larvae, 50 mL of sperm was used to fertilize the 2 L egg volume during 30 min after which this volume was filtered using a 30 μm mesh in order to remove sperm excess. Besides eggs ($\sim$ 90 μm in diameter), two endotrophic stages were used: swimming prism (24 h after fertilization: $\sim$ 120 μm in diameter) and prepluteus (48 h after fertilization: $\sim$ 250 μm in length), which were reared in 7 L cylindro conical plastic beakers. The precise number of *P. lividus* eggs and endotrophic larvae provided to feed fish larvae was estimated by 1 mL counts of the rearing volume, using a glass pipette observed through a binocular lens.

Fish larvae experiments

Two approaches were used to test the general viability of the use of *P. lividus* eggs and endotrophic larvae as first feeding of marine fish larvae. The first consisted of rearing sparids *D. sargus* and *S. aurata* larvae until 15 DAH. The second approach consisted in testing larvae's 24 h ingestion rates for five different fish species. Thus, for both approaches, four different live feed items were used: *Brachionus* spp. (the standard live feed: lorica diameter of 167 $\pm$ 3.83 μm, mean $\pm$ SEM, $n = 50$) and *P. lividus* eggs, prims and pre-plutei (the alternative live prey under analysis).

Fish eggs of all the species studied were obtained from captive fish broodstock natural spawnings.

Rearing of D. sargus and S. aurata larvae

After collecting from the egg incubators, newly hatched larvae were transferred to 20 L cylindro conical white fibreglass tanks at a density of 100 larvae L^{-1}. The system was operated in an open circuit, and before entering the tanks water passed through a cartridge filter (50 μm) and was sterilized by UV. Rearing water was maintained at 18.0 ± 0.8 °C and at a salinity of 35.6 ± 0.9 g L^{-1}. Constant slight aeration was provided assuring that oxygen was always above 80% saturation. Water flow was 0.6 L min^{-1} and the photoperiod was 14 h light: 10 h dark. Larvae were fed from mouth opening (∼ 3 DAH) to 15 DAH, and during this period a mixture of microalgae (*Nanocloropsis oculata* and *Isochrysis galbana*) was added two times per day to the rearing tanks in order to obtain a total concentration of approximately 150×10^3 *N. oculata* cells mL^{-1} and 50×10^3 *I. galbana* cells mL^{-1}.

Feeding schemes used in the rearing of D. sargus and S. aurata larvae

Four feeding experiments were chronologically performed where fish larvae were fed different live food items (Table 1). Only in the first experiment (Experiment 1 – sea urchin prey from broodstock on raw feed) *P. lividus* eggs, prisms and pre-plutei were obtained from the broodstock fed maize. These prey lead to null fish larvae survival at 15 DAH (see 'Results'), and as a result two hypotheses were formulated: the fish larvae did not ingest the alternative prey or these prey are nutritional poor. Therefore, ingestion rates were evaluated (see 'First-feeding fish larvae ingestion rates') and other sets of experiments were

Table 1 Feeding schemes used in the rearing of *Diplodus sargus* and *Sparus aurata* for the four fish larvae experiments performed

Experiment	Duration	Fish species	***Paracentrotus lividus* broods tock diet**	Feeding treatment	Feeding distribution method
1 Sea urchin prey from broodstock on raw feed	From 0 to 15 DAH	*D. sargus* and *S. aurata*	Grains of maize	1 – *Brachionus* spp 2 – *P. lividus* eggs 3 – *P. lividus* prisms	5 preys mL^{-1}, two times a day (total of 10 000 preys L^{-1} day^{-1})
2 Sea urchin prey from broodstock on prepared feed		*D. sargus* and *S. aurata*	Prepared diet with cod liver oil	4 – *P. lividus* pre-plutei 5 – Starvation	
3 Sea urchin eggs from broodstock on prepared feed – feeding frequency and combination		*D. sargus* and *S. aurata*		1 – *Brachionus* spp 2 – *P. lividus* eggs (2T) 3 – *P. lividus* eggs (3T) 4 – *P. lividus* eggs (PP) 5 – Co-feeding of *P. lividus* eggs and *Brachionus* spp (CF) 6 – Starvation	Feeding treatment 1 and 2: 5 preys mL^{-1}, two times a day (2T) (total of 10 000 preys L^{-1} day^{-1}) Feeding treatment 3: 5 preys mL^{-1}, three times a day (3T) (total of 15 000 eggs L^{-1} day^{-1}) Feeding treatment 4: 200 000 eggs day^{-1} given continuously as shown in Fig. 1 (PP) Feeding treatment 5: 5 preys mL^{-1} (CF 1:1), two times a day (CF) (total of 5000 *Brachiounus* spp. and 5000 eggs L^{-1} day^{-1})
4 Sea urchin prey from broodstock on prepared feed presented only at 8 DAH	From 8 to 15 DAH	*D. sargus*		1 – *Brachionus* spp 2 – *P. lividus* eggs 3 – *P. lividus* prisms 4 – *P. lividus* pre-plutei 5 – Starvation	5 preys mL^{-1}, two times a day (total of 10 000 preys L^{-1} day^{-1}) Until the eighth DAH *D. sargus* larvae were fed *Brachionus* spp.

3T, three times a day treatment; DAH, days after hatching; PP, peristaltic pump; CF, co-feeding.

performed in the rearing of *D. sargus* and *S. aurata* larvae. In the second experiment (Experiment 2 – sea urchin prey from broodstock on prepared feed) and in the subsequent ones, *P. lividus* broodstock were fed the enhanced prepared diet. In these first two experiments, fish larvae were fed only one of the live preys that were tested independently. As *P. lividus* eggs demonstrated to be the best alternative live prey (see 'Results'), a third experiment (Experiment 3 – sea urchin eggs from broodstock on prepared feed – feeding frequency and combination) was carried out where *P. lividus* eggs obtained from the broodstock fed the prepared diet were given to fish larvae in different combinations and frequencies (Table 1 and Fig. 1). In order to test if the low survival of fish larvae obtained with the alternative live prey in previous trials (see 'Results') was only due to the vulnerability of young first feeding larvae, a fourth experiment (Experiment 4 –sea urchin prey from broodstock on prepared feed presented only at 8 DAH) was also carried out. All *D. sargus* larvae were first fed only with *Brachionus* spp. and then the potential live preys (*P. lividus* eggs, prisms and pre-plutei obtained from the broodstock fed the prepared diet) were presented since the eighth DAH.

For all the experiments, *Brachionus* spp. was used as the larvae feed comparative treatment. *Brachionus* spp. were enriched for 24 h with docosahexanoic acid (DHA) Protein Selco (INVE Aquaculture, Belgium), and for all food items each feeding was provided at a five prey per millilitre ratio. Additionally, a starvation treatment was used in all experiments as control. All diets including the starvation treatment were tested in triplicate tanks, and at the end of the rearing period fish larvae mean survival and growth were estimated. Survival was calculated as the ratio of the total number of surviving fish larvae divided by the numbers stocked, and expressed as percentage. Larval growth was estimated as the total length increase from hatching to 15 DAH. Samples of 20 fish larvae per tank were used for the length measurements.

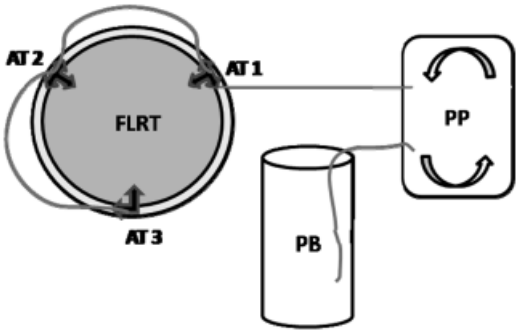

Figure 1 Schematic functioning of Feeding treatment 4 used in Experiment 3: 200 000 *Paracentrotus lividus* eggs were stocked in an aerated 7 L plastic beaker (PB) and pulled by a peristaltic pump (PP) through a capillary (3 mm internal diameter) and then distributed in the fish larvae rearing tank (FLRT) by 3 aquarium taps (AT 1, 2 and 3) placed equidistantly in the top border of the FLRT. PP flow rate was always 500 mL h^{-1}.

First-feeding fish larvae ingestion rates

Fish larvae's 24 h ingestion rates were determined for five different commercially important fish species: *D. sargus*, *D. vulgaris*, *S. aurata*, *S. senegalensis* and *D. labrax*. This evaluation was performed to better assess the general sea urchin prey acceptability by marine fish larvae and also to possibly link ingestion information with *S. aurata* and *D. sargus* survival and growth data determined previously. Gut analysis was tested previously but great difficulty was encountered to either determine the type and number of prey. Only with the rotifer diet, the mastax was sometimes observed in the gut of fish larvae.

Ten fish larvae with an age of 2 days after mouth opening were placed in a transparent glass beaker with 100 mL filtered sea water. This larval age was chosen to ensure prey ingestion by fish larvae. For instance, according to Yúfera, Polo and Pascual (1993), *S. aurata* larvae do not start to eat until 4 or 5 DAH. No aeration was provided to the beakers. Four to five preys per mL were then provided and the beakers were placed in an acclimated room with 19 °C temperature and a 14 h light: 10 h dark photoperiod for a 24-h period. After this period, the water in the beakers was filtered through a 500 μm mesh to remove the fish larvae and then this volume was filtered again through a 30 μm mesh to retain the remaining live prey. The mesh was then washed in a beaker and all this volume was scrutinized to count all the remaining live prey. Counts finished when all the volume in the beaker was observed with a binocular lens using a glass pipette. This number was then subtracted to the initial quantity of live prey given to fish larvae in order to obtain the ingestion rates, expressed as the number of prey ingested by fish larva per hour. Four types of live prey were tested for all fish species: *Brachionus* spp., *P. lividus* eggs, prisms and pre-plutei. A treatment with just ten fish larvae and no prey in the beaker was used to test fish larvae mortality, and another one just with a precise number of preys was also used to verify if the prey did

not disrupt during the 24-h period. All of these treatments were tested in triplicate. Just for *S. aurata* and *D. sargus*, an additional similar experiment was performed with 8 DAH larvae and special care was taken to avoid the recollection of *Brachionus* spp. to the beaker volume.

Statistical analysis

The 'STATISTICA 8 for Windows' software package was used for statistical analyses. The Levene statistic was used to test for homogeneity of variances for all data. Data with homogeneous variances were analysed using one-way ANOVA with Tukey's multiple comparisons to determine differences among independent factors. Data with heterogeneous variances were analysed using Kruskal–Wallis statistic followed by multiple comparisons of mean ranks for all groups (Sokal & Rohlf 1995; Zar 1999). The significance level used was $P < 0.05$. Fish larvae survival was arcsine transformed to normalize data before statistical analyses.

Results

Rearing of *D. sargus* and *S. aurata* larvae

In Experiment 1 (sea urchin prey from broodstock on raw feed), all the larvae of *D. sargus* and *S. aurata* died except when using the *Brachionus* spp. feeding treatment (mean survival at 15 DAH of 36.2% and 31.2% for *D. sargus* and *S. aurata* respectively). For the other feeding treatments, there was a difference in the day for which survival was null. For both species, all larvae died between 8–9 DAH, 10–11 DAH, 12– 13 DAH and 14–15 DAH for the starvation, pre-plutei, prisms and eggs feeding treatments respectively. Mean growth determined in Experiment 1 was 2.60 and 2.03 mm at 15 DAH for *D. sargus* and *S. aurata* larvae fed *Brachionus* spp. respectively.

In Experiment 2 (sea urchin prey from broodstock on prepared feed), similar to the results obtained in Experiment 1, the starvation treatment led to null survival between the eighth and ninth DAH for both species. However, null survival for pre-plutei treatment occurred later (13–14 DAH) for both species. For the remaining treatments, survivals are represented in Fig. 2. The *Brachionus* spp. seemed to be the better live feed treatment for both fish larvae species (45.7% and 32.0% mean survival at 15 DAH for *D. sargus* and *S. aurata* respectively). For *P. lividus* eggs and prisms, mean survivals were 5.19% and 1.58% for *D. sargus* and 4.28% and 2.19% for *S. aurata* respectively. Significant differences ($P < 0.05$) were found between prisms and *Brachionus* spp. treatment for both species. No significant differences ($P > 0.05$) in survival were found between the two fish larvae species when considering each diet treatment separately. Mean growth determined at 15 DAH in Experiment 2 for both larvae species is represented in Fig. 2 and was 1.07 and 2.49 mm for *D. sargus* larvae fed *P. lividus* eggs and *Brachionus* spp. respectively. Mean growth for *S. aurata* larvae was 0.99 and 1.98 mm when fed *P. lividus* eggs and *Brachionus* spp. respectively. For both species, significant differences ($P < 0.001$) were found in larvae growth between the two feeding treatments. Because of the reduced number of surviving fish larvae obtained with the prism feeding treatment, the mean larvae growth was not calculated in either species. When considering larvae species as the categorical predictor (factor), there were no significant differences ($P > 0.05$) in mean larvae growth considering eggs as the dependent variable. Considering *Brachionus* spp. as the dependent variable, the mean larvae growth was significantly higher ($P < 0.05$) in *D. sargus* than in *S. aurata* larvae.

Mean survivals obtained in Experiment 3 (sea urchin eggs from broodstock on prepared feed – feeding frequency and combination) for the different fish larvae diet treatments at 15 DAH are represented in Fig. 3. The *Brachionus* spp. treatment continued to be a better diet with survivals similar to the ones obtained in Experiment 2 (32.4% and 30.8% mean survival for *D. sargus* and *S. aurata* respectively). For the other diet treatments, the mean survival results obtained in a decreasing order, respectively for *D. sargus* and *S. aurata* larvae, were: 14.4% and 13.8% with the co-feeding treatment (CF); 9.50% and 7.41% with the *P. lividus* eggs given three times a day treatment (3T); 1.50% and 1.31% with the *P. lividus* eggs given two times a day treatment (2T) and 1.30% and 1.02% with the *P. lividus* eggs given by the peristaltic pump treatment (PP). Equally, all fish larvae in the starvation treatment, in Experiment 3, died between the eighth and ninth DAH for both species. Comparisons between feeding treatments for both larvae species revealed that *Brachionus* spp. treatment led to significantly higher survival ($P < 0.001$) than all the alternative treatments. The significantly lowest survivals ($P < 0.01$) were obtained with 2T and PP treatments. As in Experiment 2, no significant differences in survival ($P > 0.05$) were found between fish larvae species when considering each diet treat-

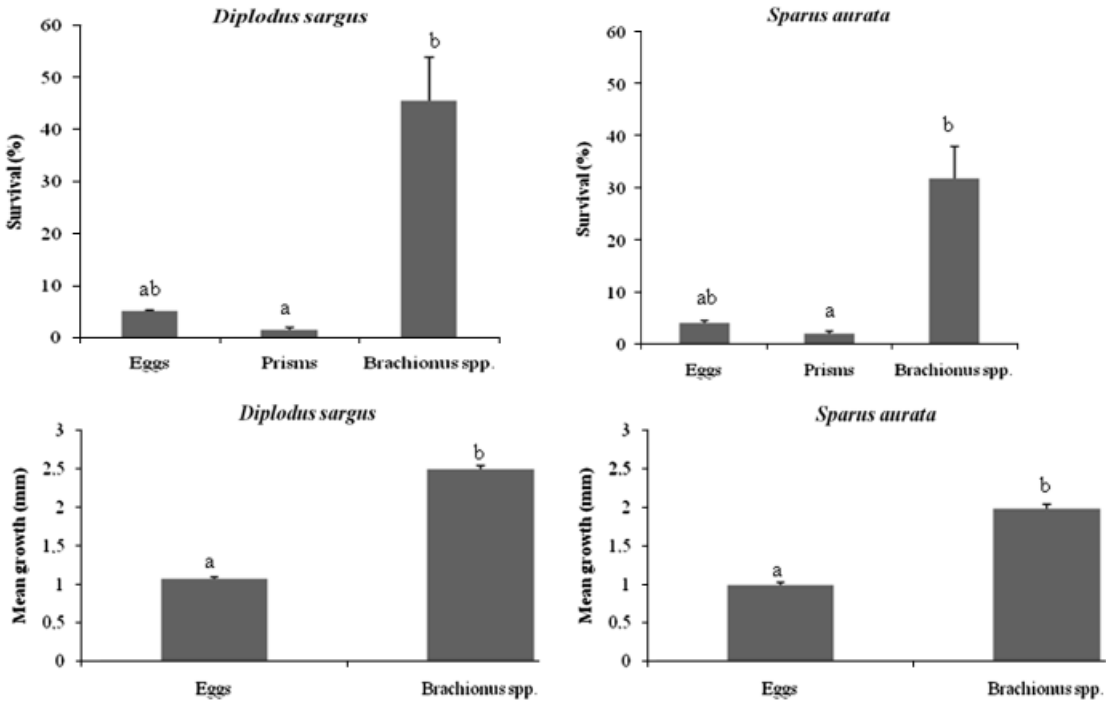

Figure 2 Mean survival (above) and mean growth (below) ± standard error of the mean of *Sparus aurata* and *Diplodus sargus* fish larvae reared from mouth opening to 15 days after hatching with different live prey (*Paracentrotus lividus* eggs, prisms and *Brachionus* spp) – Experiment 2. No data are presented for pre-plutei and starvation due to null survival obtained with these feeding treatments. No growth data are presented for prism feeding treatment due to the low number of surviving larvae. Different letters placed on the top of each bar represent significant differences between fish larvae diet treatment (Kruskal–Wallis, $P < 0.05$ for survival, and one-way ANOVA, $P < 0.05$).

ment separately. Mean growth determined in Experiment 3 for both larvae species is represented in Fig. 3 and was 1.27, 1.71 and 2.00 mm for *D. sargus* larvae fed 3T, CF (see Table 1) and *Brachionus* spp. respectively. Mean growth for *S. aurata* larvae was 1.19, 1.62 and 1.92 mm when fed 3T, CF and *Brachionus* spp. respectively. For both species, significant differences ($P < 0.001$) were found in larval growths between the three feeding treatments. Because of the reduced number of surviving fish larvae obtained with 2T and PP feeding treatments, the mean larvae growth was not calculated in either species. When considering larvae species as the categorical predictor (factor), there were no significant differences ($P > 0.05$) in mean growth between the *D. sargus* and *S. aurata* larvae considering all the diets.

In the fourth experiment (sea urchin prey from broodstock on prepared feed presented only at 8 DAH), significant higher mean survivals ($P < 0.05$) were obtained with *P. lividus* eggs and *Brachionus* spp. feeding treatments (36.2% and 35.9% respectively) (Fig. 4). Significant lower survivals ($P < 0.05$) were obtained with *P. lividus* pre-plutei and starvation treatments (5.89% and 3.84% respectively). Survival obtained with *P. lividus* prism treatment was significantly different ($P < 0.05$) from all the other treatments and had an intermediate value. In Fig. 4 are represented the *D. sargus* larvae growths at the end of the experiment (15 DAH) for the different feeding treatments. The significantly highest ($P < 0.05$) mean growth value was obtained when using *Brachionus* spp. as live feed (3.02 mm) and the significantly lowest ($P < 0.05$) was obtained using *P. lividus* eggs (1.45 mm) and starvation feeding treatments (1.63 mm). Significant ($P < 0.05$) in-between mean values were found using *P. lividus* prisms (2.06 mm) and pre-plutei (1.92 mm).

First-feeding fish larvae ingestion rates

In Fig. 5 are represented the 24 h ingestion rates for the several fish larvae species analysed. Considering the first feeding (FF) except for *D. Labrax*, there was a

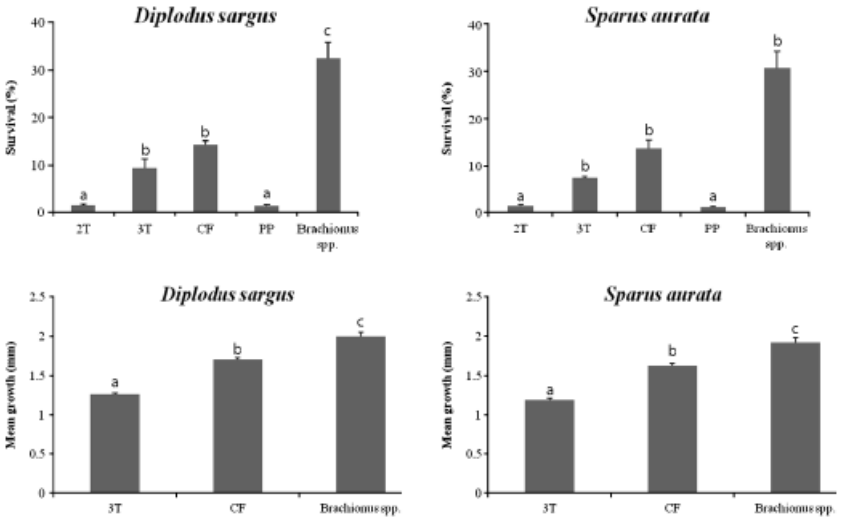

Figure 3 Mean survival (above) and mean growth (below) ± standard error of the mean of *Sparus aurata* and *Diplodus sargus* fish larvae reared from mouth opening to 15 days after hatching with different live preys [2T, 3T, CF, PP and *Brachionus* spp (see Table 1 and Fig. 1)] – Experiment 3. No growth data are presented for 2T and PP due to low number of surviving larvae obtained with these feeding treatments Different letters placed on the top of each bar represent significant differences between fish larvae diet treatment (one-way ANOVA, $P < 0.05$). 2T, prey given two times a day; 3T, prey given three times a day; CF, co-feeding (*Brachionus* spp.+*Paracentrotus lividus* eggs); PP, continuous feeding with peristaltic pump.

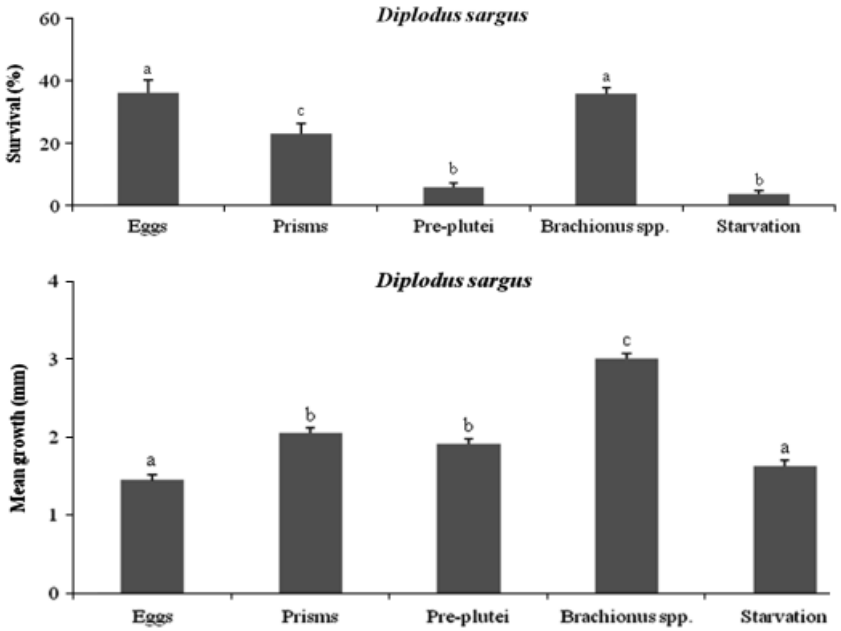

Figure 4 Mean survival (above) and mean growth (below) ± standard error of the mean of *Diplodus sargus* fish larvae reared from 8 to 15 days after hatching (DAH) with different live preys (*Paracentrotus lividus* eggs, prisms and pre-plutei, *Brachionus* spp.) and one where the fish larvae were in starvation – Experiment 4. From mouth opening until the eighth DAH larvae were fed *Brachionus* spp., including the starvation feeding treatment. Different letters placed on the top of each bar represent significant differences between fish larvae diet treatment (one-way ANOVA, $P < 0.05$).

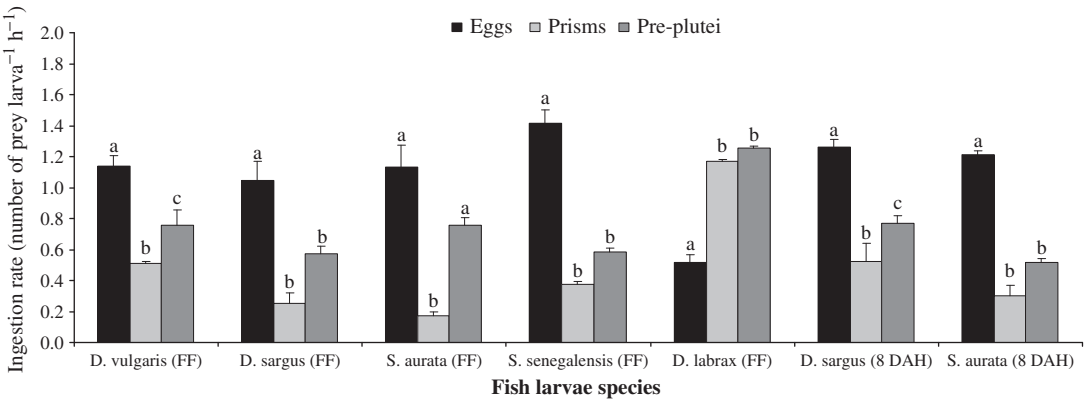

Figure 5 Mean ingestion rate (number of prey ingested larva^{-1} h^{-1}) ± standard error of the mean for five different fish larvae species using *Paracentrotus lividus* eggs, prisms and pre-plutei as live preys (FF, first-feeding; DAH, days after hatching). Different letters placed on the top of each bar represent significant differences between fish larvae diet treatment (one-way ANOVA, $P < 0.05$) for each species separately.

general tendency for higher egg ingestion rate (ranging from 1.04 to 1.42 eggs larva^{-1} h^{-1}) followed by pre-plutei (ranging from 0.57 to 0.76 pre-plutei larva^{-1} h^{-1}) and lower for prisms (ranging from 0.17 to 0.51 prims larva^{-1} h^{-1}). This higher egg ingestion rate was significant ($P < 0.05$) for all these larvae species, except when considering *S. aurata*. Lower prism ingestion rate was significant ($P < 0.05$) for *D. vulgaris* and *S. aurata*. In the other hand, *D. labrax* egg ingestion rate (0.52 eggs larva^{-1} h^{-1}) was significantly lower ($P < 0.05$) than prisms (1.17 prisms larva^{-1} h^{-1}) and pre-plutei (1.26 pre-plutei larva^{-1} h^{-1}). For *S. aurata* and *D. sargus*, 8 DAH larvae the ingestion rates followed the general tendency with a significant higher ($P < 0.05$) egg ingestion rate (1.21 and 1.26 eggs larva^{-1} h^{-1} respectively), followed by pre-plutei (0.52 and 0.77 pre-plutei larva^{-1} h^{-1} respectively) and then prisms (0.30 and 0.53 prisms larva^{-1} h^{-1} respectively), but only for *D. sargus* larvae there was a significant difference ($P < 0.05$) between prisms and pre-plutei ingestion rates. Comparing *D. sargus* and *S. aurata* fish larvae at FF and at 8 DAH, significant differences ($P < 0.05$) were only found in *D. sargus* for prisms and pre-plutei feeding treatments where the ingestion rates were significantly higher at 8 DAH. *Brachionus* spp. results were not presented because after 24 h the number of rotifers was always higher than in the beginning of the experiment, both in the rotifer feeding treatment and in the treatment with just this prey, due to the fast reproduction capacity of this species. Additionally, large variability was found in the number of rotifers among replicates, and therefore it was impossible to determine the exact ingestion rates in the rotifer feeding treatment using this methodology. After the 24 h period, for all larvae species in the control treatment just with fish larvae, the mortality was always null, and in the other control just with the alternative live prey (*P. lividus* eggs, prisms and pre-plutei) no noteworthy differences in the number of prey were observed (the error always less than 3%).

Discussion

Generally when rearing *S. aurata* and *D. sargus* fish larvae until 15 DAH, the best results for survival and growth were always obtained with the *Brachionus* spp. feeding treatment. Considering survival in all the experiments carried out in the present study, the values obtained with this diet ranged from 32.4% to 45.7% for *D. sargus* and 30.8% to 32.0% for *S. aurata*. For 45 DAH, Saavedra, Conceição, Pousão-Ferreira and Dinis (2006) found 18.7% survival in *D. sargus* larvae fed on live feed and for 25 DAH, in more recent works, Saavedra, Pousão-Ferreira, Yúfera, Dinis & Conceição (2009) and Saavedra, Barr, Pousão-Ferreira, Helland, Yúfera, Dinis & Conceição (2009) referred respectively, 6.2% and 8.6% survival. For *S. aurata* larvae, Parra and Yúfera (2001) referred 15–20% survival rate at the end of the first month of culture. Higher survival values were obtained by Parra and Yúfera (2000) where survival of *S. aurata* larvae at 15 DAH was 39.3% and 91.1% when fed 1 and 10 rotifers mL^{-1} respectively. Higher survival value

at 15 DAH (approximately 78%) was also obtained in another study by Yúfera, Fernández-Díaz, Pascual, Sarasquete, Moyano, Díaz, Alarcón, Garcia-Gallego and Parra (2000) where *S. aurata* were fed 10 rotifers mL^{-1}. Testing the effect of rearing salinity, Tandler, Anav and Choshniak (1995) obtained 11.7% survival at 32 DAH with 32.5 g L^{-1}. When testing the effect of green water in *S. aurata* larvae rearing, Papandroulakis, Divanach and Kentouri (2002) obtained 44% survival at 20 DAH. In the study of Pousão-Ferreira, Santos, Carvalho, Morais and Narciso (2003), survival of *S aurata* larvae reared until 22 DAH with *Brachionus* spp. feeding ranged approximately from 10% to 50%. These values point out the high variability of marine fish larvae survival obtained in hatcheries, but it is considered that the survival results obtained in the present work with *Brachionus* spp. feeding treatment are consistent with the expected range with the rearing conditions used.

For both species, the starvation treatment in all experiments led to null survival always between the eighth and ninth DAH. Yúfera *et al.* (1993) also obtained a peak of mortality by starvation around the ninth DAH for *S. aurata* larvae. According to the experience obtained at the hatchery of Aquaculture Research Station of IPIMAR, *D. sargus* larvae in starvation also die around the ninth DAH when yolk reserves are exhausted.

When using the alternative live prey (*P. lividus* eggs, prisms and pre-plutei), the difference in survivals obtained in Experiment 1 and 2 is noteworthy. The null survival at 15 DAH in Experiment 1, obtained with all the alternative live prey feeding treatments, must be related with nutritional deficiencies in these preys as a consequence of the *P. lividus* broodstock diet (grains of maize). Gago *et al.* (2009) found little amounts of EFA for fish larvae in *P. lividus* eggs and endotrophic larvae resulting from the broodstock fed this diet, particularly DHA. Mortality due to food rejection was not considered because of the larvae ingestion rates results. The larvae fed *P. lividus* eggs and endotrophic larvae died some days after the larvae were kept in starvation, which might indicate that fish larvae acquired some energy and raw materials through food consumption. Null survivals were first achieved with pre-plutei followed by prisms and then egg feeding treatments, which could reflect a nutritional quality increasing order, also found by Gago *et al.* (2009) when considering the lipid quantity that was related with the energy necessary to fuel *P. lividus* larval development.

In Experiment 2, the broodstock enhanced prepared diet was used to enrich *P. lividus* eggs and endotrophic larvae with EFA. In this experiment, the fish larval survival improved for the alternative live prey feeding treatments when compared with Experiment 1, particularly for eggs, but values are lower than the ones obtained with *Brachionus* spp. For preplutei, survival was still null at 15 DAH but occurred 3 days later than in Experiment 1. As Gago *et al.* (2009) showed, these results reflect higher *P. lividus* eggs and endotrophic larvae nutritional quality (particularly on polyunsaturated fatty acids) when the broodstock is fed with this enhanced prepared diet compared with grains of maize. The high variability among replicates for all diet treatments led to the inexistence of data normality, and therefore the nonparametric Kruskal–Wallis test had to be performed. This fact could explain why, in Experiment 2, the significant differences were only found for prisms and *Brachionus* spp. treatments in both species.

In spite of *P. lividus* eggs having proved to be the best alternative diet in Experiment 2, they have negative buoyancy and it was supposed that this fact leads to a minor availability of these prey to the fish larvae in the rearing tanks, because they start to fall immediately after each feeding presentation, and consequently could lead to a minor survival when compared with *Brachionus* spp., which continues to swim in the tank water column. Therefore, the Experiment 3 carried out to verify this hypothesis, showed that feeding the fish larvae two times a day (2T treatment) leads to similar survival as feeding them with the same quantity of eggs given continuously (PP treatment). However, when supplying *P. lividus* eggs three times a day (3T treatment), the fish larvae survival obtained was significantly higher for both fish species but still significantly lower than the *Brachionus* spp. treatment. These results show that, instead of negative buoyancy, lower nutritional quality seems to be the main constraint of *P. lividus* eggs in fish larvae first feeding. Better results with 3T treatment might be explained by the higher consumption of these more numerous, but still of poor quality live prey. Survival obtained with CF is significantly lower than the one obtained with *Brachionus* spp. feeding treatment, which also confirms the lower quality of *P. lividus* eggs when compared with *Brachionus* spp., because this better quality live prey were only present at half density in this feeding treatment. Considering this factor, in spite of *P. lividus* eggs and endotrophic larvae being enhanced in highly unsaturated fatty acids (HUFA) through broodstock fed

with the prepared diet that has cod liver oil as the main lipid source (Gago *et al.* 2009), the enrichment used in this study for *Brachionus* spp. (DHA Protein Selco) leads to superior HUFA quality, particularly in the DHA/EPA ratio (as by manufacturer specifications, INVE Aquaculture, Belgium).

When considering mean larvae growth in Experiments 1, 2 and 3 it is possible to conclude that *Brachionus* spp. seems to be the better diet treatment, although good results are also obtained with the CF treatment in Experiment 3. The same reasons used for survival rates can be used to explain these results and these reasons are mainly the poorer nutritional quality of *P. lividus* eggs and larvae when compared with enriched *Brachionus* spp. Besides nutritional quality, the capability of rotifers to reproduce may also explain the differences obtained both in survival and growth because prey density is always higher for this feeding treatment.

When fed *Brachionus* spp., *S. aurata* larvae growth obtained in other studies (Polo, Yúfera & Pascual 1992; Yúfera *et al.* 1993; Parra & Yúfera 2000) is slightly higher than the ones obtained in the present study but there were differences in the rearing conditions. In these studies, constant illumination, 24 h feeding and 300L rearing volume may have contributed to maximize *S. aurata* larvae growth. Nevertheless, in spite of higher tank volume but under similar rearing conditions to the ones used in the present study, Pousão-Ferreira *et al.* (2003) also obtained approximately 2 mm length increment on *S. aurata* larvae reared until 15 DAH.

Overall, in the fourth experiment where *P. lividus* eggs, prisms and pre-plutei were only presented after 8 DAH to *D. sargus* larvae fed *Brachionus* spp., the results continue to demonstrate in more developed young fish larvae the lower value of the alternative live prey compared with rotifers. In spite of similar survival rates that were obtained with *P. lividus* eggs and *Brachionus* spp. feeding treatments, the former *D. sargus* larvae mean growth is significantly lower, which may also reflect the lower *P. lividus* eggs nutritional quality. For prisms and pre-plutei feeding treatments, the survival continues to be significantly lower and the lowest survival continues to be found in the pre-plutei feeding treatment. Because rotifers have a high reproductive capability, they were observed in all the tanks at the end of the experiment. Aragão, Conceição, Fyhn and Dinis (2004) also refer that during the early fish larval stages of *S. aurata* and *S. senegalensis*, due to the low water exchange and the low larvae feeding rates, the prey may stay inside the tanks for several hours. For this reason, higher mean growth obtained with these two feeding treatments (prisms and pre-plutei) compared with eggs must be due to the lower fish larvae survival, and therefore higher rotifer availability per surviving fish larvae that continues to feed on green water. This reason may also explain why *D. sargus* larvae mean growth obtained in starvation feeding treatment was similar to the one observed in *P. lividus* eggs feeding treatment. In the literature revised for *D. sargus* larvae, growth values expressed as increases in total length were not found, and in the few studies analysed (Saavedra, Conceição *et al.* 2006; Saavedra, Pousão-Ferreira *et al.* 2009; Saavedra , Barr *et al.* 2009), dry weight was the variable determined, making direct comparison impossible.

When analysing first-feeding 24 h ingestion rates, except for *D. labrax*, there was a higher *P. lividus* egg consumption than prisms and pre-plutei. Egg slow downfall coupled with its brown-orange colour may explain this higher ingestion by very young and low motile fish larvae. Motility could also explain the lower ingestion rate for prisms that are the most active of the alternative live prey making them harder to be captured by fish larvae. Pre-plutei are not so mobile, which can explain higher consumption than prisms, but their transparency and larger size may explain why its consumption was lower than eggs. It appears that the previous explanations may also apply to *S. aurata* and *D. sargus* larvae with 8 DAH, which might indicate that feeding behaviour in these two fish larvae species does not change significantly during this period. However, for 8 DAH *D. sargus* larvae, there was a significant increase in prisms and pre-plutei consumption, which might indicate a higher ability to catch these prey.

The different pattern observed in *D. labrax* larvae ingestion rates could be the consequence of its larger mouth size and predatory activity (according to Aquaculture Research Station of IPIMAR data), which is reflected in higher prism and pre-plutei consumptions. *S. senegalensis* also have a wider mouth at first feeding than *S. aurata* and *Diplodus* spp. but its feeding behaviour is less predatory than *D. labrax* (according to Aquaculture Research Station of IPIMAR data), which might explain the dissimilarities among these two fish larvae species. These results corroborate that marine fish larvae are selective feeders both for the type and the size of prey, and moreover, this prey selectivity may play an important role during early larval development (Fyhn 1989). Rumrill, Pennington and Chia (1985) also observed a higher

predation by the red crab *Cancer productus* zoea on the sand dollar *Dendraster excentricus* eggs than on prisms and pre-plutei. Selective predation upon embryos and larvae of *D. excentricus* by several zooplanktonic predators, including fish species, were also observed by Pennington, Rumrill and Chia (1986) and Allen (2008). For *S. aurata* larvae, it was considered that prey size is the determinant factor in prey selection, both considering live and inert feed (Polo *et al.* 1992; Fernández-Díaz, Pascual & Yúfera 1994). Thus, ingestion, digestion and assimilation may depend strongly on the physical and chemical properties of the food particle irrespective of its nutritional quality (Yúfera, Fernández-Díaz & Pascual 1995).

When analysing the feeding rates of *S. aurata* larvae on microcapsules, Yúfera *et al.* (1995) referred values ranging from 3 to 35 particles larva^{-1} h^{-1} and the lower values corresponded to larvae at first feeding. Nevertheless, these ingestion values are always higher than the ones found in the present study for *P. lividus* eggs, prisms and pre-plutei. As Yúfera *et al.* (1995) considered that microcapsules are ingested at similar rates to commonly used living prey, it is supposed that the alternative live preys tested are ingested at lower rates than the *Brachionus* spp. Additionally for 6 DAH *S. aurata* larvae, Parra and Yúfera (2000) referred ingestion rates of 0.79 and 0.83 μg larva^{-1} h^{-1} when they were fed 1 and 10 rotifer mL^{-1} respectively. If these values are divided by 0.16 μg (the *Brachionus plicatilis* weight found by Theilacker & McMaster 1971), a feeding rate of approximately 5 rotifers larva^{-1} h^{-1} is obtained. This value is also indicative of a higher *Brachionus* spp. ingestion as compared with the best calculated value of 1.42 *P. lividus* eggs larva^{-1} h^{-1} found in the present study for *S. senegalensis*. In spite of some caution in these comparisons because the rearing conditions were different, this fact could also explain the lower survival and growth obtained with *P. lividus* eggs, prisms and pre-plutei feeding treatments when rearing *S. aurata* and, probably *D. sargus*, larvae until 15 DAH compared with *Brachionus* spp. feeding treatment.

The results obtained in the experiments carried out in this study suggest the conclusion that although the alternative live prey are ingested by fish larvae, which are concordant with plankton ecological studies and laboratory experiments, they cannot compete with rotifers when considering young marine fish larval survival and growth. It is suggested that nutritional aspects may be the main reason for such differences. Besides fatty acid composition, other nutrients may also play an important role. In this context, protein and amino acid composition of *P. lividus* eggs and endotrophic larvae are being investigated by our research team, and preliminary results indicate a low quantity of indispensable amino acids in the free form that appears to be vital for fish larvae survival and growth (Fyhn 1989; Rønnestad, Tonheim, Fyhn, Rojas-García, Kamisaka, Koven, Finn, Terjesen, Barr & Conceição 2003; Kvåle, Nordgreen, Tonhein, & Hamre 2007). Therefore, unless more efficient procedures are found to enrich these endotrophic preys, they showed no advantage to be used at least in marine fish first feeding.

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CHAPTER 3

The effect of sperm:egg ratio and egg concentration on egg fertilization, larvae malformations and polyspermy events in the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea)

Tiago Repolho, João Gago and Orlando Luís

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The effect of sperm:egg ratio and egg concentration on egg fertilization, larvae malformations and polyspermy events in the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea)

Abstract

This study evaluated the effect of sperm:egg ratio (10:1, 100:1, 300:1, 1000:1, 3000:1, 10000:1) and egg concentration (1, 5, 10, 20 and 50 eggs.mL^{-1}) on *Paracentrotus lividus in vitro* fertilization and subsequent embryo and 4-arm plutei development. Statistical differences were found between experimental treatments regarding the egg concentration and sperm:egg ratios used. Percent egg fertilization varied between 2.9% and 99.0%, within the experimental groups tested. A positive correlation effect was found after 90 minutes post-fertilization between egg fertilization, fertilized undeveloped eggs and first cleavage 4-cell embryos with increasing sperm:egg ratios. In opposition, normal first cleavage 2-cell segmentation was negatively correlated with sperm:egg ratios. No statistical differences, concerning larvae total length, were found between egg concentration *vs*. sperm:egg ratios experimental groups. Decreasing of post-oral arm length and normal percent larvae development were observed with increasing egg concentration, possibly related to increasing larvae culture densities. The results indicate that sperm:egg ratio is a parameter that influences percent egg fertilization, as well as segmentation stage development. Likewise, sperm:egg ratio values above 300:1, seem to induce deleterious effects over *P. lividus* segmentation stage development, regardless of egg concentration used. High percent egg fertilization (>90%) was obtained for sperm concentrations above 200 sperm cells.mL^{-1}. This study highlights the importance of performing *P. lividus in vitro* fertilization assays with well defined and appropriate egg concentration *vs*. sperm:egg ratio parameters, with special emphasis to application within research areas, where percent egg fertilization, embryo and larvae development are used as endpoint assessment tools.

Keywords: Larval development; sea urchin; *Paracentrotus lividus*; sperm:egg ratio; percent egg fertilization; polyspermy.

1. Introduction

Sea urchins represent over 750 known species (Miller and Harley, 1996; Garcia *et al*., 2000; Sotelo *et al*., 2002) and they play a key role on costal habitats as well as on ecological structure and dynamics (Gago *et al.*, 2001; Hereu *et al*., 2004).

Sea urchins are one of the most used experimental animals, among available biological models. Several reasons have contributed to their success, such as availability from supply companies, research institutions or wild caught; external fertilization and easy maintenance under captive conditions at low financial cost. Likewise their gametes, fertilization and embryo development are used to study many primordial biological processes. The easy ability to obtain gametes in large quantities, easy manipulation and *in vitro* development observation, as well as easy embryo and larvae culture, under laboratory conditions until metamorphosis, have contributed and prompt their importance as a biological model within several research areas, in order to better understand the biological mechanisms involved in development processes.

Several research areas have used sea urchins to better understand cellular, molecular and development mechanisms (Berdyshev *et al.*, 1995; Spiegler and Oppenheimer, 1995; Roccheri *et al.*, 1997; Voronina and Wessel, 2001; Thorndyke *et al.*, 2001; Dubois and Ameye, 2001; Ngo *et al.*, 2003; Aluigi *et al.*, 2008; Filosto *et al.*, 2008; Tomšić *et al.*, 2011). Likewise, embryo and larvae development have been thoroughly used as bioindicators for environmental monitoring and assessment (Fernández and Beiras, 2001; Beiras *et al.*, 2003; Pesando *et al.*, 2003; Ghirardini *et al.*, 2005; Arslan *et al.*, 2007; Caeiro *et al.*, 2009; Álvarez *et al.*, 2010; Marín *et al.*, 2010; Carballeira *et al.*, 2011a).

Fertilization and embryogenesis, as well as larvae development, are generally accepted as the most critical, sensitive and complex life phases of marine organisms. Likewise, recruitment is based upon embryo and larvae development success, which in terms of population viability is crucial. Sea urchin embryo and larvae development, are strongly dependent upon broodstock nutritional status (George *et al.*, 1990; George, 1996; Meidel *et al.*, 1999; George *et al.*, 2001; Repolho *et al.*, 2011), nutritive reserves during oocyte development (Brooks and Wessel, 2003), aquatic environmental conditions (Bressan *et al.*, 1995; Sewell and Young, 1999; Denny *et al.*, 2002; Carballeira *et al.*, 2011b), among others. The effect of some abiotic factors such as salinity on growth and development of marine invertebrates have been extensively studied, including those for echinoderms (George and Walker, 2007; Pétinay *et al.*, 2009).

Fertilization research using sea urchin as biological model started in the 19th century. Since then, several studies were conducted to study and better understand the biological mechanisms involved in sea urchin fertilization. During this phase of sea urchin reproduction, if one single spermatozoa-oocyte fusion occurs, a diploid egg develops. On the other hand, if multiple sperm-egg fusions occur, abnormal embryo development (Morgan, 1982) and consequently embryo death might occur (Wong and Wessel, 2004; Vacquier, 2011)

Several experimental variables may have direct effect on fertilization success in sea urchins. Under experimental conditions, sperm concentration, as well as sperm-egg contact time and gamete aging, were found to be determining factors in *Echinometra mathasci* fertilization success (Rahman *et al.*, 2001) in opposition to egg concentration, which revealed no significant effect over fertilization. Likewise, the same conclusions were reached for the species *Strongylocentrotus franciscanus* (Levitan *et al.*, 1991). Also sea urchin fertilization kinetics, such as sperm velocity and aging (Levitan, 2000) as well as egg longevity (Meidel and Yund, 2001; Lera and Pellegrini, 2006), play a key role in fertilization success. Likewise, culture conditions such as salinity (Carballeira *et al.*, 2011b) and seawater nature (Jonczyk *et al.*, 2001), were found to be key factors in fertilization success for two sea urchin species (*Paracentrotus lividus* and *Lytechinus pictus*) as well as for *P. lividus* larvae development.

Where sea urchin fertilization is concerned, normal embryo as well as larvae development is only achieved if a single sperm-egg nuclear fusion occurs. As soon as spermatozoa enter the oocyte, fertilization is triggered and a series of cascade events are initiated leading to embryo development (Tsaadon *et al.*, 2006). While some species possess the ability of develop eggs capable of being fertilized under sperm limiting conditions, others are able to generate eggs, which only can be fertilized at high sperm concentrations (Levitan, 2006).

Polyspermy is characterized by multiple sperm fusions with a single egg nucleus, during fertilization (Morgan, 1982). In marine invertebrates, when eggs are subject to high sperm concentrations, polyspermy might occur (Levitan, 2004; Levitan *et al.*, 2007). To overcome the possibility of this abnormal event, two polyspermic avoidance mechanisms have evolved within sea urchins (Nicotra and Schatten, 1996; Vacquier, 2011): A fast electrical block characterized by a membrane potential change (Jaffe, 1976; Wong and Wessel, 2004) followed by a slow structural block, as a result of the fertilization envelope formation and hardening (Vacquier, 2011). Thus polyspermy might still occur in the

presence of high sperm concentrations, before the structural block is completely formed (Vacquier, 2011).

When using biological models to conduct experimental research, several criteria, optimization and methodology standardization should be ensured, so results liability and accuracy are to be obtained. Likewise if monospermic sea urchin embryos are to be obtained after fertilization occurs, certain pre-experimental conditions, such as sperm egg-ratios at fertilization, have to be optimized in order to obtain reliable and feasible results.

Most of P. *lividus* embryos and larvae availability is limited to the season when this species is naturally mature. Recently, this biological constraint was addressed (Repolho *et al.*, 2011) through the development of an inert feed which allows the year round availability of mature individuals under captive conditions, as well as high percent egg fertilization (>99%) and high percent normal larvae development (85.0±1.45%) in comparison to wild organisms (82.5±1.75%).

Although *P. lividus* echinoculture have been thoroughly studied (Fenaux *et al.*, 1985a,b; Bressan *et al.*, 1995; Gosselin and Jangoux, 1998; Vaıtilingon *et al.*, 2001; Luís *et al.*, 2005; Liu *et al.*, 2007; Carboni *et al.*, 2012), experimental variables of most importance, within certain research areas, concerning the relationship between sperm-egg ratio and egg concentration, during *P. lividus* fertilization, over polyspermy events and subsequent embryo and larvae development have never been addressed. This study evaluated these factors in wild *P. lividus* and their effect over fertilization success and embryo and larva development, namely percent egg fertilization, early-stage embryo and 4-arm plutei development.

2. Material and methods

2.1 Collection of adult sea urchins

Adult *P. lividus* were collected from the wild during September-October 2011, at Cabo Raso, Portugal (38°42´30”N/9°29´11”W). Sea urchins were transported in thermal cases and immersed at all times, until they were stocked in a recirculating aquaculture system (RAS). According to salinity and water temperature measurements performed in the field, a previous adjustment of the RAS system was performed.

2.2 Adult stocking

Adult *P. lividus* (mean±standard error) with 4.9±0.085 cm (test width, TW), 2.3±0.046 cm (test height, TH) and 56.4±2.31 g (total wet weight, TWW) were allocated (±100 individuals.m^{-2}) in 400L cylindrical black fibreglass tanks (0.4 m^3 in volume and 2.51 m^2 of total submerged area, per tank), within a recirculating aquaculture system (RAS), comprising 3.48 m^3 total water volume (Luís *et al.*, 2005).

Water filtration was performed through mechanical (Glass Wool), physical (Jetskim 200, Schuran Gbr, Germany) and biological (ouriço®, Fernando Ribeiro Lda, Portugal) filters as well as UV sterilization (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany). Aeration was performed through an air compressor (Hiblow air pump HP-80, Techno Takatsuki Co. Ltd, Japan). Water temperature was kept through automatic thermoregulation by means of water heaters (1000W - STIU1, Electrifor SA., Barcelona, Spain) and a chilling system (Frimar, Fernando Ribeiro Lda, Portugal). Water circulation, within the RAS, was performed by means of a magnetic pump (Iwaki MX-70VM-13, Iwaki co, Ltd, Japan). Water flow within each culture tank was kept at 8.5$Lmin^{-1}$ (55% renewal rate$hour^{-1}$). Culture parameters were as follows: Salinity (35±1 PSU); temperature (18±1°C); photoperiod (14:10; L:D). Illuminance was maintained at 700 Lux (Gossen Lunasis 3, P. Gossen & Co., Erlangen, Germany), through overhead fluorescent lighting (Digilamp FL-40SS/36 Daylight, 6400K, 2450 lumen). Adult sea urchins were kept, within the culture system, without food supply, until experimental assays were performed, although never above 15 days before being tested.

2.3 Spawning induction, egg and sperm collection

Prior to spawning induction, adult sea urchins were removed from culture tanks and rinsed with seawater for debris removal. Each individual was injected with 0,5 mL (TW<4.9 cm) or 1 mL (TW>5.0 cm) KCl solution (0.5M), through the peristomial membrane, to induce spawning. A minimum of 3 males and 3 spawning females were used for each experimental assay. During spawning, *P. lividus* females were completely immersed, with aboral region facing downwards, in 400 mL glass beakers, during a maximum of 30 minutes (Leahy, 1986). Sperm collected in dry, by means of a micropipette (Pipetman classic™, Gilson Inc., USA) and stored in 1.5 mL eppendorf vials. Afterwards, sperm was mixed in seawater and spermatozoa mobility was checked under 400x optic microscopy (Axiostar Plus, Carl Zeiss, Gottingen, Germany). Oocytes were sieved through a 120μm mesh, for debris removal, and mixed afterwards. Oocyte and spermatozoa concentrations were checked for each pool.

Oocyte concentration was checked by observing three 1mL samples, under 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany). Spermatozoa concentration was checked through haemacytometer observations (Bürker chamber, Germany), under 400x optic microscopy (Axiostar Plus, Carl Zeiss, Gottingen, Germany). Seawater used for fertilization procedures was filtered (0.35 μm) and UV sterilised (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany).

2.4 *in vitro* fertilization and percent egg fertilization

A total of five fertilization experimental trials were performed, each comprising one oocyte concentration (1, 5, 10, 20 and 50 oocytes.mL^{-1} in seawater) with six sperm:egg ratios (10:1; 100:1; 300:1; 1000.1, 3000.1; 10000:1). Three replicates were performed per each of the oocyte concentration and sperm:egg ratio tested. Fertilization assays were conducted in 800 mL glass beakers (Base area (BA)=63.6 cm^2). Oocytes and sperm were added, at desired concentration, to glass beakers, by means of a micropipette (Pipetman classic™, Gilson Inc., USA). After 15 minutes, 100 mL of each fertilization beaker was passed through a 30μm-immersed mesh strainer and gently rinsed with seawater, for sperm removal. Afterwards, sieved eggs and embryos were immediately transferred to 100 mL glass beakers (BA=15,2 cm^2), for larvae development until 72h post-fertilization. The same procedure was performed for remaining 700 mL of volume, where eggs and oocytes were transferred to cleaned glass beakers. After 90 minutes, the eggs and embryos present in this 700 mL volume were sieved through a 30μm mesh immersed strainer and preserved in neutralised formaldehyde (4%). Percent egg fertilization (presence or absence of fertilization membrane) and undeveloped fertilized eggs, 2 and 4-cell stage embryos were checked in approximately 100 oocytes/eggs, for each of the replicates (figure 1). Samples were randomly numbered, as well as larvae development beakers. Sample identification was decoded after percent egg fertilization and segmentation stage development was assessed. Fertilization was performed with filtered (0.35 μm) and UV sterilized (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany) seawater, at 18±1°C, 35±1 PSU and 8.2±1 (pH). All observations were performed under 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany).

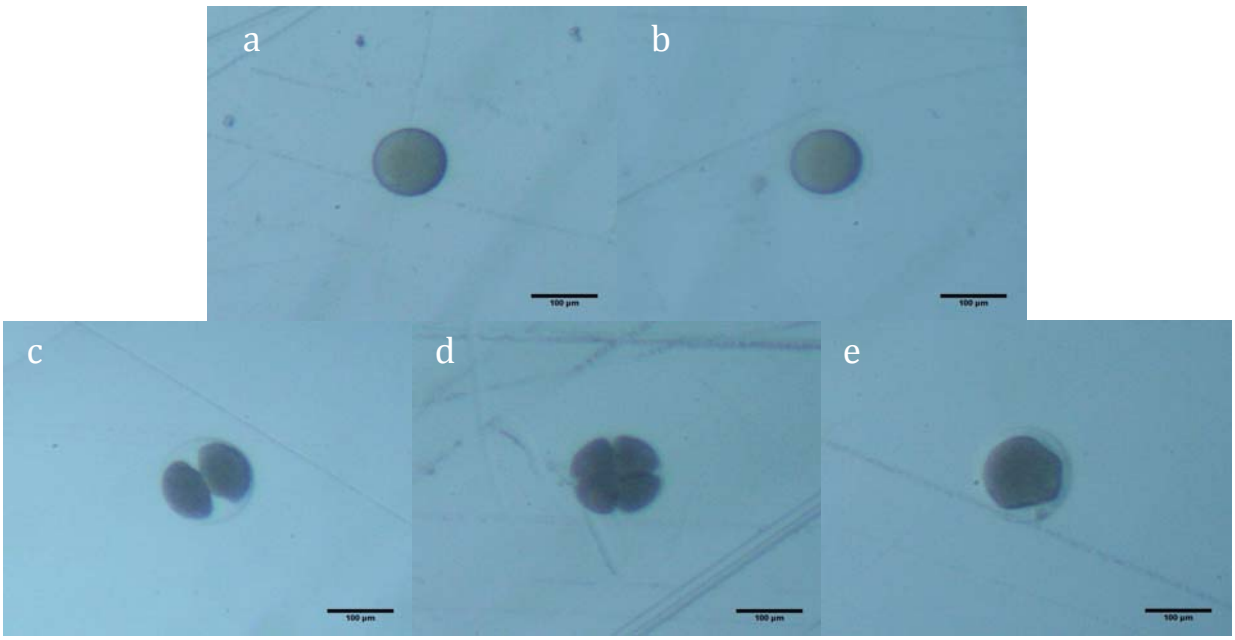

Figure 1 *Paracentrotus lividus* oocyte, eggs and embryos (90 minutes post-fertilization) as criteria for percent egg fertilization and segmentation stage development determination. a: oocyte; b, e: undeveloped fertilized egg; c: 2-cell embryo; d: 4-cell embryo.

2.5 Larvae culture assays

Plutei larviculture was performed in 100 mL glass beakers, for a period of 72h, in filtered (0.35 μm) and UV sterilised (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany) static seawater, without aeration. Culture temperature was 18±1°C and salinity 35±1 PSU. Illuminance was maintained at 700 Lux (Digilamp FL-40SS/36 Daylight, 6400K, 2450 lumen), during a 14:10 (L:D) artificial light cycle. Glass beakers used for larvae development were randomly numbered. Sample identification was only decoded after performing plutei observation for analyzed parameters. Water temperature was maintained by partial-immersing the culture glass beakers within a thermo-regulated RAS, equipped with water heaters (1000W - STIU1, Electrifor SA., Barcelona, Spain) and a chilling device (Hailea HC-150A, Guangdong Hailea Group Co., Ltd, China). Temperature was crosschecked with a digital thermometer (predictor IT808 digital thermometer, Omega Pharma, Netherlands), twice a day.

After 72h, neutralized formaldehyde (4%) was added to larviculture glass beakers. A total of 90 larvae, 30 per replicate, were measured for each of the initial egg concentration *vs* sperm:egg ratio experiment, with the exception for: 1 egg.mL^{-1} with 100:1 (total of 22 larvae); 5 egg.mL^{-1} with 10:1 (total of 20 larvae), 10 egg.mL^{-1} with 10:1 (total of 22 larvae) and 20 egg.mL^{-1} with 10:1 (62 larvae). For each of the replicates, larvae were measured with a micrometric eye lens, for total length (TL) and post-oral arm length (POL). Total length was

defined as the distance (µm) between the apical and the post-oral arm extremity and post-oral arm length as the distance (µm) between the basis of the mouth and the post-oral arm extremity (figure 2a).

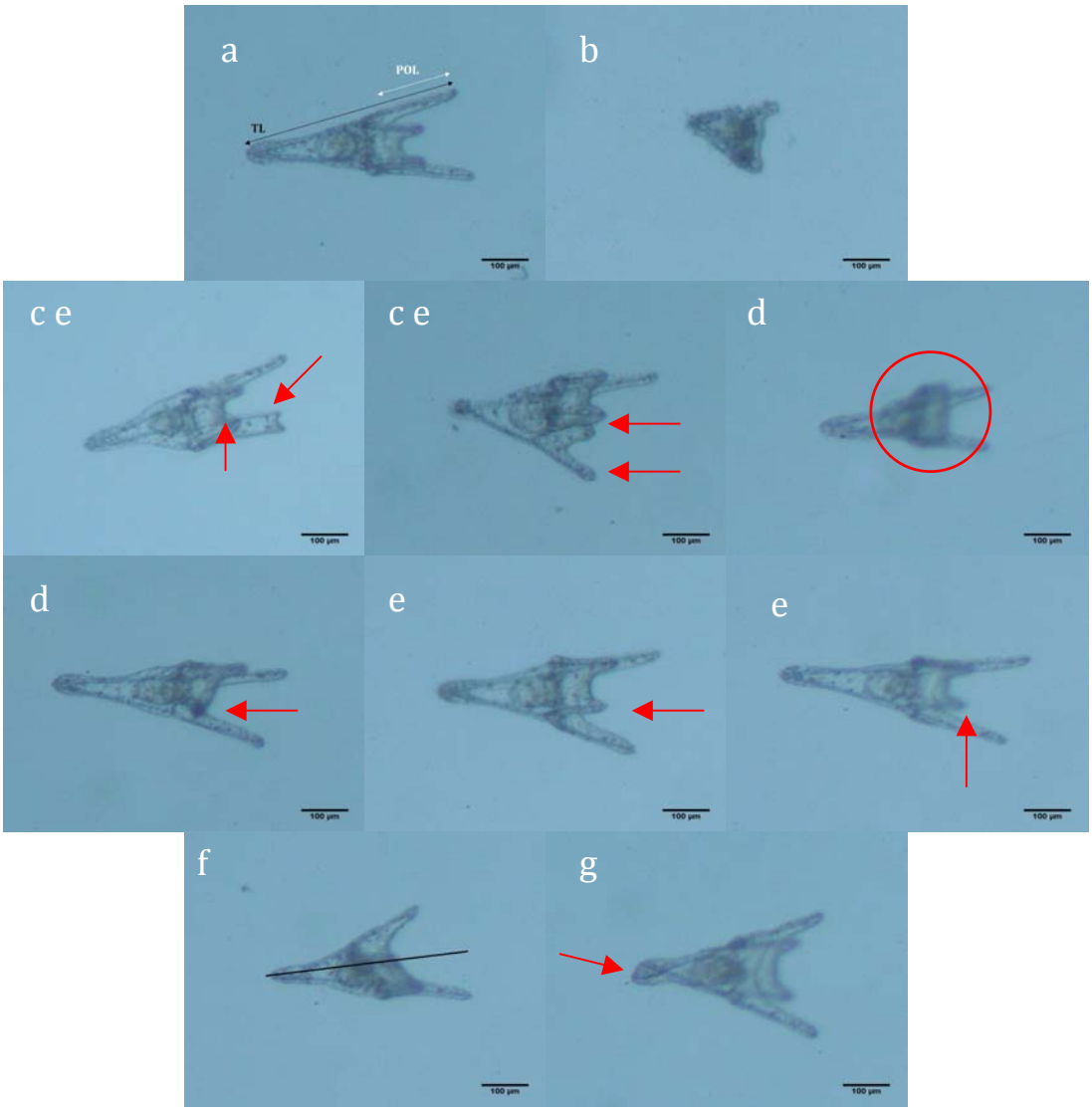

Figure 2 Plutei larvae (72h post-fertilization) with normal (a) and abnormal (b–f) development. a: Normal plutei; b: Delayed development "Apollo" shaped larvae; c: Deformed arms; d: Absence of arms; e: Asymmetric sized arms; f: Morphological asymmetry over the longitudinal axis; g: Crossed body rods.

Normal *vs.* abnormal larvae development was checked, through the presence or absence of larvae malformations: Plutei larvae (4-arm) were identified as abnormal when the following observations were made: Delayed development at 72h; presence of deformed arms; asymmetric sized arms; arms absence; morphological asymmetry over anterior-posterior longitudinal axis; "apollo" shaped larvae; crossed body rods. (figure 2). Larvae without any of the above mentioned characteristics were classified as normal (figure 2). All

observations were performed under 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany), using a micrometric eye lens (PI 10x/18, Carl Zeiss, Gottingen, Germany).

2.6 Statistical analysis

Percentage data was transformed (arcsine, ln), prior to statistical analysis (SigmaStat and SigmaPlot software, Systat Sofware Inc., Windows version 3.10). Whenever ANOVA assumptions where observed, statistical analysis (ANOVA) was applied, prior to multiple comparison analysis (Tukey). Non-parametric analysis (Kruskal-Wallis) was applied whenever data did not comply with ANOVA assumptions and multiple comparisons, between different experimental treatments, were performed using Tukey test and Dunn´s method. Spearman rank order correlation was applied for correlation analysis between percentage data (percent egg fertilization, percent segmentation stage development, normal percent larvae development) *vs*. egg concentrations and sperm:egg ratios, used in the experimental trials.

3. Results

3.1 Percent egg fertilization

Mean values for percent egg fertilization are shown in table 1. Statistical differences (ANOVA, Tukey test, $P<0.05$) were found in all treatments where the same egg concentration and different sperm:egg ratios were used. Significant differences (ANOVA, Tukey test, $p<0.05$) were found between treatment with 1 egg.mL^{-1} and 10:1 *vs*. remaining sperm:egg ratios, treatment with 5 egg.mL^{-1} and 10:1 sperm:egg ratio *vs*. remaining sperm:egg ratios, treatment with 10 egg.mL^{-1} and 10:1 *vs*. remaining sperm:egg ratios and treatment with 20 egg.mL^{-1} and 10:1 sperm:egg ratio *vs*. remaining sperm:egg ratios. The highest percent egg fertilization value (99.0%) was found for the combination of 1 egg.mL^{-1} and 10000:1 sperm:egg ratio, while the lower result (2.9%) was denoted at the same egg concentration and 10:1 sperm:egg ratio experiment.

Table 1 Mean values for percent egg fertilization (%) ± standard error for *P. lividus*, concerning fertilization assays. Mean values followed by different letters represent statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments with the same egg concentration and different sperm:egg ratios. Top row indicates sperm:egg ratios. First column indicates egg concentration (eggs.mL^{-1}).

	10:1	100:1	300:1	1000:1	3000:1	10000:1
1	**2.9** ± 0.54 b	**13.8** ± 0.666 c	**97.6** ± 0.422 a	**97.6** ± 0.274 a	**98.8** ± 0.299 a	**99.0** ± 0.0158 a
5	**6.7** ± 0.56 d	**96.8** ± 0.621 ab	**97.1** ± 0.450 ab	**96.0** ± 0.852 a	**98.8** ± 0.330 c	**98.3** ± 0.319 bc
10	**15,7** ± 0.625 c	**95.4** ± 0.667 b	**97.7** ± 0.275 a	**97.1** ± 0.714 ab	**97.1** ± 0.454 ab	**97.3** ± 0.477 ab
20	**90.6** ± 0.607 d	**96.0** ± 0.536 c	**98.0** ± 0.244 ab	**96.6** ± 0.274 bc	**98.4** ± 0.308 a	**98.0** ± 0.579 a
50	**94.8** ± 0.446 b	**95.4** ± 0.374 b	**96.7** ± 0.784 bc	**97.5** ± 0.402 ac	**98.5** ± 0.320 a	**98.9** ± 0.264 a

Likewise, statistical differences (ANOVA, Tukey test, $p<0.05$) were denoted when comparing treatments for the same sperm:egg ratio with different egg concentrations, with the exception for the 300:1 and 1000:1 sperm:egg ratio experimental groups, where no statistical differences were found between egg concentration experimental groups.

Table 2 Mean values for percent egg fertilization (%) ± standard error for *P. lividus*, concerning fertilization assays. Mean values followed by different letters represent statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments with the same sperm:egg ratio and different egg concentrations. Top row indicates egg concentration (eggs.mL^{-1}). First column indicates sperm:egg ratios.

	1	5	10	20	50
10:1	**2.9** ± 0.54 a	**6.7** ± 0.56 a	**15,7** ± 0.625 b	**90.6** ± 0.607 c	**94.8** ± 0.446 d
100:1	**13.8** ± 0.666 a	**96.8** ± 0.621 b	**95.4** ± 0.667 b	**96.0** ± 0.536 b	**95.4** ± 0.374 b
300:1	**97.6** ± 0.422	**97.1** ± 0.450	**97.7** ± 0.275	**98.0** ± 0.244	**96.7** ± 0.784
1000:1	**97.6** ± 0.274	**96.0** ± 0.852	**97.1** ± 0.714	**96.6** ± 0.274	**97.5** ± 0.402
3000:1	**98.8** ± 0.299 bc	**98.8** ± 0.330 bc	**97.1** ± 0.454 a	**98.4** ± 0.308 ac	**98.5** ± 0.320 ab
10000:1	**99.0** ± 0.0158 bc	**98.3** ± 0.319 bc	**97.3** ± 0.477 a	**98.0** ± 0.579 ac	**98.9** ± 0.264 ab

Percent egg fertilization values above 90% where always observed with the exception for 1, 5 and 10 egg.mL^{-1} concentration at 10:1 and 1 egg.mL^{-1} concentration at 100:1 sperm:egg ratio treatments.

Correlation results between analyzed variables are shown in table 3. A positive correlation ($r_s = 0.791$, $p<0.001$) was found between percent egg fertilization and sperm:egg ratio variables.

Table 3 Spearman rank order correlation between percent egg fertilization, egg concentration and sperm:egg ratios, concerning percent egg fertilization experimental assays.

	Egg concentration	Percent egg fertilization	Sperm:egg ratio
Egg concentration	----------	rs = 0.0408	rs = 0.000
	----------	p = 0.702	p = 1.000
Percent egg fertilization	----------	----------	rs = 0.791
	----------	----------	p = 0.000
Sperm:egg ratio	----------	----------	----------
	----------	----------	----------

3.2 Percent segmentation stage development

Results of mean values for *P. lividus* percent 2–cell segmentation development, after 90 minutes post-fertilization are shown in figures 3 and 4. Statistical differences (ANOVA, Tukey test, $p<0.05$) were found for different egg concentration groups within the same sperm:ratio, with the exception for the experimental group when 300:1 sperm:egg ratio was applied. A decrease in 2–cell segmentation development results is observed between different egg concentration treatments groups at the 3000:1 and 10000:1 sperm:egg ratios for 20 egg.mL^{-1} towards 50 egg.mL^{-1} experimental groups, within all range of sperm:egg ratios tested. The highest percentage of 2-cell segmentation development (98.1%) was obtained for the combination of 20 egg.mL^{-1} and 300:1 sperm:egg ratio. Experimental treatments with 1 and 5 egg.mL^{-1} at 10:1 sperm:egg ratio were significantly lower (ANOVA, Tukey test, $p<0.05$) in comparison to remaining sperm:egg ratios tested. Likewise and for treatments with 20 and 50 egg.mL^{-1} at 10000:1 sperm:egg ratio, significantly lower (ANOVA, Tukey test, $p<0.05$) 2-cell development percentage was obtained, in comparison with remaining sperm:egg ratios tested.

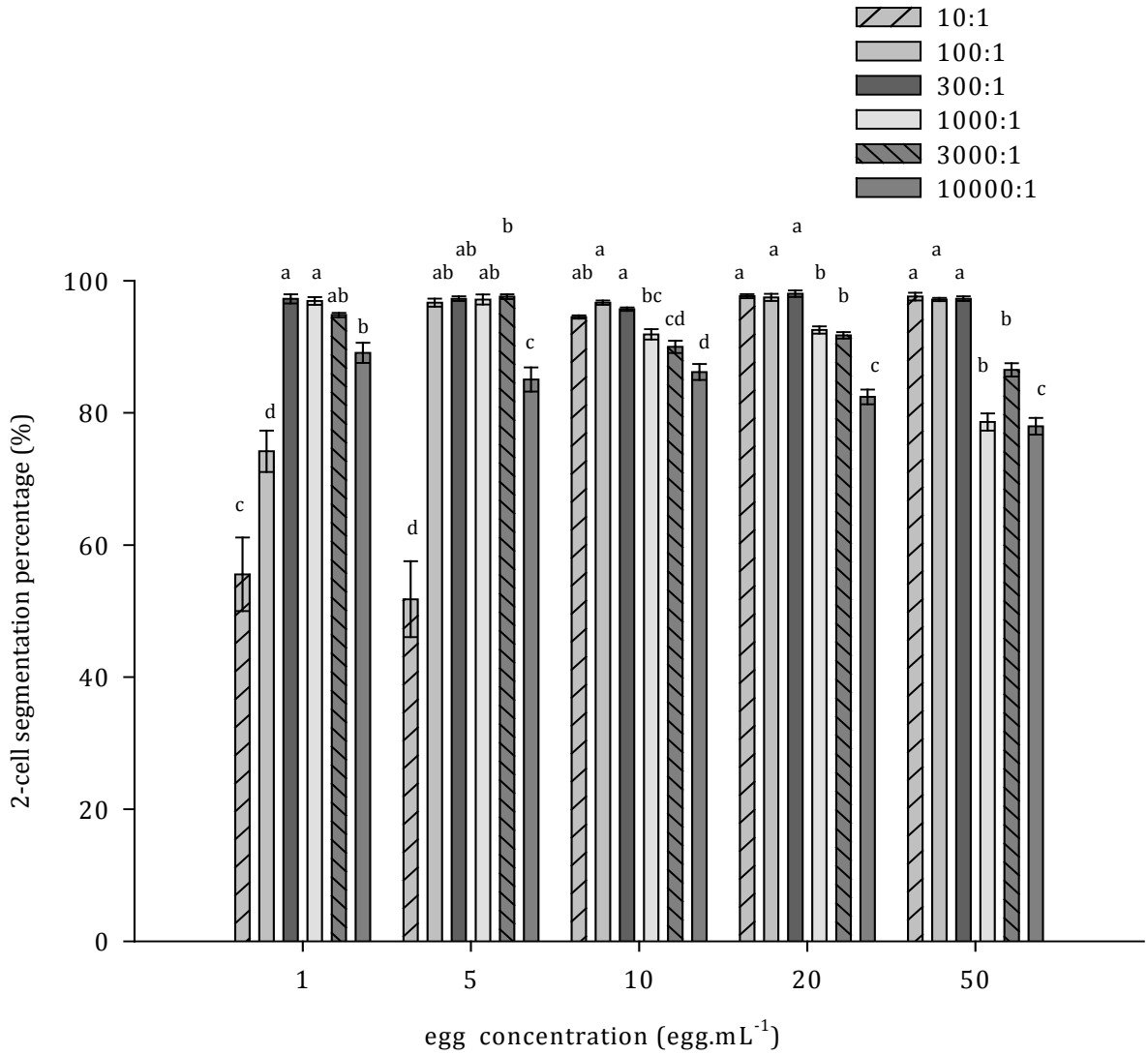

Figure 3 Mean values for 2-cell segmentation stage development (%) ± standard error, of *P. lividus,* after 90 minutes post-fertilization. Different letters at the top of the bars, represent statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments within the same egg concentration.

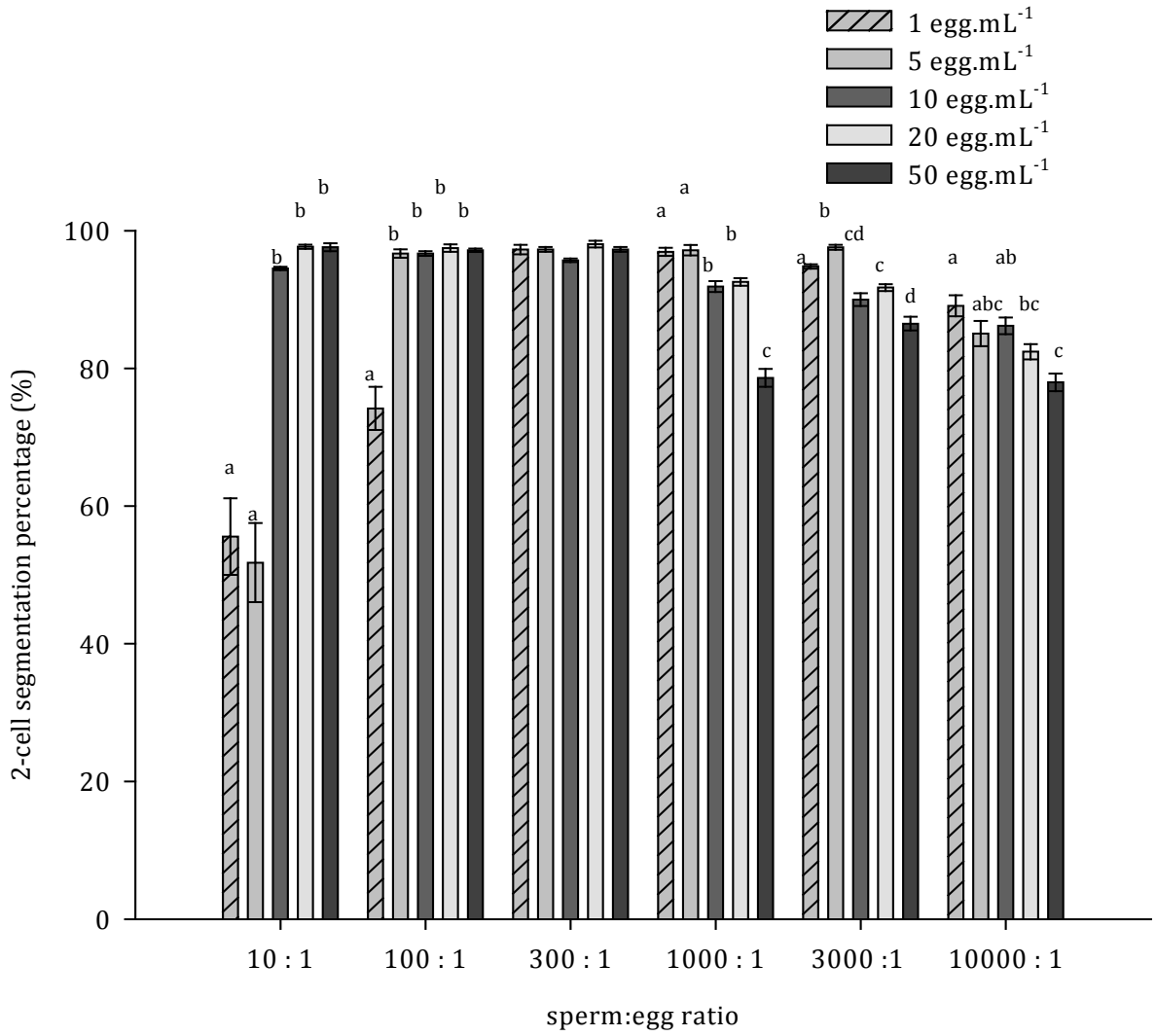

Figure 4 Mean values for 2-cell segmentation stage development (%) ± standard error, of *P. lividus,* after 90 minutes post-fertilization. Different letters at the top of the bars, represent statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments within the same sperm:egg ratio.

Correlation results between 2-cell segmentation development, egg concentration and sperm:egg ratios, are presented in table 6. A negative statistical correlation ($rs = -0.305$, $p<0.05$) was found between 2-cell segmentation and sperm:egg ratio variables, which indicates a tendency of 2-cell segmentation to decrease while sperm:egg ratio increases.

Table 6 Spearman rank order correlation between 2-cell segmentation development, egg concentration and sperm:egg ratios.

	Egg concentration	2-cell segmentation	Sperm:egg ratio
Egg concentration	----------	rs = 0.0856	rs = 0.000
	----------	p = 0.422	p = 1.000
2-cell segmentation	----------	----------	rs = -0.305
	----------	----------	p = 0.00355
Sperm:egg ratio	----------	----------	----------
	----------	----------	----------

Results of mean values for *P. lividus* percent 4–cell segmentation development, after 90 minutes post-fertilization are shown in figure 5. Statistical differences (Kruskal-wallis, Tukey test, $p<0.05$) were only found between different egg concentration groups at 1000:1 and 10000:1 sperm:egg ratios. For experimental groups concerning 1, 5, 10, 20 and 50 eggs.mL^{-1} (10:1, 100:1 and 300:1 sperm:egg ratios), 1 and 5 eggs.mL^{-1} (1000:1 sperm:egg ratio) and 1, 5, 20, 50 eggs.mL^{-1} (10000:1 sperm:egg ratio), no 4-cell segmentation was observed.

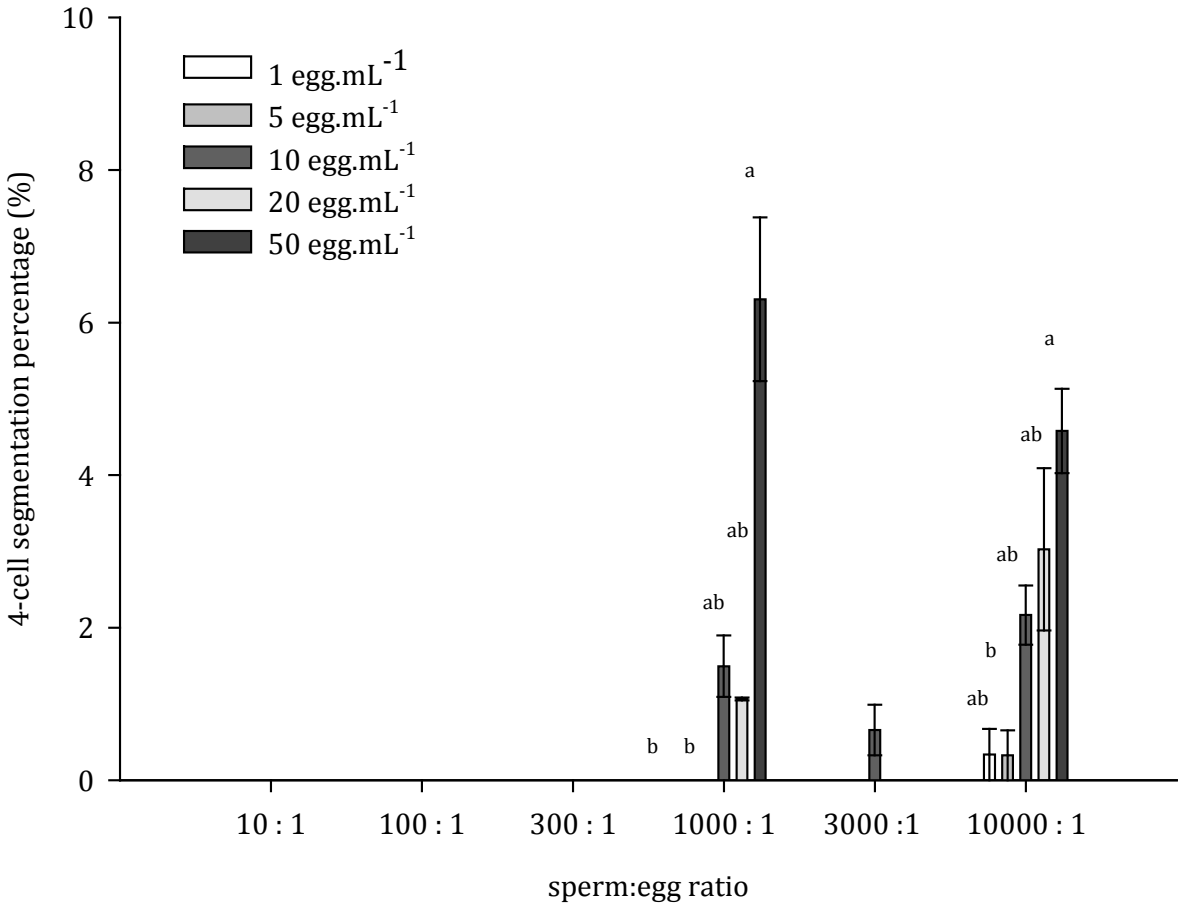

Figure 5 Mean values for 4-cell segmentation stage development (%) ± standard error, of *P. lividus,* after 90 minutes post-fertilization. Different letters at the top of the bars, represent statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) between treatments between treatments within the same sperm:egg ratio.

Correlation results between 4-cell segmentation development, egg concentration and sperm:egg ratios, are presented in table 7. Positive statistical correlations were found between 4-cell segmentation *vs*. sperm:egg ratio ($rs = 0.526$, $p<0.001$) and 4-cell segmentation *vs*. egg concentration ($rs = 0.315$, $p<0.05$) variables.

Table 7 Spearman rank order correlation between 4-cell segmentation development, egg concentration and sperm:egg ratios.

	Egg concentration	4-cell segmentation	Sperm:egg ratio
Egg concentration	----------	rs = 0.315	rs = 0.000
	----------	p = 0.00262	p = 1.000
4-cell segmentation	----------	----------	rs = 0.526
	----------	----------	p = 0.000
Sperm:egg ratio	----------	----------	----------
	----------	----------	----------

Results of mean values for *P. lividus* fertilized undeveloped eggs, after 90 minutes post-fertilization are shown in figures 6 and 7. Statistical differences (ANOVA, Tukey test, $p<0.05$) were found between all egg concentration treatment groups at different sperm:egg ratios, with the exception for 1, 5, 10, 20 and 50 eggs.mL^{-1} at 300:1 and 10000:1 sperm:egg ratios. Additionally, a general increment of undeveloped fertilized eggs can be observed, for all egg concentration treatments groups, between 3000:1 and 10000:1 sperm:egg ratios. Results concerning treatment 50 eggs.mL^{-1} (1000:1 and 3000:1 sperm:egg ratios) were significantly higher (ANOVA, Tukey test, $p<0.05$) in comparison to remaining egg concentration treatments while significant differences (ANOVA, Tukey test, $p<0.05$) for the same treatment at 10:1 sperm:egg ratio were only found with 1, 5 and 10 eggs.mL^{-1} experimental groups. Likewise, significant differences (ANOVA, Tukey test, $p<0.05$) were found for treatments with 1 and 10 eggs.mL^{-1} (10000:1 sperm:egg ratio) *vs*. 10:1, 100:1, 300:1 and 1000:1 experimental sperm:egg ratio groups. Additionally, treatment 5 eggs.mL^{-1} (10:1 sperm:egg ratio) was significantly higher (ANOVA, Tukey test, $p<0.05$) in comparison to remaining sperm:egg ratio treatments.

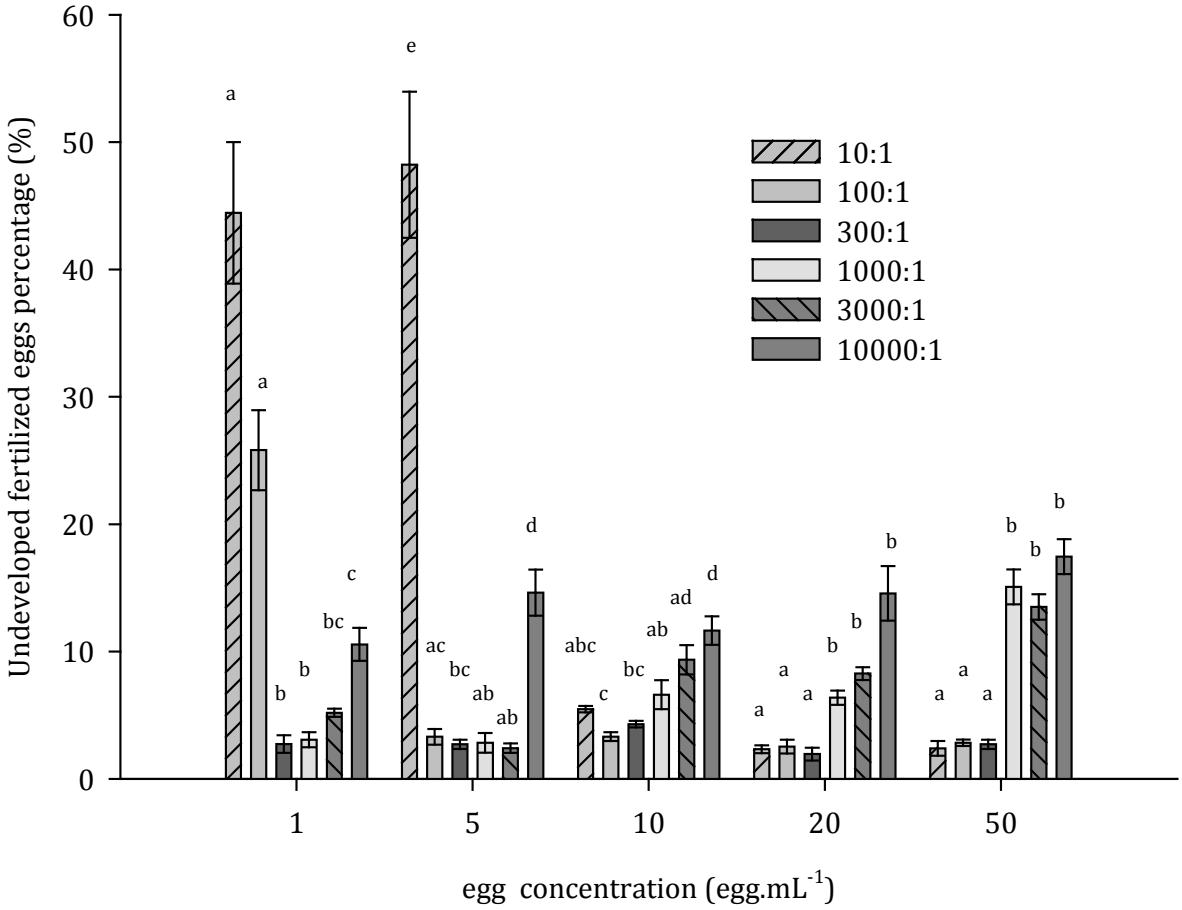

Figure 6 Mean values for fertilized undeveloped eggs percentage (%) ± standard error, of *P. lividus,* after 90 minutes post-fertilization. Different letters at the top of the bars, represent statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments within the same egg concentration.

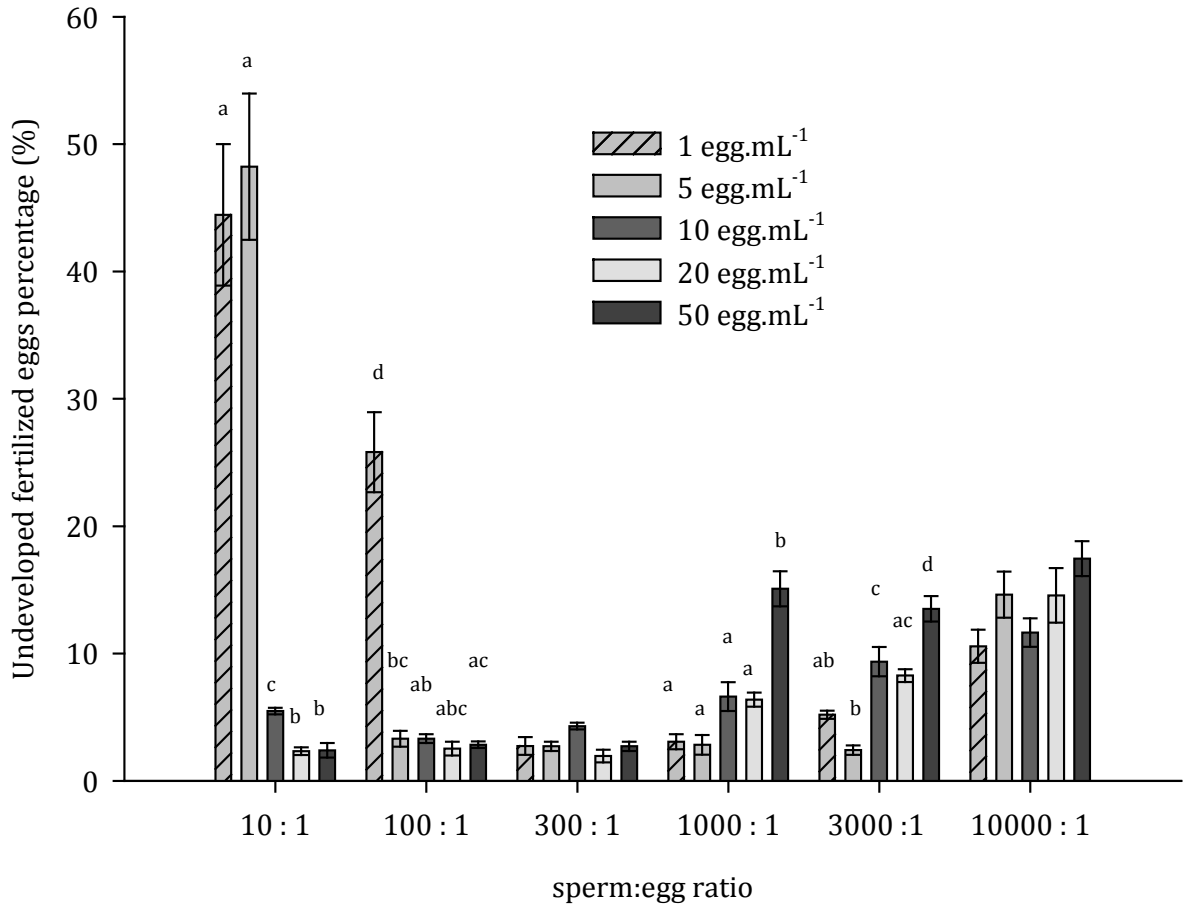

Figure 7 Mean values for fertilized undeveloped eggs percentage (%) ± standard error, of *P. lividus,* after 90 minutes post-fertilization. Different letters at the top of the bars, represent statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments within the same sperm:egg ratio.

Correlation results between fertilized undeveloped eggs development, egg concentration and sperm:egg ratios, are presented in table 8. A positive statistical correlation ($rs = 0.304$, $p<0.05$) was found between fertilized undeveloped eggs *vs*. sperm:egg ratio, indicating a tendency of fertilized undeveloped eggs to increase together with increasing sperm:egg ratios.

Table 8 Spearman rank order correlation between fertilized undeveloped eggs, egg concentration and sperm:egg ratios.

	Egg concentration	Fertilized undeveloped eggs	Sperm:egg ratio
Egg concentration	----------	rs = -0.102	rs = 0.000
	----------	p = 0.337	p = 1.000
Fertilized undeveloped eggs	----------	----------	rs = 0.304
	----------	----------	p = 0.00371
Sperm:egg ratio	----------	----------	----------
	----------	----------	----------

3.3 Larvae development

3.3.1 Larvae total length

Results concerning total larvae length of *P. lividus* four-armed plutei are shown in table 9. No statistical differences (Kruskal-Wallis, p>0.05) were found between single egg concentration *vs*. different sperm:egg ratios and equal sperm:egg ratio *vs*. different egg concentration treatments.

Table 9 Mean values for total length (µm) ± standard error of *P. lividus* four-armed plutei, after 72h post-fertilization. Top row indicates egg concentration (eggs.mL^{-1}). First column indicates sperm:egg ratios. For the experimental trial concerning 1egg.mL^{-1} and 10:1 (sperm:egg ratio), results are not shown due to the absence of larvae within the replicate beakers.

	1	5	10	20	50
10:1	-------	**488** ± 4.24	**490** ± 4.47	495 ±2.64	**493** ± 2.55
100:1	**491** ± 3.02	**492** ± 1.97	**489** ± 1.98	**494** ±2.03	**489** ± 2.07
300:1	**493** ± 1.91	**494** ± 1.92	**491** ± 1.82	**492** ±2.58	**489** ± 2.14
1000:1	**492** ± 1.80	**487** ± 2.16	**490** ± 1.97	**491** ±2.18	**490** ± 2.07
3000:1	**493** ± 1.92	**489** ± 2.01	**486** ± 2.12	**491** ±1.97	**485** ± 2.13
10000:1	**494** ± 1.75	486 ± 2.15	**488** ± 2.05	**493** ±2.11	**488** ± 2.11

3.3.2 Larvae post-oral arm length

Mean values for post-oral arm length of *P. lividus* larvae are shown in table 10. Statistical differences (Kruskal-Wallis, Tukey test, p<0.05) were found in treatments comparing 3000:1 sperm:egg ratio *vs*. different egg concentrations. No statistical differences (Kruskal-Wallis, p>0.05) were found when comparing treatments with single egg concentration *vs*. different sperm:egg ratios treatments.

Table 10 Mean values for post-oral arm length (µm) ± standard error of *P. lividus* four-armed plutei, after 72h post-fertilization. Mean values followed by different letters represent statistical differences (Kruskal-Wallis, Tukey test, p<0.05) between treatments with the same sperm:egg ratio. Top row indicates egg concentration (eggs.mL^{-1}). First column indicates sperm:egg ratio. For the experimental trial concerning 1egg.mL^{-1} and 10:1 (sperm:egg ratio), results are not shown due to the absence of larvae within the replicate beakers.

	1	5	10	20	50
10:1	--------	**195** ±2.65	**193** ±2.34	**194** ±1.23	**190** ±1.20
100:1	**193** ±2.67	**193** ±1.16	**192** ±1.09	**194** ±0.911	**194** ±0.952
300:1	**195** ±1.14	**193** ±1.6 4	**195** ±1.26	**191** ±1.17	**195** ±1.08
1000:1	**195** ±1.23	**192** ±1.11	**192** ±1.14	**194** ±1.16	**190** ±1.10
3000:1	**195** ±1.05 ab	**190** ±1.09 b	**193** ±1.13 ab	**196** ±0.930 a	**191** ±1.11 b
10000:1	**195** ±1.35	**192** ±1.18	**193** ±1.18	**191** ±1.12	**193** ±1.10

Mean pooled data values for post-oral arm length of *P. lividus* larvae are shown in figure 8. Statistical differences (Kruskal-Wallis, Dunn´s method, $p<0.05$) were found between treatments of 1 egg.mL^{-1} *vs*. 50 egg.mL^{-1}.

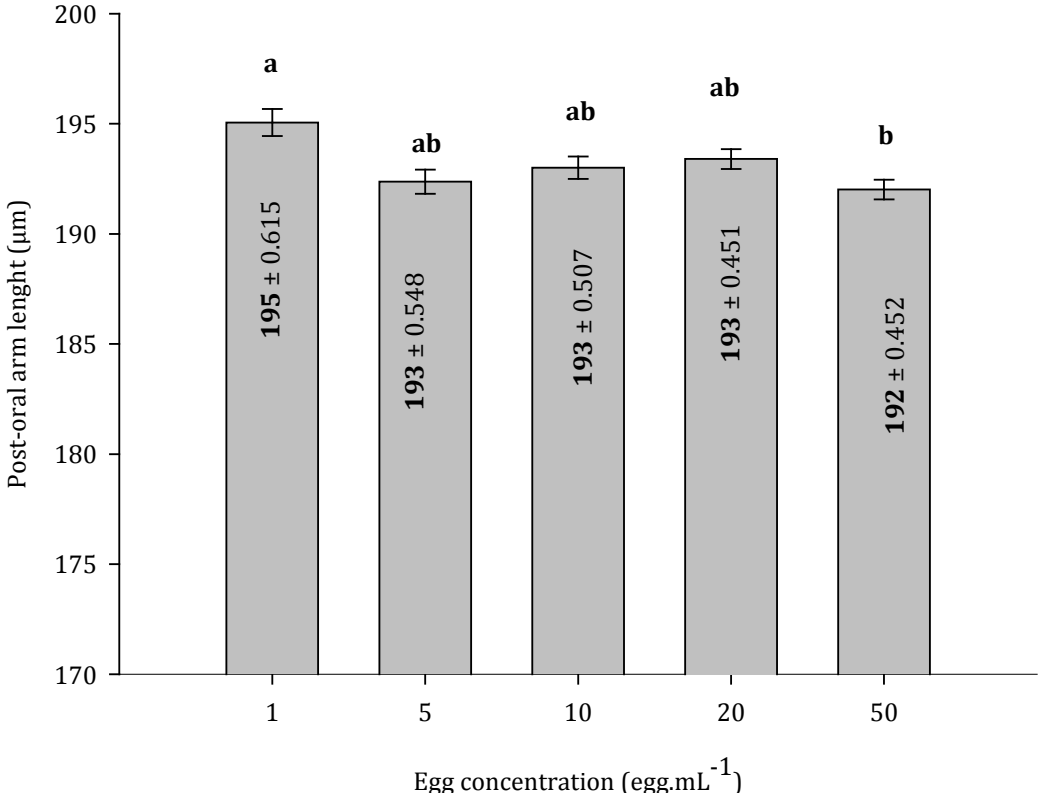

Figure 8 Average post-oral arm length (µm) ± standard error of *P. lividus* four-armed plutei, after 72h post-fertilization. Each egg concentration corresponds to pooled data from results obtained for different sperm:egg ratios among the same egg concentration treatment. Different letters placed at the top of the bars indicate statistical differences (Kruskal-Wallis, Dunn´s method, $p<0.05$) between treatments.

3.3.3 Normal percent larvae development

Results concerning normal percent larval development of *P. lividus* four-armed plutei are shown in table 11. Statistical differences (ANOVA, Tukey test, $p<0.05$) were found between different egg concentrations, for the same sperm:egg ratios. No statistical differences (ANOVA, Tukey test, $p>0.05$) were found between equal egg concentration *vs*. different sperm:egg ratios treatments.

Table 11 Mean values for normal percent larval development of *P. lividus* four-armed plutei (µm) ± standard error, after 72h post-fertilization. Mean values followed by different letters represent statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments with the same sperm:egg ratio. Top row indicates egg concentration (eggs.mL^{-1}). First column indicates sperm:egg ratios. For the experimental trial concerning 1egg.mL^{-1} and 10:1 (sperm:egg ratio), results are not shown due to the absence of larvae within the replicate beakers.

	1	5	10	20	50
10:1	--------	**84.9** ±0.794 ab	**86.3** ±0.595 b	**78.9** ±2.20 ac	**77.8** ±2.32 c
100:1	**82.1** ±3.57 ab	**81.1** ±1.12 ab	**84.4** ±1.31 a	**82.2** ±1.19 ab	**74.4** ±1.12 b
300:1	**86.7** ±1.93 a	**83.3** ±1.93 ab	**81.1** ±1.12 ab	**81.2** ±1.15 ab	**77.8** ±1.21 b
1000:1	**83.3** ±1.92 ab	**85.5** ±1.17 a	**83.4** ±1.95 ab	**81.1** ±2.22 ab	**75.6** ±2.26 b
3000:1	**82.3** ±1.13 b	**83.3** ±1.41 b	**82.**2 ±1.11 b	**80.1** ±1.93 ab	**73.3** ±1.82 a
10000:1	**85.6** ±1.01 a	**84.4** ±2.23 ab	**83.4** ±1.62 ab	**78.9** ±2.02 ab	**75.5** ±1.10 b

Mean pooled data values for normal percent larval development of *P. lividus* larvae are shown in figure 9. Statistical differences (Kruskal-Wallis, Dunn´s method, $p<0.05$) were found between treatments 50 egg.mL^{-1} *vs*. 1, 5 and 10 egg.mL^{-1}.

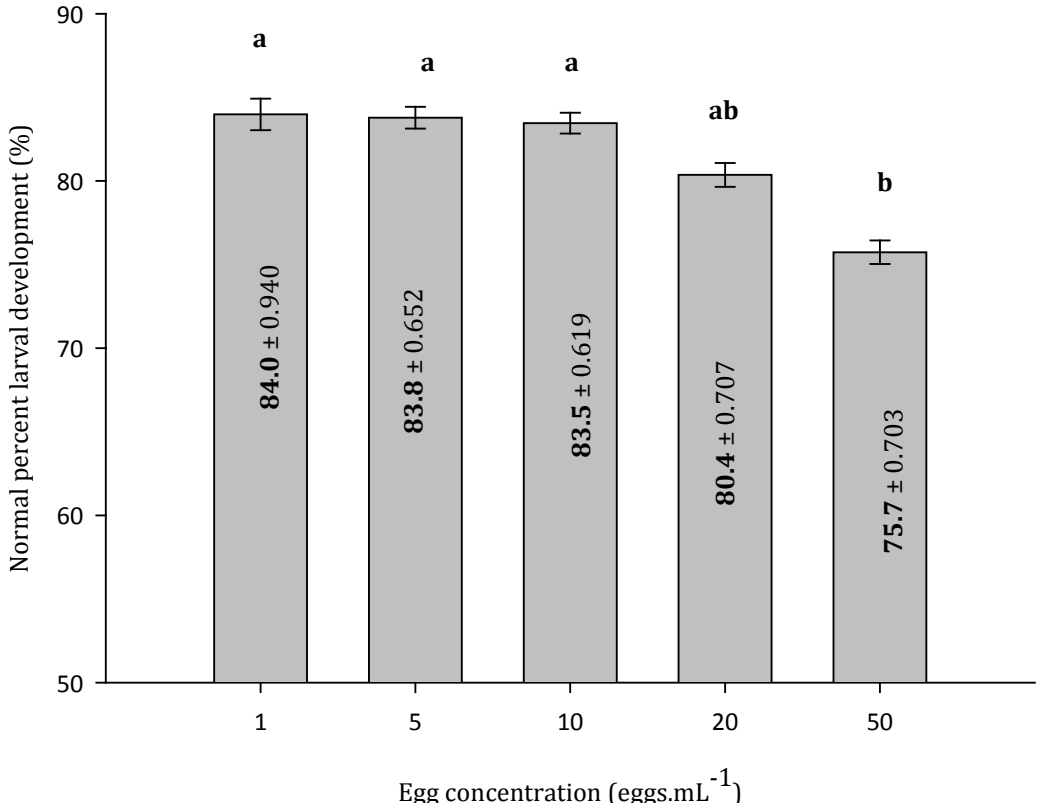

Figure 9 Average values for normal percent larval development (%) ± standard error of *P. lividus* four-armed plutei, after 72h post-fertilization. Each egg concentration corresponds to pooled data from results obtained for different sperm:egg ratios among the same egg concentration treatment. Different letters placed at the top of the bars indicate statistical differences (Kruskal-Wallis, Dunn´s method, $p<0.05$) between treatments.

Correlation between normal percent larvae development, egg concentration and sperm:egg ratios, is presented in table 12. A negative statistical correlation (rs = -0.652, p<0.001) was found between percent normal larvae development *vs*. egg concentration.

Table 12 Spearman rank order correlation between normal percent larvae development, egg concentration and sperm:egg ratios.

	Egg concentration	Normal percent larvae development	Sperm:egg ratio
Egg concentration	----------	rs = -0.652	rs = -0.0739
	----------	p = 0.000	p = 0.496
Normal percent larvae development	----------	----------	rs = -0.0189
	----------	----------	p = 0.862
Sperm:egg ratio	----------	----------	----------
	----------	----------	----------

4. Discussion

4.1 Percent egg fertilization

It is well known that several factors affect egg fertilization in sea urchins (Levitan *et al.*, 1991). Sperm age and dilution, egg concentration as well as sperm-egg contact time or abiotic conditions can produce beneficial or detrimental effects over external fertilization success within these organisms.

In our experiments, we used *P. lividus* gametes obtained from several females and males individuals, in order to diminish or even avoid influence derived from possible maternal or paternal effects, such as nutritional or even genetic. Furthermore sea urchins were collected at the same time in the same area.

Our results show that percent egg fertilization varied from 2.9 % (1 egg.mL^{-1} and 10:1 sperm:egg ratio treatment) to 99.0 % (1 egg.mL^{-1} and 10000:1 sperm:egg ratio treatment), within the experimental variables tested. Previous studies (Levitan *et al.*, 1991) have concluded that low sperm concentrations (47 sperm cells.mL^{-1}) cannot sustain fertilization success in *Strongylocentrotus franciscanus*. In our experiments, low percent egg fertilization results, below 15.7 %, were obtained for 1, 5, 10 eggs.mL^{-1} with 10:1 and 1 egg.mL^{-1} with 100:1 sperm:egg ratios. These results could be explained due to the low sperm concentration (number of sperm cells.mL^{-1} of volume) that could have possibly unable higher fertilization rates values.

With the exception for 300:1 and 1000:1 sperm:egg ratios *vs.* different egg concentration treatments, significant effects were found on percent egg fertilization over remaining experimental trials, where combinations of same sperm:egg ratio *vs*. different egg concentrations were tested. A positive correlation was found between percent egg fertilization and sperm:egg ratio, which indicates a significant relationship between these two variables.

Percent egg fertilization was always above 90%, with the exception for treatments with 1, 5, 10 eggs.mL^{-1} with 10:1 and 1 egg.mL^{-1} with 100:1 sperm:egg ratio groups. According to results, it seems plausible to assume that sperm concentrations below 100 sperm cells.mL^{-1}, could have a deleterious effect over fertilization success outcome, while concentrations above 200 sperm cells.mL^{-1} enable fertilization rates above 90%, whatever egg concentration is used.

Likewise, since no significant correlation was found between egg concentration and percent egg fertilization, the range of egg concentrations tested seems not to have a significant effect on percent egg fertilization results, what agrees with previous studies concerning *Strongylocentrotus franciscanus* (Levitan *et al.*, 1991) and *Echinometra mathasci* (Rahman *el al.*, 2001). Sperm-egg contact time as well as sperm age are also important factors that influence fertilization success (Levitan *et al.*, 1991; Levitan, 2000), but in the present study these parameters were standardized for all treatments and were not individually addressed.

4.2 Percent segmentation stage development

In order to evaluate percent segmentation stage development as possible polyspermy determination endpoint, we have assessed percentage of fertilized undeveloped eggs, 2 and 4-cell embryo segmentation stage after 90 minutes post-fertilization, resulting from *P. lividus* fertilization events at different sperm:egg ratios and egg concentrations. Our results show that 2-cell segmentation development varied from 51.8 % (5 egg and 10:1 sperm:egg ratio) to 98.1 % (20 egg.mL^{-1} and 300:1 sperm:egg ratio), within the experimental combinations tested.

The lowest 2-cell segmentation development values were obtained for the combinations of 1-5 eggs.mL^{-1} with 10:1 sperm:egg ratio and 1 eggs.mL^{-1} with 100:1 sperm:egg ratio. However and by comparison with remaining treatments and overall results, these results are likely to be a consequence of the small sample size of fertilized eggs

analyzed within these treatments, due to the low percent egg fertilization results, rather than a polyspermy outcome event. Our data also shows an overall decrease within the number of 2-cell segmented embryos for the different egg concentration groups tested, from 3000:1 to 10000:1 sperm:egg ratios and for 20 egg.mL^{-1} towards 50 egg.mL^{-1} experimental groups, within all range of sperm:egg ratios tested. Additionally and whereas 4-cell and undeveloped fertilized eggs are concerned, we observed a simultaneous increase within the percentage of these parameters, from 3000:1 to 10000:1 sperm:egg ratio and for 20 egg.mL^{-1} towards 50 egg.mL^{-1} experimental groups, although for some of the experimental egg concentration and sperm:egg ratios analyzed, 4-cell stages were not observed. Levitan *et al* (2007) addressing the risk of polyspermy in *Strongylocentrotus purpuratus, Strongylocentrotus franciscanus* and *Strongylocentrotus droebachiensis* observed that under excess sperm concentrations, sea urchin eggs were sometimes as he termed "obliterated", leading them to conclude that egg death could have been due to excess sperm-egg collisions instead of excess fusion events. Within our experiments, we observed undeveloped fertilized eggs that did not present a spherical morphology (check Figure 1e). Whether or not, egg development stall was due to excess sperm-egg fusions or collisions, we cannot precise it because normal and abnormal shaped undeveloped fertilized eggs were all accounted together.

A negative correlation was found between 2-cell segmentation development *vs*. sperm:egg ratio, which indicates a significant relationship between these two variables. Additionally, positive correlations were found between 4-cell segmentation development *vs*. sperm:egg ratio and fertilized undeveloped eggs development *vs*. sperm:egg ratio, which possibly indicates that increasing sperm:egg ratios could have an effect on *P. lividus* egg segmentation, which could be linked to polyspermic events during fertilization.

Our results also show an overall decrease in 2-cell egg segmentation within all egg concentration treatment groups beyond the 3000:1 sperm:egg ratio tested. Concerning egg concentration effect over percent segmentation stage development, 4-cell segmented eggs were the only parameter whereas a positive correlation was found. In this case, it seems that increasing numbers of 4-cell stage embryos could possibly derive from increasing sperm concentration and consequently be a result of polyspermic events that occurred during fertilization. Although sea urchin early live stages segmentation is quite synchronous, 4-cell staged embryos, should not be observable at 90 minutes post-fertilization, under the experimental conditions used in this study. In this way, it seems plausible to say that the observed 4-cell embryos could indicate polyspermy during fertilization.

The obtained results allow us to conclude that sperm:egg ratio should be the main factor influencing percent segmentation stage development in *P. lividus*.

4.3 Larvae development

In this section, we aimed to check if different sperm:egg ratios and egg concentrations used during *P. lividus in vitro* fertilization, could have direct influence over 4-arm plutei total length, post-oral arm length and normal staged 4-arm plutei percentage, as a result of possible abnormal development of polyspermic embryos. The results show no effects of egg concentrations *vs*. sperm:egg ratios tested over larvae total length values. With the exception for the 3000:1 sperm:egg ratio *vs*. different egg concentration treatments, no statistical differences were found whereas post-oral-arm length is concerned, but no logical explanation could be found in order to explain this result.

Whenever more than one sperm cell fuses an egg, resultant embryo development stops and polyspermic zygote dies (Wong and Wessel, 2004; Vacquier, 2011). Our results sustain this hypothesis once a near absence of statistical differences on larvae total length and post-oral arm length was observed between treatments, which lead us to conclude that these larvae biometric parameters cannot be used as endpoint to determine if polyspermic events have occurred or not. In opposition, statistical differences were found when pooled data concerning same egg concentration for post-oral arm length parameter were analyzed, namely 50 eggs.mL^{-1} *vs*. 1 egg.mL^{-1} treatments. These results indicate that observed differences could be possibly due to delayed growth of plutei post-oral arms and damage, both as a consequence of different culture densities, with special emphasis to 50 eggs.mL^{-1} treatment.

Results concerning percent normal larvae development showed statistical differences ($p<0.05$) between treatments opposing sperm:egg ratios *vs*. different egg concentrations. Likewise a statistically negative correlation was found between egg concentration *vs*. percent normal larvae development, in opposition to sperm:egg ratios *vs*. percent normal larvae development. Using the same rationale as for results obtained from larvae biometric measurements, we can conclude that the obtained data is possibly correlated with culture conditions, i.e. larvae density, instead of larvae that developed from polyspermic embryos, once it is well known that resulting zygotes eventually die. Additionally and to sustain this conclusion, an overall decrease in normal percent larvae development was observed when

pooled data from the same egg concentrations with different sperm:egg ratios was compared (1, 5, 10, 20 and 50 eggs.mL^{-1}).

Another factor that could possibly explain the obtained results, concerning normal percent larvae development, could have been related with the nutritional status of the wild *P. lividus* adults used to perform the experiment and consequently their gametes. It is well known that gamete quality and larvae development is strongly dependent upon adult nutritional status (George *et al.*, 1990; Meidel *et al.,* 1999). However in our study, *P. lividus* adults were collected during the same time scale, geographical area and spawning season. Likewise, similar values for normal larvae total length (520±15,6 μm) were obtained in a previous study (Repolho *et al.*, 2011), with individuals collected within the same annual time frame, location and reproductive season. Although a biochemical analysis of gametes, i.e. eggs, was not performed, these assumptions led us to conclude that it seems less likely that normal percent larvae development results obtained in the present study, could be related or due to natural nutritional deficiencies.

5. Conclusions

Considering the results obtained in this work, we can conclude that sperm:egg ratio is a fertilization parameter that influences percent egg fertilization and percent segmentation stage development in *P. lividus*. Egg concentration (1, 5, 10, 20 and 50 eggs.mL^{-1}) is not statistically correlated with percent egg fertilization but could have a negative impact on larvae development, i.e post-oral arm length and normal 4-arm plutei percentage. Results indicate that normal percent larvae development decreased as a possible consequence from increasing culture densities. High percent egg fertilization values, above 90%, were obtained for sperm concentrations above 200 sperm cells.mL^{-1}. *Paracentrotus lividus* 2-cell egg segmentation was negatively correlated with increasing sperm:egg ratio. In opposition, 4-cell egg segmentation and undeveloped fertilized eggs were positively correlated with increasing sperm:egg ratios.

Among the parameters tested, sperm:egg ratio values above 300:1, seem to produce a deleterious effect over percent segmentation egg development, regardless of egg concentration used. This study highlights the importance of performing fertilization assays of *P. lividus*, with appropriate egg concentration *vs*. sperm:egg ratio, specially when certain parameters, such as fertilization rates and embryo, larvae development are used as endpoint

assessment tools, in scientific areas such as aquatic ecotoxicology or pharmaceutical toxicology.

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CHAPTER 4

Culture conditions of the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) exotrophic plutei larvae: Implications to larvae production

Tiago Repolho, João Gago and Orlando Luís

Submitted to *Aquaculture*

Culture conditions of the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) exotrophic plutei larvae: Implications to larvae production

Abstract

This study evaluated the effects of different *Paracentrotus lividus* larviculture biotic and abiotic parameters on larvae stage, development and survival, during an 18-day culture period. Additionally, the possibility to mass-produce *P. lividus* 4-arm plutei (40 plutei.mL^{-1}) until 5 and 10 days post-fertilization (DPF), using *D. tertiolecta* and 2 microdiets (Frippak 1#CAR; Lanzy spirulina+) as plutei feed, besides the effect of Algamac (3050 flake) enrichment, over plutei survival (5 and 10 DPF), were assessed. Abiotic culture conditions (aeration, culture water renovation) and the utilization of different feeds (*Dunaliella tertiolecta* and two microencapsulated/microbound diets), feeding regimes (single feed or mixed) and stocking densities (1.5, 3, 5, 7.5, 10 and 20 plutei.mL^{-1}) were studied to assess 4, 6 and 8-arm plutei development and survival. Survival until 18 days post-fertilization (DPF) was only achieved when aeration and a water renewal (every 2 or 4 days) of culture vessels was performed. Our results show that water renewal of culture media is an essential procedure to sustain larvae survival and development, until 18 DPF. Statistical differences were found between feed experimental groups. All feeds, single (*Dunaliella tertiolecta*, Frippak #1CAR, Lanzy spirulina+) or mixed provided, supported *P. lividus* larvae development and survival until 18 DPF. Microalgae *D. tertiolecta* proved to be the most appropriate diet, in terms of plutei survival (51.1-53.3%) at 18 DPF, as well for 6 (92.8% - 93.3%) and 8-arm (96.4% - 97.8%) plutei stage development, at 12 and 18 DPF respectively. A negative effect on larvae survival was found with increased initial larvae culture densities. Increasing *P. lividus* plutei culture densities proved to have deleterious effects over larvae survival beyond 12 DPF. The best combination in terms of plutei development and survival at 18 DPF was achieved using an initial culture density of 1,5 larvae.mL^{-1}. High mortality was observed for Algamac enrichment treatments, with plutei survival rates varying between 6.31 - 8.75% (5 DPF) and 3.13 - 4.38% at 10 DPF. The microdiets Frippak (1#CAR) and Lanzy (spirulina+) treatments did not sustained larval survival until 10 DPF, at 40 plutei.mL^{-1} initial culture densities.

Mass production of *P. lividus* exotrophic plutei at higher densities (20 plutei.mL^{-1}), until 18 DPF is possible but with associated high mortality. Further development of artificial

feeds is needed to enhance *P. lividus* larvae growth and development, thus reducing associated hatchery costs where *P. lividus* larviculture is concerned.

Keywords: Larval development; sea urchin; *Paracentrotus lividus*; percent egg fertilization; *Dunaliella tertiolecta*; plutei.

1. Introduction

Over the years, biological models such as sea urchin embryo and larvae have received increased attention by the international research community. As bioindicators applied in environmental assessment (Caeiro *et al.*, 2009; Pétinay *et al.*, 2009; Álvarez *et al.*, 2010; Marín *et al.*, 2010; Fabbrocini and D'Adamo, 2011; Carballeira *et al.*, 2011), prediction of ocean acidification effects at organism and ecosystem levels (O'Donnell *et al.*, 2009; Byrne *et al.*, 2009; Clark *et al.*, 2009; O'Donnell *et al.*, 2010; Martin *et al.*, 2011; Reuter *et al.*, 2011) or used to study cellular, molecular and development mechanisms underlying biological processes (Ernst, 1997; Roccheri *et al.*, 1997; Voronina and Wessel, 2001; Dubois and Ameye, 2001; Vickery *et al.*, 2001; Briggs and Wessel, 2006; Thurber and Epel, 2007; Aluigi *et al.*, 2008; Agnello and Roccheri, 2010; Dale *et al.*, 2010; Tomšić *et al.*, 2011), sea urchin early development stages have proven their unquestionable importance as biological tools used in fundamental and applied science.

Sea urchin embryos and larvae are considered the most sensitive and complex development stages of these marine invertebrates, through out their life cycle. It is well known that sea urchin gamete size and quality, as well as larval development, survival and metamorphosis are strongly determined by both parental as well as larval nutritional status (George *et al.*, 1990; Bertram and Strathmann, 1998; Meidel *et al.,* 1999). Besides nutritional requirements, echinoderm larval development is directly dependent upon skeleton formation and plasticity, ciliary apparatus and digestive tract development, as well as environmental factors (Strathmann *et al*. 1992; Meidel *et al.*, 1999; McEdward and Herrera, 1999; Sewell *et al.*, 2004; George and Walker, 2007; Kashenko, 2007; Byrne *et al.*, 2008a; Byrne *et al.*, 2008b).

To date, larvae development, under captive conditions of several echinoderm species, has been thoroughly studied, including *P. lividus* (Fenaux *et al.*, 1985b; Gosselin and Jangoux, 1998; Vaïtilingon *et al.*, 2001; Liu *et al.*, 2007; Carboni *et al.*, 2012).

Once energy requirements of exotrophic larvae are mainly dependent of exogenous feeding, diets as well as feeding regimes are of crucial importance for the development, stage synchrony and survival to metamorphosis of functional planktotrophic feeding larvae. Likewise, if one desires to culture under captive conditions an aquatic species (i.e. sea urchin), several criteria have to be addressed. Development and optimization of embryo, larvae, juvenile and adult culture conditions, as well as diet (natural or artificially processed) and feeding regimes are of critical importance, if culture success is to be obtained.

Sea urchin larvae normally fed upon live unicellular organisms such as microalgae. The use of microalgal species as live feed for early life stages of these marine organisms, presents some advantages over artificial processed microdiets, such as: extended life span (once they are live organisms) and easier acceptance by predator larvae (Liu *et al.*, 2007). In this context, several microalgae species have been used in *P. lividus* larviculture, including *Dunaliella tertiolecta* (Liu *et al.*, 2007; Carboni *et al.*, 2012), *Monocrysis lutheri* (Fenaux *et al.*, 1985a), *Phaeodactylum tricornutum* (Grosjean *et al.*, 1996; Gosselin and Jangoux, 1998; Vaïtilingon *et al.*, 2001), *Cricosphaera elongata* and *Pleurochrysis carterae* (Carboni *et al.*, 2012).

Over the years, formulated microfeeds have received increased attention as an alternative feed source for marine larviculture. Cost effectiveness, less labour to produce and maintain, easily long-term storable and immediately available, customized biochemical composition according to species energetic and nutritional requirements and increased reduction in feed pathogenic contamination normally associated with live feed cultures, are some of the key factors that these microdiets possess *vs.* live feeds. Likewise, they can be used to incorporate other dietary components aimed to enhance disease resistance (probiotics and immunostimulants), promote growth or simply improve feed acceptability. But despite recent developments, further research is needed to overcome problems involved in the use of microdiets as larvae feeds, such as their water stability, nutrient leaching, palatability, digestibility, spatial availability and supply methodology under culture conditions, once they tend to sink upon deliver.

The complete larval development of a sea urchin species (*Lytechinus variegatus*) using a microencapsulated feed was achieved by George *et al.* (2004). Liu *et al.* (2007) accomplished the same for *P. lividus* but faster growth and a reduction in time necessary for larvae to reach competency was only achieved when using *Dunaliella tertiolecta* as live feed. Additionally, the microfeeds used only supported normal larvae development when delivered at a high feeding frequency. Where echinoplutei culture is concerned, further

development of artificial microfeeds is needed in order to overcome problems like adequacy of their biochemical composition for development stages energetic and nutritional needs. Once exotrophic plutei are known to selectively ingest feed particles upon flavor (Rassoulzadegan and Fenaux, 1979; Rassoulzadegan *et al.*, 1984; Pedrotti, 1995), the inclusion of attractants as also been suggested (Liu *et al.*, 2007).

During the last two decades, a growing interest in the aquaculture of *P. lividus* as been observed (Frantzis and Grémare, 1992; Fernandez, 1997; Basuyaux and Blin, 1998; Spirlet *et al.*, 1998; Fernandez and Boudouresque, 2000; Spirlet *et al.*, 2001; Shpigel *et al.*, 2004; Shpigel *et al.*, 2005; Shpigel et al., 2006; Cook and Kelly, 2007a, 2007b; Liu *et al.*, 2007; Symonds *et al.*, 2007; Pantazis, 2009; Carboni *et al.*, 2012), mainly driven by the overexploitation of natural stocks (Fernandez, 1997; Pais *et al.*, 2007) and increasing demand and commercial value of its gonads (Symonds *et al.*, 2007). Likewise, considerable effort has been applied in the development of culture methodology, behavior studying, feeding and nutritional requirements of *P. lividus* larvae (Rassoulzadegan and Fenaux, 1979; Fenaux *et al.*, 1985b; Bressan *et al.*, 1995; Gosselin and Jangoux, 1998; Vaïtilingon *et al.*, 2001; Liu *et al.*, 2007; Pétinay et al., 2009; Carboni *et al.*, 2012). Nevertheless, research aimed to ascertain nutritional needs of *P. lividus* larval stages needs further development (Liu *et al.*, 2007; Carboni *et al.*, 2012). Sea urchin larvae development and survival are intrinsically dependent upon several culture parameters such as water quality, temperature, type of feed and availability, feeding regimes and larval culture density.

Embryonic and larvae culture densities are of crucial importance for sea urchin survival to metamorphosis, both for basic research studies and large-scale echinoculture production. Several authors have already addressed this echinoculture constraint. Jimmy *et al.* (2003) have studied the large-scale production of *Echinus esculentus* in terms of quality and quantity effects of larval diet on survival, morphology and metamorphosis success. Also Buitrago *et al.* (2005) assessed the effect of larvae culture densities (0.25, 0.5 and 1 larvae mL-1), fed with *Chaetoceros muelleri*, to evaluate mass production of competent *Lytechinus variegatus* larvae. To date *P. lividus* early life stages development has been based upon different embryo and larvae culture densities (Rassoulzadegan and Fenaux, 1979; Fenaux *et al.*, 1985a, 1985b; Bressan *et al.*, 1995; Grosjean *et al.*, 1996; Gosselin and Jangoux, 1998; Vaïtilingon *et al.*, 2001; Liu *et al.*, 2007; Pétinay *et al.*, 2009; Carboni *et al.*, 2012).

Depending on research objectives, if sea urchin larvae development and survival are not to be compromised, embryo and larvae culture densities need to be established. Besides

one can hypothesize that higher larviculture densities can increase larvae collision events and subsequent malformations, leading to abnormal plutei development.

In this study, our aim was to test the effects of different *P. lividus* larviculture biotic and abiotic parameters on larvae stage, development and survival, during an 18-day culture period. We therefore inferred the implications of different feeds (microalgae and microencapsulated/microbound diets), feeding regimes (single feed or mixed), stocking densities (1.5, 3, 5, 7.5, 10 and 20 plutei.mL^{-1}) and abiotic culture conditions (aeration; culture water renovation) over the 4, 6 and 8-arm plutei development and survival. We also discuss the possibility to mass-produce *P. lividus* exotrophic larvae.

2. Material and methods

2.1 Collection of adult sea urchins

Adult sea urchins of the species *P. lividus*, were collected from wild populations during February-March 2011, at Cabo Raso, Portugal (38°42´30”N/9°29´11”W). Selection of collection site was based upon criteria described elsewhere (Repolho *et al.*, 2011). Sea urchins were transported in thermal cases and completely immersed, at all times, until they were stocked in the captive rearing system.

2.2 Adult rearing system

A salinity and temperature adjustment, in the recirculating aquaculture system (RAS) was performed, according to field measured parameters. Adult *P. lividus* (mean ± standard error) with 4.8±0.054 cm (test width, TW), 2.3±0.036 cm (test height, TH) and 54.0±1,54 g (total wet weight, TWW) were randomly stocked (±100 individuals.m^{-2}) in eight 400L black fiberglass tanks (0.4 m^3 in volume and 2.51 m^2 of total submerged area, per tank) within the RAS (3,48 m^3 total volume), equipped with a magnetic pump (Iwaki MX-70VM-13, Iwaki co, Ltd, Japan) for water circulation. Water flow per each culture tank was set to 8.5L.min^{-1} (55% water renewal.hour^{-1}).

The RAS was equipped with biological (ouriço®, Fernando Ribeiro Lda, Portugal), mechanical (Glass Wool) and physical (Jetskim 200, Schuran Gbr, Germany) filters, as well as UV sterilization (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany). Water

temperature was maintained through an automatic thermoregulation system (Frimar, Fernando Ribeiro Lda, Portugal).

Aeration was performed by means of an air blower (Hiblow air pump HP-80, Techno Takatsuki Co. Ltd, Japan). Culture parameters were as follows: Temperature (18±1 °C, Luís *et al.*, 2005), photoperiod (14:10 (L:D), Luís *et al.*, 2005) and salinity (35±1 PSU). Illuminance was set to 700 Lux. Artificial illumination was mechanically timed and provided to each tank by overhead fluorescent light bulbs (Digilamp FL-40SS/36 Daylight, 6400K, 2450 lumen). Temperature (predictor IT808 digital thermometer, Omega Pharma, Netherlands) was daily checked. A 5% RAS water change was performed once a week, while cleaning bottom tank debris.

2.3 Adult feeding

After field collection, adult individuals were maintained in the culture system without feed supply for a period of four weeks. After inanition period, feeding was supplied twice a week, during a 12-week time period, by scattering pelleted inert feeds over adult sea urchins. Adult feed composition in described elsewhere (Gago *et al.*, 2009). The supplied dry pelleted feed was based upon a vegetable based protein source (wheat and maize flour mix), with cod liver oil as surplus lipid source, including low-chain polyunsaturated fatty acids.

2.4 Spawning induction, egg and sperm collection

Adults *P. lividus* were induced to spawn, by means of a 0,5 mL (TW<4.9 cm) or 1 mL (TW>5.0 cm) KCl injection (0.5M) through the peristomial membrane. Afterwards they were placed individually in 400 mL glass beakers, filled with filtered (0.35 μm) and UV sterilized seawater. During spawning procedures, sea urchins were kept completely immersed and for a maximum of thirty minutes (Leahy, 1986). Each spermatozoa spawning was checked for mobility under 400x optic microscopy (Axiostar Plus, Carl Zeiss, Gottinngen, Germany) observations. Oocytes and spermatozoa were pooled separately, according to gamete type.

Gamete concentrations were estimated for each pool: Oocyte concentration was checked by observing three 1mL sub samples, under 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany); spermatozoa concentration was checked through haemacytometer observations (Bürker chamber, Germany), under 400x optic microscopy (Axiostar Plus, Carl Zeiss, Gottinngen, Germany).

2.5 *in vitro* fertilization

Fertilization assays were performed in 800 mL glass beakers (Base area (BA) = 63,6 cm^2), at a 3000:1 sperm:egg ratio. After fifteen minutes, three samples of 10mL were taken from the fertilization beakers and preserved in neutralized formaldehyde (4%). Percent egg fertilization was determined, by observing presence or absence of fertilization membrane, under 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany). A total of 3 samples of 1000 eggs/oocytes were observed. Eggs/oocytes present in the remaining volume, were sieved (30 μm), gently rinsed with seawater for sperm removal and inoculated in 5000 mL plastic beakers, for 24h, at 18±1°C, with gentle aeration (approximately 30 air bubbles.min^{-1}), until swimming gastrula developed. After 24h post-fertilization, three 1 mL samples were fixed in neutralized formaldehyde (4%) and gastrula concentration was determined, under 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany). Seawater used for fertilization procedures and embryo development was filtered (0.35 μm) and UV sterilized (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany).

2.6 Larval development assays

2.6.1 Larvae culture

Larval development assays were performed, according to pre-established conditions, comprising 3 replicates for each of the larviculture-tested conditions (table 1). Within experiment 1, from now onwards termed "culture conditions experiment", we have tested the effect of water change and aeration on *P. lividus* larvae development and survival until 18 DPF. For experiment 2 (feed experiment), we have analysed the effect of artificial (microdiets) and natural (*D. tertiolecta*) diets over *P. lividus* plutei development and survival, until 18 DPF. Finally in experiment 3, termed as plutei culture density experiment, we have investigated the possibility to mass-produce plutei, until 18 DPF. For experiment 2 and 3, we used the culture conditions which enabled better higher survival rates until 18 DPF (tested in culture conditions experiment), namely with aeration and water change every 2 days (W2).

Physicochemical parameters were as follows: Salinity (35±1 PSU); Temperature (18±1°C); Photoperiod (14h:10h, L:D); Illuminance (700 Lux). Sea urchin larviculture was

performed in 7L cylindro-spherical transparent PVC vessels (Fernando Ribeiro Lda, Portugal). Water temperature was thermo-regulated within a closed water recirculating system, equipped with a chilling device (Hailea HC-150A, Guangdong Hailea Group Co., Ltd, China) and water heaters (200W aquarium heater, Jager 3604, Eheim, GmbH & Co, Germany). Where vessels were placed, temperature (predictor IT808 digital thermometer, Omega Pharma, Netherlands) was checked daily. In those treatments where aeration was applied, approx. 60 air bubbles.min^{-1} aeration rate was used, within each larviculture vessel. Photoperiod was mechanically timed and fluorescent daylight bulbs (Digilamp FL-40SS/36, 6400K, 2450 lumen) were used. Filtered (0.35 µm) and UV sterilized (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany) seawater was used for larvae rearing.

Table 1 *Paracentrotus lividus* larviculture experimental trials.

Experiment	PluteimL^{-1}	Feeding regime	Culture conditions	Observed parameters
1	1,5	D	NN; WN; N2; W2; N4; W4	LS; ST
2	1,5	D; F; L; DF; DL; FL	W2	LS; TL; PO; BW; ST
3	1.5; 3; 5; 7.5; 10; 20	D	W2	LS; TL; PO; BW; ST

NN - Without aeration and water change; WN: With aeration and without water change; N2: Without aeration and with water change every 2 days; W2: With aeration and water change every 2 days; N4: Without aeration and with water change every 4 days; W4: With aeration and water change every 4 days; LS – Larvae survival; TL: Total length; PO: Post-oral arm length; BW: Body width; ST: Plutei stage determination (4-arm, 6-arm and 8-arm larvae).
D: *D. tertiolecta* 2x day, every two days.
F: Frippak (#1CAR), 3x day, at $^1/_3$ daily ration each, every day.
L: Lanzy (spirulina+), 3x day, at $^1/_3$ daily ration each, every day.
FL: ½ Frippak (#1CAR) + ½ Lanzy (spirulina+), 3x day, at $^1/_3$ daily ration each, every day.
DF: ½ *D. tertiolecta* + ½ Frippak (#1CAR,) 3x day, at $^1/_3$ daily ration each, every day.
DL: ½ *D. tertiolecta* + ½ Lanzy (spirulina+), 3x day, at $^1/_3$ daily ration each, every day.

Experiments 2 and 3 were performed with W2 culture conditions, following the best results obtained concerning *P. lividus* survival and development (see results), in comparison to NN, WN, N2, N4 and W4 experimental treatments.

2.6.2 Larvae feeding

Three different feeds, single or combined, were presented to plutei larvae: The microalgae *Dunaliella tertiolecta* (Chlorophyta: Clorophyceae) and two 5-30µm particle size microencapsulated/microbound feeds (Frippak #1CAR and Lanzy spirulina+, INVE Ltd, Thailand). Microalgae was grown in Conway culture medium, with filtered (0.35 µm) and autoclaved seawater.

Microalgae cell counting (Bürker chamber, Germany), under 400x optic microscopy (Axiostar Plus, Carl Zeiss, Gottinngen, Germany), was daily performed to access culture concentration prior to larvae feeding.

Precise particle concentration in each formulated feeds was accessed through a Coulter Counter® (model ZM, Coulter Electronics Ltd, England), with a 140 µm cell aperture, coupled to a multichannel particle analyzer (Coulter® Channelyser® 256, Coulter Electronics Ltd, England). Microencapsulated/Microbound rations were daily weighted (Precisa 40SM-200A, PAG Oerlikon AG, Zurich, Switzerland) and mixed with seawater in a 100 mL glass beaker, prior to daily feeding regimes. Feed was delivered to larvae according to pre-established larviculture conditions (Table 1).

Daily feed ration was 1500 cells/particles.mL^{-1} (4-arm plutei), 4500 cells/particles.mL^{-1} (6-arm plutei) and 7500 cells/particles.mL^{-1} (8-arm plutei). In the plutei culture density experiment, daily feed ration was standardized and proportionally incremented according to initial plutei culture density.

2.6.3 Larval survival, growth and development

Paracentrotus lividus larviculture trials ended after 18 DPF. Survival was recorded for each of the larval development trials, at 6, 9, 12, 15 and 18 DPF. Fifteen larvae were randomly sampled from each replicate tank of each treatment, and preserved in neutralized formaldehyde (4%) for biometric parameters measurement (total length; post-oral arm length; body width) and larvae stage determination (6 and 8-arm plutei) (figure 1). All observations were performed under 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany), using a micrometric eye lens (PI 10x/18, Carl Zeiss, Gottingen, Germany)

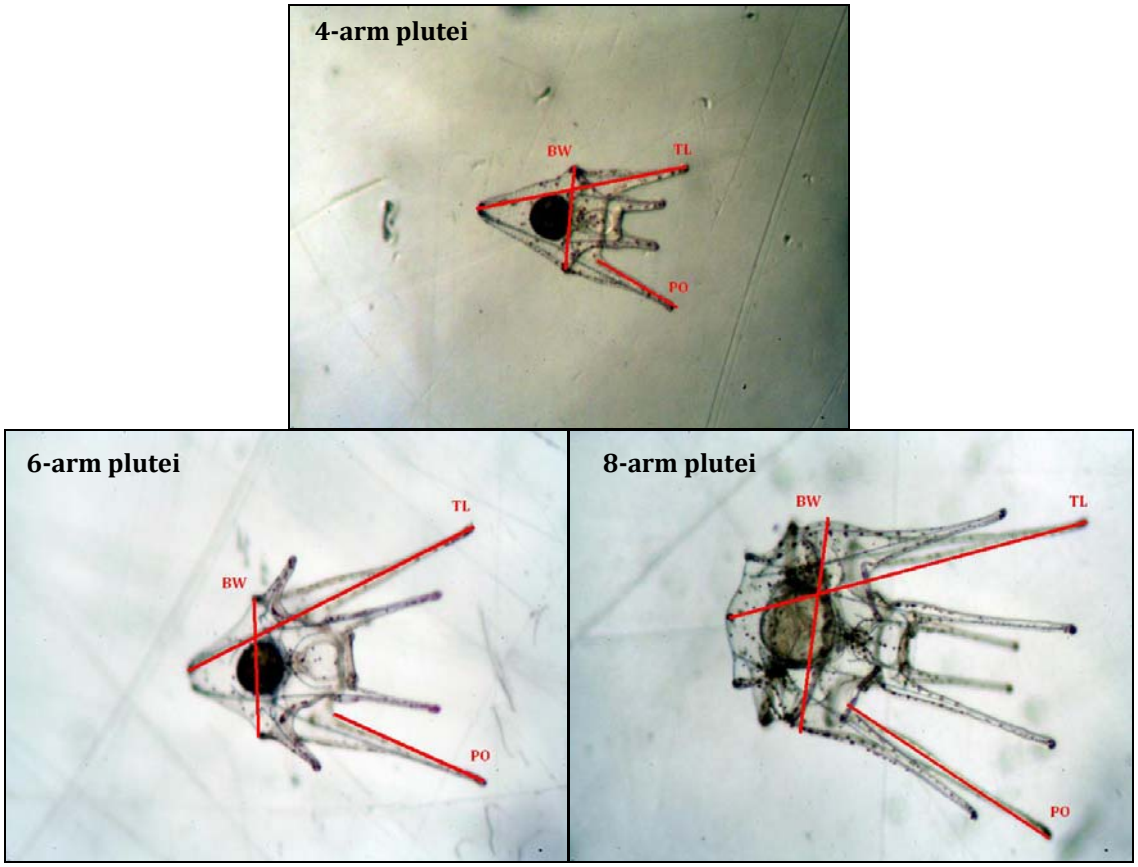

Figure 1 Schematics of biometric measurements performed on *P. lividus* larvae (4-arm, 6-arm and 8-arm plutei). TL: Total length; PO: Post-oral arm length; BW: Body width.

2.7 Plutei mass culture and enrichment

In order to assess the possibility to mass culture and nutritionally enrich *P. lividus* larvae, as commonly practiced for *Artemia* spp. metanauplii, an additional set of experiments was performed (table 2). Within these experiments (4 a-e), we used the culture conditions which enable better survival rates until 18 DPF (culture conditions experiment), namely with aeration and water change every 2 days (W2). Remaining culture conditions (temperature, illuminance, photoperiod, salinity, culture vessels) are described in the larvae culture section (2.6.1). Concentration of Algamac (3050 Flake) for plutei enrichment was provided according to the same manufacturer instructions described for *Artemia* spp. nauplii (0.2g/liter/100.000 nauplii/12h enrichment period). Additional experimental trials were performed where Algamac concentration was ½, ¼ and $^1/_8$ of standard concentration, while performing the same enrichment period (0.2g/liter/100.000 nauplii).

Table 2 – Experimental trials concerning *P. lividus* larvae mass production and enrichment. S: Survival; DPF: Days post-fertilization.

Experiment	Feed provided	Feed concentration	Sampling days	Parameters analysed
4a	*Dunaliella tertiolecta*	40.000 cells.mL^{-1}	5, 10 DPF	S
4b	Frippak #1CAR	40.000 particles.mL^{-1}	5, 10 DPF	S
4c	Lanzy spirulina+	40.000 particles.mL^{-1}	5, 10 DPF	S
4d	*D. tertiolecta* (2 ->4 DPF) 12h Algamac 3050 (4 ->5 DPF)	40.000 cells.mL^{-1} Standard, ½, ¼, $^1/_8$	5 DPF	S
4e	*D. tertiolecta* (2 ->9 DPF) 12h Algamac 3050 (9 -> 10 DPF)	40.000 cells.mL^{-1} Standard, ½, ¼, $^1/_8$	10 DPF	S

2.8 Statistical analysis

Arcsine transformation was applied to percentage data, before statistical analysis. One-way ANOVA was applied for statistical analysis (data normality and variance homogeneity observed) and multiple comparison analysis was performed (Tukey). Whenever ANOVA assumptions were not observed, Kruskal-Wallis non-parametric analysis was applied as well as multiple comparisons testing between different experimental treatments data (Tukey). Pearson rank order correlation was applied for correlation analysis between larvae density *vs*. survival in the larvae culture density experiment. Statistical analysis was performed using SigmaStat and SigmaPlot (Systat Sofware Inc., Windows version 3.10) software.

3. Results

3.1 Culture conditions experiment

3.1.1 Larval survival

Larval survival at 6, 9, 12, 15 and 18 DPF, concerning the culture conditions experiment is shown in figure 2. Statistical differences (ANOVA, Tukey test, $p<0.05$) were found between experimental treatments, at different sampling time periods. Survival until 18 DPF was only achieved for the N2, W2, N4 and W4 treatments. The highest value concerning survival at 18 DPF was found for the W2 treatment (52.9±1.94 %), while the

lowest result was obtained for the N4 experimental treatment (27.6±1.60 %). Statistical differences (ANOVA, Tukey test, $p<0.05$) were found between treatment W2 *vs*. N2, N4 and W4 at 15 and 18 DPF. Treatments NN and WN, only supported larvae survival until 9 and 12 DPF, respectively.

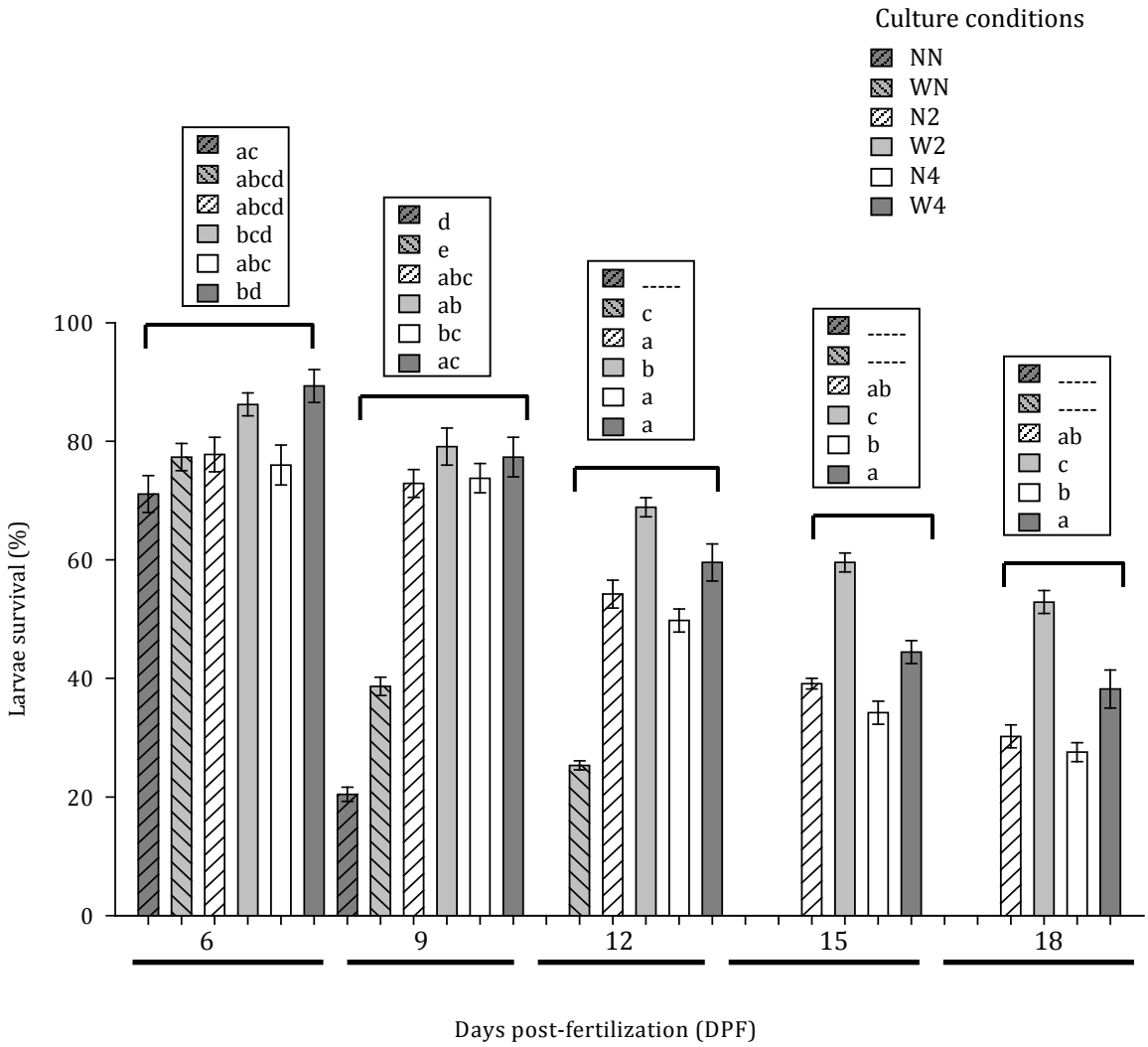

Figure 2 Results of mean values for *P. lividus* plutei survival (%) ± standard error, at 6, 9, 12, 15 and 18 DPF, regarding to different culture conditions treatments. Different letters placed at the Tables on top of the bars indicate statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments at the same sampling time period. NN: Without aeration and without culture water change; WN: With aeration and without culture water change; N2: Without aeration and with culture water change every 2 days; W2: With aeration and culture water change every 2 days; N4: Without aeration and with culture water change every 4 days; W4: With aeration and culture water change every 4 days.

3.1.2 Larval stage

Results concerning *P. lividus* 6 and 8-arm plutei percentage (%) ± standard error at 12 and 18 DPF are shown in figure 3. In all treatments, 100% of the larvae were on 4-arm stage at 3 and 6 DPF. At 12 DPF, 93.2% and 91.1% of the larvae were on 6-arm stage for W2

and W4 treatments respectively, whereas 86.7% for N2, 68.9% for W4 and only 13.3% for WN treatment, were observed. No statistical differences (Kruskal-Wallis, Tukey test, $p>0.05$) were found between N2, W2, N4 and W4 experimental treatments at 18 DPF. Statistical differences (ANOVA, Tukey test, $p<0.05$) were found between treatments WN vs. W2 and N2 experimental groups. At 18 DPF, 100% of larvae in treatments W2 and W4 were in the 8-arm plutei stage, while 84.4% and 80.0% of the larvae have reached the same development stage in N2 and N4 treatments respectively. Treatments NN and WN did not support larvae development beyond 4-arm plutei stage.

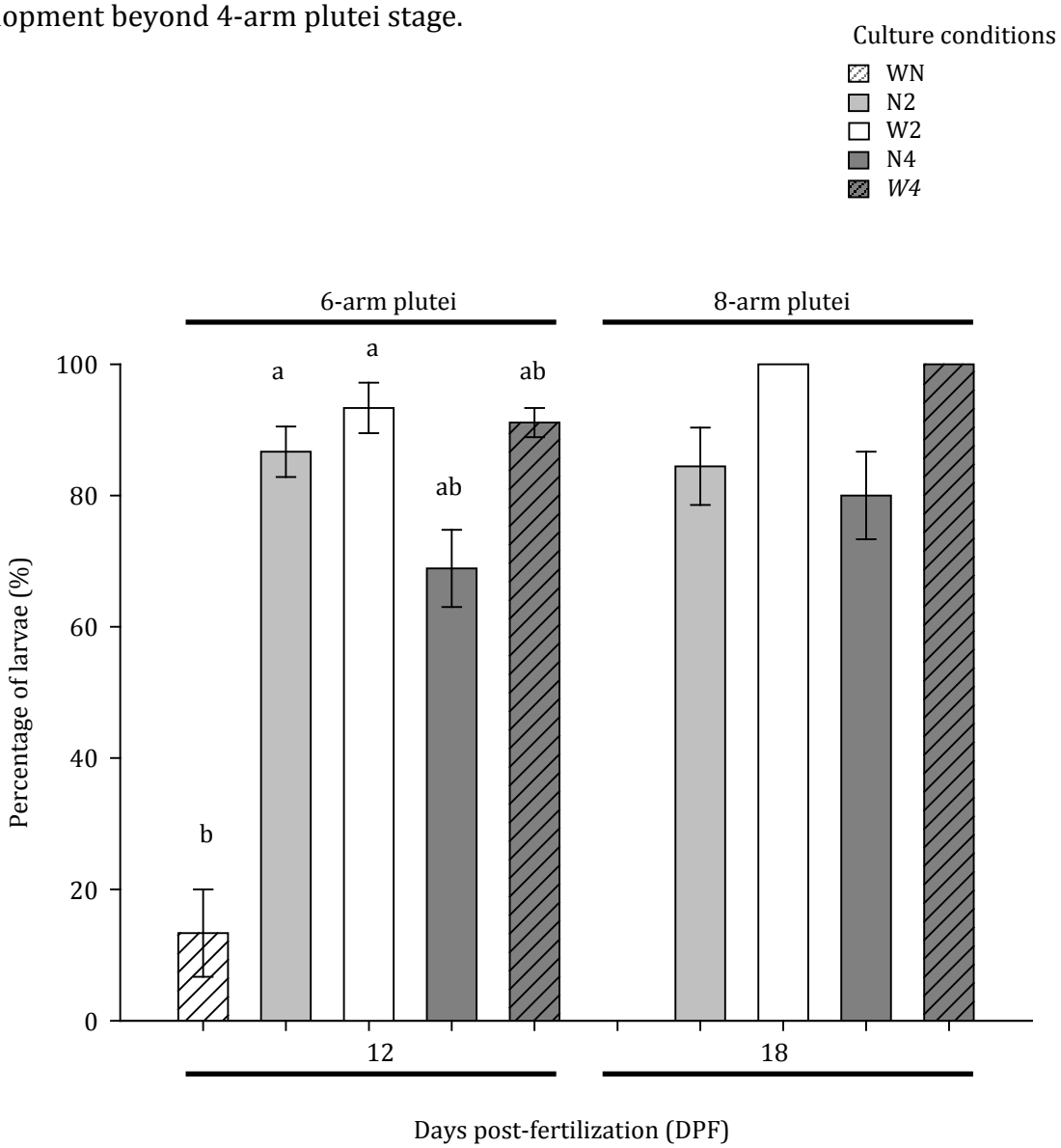

Figure 3 Mean values for *P. lividus* 6 and 8-arm plutei stage percentage (%) ± standard error at 12 and 18 DPF, concerning culture conditions experiments. N2: Without aeration and with culture water change every 2 days; WN: With aeration and without culture water change; W2: With aeration and culture water change every 2 days; N4: Without aeration and with culture water change every 4 days; W4: With aeration and culture water change every 4 days.

3.2 Feed experiment

3.2.1 Larval survival

Mean values of larval survival at 6, 9, 12, 15 and 18 DPF, concerning the feed experiment is shown in figure 4. Statistical differences (ANOVA, Tukey test, $p<0.05$) were found between experimental treatments, at different sampling time periods namely 12, 15 and 18 DPF.

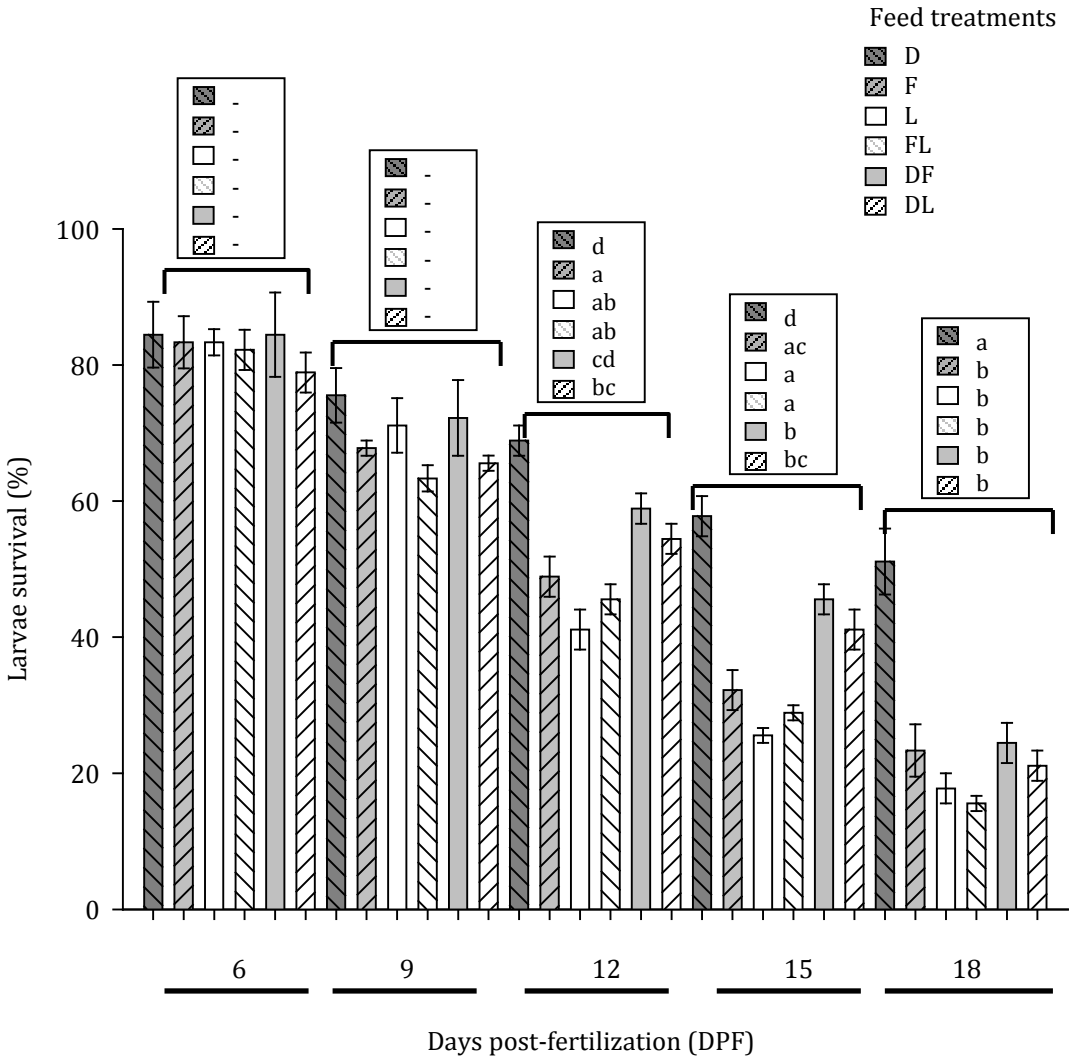

Figure 4 Mean values for *P. lividus* plutei survival (%) ± standard error, at 6, 9, 12, 15 and 18 DPF, concerning feed experimental treatments. Different letters placed at the tables on top of bars indicate statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments at the same sampling time period. D: *D. tertiolecta*; F: Frippak#1CAR; L: Lanzy spirulina+; FL: ½ Frippak + ½ Lanzy: DF: ½ *D. tertiolecta* + ½ Frippak; DL: ½ *D. tertiolecta* + ½ Lanzy.

The highest larvae survival value, at 18 DPF, was obtained for treatment D (51.1±4.84 %), in opposition for the lowest result for FL treatment (15.6±1.11 %). F and L treatments resulted in 23.3±3.85 % and 17.8±2.22 % survival, respectively. From 15 DPF onwards, treatment D was statistically different (ANOVA, Tukey test, $p<0.05$) in comparison to

remaining treatments. No statistical differences (ANOVA, Tukey test, p>0.05) were found between treatments F, L and FL, as well as DF and DL, during the entire experimental period.

3.2.2 Larval stage

Results concerning *P. lividus* 6 and 8-arm plutei stage percentage (%) ± standard error at 12 and 18 DPF are shown in figure 5. Statistical differences (ANOVA, Tukey test, p<0.05) were found between treatment D *vs.* artificial feed treatments (F, L and FL) at 12 DPF. Likewise statistical differences (ANOVA, Tukey test, p<0.05) were found between treatment D and remaining feed treatments (F, L and FL) at 18 DPF. Within all treatments, 100% of the larvae, were on 4-arm stage at 3 and 6 DPF.

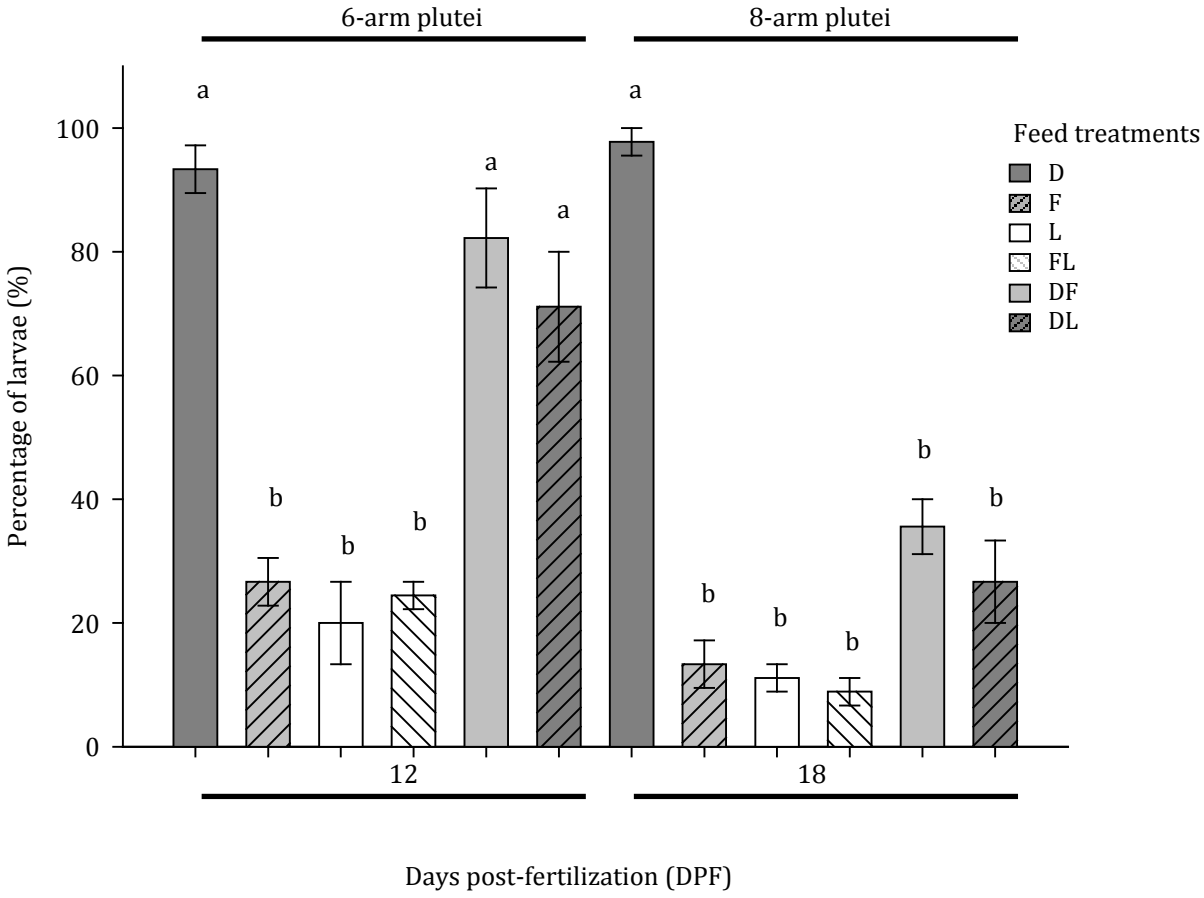

Figure 5 Mean values for *P. lividus* 6 and 8-arm plutei stage percentage (%) ± standard error at 12 and 18 DPF, concerning feed experimental treatments. Different letters placed at the top of bars indicate statistical differences (ANOVA, Tukey test, p<0.05) between treatments, at the same sampling time period (12 and 18 days PF). D: *D. tertiolecta*; F: Frippak#1CAR; L: Lanzy spirulina+; FL: ½ Frippak + ½ Lanzy: DF: ½ *D. tertiolecta* + ½ Frippak; DL: ½ *D. tertiolecta* + ½ Lanzy.

At 12 DPF, a higher percentage of larvae were on 6-arm stage for treatment D (93.3 %) in comparison to F (26.5%), L (20.0%) and FL (24.4%) treatments. 97.8 % of larvae in

treatments D were on the 8-arm stage at 18 DPF, in opposition to lower percentages of 13.3%, 11.1%, 8.9%, 35.6% and 26.7% for F, L, FL, DF and DL treatments, respectively.

3.2.3 Larval morphology

Results concerning *P. lividus* larvae total length (µm) ± standard error at 3, 6, 12 and 18 DPF, during experimental feeding trials, are show in figure 6. Statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) were found between D, DF and DL *vs.* F, L and FL treatments at 6, 12 and 18 DPF. The highest mean values for larvae total length was obtained at 12 DPF for DF (820±6.78 µm), DL (794±5.69 µm) and D (793±6.09 µm) treatments. Mean higher values of 743±7.29 µm, 727±5.74 µm, 711 ± 6.34 µm were obtained for treatments DL, DF and D in comparison to 658±5.05 µm, 648 ±4.95 µm and 630±4.48 µm for treatments FL, F and L, at 18 DPF. A general increase in larvae total length was observed for all treatments until 12 DPF. Between 12 and 18 DPF, a decrease in larvae total length was also observed for all treatments with the exception for treatment FL.

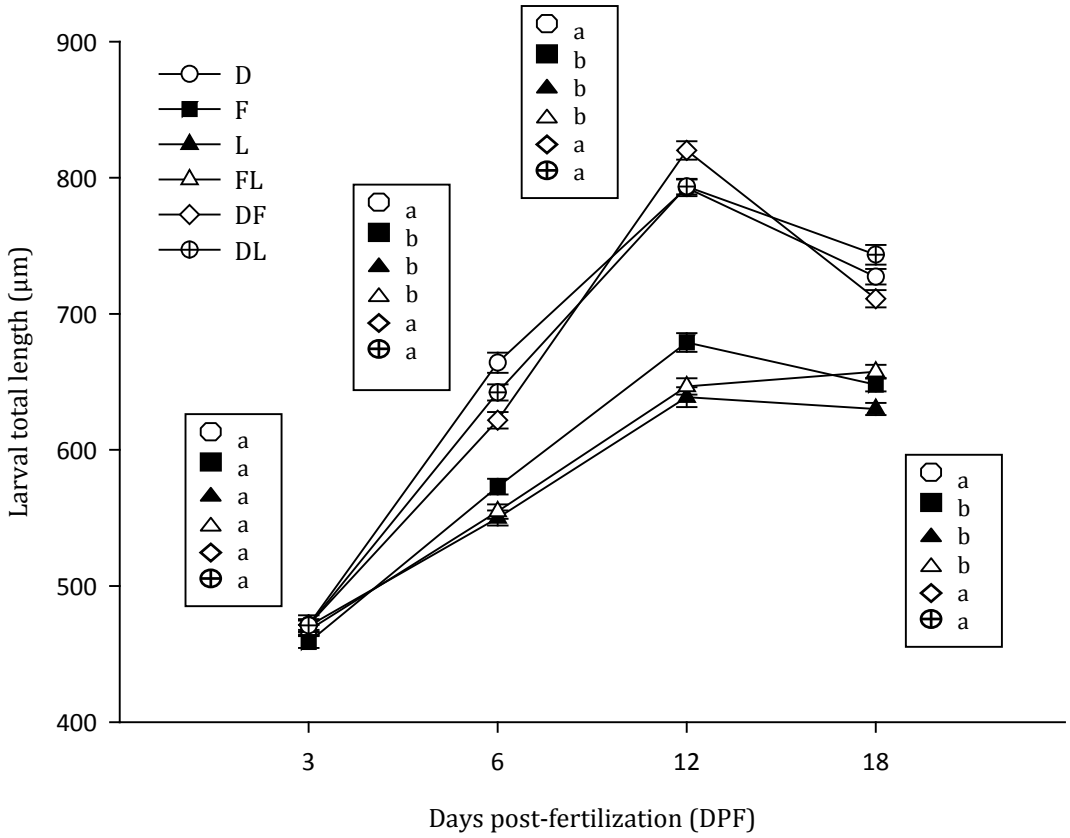

Figure 6 Mean values for *P. lividus* larvae total length (µm) ± standard error at 3, 6, 12 and 18 DPF, concerning feed experimental trials. Different letters placed at tables indicate statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) between treatments, at the same sampling time period. D: *D. tertiolecta*; F: Frippak#1CAR; L: Lanzy spirulina+; FL: ½ Frippak + ½ Lanzy: DF: ½ *D. tertiolecta* + ½ Frippak; DL: ½ *D. tertiolecta* + ½ Lanzy.

Results concerning *P. lividus* larvae post-oral arm length (µm) ± standard error at 3, 6, 12 and 18 DPF, during experimental feeding trials, are show in figure 7. Statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) were found between treatments, at different sampling time periods (3, 6, 12 and 18 DPF). Treatments DF and DL showed similar post-oral arm length values at 12 and 18 DPF and were statistically different (Kruskal-Wallis, Tukey test, $p<0.05$) in comparison to D, F, L and FL treatments. No statistical differences (Kruskal-Wallis, Tukey test, $p>0.05$) were found between F *vs.* L and FL treatments, during the entire experimental trial. DL and DF showed the highest post-oral arm length values at 12 DPF (362±3.56 µm and 377±3.69 µm) and 18 DPF (320±3.36 µm and 292±3.48 µm). A general increase in post-oral arm length values was observed from 3 until 12 DPF, followed by an overall decrease until 18 DPF.

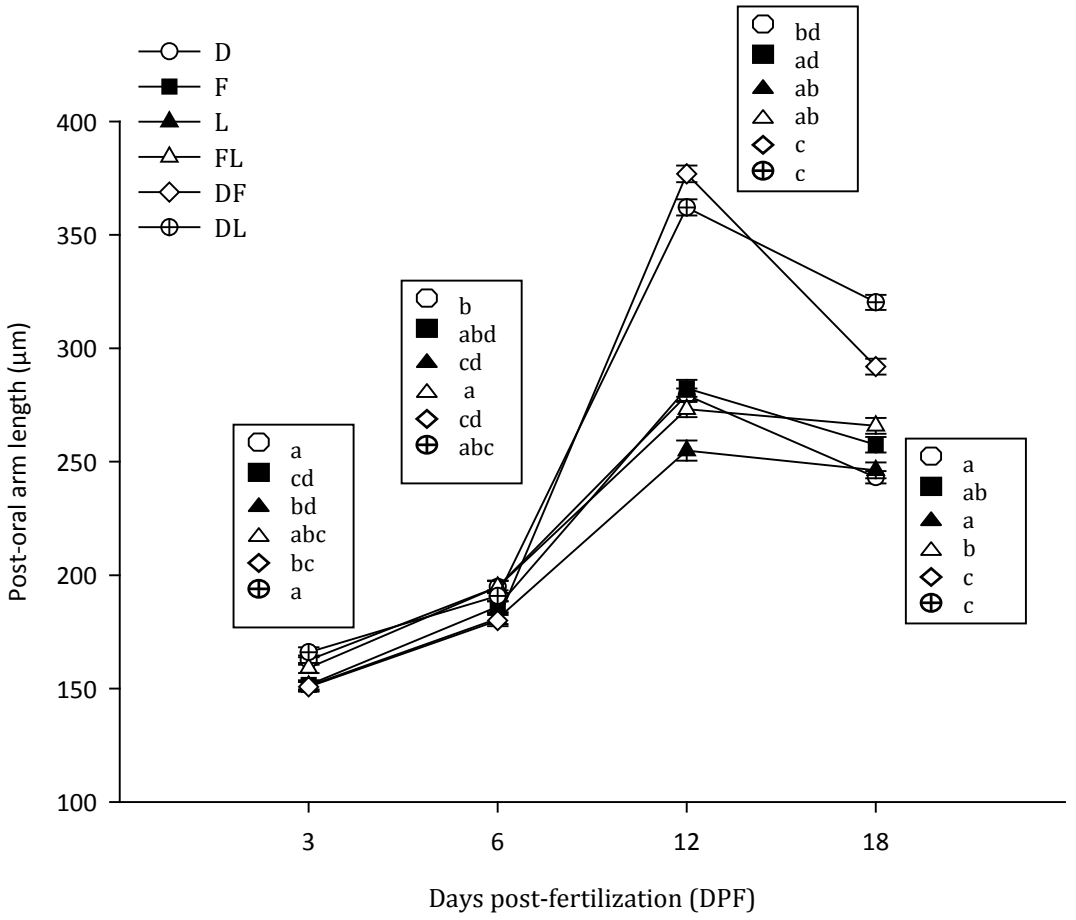

Figure 7 Mean values for *P. lividus* larvae post-oral arm length (µm) ± standard error at 3, 6, 12 and 18 DPF, concerning feed experimental trials. Different letters placed at Tables indicate statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) between treatments, at the same sampling time period. D: *D. tertiolecta*; F: Frippak#1CAR; L: Lanzy spirulina+; FL: ½ Frippak + ½ Lanzy: DF: ½ *D. tertiolecta* + ½ Frippak; DL: ½ *D. tertiolecta* + ½ Lanzy.

Results concerning *P. lividus* larvae body width (μm) ± standard error at 3, 6, 12 and 18 DPF, during experimental feeding trials, are show in figure 8. Statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) were found between treatments, at different sampling time periods (3, 6, 12 and 18 DPF). Treatments DF and DL showed similar body width values at 3, 6, 12 and 18 DPF and were statistically different (Kruskal-Wallis, Tukey test, $p<0.05$) in comparison to F, L and FL treatments at 12 and 18 DPF. Likewise treatment D was statistically different (Kruskal-Wallis, Tukey test, $p<0.05$) at 18 days PF, showing the highest body width value of 473±4.43 μm, while the lowest results were obtained for treatment, F, L and FL with 243±2.88 μm, 223±2.52 μm and 217±2.42 μm respectively. DF and DL treatments showed similar and non- statistically different values (Kruskal-Wallis, Tukey test, $p>0.05$) at 3, 6, 12 and 18 DPF. Likewise the same was observed between treatments F, L and FL at 12 and 18 days PF. A general increase in body width values was observed from 3 until 18 DPF, regardless of treatment.

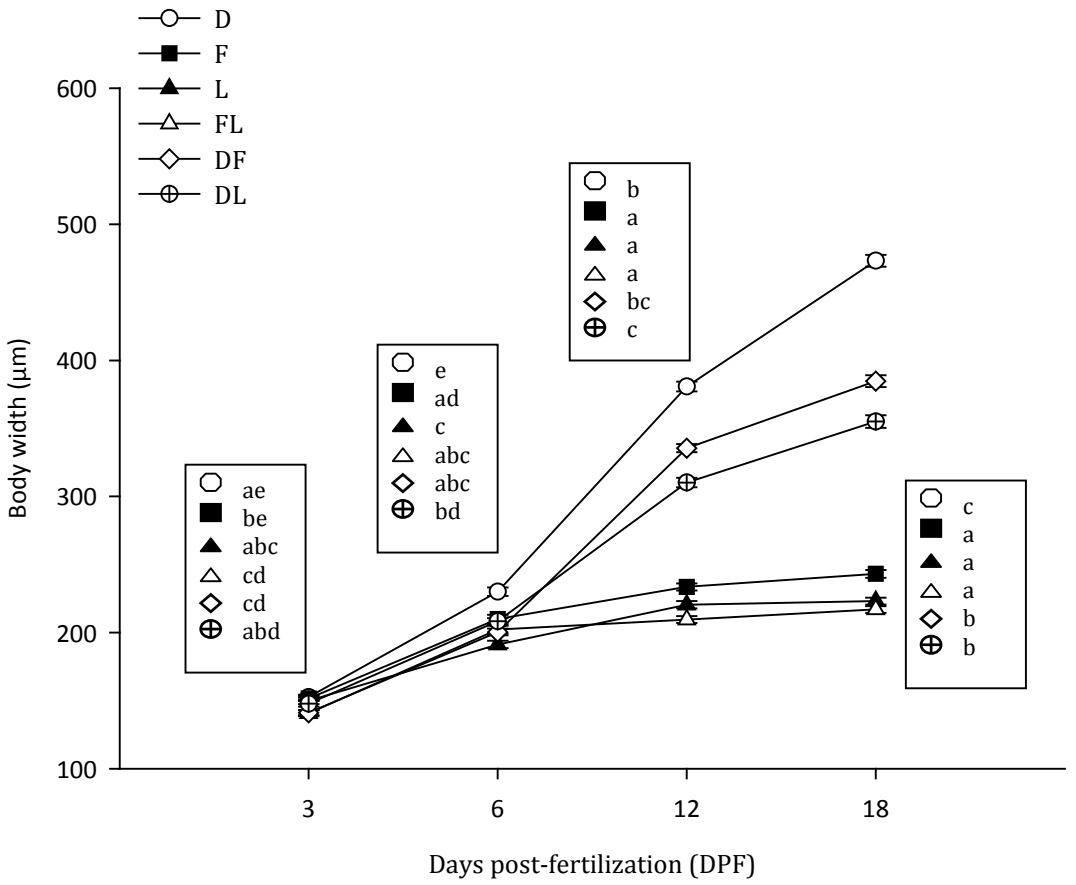

Figure 8 Mean values for *P. lividus* larvae body width (μm) ± standard error at 3, 6, 12 and 18 DPF, concerning feed experimental trials. Different letters placed at tables indicate statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) between treatments, at the same sampling time period. D: *D. tertiolecta*; F: Frippak#1CAR; L: Lanzy spirulina+; FL: ½ Frippak + ½ Lanzy: DF: ½ *D. tertiolecta* + ½ Frippak; DL: ½ *D. tertiolecta* + ½ Lanzy.

3.3 Plutei culture density experiment

3.3.1 Larval survival

Results of larval survival at 6, 9, 12, 15 and 18 DPF, concerning experimental trials with different plutei culture densities are shown in figure 9. Statistical differences (ANOVA, Tukey test, $p<0.05$) were found between experimental treatments, at different sampling time periods (6, 9, 12, 15 and 18 DPF). Statistical differences (ANOVA, Tukey test, $p<0.05$) were found between 1.5 plutei.mL^{-1} *vs.* remaining treatments, at 12, 15 and 18 DPF. No statistical differences (ANOVA, Tukey test, $p>0.05$) were found for survival results concerning 3 and 5 plutei.mL^{-1} at 6, 9, 10, 12, 15 and 18 DPF. At 9, 12 and 15 DPF, treatment with 20 plutei.mL^{-1}, was statistically different (ANOVA, Tukey test, $p<0.05$) from remaining experimental groups.

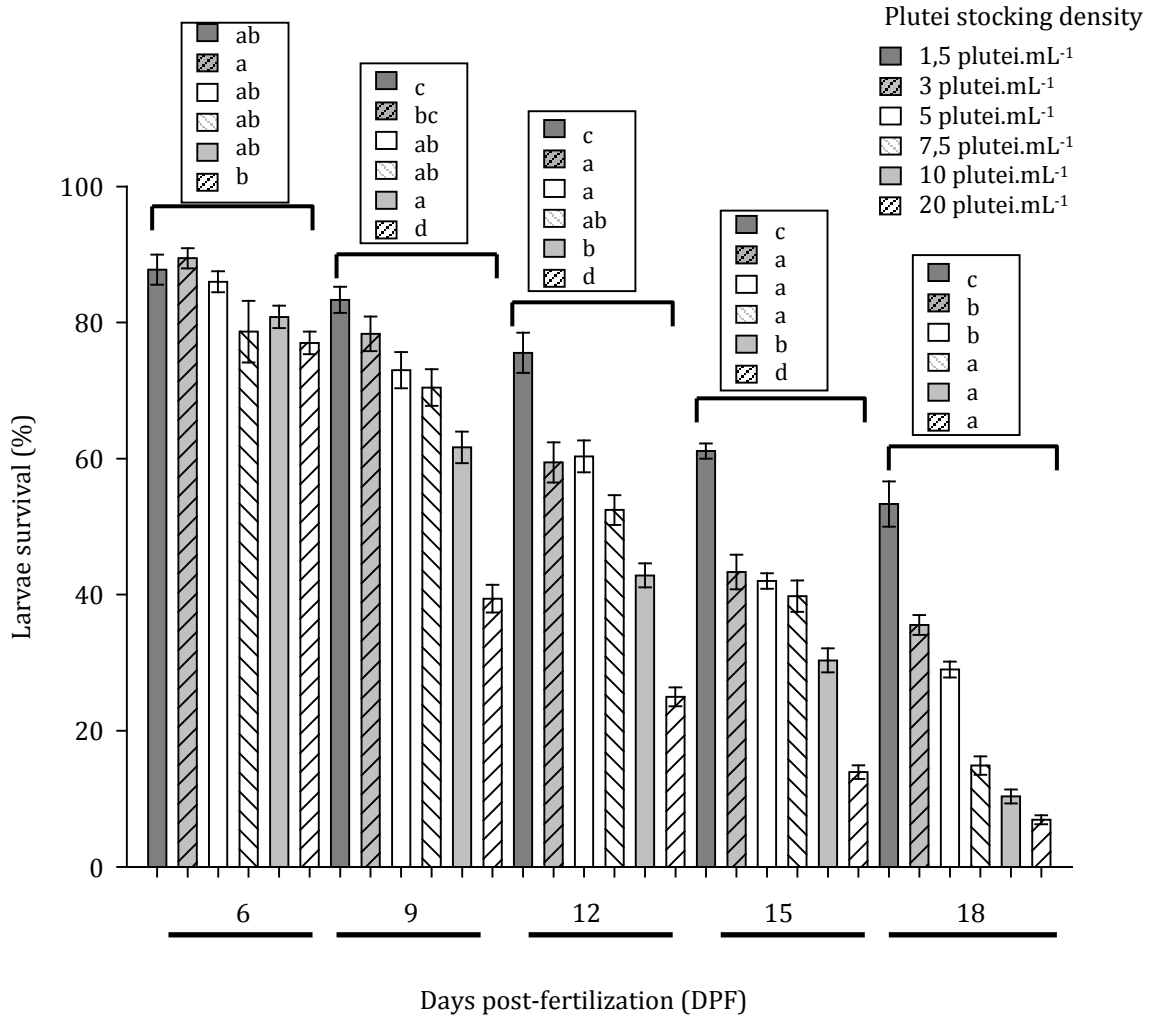

Figure 9 Mean values for *P. lividus* larvae survival (%) ± standard error, at 6, 9, 12, 15 and 18 DPF, concerning experimental trials with different plutei culture densities. Different letters placed in tables on top of bars indicate statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments at the same sampling time period. X-axis values refer to treatments with initial plutei concentration (plutei.mL^{-1}) of 1.5, 3, 5, 7.5, 10 and 20 larvae at 6, 9, 12, 15 and 18 DPF, respectively.

Treatment with initial larvae density of 1.5 plutei.mL^{-1} achieved the highest survival value (53.3±3.32 %) at 18 DPF while the lowest survival results were obtained for treatments of 7.5 plutei.mL^{-1} (14.9±1.36 %), 10 plutei.mL^{-1} (10.3±1.01 %) and 20 plutei.mL^{-1} (6.9±0.65 %).

Correlations between larvae survival, density and experimental time frame are presented in table 3. Negative statistical correlations were found between larvae survival *vs*. larvae density (rs = -0.483, p<0.001) and larvae survival *vs*. experimental time frame (rs = -0.826, p<0.001).

Table 3 Pearson rank order correlation between larvae survival, density and experimental time frame.

	Larvae density	Experimental time frame	Larvae survival
Larvae density	----------	rs = 0.000	rs = -0.483
	----------	p = 1.000	p = 1.44 e $^{-6}$
Experimental time frame	----------	----------	rs = -0.826
	----------	----------	p = 1.205e $^{-23}$
Larvae survival	----------	----------	----------
	----------	----------	----------

3.3.2 Larval stage

Results from plutei culture densities experiments, regarding mean values of *P. lividus* 6 and 8-arm plutei stage percentage (%) ± standard error at 12 and 18 DPF are shown in figure 10. No statistical differences (ANOVA, Tukey test, p>0.05) were found between treatments at 12 and 18 DPF. In all treatments, 100% of the larvae were on 4-arm stage at 3 and 6 days PF. At 12 DPF, 1.5 plutei.mL^{-1} treatment presented 92,8% of larvae on the 6-arm stage, in opposition to the value obtained for treatment with 5 plutei.mL^{-1} (73,4%). At 18 DPF, 96,4% of plutei within 1.5 plutei.mL^{-1} treatment were on 8-arm stage, in comparison to 86,7% for 3 plutei.mL^{-1} treatment, 91.1% for 5 plutei.mL^{-1} treatment, 75,6% for 7.5 plutei.mL^{-1} treatment, 77,8% for 10 plutei.mL^{-1} treatment and 95,6% for treatment with 20 plutei.mL^{-1}.

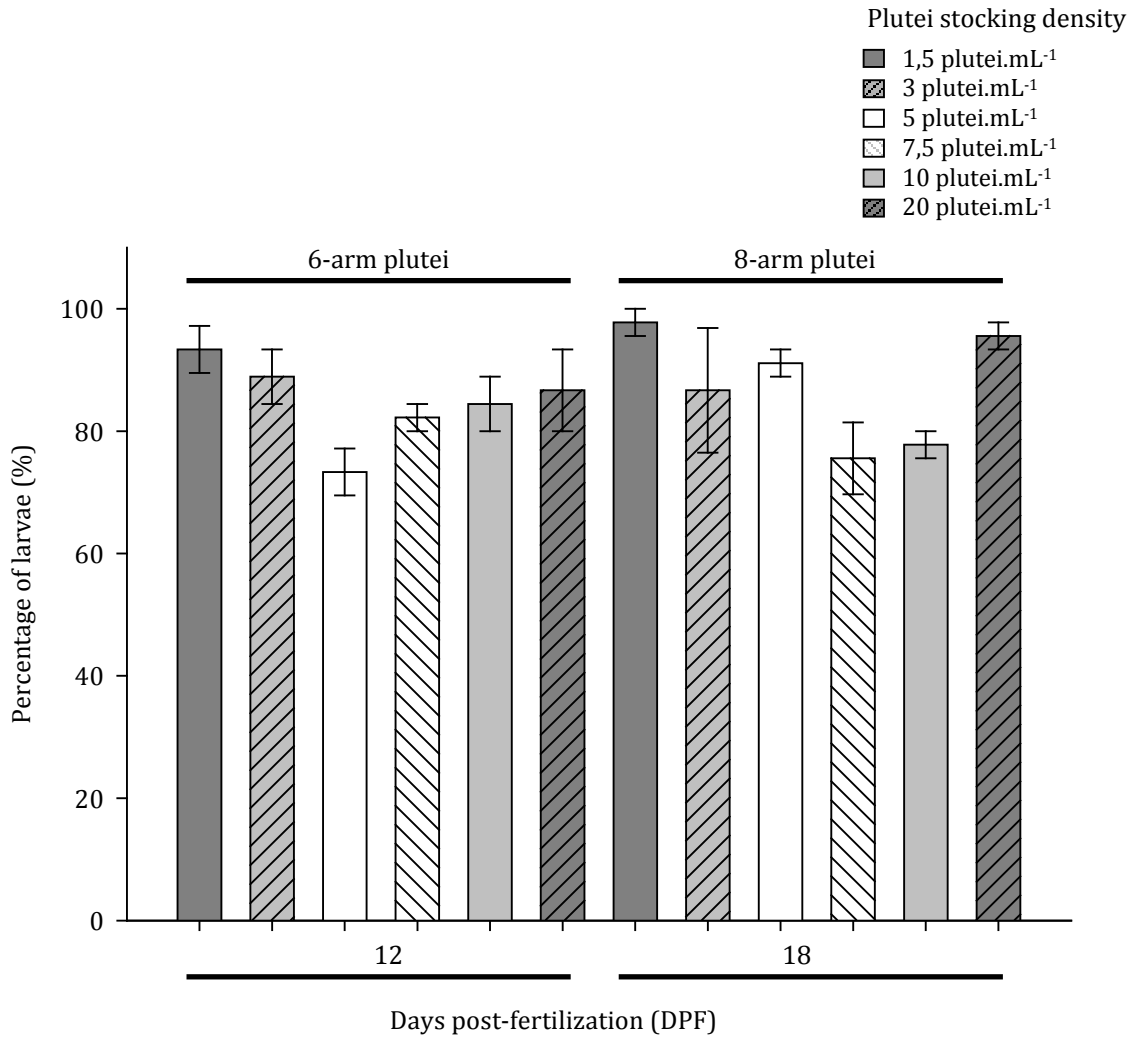

Figure 10 Mean values for *P. lividus* 6 and 8-arm stage plutei percentage (%) ± standard error at 12 and 18 DPF, concerning experiments performed with different plutei culture densities. X-axis values correspond to treatments with initial plutei concentration (plutei.mL^{-1}) of 1.5, 3, 5, 7.5, 10 and 20 larvae at 12 and 18 DPF, respectively.

3.3.3 Larval morphology

Results concerning *P. lividus* larvae total length (µm) ± standard error at 3, 6, 12 and 18 DPF, concerning experiments performed with different plutei culture densities, are show in figure 11. Statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) were found between all treatments at 12 and 18 DPF. Likewise, treatment with initial plutei concentration of 20 larvae.mL^{-1} was statistically different (Kruskal-Wallis, Tukey test, $p<0.05$) in comparison to remaining experimental treatments at 12 DPF. Treatments with initial plutei concentration of 3, 5 and 7.5 larvae.mL^{-1} presented similar and non-statistically different total length results (Kruskal-Wallis, Tukey test, $p>0.05$) at 3, 6, 12 and 18 DPF. A general increase in larvae total length was observed for all treatments until 12 DPF, followed by a total length

decrease between 12 and 18 DPF, with the exception for treatment with 20 larvae.mL^{-1} (692±8.76 µm at 12 DPF and 690±5.92 µm at 18 DPF).

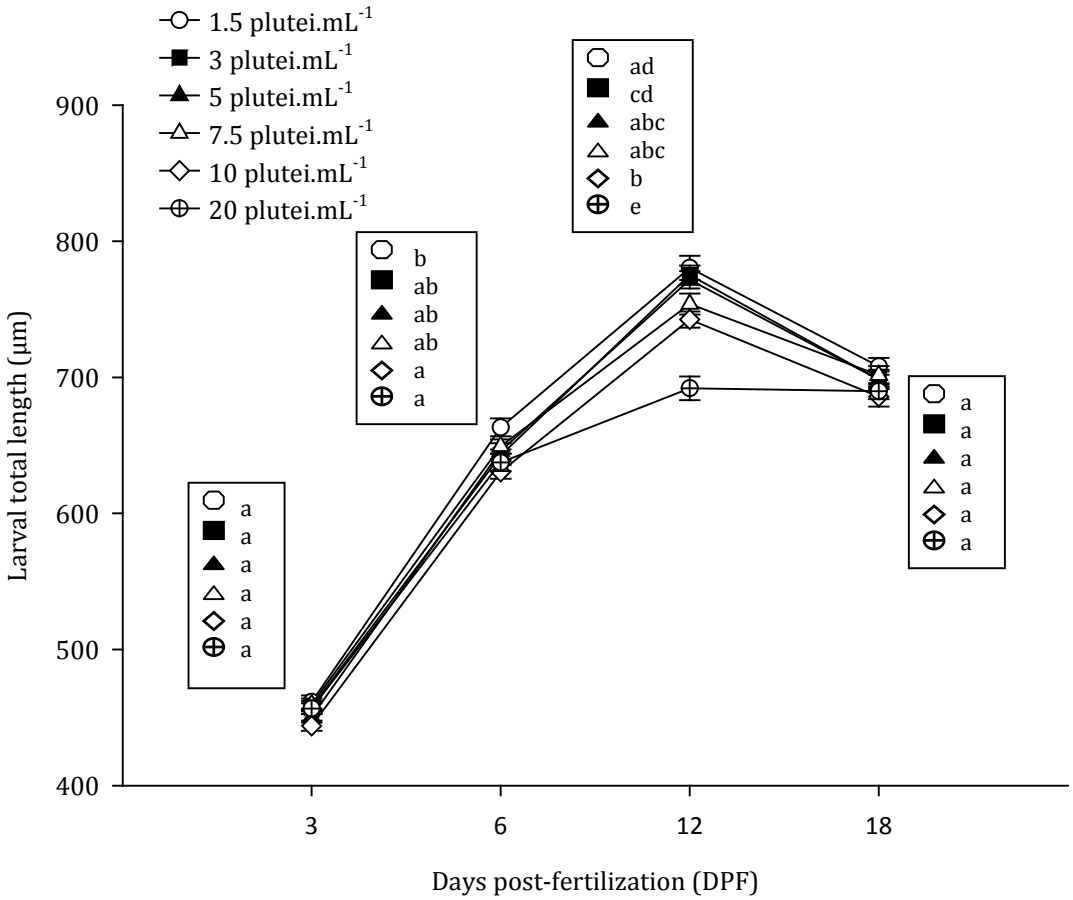

Figure 11 Mean values for *P. lividus* larvae total length (µm) ± standard error at 3, 6, 12 and 18 DPF, concerning experiments performed with different plutei culture densities. Different letters placed at tables indicate statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) between treatments, at the same sampling time period.

Results concerning *P. lividus* larvae post-oral arm length (µm) ± standard error at 3, 6, 12 and 18 DPF, during experiments performed with different plutei culture densities, are show in figure 12. Statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) were found between all treatments, at 3, 12 and 18 DPF. Treatments with initial plutei concentration of 1.5, 3 and 5 larvae.mL^{-1} showed similar and non-statistically different (Kruskal-Wallis, Tukey test, $p<0.05$) post-oral arm length values at 3, 6, 12 and 18 DPF. A general increase in larvae post-oral arm length was observed for all treatments until 12 DPF followed by a general decrease between until 18 DPF.

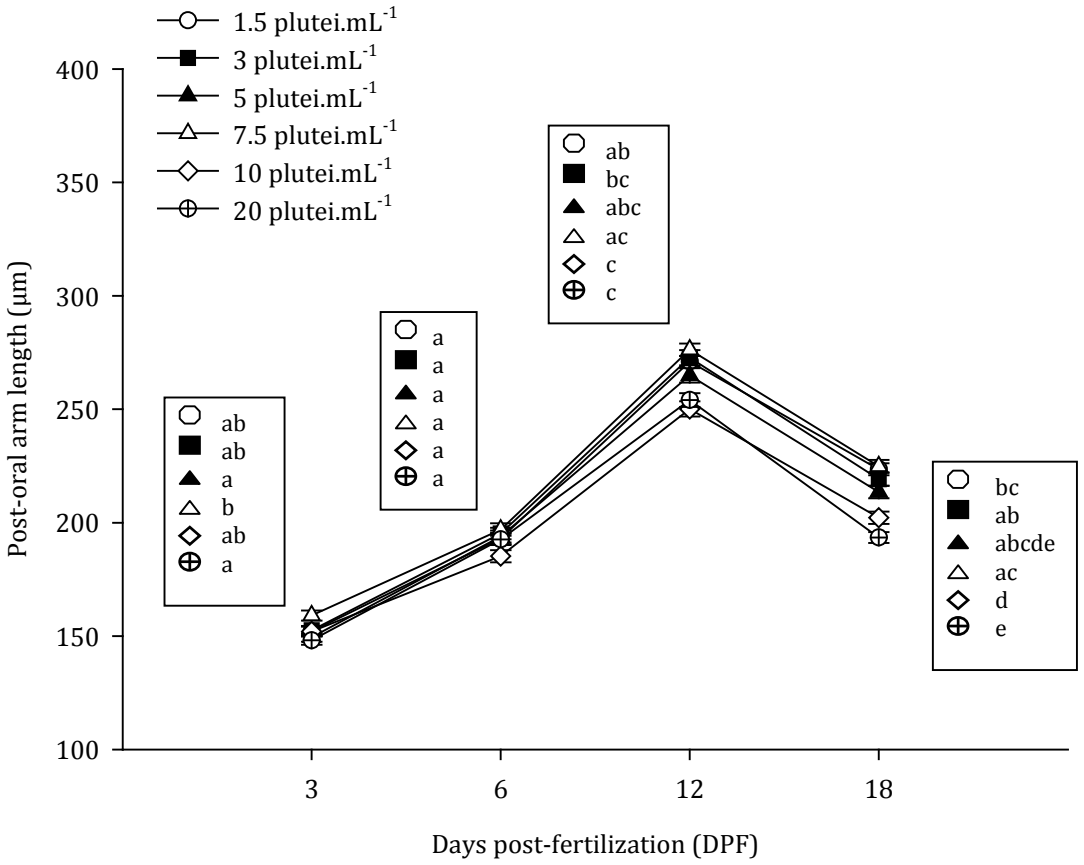

Figure 12 Mean values for *P. lividus* larvae post-oral arm length (µm) ± standard error at 3, 6, 12 and 18 DPF, experiments performed with different plutei culture densities. Different letters placed at tables indicate statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) between treatments, at the same sampling time period.

Results concerning *P. lividus* larvae body width (µm) ± standard error at 3, 6, 12 and 18 DPF, regarding experiments performed with different plutei culture densities, are show in figure 13. Statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) were found between treatments, at 3, 12 and 18 DPF. With the exception for 18 DPF, treatments with initial larvae concentration of 1.5, 3 and 5 larvae.mL^{-1}, presented similar and non-statistically different (Kruskal-Wallis, Tukey test, $p>0.05$) body width values. An overall increase in body width values was observed for all treatments until 18 DPF, with the highest values obtained for treatment with initial plutei concentration of 20 larvae.mL^{-1} (505±5.57µm), 10 larvae.mL^{-1} (492±5.67µm) and 1.5 larvae.mL^{-1} (489±6.91 µm) in comparison to the lowest for treatment with 7.5 larvae.mL^{-1} (400±5.04 µm).

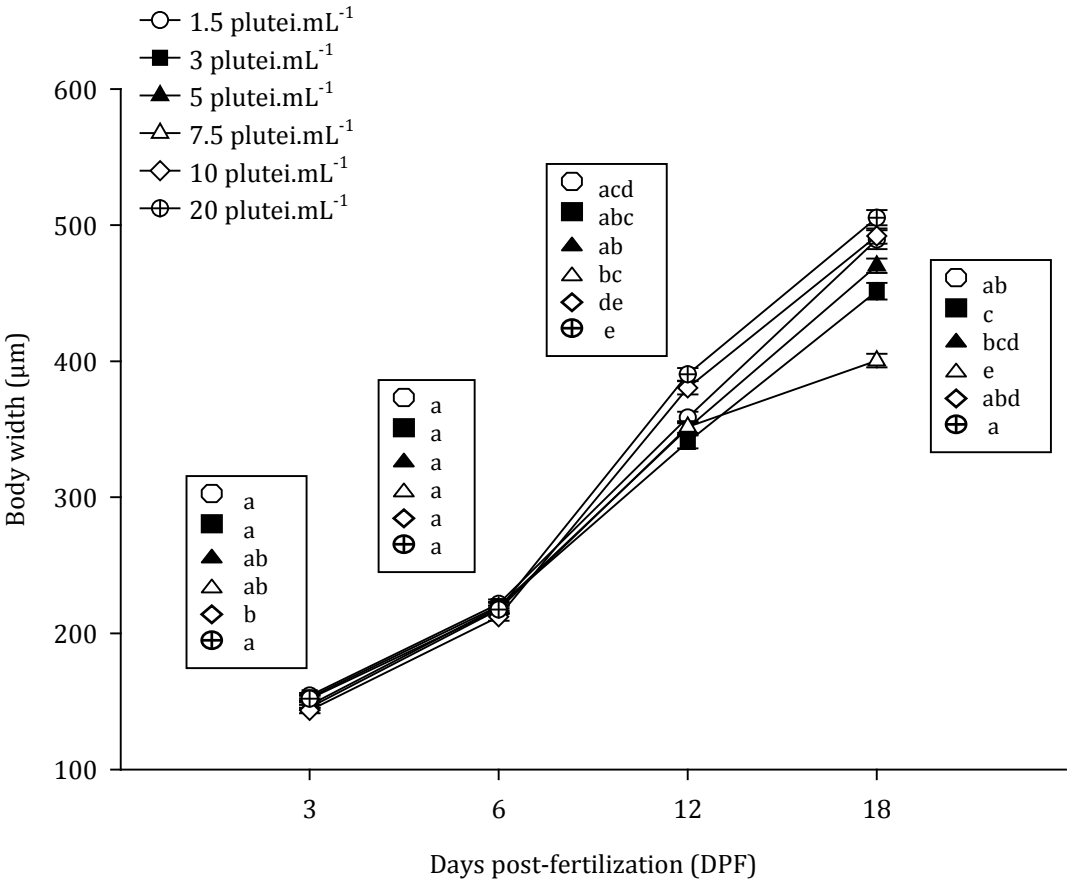

Figure 13 Mean values for *P. lividus* larvae body width (µm) ± standard error at 3, 6, 12 and 18 DPF, experiments performed with different plutei culture densities. Different letters placed at Tables indicate statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) between treatments, at the same sampling time period.

3.4 Plutei mass culture and enrichment

Results concerning larvae mean survival rates ± standard error) of *P. lividus*, within feeding treatments with *D. tertiolecta*, microencapsulated/microbound feeds (Frippak 1#CAR/Lanzy spirulina+) and enrichment with Algamac (3050 flake), at 5 and 10 DPF, are shown in figure 14. Statistical differences (Kruskal-Wallis, Tukey test, $p<0.05$) were found between treatments with *D. tertiolecta vs*. Algamac 3050 (standard ration) and Algamac 3050 (1/2), at 5 DPF. Likewise, statistical differences (ANOVA, Tukey test, $p<0.001$) were denoted at 10 DPF, between *D. tertiolecta vs*. Algamac treatments. The highest survival rates at 5 and 10DPF were obtained for the *D. tertiolecta* treatment, 56.3±2.37 % and 32.1±0.80 %, respectively.

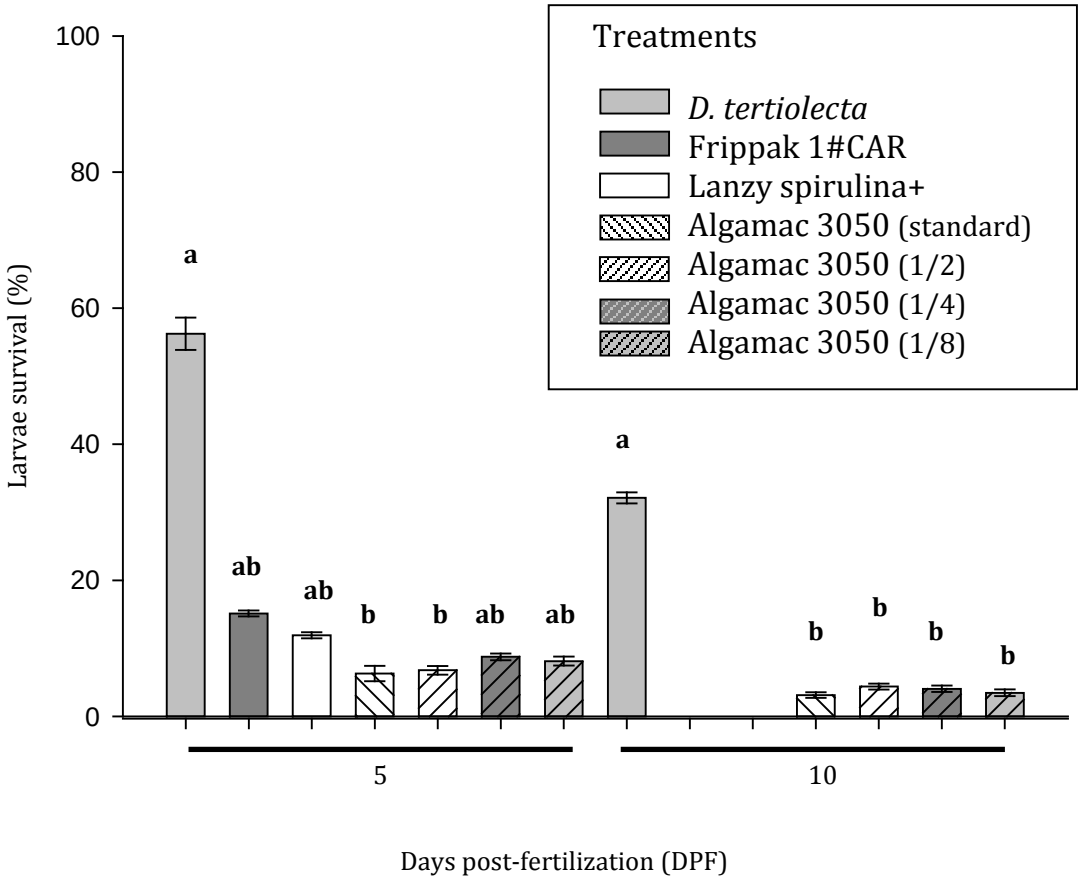

Figure 14 Mean values for larvae survival rates ± standard error of *P. lividus*, concerning feeding treatments with *D. tertiolecta*, microencapsulated/microbound feeds and enrichment with Algamac 3050, at 5 and 10 DPF. Different letters at the top of the bars, indicate statistical differences ($p<0.05$) between treatments, at the same sampling time.

Frippak and Lanzy treatments did not sustained larval survival at 10 DPF. High mortality was observed for Algamac treatments, where survival rates varied between 6.31±1.12 % - 8.75±0.484% at 5 DPF and 3.13±0.405 % - 4.38±0.448 % at 10 DPF.

4. Discussion

4.1 Culture conditions experiment

For the experimental treatments tested, larvae survival at 18 DPF, was only achieved for the W2 (with aeration and water change every 2 days), W4 (with aeration and water change every 4 days), N2 (without aeration and with water change every 2 days) and N4 (without aeration and with water change every 4 days) treatments, in opposition to treatments WN (with aeration and without water change) and NN (without aeration and

without water change). Treatment NN was only able to sustain plutei survival until 9 DPF, while culture conditions on treatment WN enabled plutei survival until 12 DPF. Additionally, a higher larvae survival at 15 and 18 DPF was found for treatment W2 in comparison to treatments W4, N2 and N4. These results suggest that abiotic culture conditions, such as regular renewal of culture water, preferably every 2 days, avoids or minimizes deleterious effects on water quality thus improving larvae survival outcome. In the same context, a possible build up of microalgae metabolites and/or harmful physical-chemical water parameters, as a result of organic matter decay, could have contributed to increased larvae mortality, specially in treatments NN and WN, thus restricting larvae development and survival on these treatments beyond 9 and 12 DPF respectively.

In treatments W2 and W4, 100% of larvae were 8-armed plutei at 18 DPF, while 84,4% and 80,0% reached the same development stage for N2 and N4 experimental groups. Once plutei larvae tended to aggregate on the surface of the larvivulture vessels without aeration (N2 and N4), water mixing induced by aeration could have enabled more efficient larvae dispersion and microalgae distribution through the rearing water column, thus improving larvae development and survival outcome.

Through out the entire sampling time frame, observed larvae had always-full greenish colored stomachs, which indicated feed acceptability by plutei during its development. A lower value for larvae survival at 18 DPF, for the W2 treatment was obtained in comparison to previous studies (Liu *et al.*, 2007). Although feeding rate and frequency, culture density and water temperature were similar, certain culture parameters such as aeration or percentage of water change could have influenced such differences. In our experiments and in order to avoid possible damage to plutei larvae and thus reduce survival, a 75% water change was performed every two days, always maintaining plutei larvae in the culture tanks. If by applying this procedure, physical damage of larvae could have been reduced, deleterious effects caused by a not complete water change of culture tanks, as Liu *et al*. (2007) did, could also have influenced survival.

4.2 Feed experiment

Following the findings of the culture conditions experiment, we have analysed the effect of live and inert, single or mixed feeds on *P. lividus* larvae development, survival and morphology (total length, post-oral arm length and body width). No differences in larvae survival among treatments were found at 6 and 9 DPF. After 15 DPF, treatment D was

statistically different from remaining experimental groups, reaching a 51.1% survival at 18 DPF, in opposition to 15.6% for FL experimental treatment. At 12 and 18 DPF, 93.3% and 97.8% of the larvae in treatment D were on 6 and 8-arm plutei stage, in comparison to 26.7% and 13.3% for treatment F, 20.0% and 11.1% for treatment L, 24.4% and 8.89% for treatment FL, 82.2% and 35.6% for treatment DF and 71.1% and 26.7% for treatment DL. At 12 DPF, statistically significant effects were found between D *vs*. F, L and FL 6-arm staged pluteus percentage, while at 18 DPF those effects were found between treatments D and remaining experimental groups. These results show that although formulated feeds sustain larvae development and survival, *D. tertiolecta* as well as mixed feed combinations of both this microalgae and artificial feeds (DF and DL) allow better results in terms of *P. lividus* larviculture, namely plutei survival and development. However, no statistically significant effects were obtained, in terms of larvae survival and 8-arm plutei stage percentage, at 18 DPF, between F, L, FL, DF and DL treatment groups. Results concerning F and DF *vs*. L and DL treatments were consistently higher at 12, 15 and 18 DPF survival rate as well as for 6 and 8-arm plutei percentage at 12 and 18 DPF. Assumptions can be made, regarding the biochemical proximal composition of both Frippak (#1CAR) and Lanzy (spirulina+). Besides its physiological function, ascorbic acid influences vertebrate growth and development (Cahu *et al.*, 2003). Likewise, among marine organisms, polyunsaturated fatty acids (PUFA) are well known for their essential role in development as well as its direct relation with survival. Phospholipds on the other hand, are a major and structurally important component of cell membranes. Fripakk (1#CAR) contains higher values of vitamine C and E in comparison to Lanzy (spirulina+), namely 4500 ppm and 500 ppm *vs*. 370 ppm and 370 ppm. It also contains higher values for total HUFA n-3 (mg/gdw), phospholipids (mg/gdw) and 3 times more total lipids (%). Thus, the higher levels of both n-3 highly unsaturated fatty acids and phospholipids, besides lipid percentage, could have also contributed to higher larvae survival and development within treatment F, in comparison to treatment L. Although artificial feeds and microalgae, provided in single (F, D) or mixed form (FL, DF, DL), sustained larvae development and survival of *P. lividus*, these feeds were unable to surpass *D. tertiolecta* treatments.

These findings oppose those obtained by Liu *et al.*, 2007, in what survival of F and L *vs*. D treatments, are concerned. One plausible explanation for these results could be either related with feed proximal composition or particle size. While Lanzy ZM (used by Liu *et al.*, 2007) *vs*. Lanzy spirulina+ composition is nutritionally different in terms of lipid (%), total HUFA n-3, phospholipds and vitamins (C and E), the same does not occur for Frippak CD

(used by Liu *et al.*, 2007) *vs*. Frippak #1CAR. Likewise and since the artificial feeds used in the present study, contain algae extracts, it would be predictable to be more palatable and enhance larvae survival and development, what also did not occur. The artificial diets used in our study were smaller (5-30μm range), than the ones, in 30-90 μm range, used by Liu *et al.* (2007). As a result, a slower sedimentation rate and water column suspension for longer periods could be expectable (Cara *et al.*, 2007). But if this was the case, then better survival results would be obtained, empirically speaking. The only logical explanation although not experimentally confirmed was that more energy could have been allocated towards suspension feeding of smaller particles *vs*. larger ones, with consequent energy reduction towards somatic growth and development. Likewise and since only a 75% water renewal of larviculture tanks was performed, every two days, a possible explanation for the obtained results could be associated with the influence that certain levels of unmonitored physical-chemical parameters, derived from degradation and leaching of nutrients from artificial diets, could have pose in terms of water quality and subsequent negative influence over larvae survival and development within these treatments (Muir and Sutton, 1994).

Also and during sampling days, plutei fed with *D. tertiolecta* had at all times full stomachs, in contrast to less evident ones for treatments DF and DL, as a result of an observed less green colored contents. For larvae feeding F, L and FL, findings were harder to observe, although we could confirm, the presence of feed particles but at a smaller scale than D, DF and DL experimental groups. These observations suggest that, and in accordance with Liu *et al.* (2007), some kind of *P. lividus* plutei feed selectivity might have occurred, towards *D. tertiolecta*.

Total length (TL), post-oral arm length (PO) and body width (BW) varied between experimental treatments, during the entire larvae culture time frame. A slower growth rate, after 3 DPF was observed among treatments F, L and FL *vs*. D, DF and DL. Larvae D, DF and DL always showed higher TL, PO and BW values at 6, 12 and 18 DPF in comparison to remaining treatments. These findings are also supported by results obtained for percentage of 6 and 8-arm plutei at 12 and 18 DPF. These results in treatments F, L and DF had a reflection on body width, during the experimental time period. Body width was highest for treatment D at 18 DPF, followed in order of magnitude by DF, DL, F, L and FL, which reflect better-feed intake and acceptability and consequently energy allocation towards larvae development. Nevertheless, the results of this experiment follow the general trend concerning *P. lividus* larvae development, described in previous studies, where *D. tertiolecta* was used as live feed for plutei (Liu *et al.*, 2007; Carboni *et al.*, 2012).

4.3 Plutei culture density experiment

In this experiment we have analysed the effect of *P. lividus* larvae culture densities over survival and plutei morphology (total length, post-oral arm length and body width). A correlation effect was found between larvae density *vs*. survival, over the entire experimental period. Results show that there is a negative effect over plutei survival, with increasing larvae culture densities, from 3 to 18 DPF. Buitrago *et al.* (2005) investigated the effect of larvae culture densities (0.25, 0.5 and 1 larvae.mL^{-1}) on larvae survival, length, dry weight and stage index in order to evaluate mass production of competent *Lytechinus variegatus* larvae. They concluded that competent larvae could be produced with more than 75% survival within a 13 days culture period, at initial culture densities of 0.25–1 larvae.mL^{-1}.

Higher larvae survival of 1.5 plutei.mL^{-1} was observed from day 6 to 18 DPF, in comparison to remaining treatments. At 18 DPF, treatment with initial larvae density of 1.5 plutei.mL^{-1} reached the highest survival (53.3±3.32 %) while the lowest survival result was obtained for treatment of 20 plutei.mL^{-1} (6.9±0.65 %). No effect concerning larvae survival was found between treatments with initial larvae concentration of 3 and 5 plutei.mL^{-1}, during the entire experimental period.

Larvae in all treatments always showed full stomachs, during the entire rearing trial, indicating microalgae feed acceptance. No apparent effect of larvae culture density over 6 and 8-arm plutei stage percentage at 12 and 18 DPF was observed.

Larvae in treatment 20 plutei.mL^{-1} showed a slower growth rate, in terms of total length, from the 6 DPF until the end of the experimental trial, in comparison to remaining experimental groups. Between 12 and 18 DPF, the observed decrease of post-oral arm length and increased values for body width within this treatment *vs*. remaining groups, indicates that larvae in this treatment could have shifted energy allocation from growth needs towards development, i.e. earlier metamorphosis, possibly due to microalgae feed abundance.

Although survival results for treatment with initial 1.5 plutei.mL^{-1} at 18 DPF, fall below those obtained by Liu *et al.* (2007), values for larvae total length during the entire experimental period were higher. Since the same microalgae species was used to feed *P. lividus* larvae, one possible explanation would be the nutritional quality of *D. tertiolecta* used in this study. Macro and micronutrients medium concentration, salinity, temperature, pH as

well as light intensity, are some of the parameters that can influence microalgae growth and biochemical constitution. Although *D. tertiolecta* is known to have low nutritional quality, in terms of fatty acid content (Payne and Rippingale, 2000; Nevejan *et al.*, 2003; George *et al.*, 2008;Carboni *et al.*, 2012), no comparison can be made once direct biochemical analysis of the *D. tertiolecta* used in the present work was not performed. Nevertheless, the findings presented in this experiment, concerning *P. lividus* larvae development at initial concentration of 1.5 plutei.mL^{-1}, follow the same trend of those obtained by Liu *et al.* (2007).

4.4 Plutei mass culture with final enrichment

Concerning the plutei mass culture and final enrichment experiments, we checked larvae performance and survival of *P. lividus* exotrophic plutei, when fed with the microalgae *D. tertiolecta*, different artificial microfeeds (Frippak 1#CAR and Lanzy spirulina+) and as final feed complement different concentrations of a commercial available enrichment product (Algamac 3050 flake). *Dunaliella tertiolecta* treatments showed relatively high survival rates at 5 and 10 DPF. What Algamac enrichment experiment is concerned, high 4-arm plutei mortality was observed, at 5 and 10 DPF, after the 12h enrichment was performed. In addition, our results show no statistical differences on plutei survival rates within treatments, within Algamac treatments, used at 5 and 10 DPF. These results lead us to conclude that this enrichment product has deleterious effects over *P. lividus* survival. Additionally, we have observed morphological alterations (shortening of arms) of plutei subject to Algamac enrichments, which in turn can indicate possible chemical alterations in culture water.

Where artificial feeds are concerned, low survival rates were obtained at 5 DPF and null survival at 10 DPF. Liu *et al.*, (2007) were able to grow *P. lividus* larvae until metamorphosis, when larvae were fed with Frippak #2 CD and Lanzy ZM, but these authors aim was not to perform mass-production of plutei larvae as live feed. In addition, they used an initial stocking density of 1.5 plutei.mL^{-1} in comparison to our 40 plutei.mL^{-1}. When *D. tertiolecta* was used as larvae feed, our results show a survival rate of 56.3% at 5 DPF and 32.1% at 10 DPF. Although the same culture conditions were used, it seems that, not only higher stocking densities have a negative impact over plutei survival over time, as deterioration of water quality, even with every 2 days water change of culture media, might have occurred, for microdiet feeding treatments. In this manner, while microalgae are live feed and are able to maintain alive for a prolonged time, artificial diets do not possess that

characteristic, which could have lead, in our case, to increased decay in water quality, thus increasing noxious metabolites which in turn have compromised larval survival.

5. Conclusion

From the results presented in this study, it may be concluded that culture water renewal (every 2 days) during *P. lividus* larviculture is an essential procedure to sustain plutei survival and development, until 18 DPF. Additionally, *P. lividus* plutei development and survival to 18 DPF, was supported by all feeds tested, single or mixed provided (*Dunaliella tertiolecta*, Frippak #1CAR, Lanzy spirulina+). *Dunaliella tertiolecta* was the employed plutei live feed which enabled the best results, considering larvae survival at 18 DPF, as well as 6 and 8-arm plutei stage development, at 12 and 18 DPF respectively. Increasing *P. lividus* plutei culture densities proved to have deleterious effects over plutei survival, during an 18-day culture period. The best combination in terms of plutei development and survival to 18 DPF was achieved using an initial culture density of 1.5 plutei.mL^{-1}. Within experiments where 40 plutei.mL^{-1} initial culture densities were used, Algamac enrichment produced high plutei mortality at 5 and 10 DPF. Additionally and for microdiets Frippak (1#CAR) and Lanzy (spirulina+) treatments, low plutei survival was observed at 5 DPF, 15,1% and 11,9% respectively, with null survival being observed at 10 DPF. Mass production of *P. lividus* plutei at higher stocking densities (>1.5 plutei.mL^{-1}) until 18 DPF is possible but with a progressive increasing mortality rate associated.

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CHAPTER 5

Potential of the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) exotrophic larvae as live feed for rearing marine decapod crustacean larvae

Tiago Repolho, João Gago and Orlando Luís

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Potential of the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) exotrophic larvae as live feed for rearing marine decapod crustacean larvae

Abstract

A set of 2 experiments was performed in order to evaluate the potential of *Paracentrotus lividus* exotrophic larvae, as live feed for decapod crustacean larviculture. In the first experiment, 24h ingestion rates were checked for five crustacean larvae (*Lysmata seticaudata*, *Maja brachydactyla, Palaemon elegans*, *Pachygrapsus marmoratus* and *Xantho incisus*) over a set of live preys, consisting in sea urchin *P. lividus* early life development stages (oocytes; gastrulas; 3 and 5 days post-fertilization (DPF) 4-arm plutei, 12 DPF 6-arm plutei and 18 DPF 8-arm plutei) and *Artemia* spp. (newly hatched nauplii and 24h metanauplii). *Lysmata seticaudata* was the only species that ingested all of the live prey tested. With the exception of this species, no ingestion on 6 and 8-arm plutei was observed in the remaining crustacean larvae species. For newly hatched nauplii and 24h *Artemia* spp. metanauplii live feeds, no ingestion was observed by *P. marmoratus* and *X. incisus* larvae. *Paracentrotus lividus* oocytes were ingested by all of the crustacean species tested.

In the second experiment, we checked the possibility to use 4-arm plutei as live prey for *Lysmata seticaudata*, *Maja brachydactyla* and *Pachygrapsus marmoratus* larval rearing versus *Artemia* spp. naupliar stages. Complete larval development and survival until metamorphosis was only achieved for *Lysmata seticaudata* when newly hatched *Artemia* spp. nauplii were used as live prey. For *Maja brachydactyla*, development until megalopa stage was achieved with *Artemia* spp. life preys. *Paracentrotus lividus* exothrophic 4-arm plutei sustained *L. seticaudata* larvae survival to 28 days after hatch (DAH) and development until zoea IV stage. For *M. brachydactyla*, larvae survived until 12 DAH when fed *P. lividus* 4-arm plutei live preys but were unable to sustain development beyond zoea II. When fed with *P. lividus* 4-armed plutei, *P. marmoratus* larvae were unable to reach zoea II stage and survival was null at 9 DAH. Inadequate fatty acid profile of *P. lividus* live prey and possible chemical or structural defences against predation are suggested as factors which might have affected feed acceptance by crustacean larvae towards this live prey. Although evidence of *P. lividus* 4-arm plutei consumption by crustacean larvae is presented, they were unable to out compete *Artemia* spp. performance as live feed, in terms of larvae development until metamorphosis and survival.

Keywords: *Paracentrotus lividus*; exotrophic larvae; live preys; decapod crustacean; larviculture; fatty acids.

1. Introduction

Temperate waters brachyuran and caridean species are marine crustaceans of commercial interest in Europe, whose culture development has been mainly driven by natural stock declines partially due to human seafood supply, live bait and ornamental trade demand.

Like most marine invertebrates, larviculture of marine crustacean decapods is dependent upon several factors of crucial importance to sustain larval survival and development, under captive conditions: Abiotic culture conditions, type of exogenous feeds, feeding regime and maternal provisioning of energetic and nutritional egg components.

The larviculture of these species has been mainly dependent upon the use of *Artemia* spp., as live feed. One of the main problems associated with the use of *Artemia* spp. as live prey is the natural low nutritional value of this zooplankton species. Over the years, this issue has been thoroughly addressed and partially overcomed through the application of bioencapsulating techniques with specific enrichment products, such as lipid emulsions (Sorgeloos *et al.*, 1998), in order to satisfy predator species nutritional needs.

Microprocessed feeds have increasingly been used in marine crustacean larviculture (Kumlu and Jones, 1995; D'Abramo, 2003; Holme *et al.*, 2006; Andrés *et al.*, 2011). Holme *et al.* (2006) concluded that total replacement of *Artemia* spp. by an experimental microbound diet resulted in poor survival of *Scylla serrata* zoea, but co-feeding of the same microdiet with *Artemia* spp. (50%/50%) could enhance larval development and survival. In another study, Andrés *et al.* (2011) found that *Maja brachydactyla* larvae survival was not significantly affected when live prey was replaced (up to 50%) by a formulated microbound diet. However some problems persist and need to be solved if total replacement of live preys by microbound feeds is to be achieved.

Nevertheless and despite the recent advances in microdiets formulation (Jones, 1998; Yúfera *et al.*, 1995; Yúfera *et al.*, 1999; Yúfera *et al.*, 2000; D'Abramo, 2002; Cara *et al.*, 2007; Holme *et al.*, 2009;) and evaluation of other live preys as live feeds (Kumlu *et al.*, 1998; Evjemo *et al.*, 2003; Helland *et al.*, 2003; Ricci *et al.*, 2003; Schlechtriem *et al.*, 2004; Meeren *et al.*, 2008;), *Artemia* spp. still remains as one the most comprehensive and generally used live feed to be used in marine larviculture.

Early developmental stages (eggs, embryos and larvae) of marine invertebrates are subject to intense predation in their natural environment, thus considered one of the most important causes of their mortality (Pennington *et al.,* 1986; Allen, 2008). To avoid predation, several defensive adaptations by echinoderm embryos and larvae have been suggested such as morphological structures, chemical defences, behavioural and species reproductive patterns (Iyengar and Harvell, 2001; Lindquist, 2002; Burdett-Coutts and Metaxas, 2004; Metaxas and Burdett-Coutts, 2006; Vaughn and Strathmann, 2008; Vaughn, 2010). Plutei predation by planktonic species has also been suggested (Rumrill, 1990) and laboratory findings confirm this hypothesis (Rumrill *et al.* 1985; Pennington *et al.,* 1986; Allen, 2008; Gago *et al.*, 2010). Pennington *et al.* (1986) tested predation rates of *Dendraster excentricus* embryonic and larval stages by eleven predators. They concluded that fish showed high consumption rates of plutei larvae in opposition to invertebrate predators, but a constant predation pattern over the different prey stages by zooplanktonic predators was not found.

Johnson and Shanks (1997) assessed predation rates of *Upogebia pugettensis* zoea and two leptoniedusa upon *Strongylocentrotus purpuratus* blastulae and echinoplutei, concluding that this event mainly occurred at non-natural high prey densities, supposedly reflecting unnatural prey-predator conditions. Also, Allen and McAlister (2007) studied the predation risk of agarose baits flavoured with *Dendraster excentricus* eggs in natural habitats, concluding that it was highest in benthic habitats. Likewise (Allen, 2008) studied, under laboratory conditions, the predation by crab zoea and megalopa, besides other predators (postlarval fish, chaetognaths and tunicates), upon different sized *Dendraster excentricus* larvae. He observed that all predators consumed live preys of different size and age, with crustacean larval stages, besides adult chaetognaths, being capable of consuming plutei larvae. Additionally, this author reached the conclusion that predation rates were dependent upon the type of predator, besides prey size and development stage. Echinoderms are a group of marine organisms, which inhabit oceans at various depths (Miller & Harley, 1996). They represent almost 7000 species (Barnes and Crook, 2001) of those more than 750 are sea urchins (Sotelo *et al*., 2002).

Sea urchins play an important role in structural organization of subtidal communities as well as macroalgae control (Vadas *et al.*, 2000). Moreover, echinoplutei importance within marine zooplankton composition and biomass has been emphasized (Fransz *et al.,* 1984; Lindley *et al.,* 1995; López *et al.,* 1998). Although predation may vary according to predator

and/or prey development stage, ecological and experimental data, suggests that sea urchin plutei could be preyed by marine organisms.

The purple sea urchin *Paracentrotus lividus* is a species most commonly found in rocky shores, whose geographical distribution spans from the North-east Atlantic Ocean to the Mediterranean Sea (Spirlet *et al*., 2001). Juvenile and adult *Paracentrotus lividus* predators include finfish species such as *Diplodus sargus*, *Diplodus vulgaris* and *Coris julis* (Sala, 1997; Sala and Zabala, 1996; Guidetti, 2003), starfish *Marthasterias glacialis* (Bonaviri *et al.*, 2009) and crustaceans species such as *Maja squinado* (Bernardéz *et al.*, 2000).

When investigating the potential of a zooplanktonic species, as live feed for marine aquaculture, several criteria have to be primarily addressed, such as independence of natural and seasonal reproductive restraints, nutritional relevance as live prey, biometry and mass production feasibility. Likewise live prey acceptance and preference is a crucial parameter in the establishment of feasible options for larval diets, before culture of a particular species is to proceed.

Over the last decades, *P. lividus* larviculture has increasingly been studied (Fenaux *et al.*, 1985; Gosselin *et al.*, 1998; Vaıtilingon *et al.*, 2001; Liu *et al.*, 2007; Carboni *et al.*, 2012).

Besides two studies that emphasize the hypothesis of early life planktonic stages of sea urchins as live feeds in marine aquaculture (Luís *et al.*, 2005) for *P. lividus* and Hubbard *et al.*, (2003) for *Lytechinus variegatus*), only (Gago *et al.*, 2010) have conducted marine larviculture trials using *P. lividus* eggs and plutei as live preys for marine fish larvae. Size similarity in comparison to *Brachionus* spp. and *Artemia* spp., simple larvae culture and spawning induction techniques, as well as easy broodstock maintenance under captive conditions and large year-round spawnings, are some of the highlighted characteristics, which sustains the hypothesis of *P. lividus* potential as live prey for marine aquaculture (Luís *et al.*, 2005).

Fatty acid profiles of *P. lividus* exotrophic plutei have only been recently addressed but were mainly aimed to echinoculture purposes (Liu *et al.*, 2007; Carboni *et al.*, 2012).

Likewise, fatty acids nutritional quality of this species eggs and endotrophic larvae (Gago *et al.*, 2009), have already been studied. These authors improved nutritional quality of these species early life stages, through manipulation of broodstock diets composition, in order to assess its potential as live prey in marine fish larviculture. Also (Repolho *et al.*, 2011) studied amino acids composition of eggs derived from captive feed *P. lividus* broodstock to assess normal percent larvae development of endotrophic plutei.

To our knowledge, the potential of *P. lividus* plutei as live prey for marine crustacean larviculture, has never been studied. Therefore we checked live feed acceptance and preference of six *Paracentrotus lividus* early life stages (oocytes; blastula; 4-arm plutei with 3 days post-fertilization (DPF); 4-arm plutei 5 DPF; 6-arm plutei 12DPF; 8-arm plutei 18 DPF) and *Artemia* spp. (newly hatched nauplii and 24h metanauplii) as live preys upon a range of five crustacean predator zoea I larvae (monaco shrimp *Lysmata seticaudata* (Decapoda; Hippolytidae), Atlantic Ocean spiny spider crab *Maja brachydactyla* (Decapoda; Majidae), rockpool prawn *Palaemon elegans* (Decapoda; Palaemonidae), marbled rock crab *Pachygrapsus marmoratus* (Decapoda; Grapsidae) and montagu's crab *Xantho incisus* (Decapoda; Xanthidae), through 24h ingestion rate analysis. Futhermore, we have evaluated the potential of *P. lividus* exotrophic larvae *versus Artemia* spp. naupli as live feeds, for the larviculture of three marine decapod crustacea (*Lysmata seticaudata*, *Maja brachydactyla* and *Pachygrapsus marmoratus*), in terms of zoea survival. Fatty acids profile of live preys was performed in order to further discuss results and compare findings with previous larviculture experimental research performed with these crustacean species.

2. Material and methods

2.1 Adult sea urchins collection and stocking

Adult *P. lividus* (mean ± standard error) with 4.8±0.044 cm (test width, TW), 2.4±0.42 cm (test height, TH) and 55.6±1.35 g (total wet weight, TWW) were collected from the wild, in the western coast of Portugal (Cabo Raso, 38°42´30”N/9°29´11”W), between May and October 2011. Sea urchins were randomly stocked (±100 individuals.m^2) in 400L cylindrical black fibreglass tanks (2.51 m^2 of total submerged area, per tank), integrated in a 3,48m^3 recirculating aquaculture system (RAS), at 18±1^oC (Luís *et al.*, 2005), 35±1 PSU (Radenac *et al.*, 2001), 700 Lux of illuminance and 14:10 (L:D) light cycle (Luís *et al.*, 2005). System aeration was maintained through an air compressor (Hiblow air pump HP-80, Techno Takatsuki Co. Ltd, Japan) and water circulation by means of a magnetic pump (Iwaki MX-70VM-13, Iwaki co, Ltd, Japan). Fluorescent lightning bulbs (Digilamp FL-40SS/36 Daylight, 6400K, 2450 lumen) provided artificial illumination over each tank. Water turnover, within culture tanks, was 55% $hour^{-1}$. RAS was equipped with biological (ouriço®, Fernando Ribeiro Lda, Portugal), mechanical (Glass Wool) and physical (Jetskim 200, Schuran Gbr, Germany) filters, as well as, UV disinfection (Aqua-UV, Model AN-55, De Bary Aquaristik,

Deizisau, Germany). Water temperature was controlled through an automatic system linked to water heaters (1000W - STIU1, Electrifor SA., Barcelona, Spain) and a chilling system (Frimar, Fernando Ribeiro Lda, Portugal). Culture parameters were checked daily: Temperature (predictor IT808 digital thermometer, Omega Pharma, Netherlands); salinity (V2 refractometer, Tropical marine centre, UK); and illuminance (Gossen Lunasix 3, P. Gossen & Co., Erlengen, Germany). A 5% RAS water change was performed once a week.

Upon collection, adult *P. lividus* were kept, without food supply, within the culture system and for a time period not exceeding 15 days, , until being used for gamete collection.

2.2 *In vitro* fertilization

Paracentrotus lividus spawning was induced by an injection of 0,5 mL (TW<4.9 cm) or 1 mL (TW>5.0 cm) KCl injection (0.5M), through the peristomial membrane. After sea urchin spawning, gametes were sieved (120µm), for debris removal. Sperm mobility and concentration were checked under 400x optic microscopy (Axiostar Plus, Carl Zeiss, Gottinngen, Germany), by means of haemacytometer observations (Bürker chamber, Germany). Oocyte concentration was checked under a 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany). Oocytes and spermatozoa were mixed, in 1000 mL glass beakers, at a 3000:1 sperm/egg ratio. After 15 minutes, eggs/oocytes were sieved (30µm) through an immersed mesh filter, for sperm removal. Percent egg fertilization was always above 98%. Eggs were inoculated in 5000 mL plastics beakers, with gentle bottom to surface aeration (approximately 30 air bubbles.min^{-1}), by means of a glass pipette, at 19±1°C, until swimming gastrula developed. Filtered (0.35 µm) and UV sterilized (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany) seawater (35±1 PSU) was used for all spawning and fertilization assays.

2.3 Live feed production

2.3.1 *Paracentrotus lividus* larvae

Paracentrotus lividus gastrula concentration (24h post-fertilization) was determined through 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany) observations. Inoculation of swimming gastrula in larval culture tanks was performed, according to desired concentration. Plutei culture (40 plutei.mL^{-1}) was performed in 7L cylindro-

spherical transparent PVC vessels (Fernando Ribeiro Lda, Portugal), filled with filtered (0.35µm), UV sterilized (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany) and aerated (approximately 60 air bubbles.min^{-1}) seawater. Microalgae *Dunaliella tertiolecta*, was added to plutei culture, according to larvae culture density, based upon a daily feeding of approximately 40.000 cells.mL^{-1} (stocking density of 40 plutei.mL^{-1}). Additionally, approximately 130.000 cells.mL^{-1} was added to culture bins at 48h and 96h post-fertilization, 12h before harvesting plutei, to be used as live prey. Culture conditions were as follows: Salinity (35±1 PSU); temperature (19±1°C); photoperiod (14h:10h, L:D); pH (8.2±1) and illuminance (700 Lux). Culture tanks were half immersed in a recirculating freshwater system, equipped with an automatic temperature control system. Larvae culture aeration was maintained through an air blower (Hiblow, 220). Artificial illumination was provided by mechanical timed daylight fluorescent bulbs (Digilamp FL-40SS/36, 6400K, 2450 lumen). Air flux to each larvae culture tank was regulated through two air valves: One located at the exit of the air blower and the second (6mm diameter immersed opening aperture) located at the basis (middle) of the larval development tank.

2.3.2 *Artemia* spp.

Artemia spp. (Great Salt Lake *Artemia* cysts, Sanders Brine Shrimp Company, United States) hatching and culture (40 artemia.mL^{-1}) was performed in 7L cylindro-spherical transparent PVC vessels (Fernando Ribeiro Lda, Portugal), filled with filtered (0.35µm), UV sterilized (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany) and vigorously aerated seawater. Brine shrimp culture concentration was volumetrically checked. *Artemia* spp. nauplii were used as live preys, newly hatched and 24h fed *Dunaliella tertiolecta*. Microalgae were added to culture bins, 12h before harvesting *Artemia* spp. metanauplii. The concentration of *D. tertiolecta* provided was approximately 130.000 cells.mL^{-1}. Culture parameters were as follows: Salinity (35±1 PSU); temperature (26±1°C); photoperiod (24h light). Permanent artificial illumination was provided (Digilamp FL-40SS/36 Daylight, 6400K, 2450 lumen).

2.4 Microalgae

2.4.1 *Dunaliella tertiolecta*

The microalgae *Dunaliella tertiolecta* (Chlorophyta: Chlorophyceae) was grown in Conway culture medium in filtered (0.35 µm) and autoclaved seawater. Microalgae concentration was checked daily (Bürker chamber, Germany) and delivered to sea urchin plutei and *Artemia* spp., by means of a micropipette (Pipetman classic™, Gilson Inc., USA).

2.5 Fatty acid composition of live prey

Three replicates of 3 and 5 days post-fertilization (DPF) plutei larvae (fed with *Dunaliella tertiolecta*) and 24h *Artemia* spp. fed *D. tertiolecta*, were analysed for total lipids and fatty acids contents.

Plutei larvae and *Artemia* spp. were sieved through a 60µm mesh strainer. Live feed samples were gently rinsed with filtered seawater (0.35µm), followed by freshwater (5µm). Excess water was removed, through filter paper absorption. Samples were stored in 1.5mL eppendorf tubes, at -20ºC for 24h. Afterwards they were lyophilised using a vacuum pump (RV12, Edwards, England) connected to a refrigerated condensation trap (RT400, Savant Instruments Inc., New Your, USA) and a vapor trap (Vapornet VN100, Savant Instruments Inc., New Your, USA), for 24h. After lyophilization, eppendorfs were filled with nitrogen gas and stored at -80°C (FS500 –86ºC ULT Freezer, Fisher Scientific), for posterior total lipids and fatty acids extraction. Lipids extraction was performed following Bligh and Dyer (1959) and described in detail elsewhere (Gago *et al*., 2009). Recovery of fatty acid methyl esters (FAME) was performed with 2 mL N-Heptane. Peak areas were determined using the Varian software and the quantification was done using C19:0 as internal standard.

2.6 Adult crustacean collection, maintenance and spawning

Crustacean species were chosen based on the following criteria: As they cohabit and share the same rocky shore ecosystem, they could possibility represent, in particular their larvae stages, potential predators of *P. lividus* early development stages.

Wild ovigerous females of the species *Lysmata seticaudata* (Risso, 1816) and *Maja brachydactyla* (Balss, 1922) were commercially acquired (Lusoreef lda, Portugal; Viveiros PescaVerde, Portugal), while *Palaemon elegans* (Rathke, 1837), *Pachygrapsus marmoratus* (Fabricius, 1787) and *Xantho incisus* (Leach, 1814) were field collected during low tides at Cabo Raso (38°42´30”N/9°29´11”W), Cascais, Portugal (table 1). Specimens were transported, immersed in thermal cases, into a recirculating aquaculture system (RAS), equipped with automatic thermo-regulation, by means of water heaters (200W aquarium

heater, Jager 3604, Eheim, GmbH & Co, Germany) and a chilling system (Hailea HC-1000A, Guangdong Hailea Group Co., Ltd, China). RAS temperature was previously set in order to avoid thermal shock to collected organisms. Culture parameters were as follows: 20±1ºC (temperature), 35±1 PSU (salinity) and 8.2±1(pH). RAS seawater was filtered (0.35µm) and UV sterilized (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany). A 10% water change (twice a week) of the culture system was performed. *Lysmata seticaudata* females were stocked into 17L rectangular glass culture tanks (0.08m^2 of bottom submerged area), with water flow rate of 0.3L.min^{-1} and kept in complete darkness. Adult females were daily feed with chopped shrimp meat. Each culture tank was divided by a 0.75x0.75 cm mesh (physical barrier between broodstock and newly hatched larvae), in order to sustain 4/5 of submerged volume available to adults and remaining 1/5 to hatched larva.

Maja brachydactyla, *P. elegans*, *P. marmoratus* and *X. incisus* ovigerous females were stocked into 50L (*M. brachydactyla*) and 17L (*P. elegans*, *P. marmoratus* and *X. incisus*) rectangular glass culture tanks, (0.24m^2 and 0.08m^2 of bottom submerged areas respectively), with a water flow rate of 0.3L.min^{-1} and in complete darkness. Females were daily feed with live adult sea urchins (*M. brachydactyla*) and chopped shrimp meat (*P. elegans*, *P. marmoratus* and *X. incisus*). Outlet drains from culture tanks were equipped with larvae collectors (450µm size mesh), which were checked and cleaned from debris, on a daily basis. Temperature and salinity were monitored daily. Nitrite levels (Profi-Test Kits, Salifert) were kept below detection levels. Nitrates levels were controlled throw weekly RAS water changes.

Table 1 – Total length, standard length, carapace length and width, total wet weight and stocking density of adult decapod crustacean females. First column indicates decapod crustacean species.

Species	Total length (TL) (cm)	Standard length (SL) (cm)	Carapace length (CL) (cm)	Carapace width (CW) (cm)	Total wet weight (TWW) (g)	Stocking density (females.m^2)
Lysmata seticaudata	**3.7** ± 0.060	**3.4** ± 0.065	----------	----------	**1.3** ± 0.044	±100 females m^2
Maja brachydactyla	----------	----------	**14.0** ± 0.358	**12.3** ± 0.350	**797.7** ± 59,40	±4 females m^2
Palaemon elegans	**3.9** ± 0.053	**3.2** ± 0.047	----------	----------	**0.9** ± 0.052	±100 females m^2
Pachygrapsus marmoratus	----------	----------	**1.6** ± 0.072	**1.7** ± 0.075	**2.5** ± 0.33	±100 females m^2
Xantho incisus	----------	----------	**1.2** ± 0.034	**1.8** ± 0.046	**2.1** ± 0.15	±100 females m^2

Mean values for TL, SL, CL, CW and TWW are expressed in cm ± standard error.

Hatched larvae were collected into 1000 mL glass beakers. Strong overhead beaker illumination was set and larvae that showed positive photo taxis were sorted to randomly stock larviculture tanks.

2.7 Crustacean larvae daily ingestion pattern

Crustacean larvae ingestion measurements were performed for five species of crustacean decapods: *Lysmata seticaudata* and *Palaemon elegans* (Caridea); *Maja brachydactyla*, *Pachygrapsus marmoratus* and *Xantho incisus* (Brachyura). For each predator species, eight different live preys were tested: *P. lividus* development stages (oocytes; gastrulas; 4-arm plutei 3 and 5 DPF; 6-arm plutei 12 DPF; 8-arm plutei 18 DPF) and *Artemia* spp. (newly hatched nauplii and 24h metanauplii). One stage I crustacean larvae was placed in a transparent rectangular plastic container (11.2cm^2 bottom area), filled with 10 mL of seawater, without aeration. A total of 10 replicates for each one crustacean larva/live prey trial were performed as well as two negative controls: One with one crustacean larva and without live prey (n=10); another with live prey and without crustacean larvae (n=5). A 5 prey.mL^{-1} concentration was used for ingestion experiments. Experimental parameters were as follows: 20±1^{o}C (temperature), 35±1 PSU (salinity), 700 Lux (Illuminance). Light cycle was artificially maintained at 14:10 (L:D), through daylight illumination (Digilamp FL-40SS/36, 6400K, 2450 lumen). Seawater was filtered (0.35µm) and UV sterilized (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau, Germany). Ingestion trials were performed during a 24h time period, after which live prey not ingested, for each of the experimental trials, were counted. Each replicate was observed under 50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany). The daily ingestion ration was expressed as the number of live prey ingested by each crustacean larva, per hour.

2.8 Crustacean larvae culture

Based upon ingestion rate results and the aim of our study (potential of *Paracentrotus lividus* exotrophic larvae as live feed for rearing of marine decapod crustacean larvae), three out of the five decapod crustacean larvae as predators (*Lysmata seticaudata* (caridea)*, Maja brachydactyla* and *Pachygrapsus marmoratus* (Brachyura)) and four live preys (3 and 5 DPF 4-arm *P. lividus* plutei, newly hatched nauplii and 24h metanauplii *Artemia* spp.) were selected to conduct crustacean larviculture trials. Crustacean larvae were stocked at a 10

larva.L^{-1} density. Concerning *P. lividus* plutei dispersal within crustacean larviculture tanks, a pre-trial was performed in order to access the relation of this culture parameter with water renewal rate and aeration, within larviculture tanks. Since a large quantity of *P. lividus* 4-arm plutei were observed to aggregate and consequently partially clogging outlet drain filters at flow rates higher than 0.2Lmin^{-1}, two different experimental sets where performed: One where water renewal in larviculture tanks was constant (0,5 L.min^{-1} for newly hatched *Artemia* spp. nauplii (Artemia NH) and 24h metanauplii (Artemia 24h); 0.2 L.min^{-1} for 4-arm plutei with 3 DPF (P3D) and 5 DPF (P5D)) and another where water renewal within crustacean larviculture tanks was arrested during a daylight time period of approximately 8 hours (only for 4-arm plutei with 3 DPF (P3ID) and 5 DPF (P5ID) treatments). Only in treatments with *P. lividus* plutei as live preys, aeration of crustacean larviculture tanks was performed, by means of a partially submerged glass pipette, whose extremity was placed at the bottom of the culture tanks and allowed a slow release of approximately 30 air bubbles.min^{-1}, directed from bottom to surface. Live preys selection was based upon crustacean larvae ingestion rates and biometry similarity (total length) between sea urchin development stages and *Artemia* spp. (3DPF 4arm plutei *vs*. newly hatched nauplii and 5DPF 4-arm plutei *vs*. 24h metanauplii). Crustacean larviculture was performed in white cylindro-conical fiberglass tanks. A NITEX™ mesh screen (150 µm mesh size sieve) was placed in the outlet drain, of the culture tanks. Every day, mesh screens were replaced (480µm mesh size sieve) to allow live prey flushing, for a time period of approximately 2 hours. Uneaten live preys were collected in a 50 µm drain filter, filled with glass wool fibber. After flushing, cleaned 150µm mesh screens were placed in the outlet drain and live prey feeding was performed (1x day) and at a density of approximately 5 preys.mL^{-1}. A more detailed description of the flushing procedure is described in Calado *et al.*, (2003).

Culture conditions were as follows: 20±1^{o}C (temperature), 35±1 PSU (salinity), 700 Lux (Illuminance); 14:10 (L:D light cycle)) and 8.2±1(pH). Overhead artificial illumination (daylight fluorescent bulbs, Digilamp FL-40SS/36, 6400K, 2450 lumen) was mechanically timed according to light cycle settings. For the species *L. seticaudata*, 10 randomly sampled larvae were checked for development stage determination (Calado *et al.*, 2004) at 15, 22 and 29 DAH. Survival rate was determined at 8, 15, 22, 29 and 31-34 DAH for treatments with *Artemia* spp. and at 8, 15 and 22 DAH for treatments with *P. lividus* 4-arm plutei as live preys. Time (DAH) for settlement of the first post-larvae, was recorded for each replicate, within newly hatched *Artemia* spp. nauplii treatment. Day (DAH) of 100% mortality, for treatments with *P. lividus* plutei as live prey was also recorded. For the species *M.*

brachydactyla, larvae survival was determined at 5 and 10 DAH. Larval development stage determination (ten randomly sampled larvae), was recorded at 5 DAH and 10 DAH, for all larvae within each replicate tank per treatment. Percentage of larvae in the megalopa stage, was recorded at 10 DAH. Larvae stage determination was performed according to Guerao *et al.* (2008, 2010). Time (DAH) for the appearance of the first megalopa, was recorded for each replicate, within newly hatched nauplii and 24h *Artemia* spp. metanauplii treatments. The day (DAH) when 100% crustacean larvae mortatility was observed for treatments with *P. lividus* plutei as live prey was also recorded. For the species *P. marmoratus*, 10 randomly selected larvae were checked for development stage determination at 5 DAH. Survival rate was determined at 5 DAH, for treatments with *P. lividus* 4-arm plutei as live preys, according to Cuesta and Rodriguez (1999, 2000). Culture tanks were cleaned and filled with pre-conditioned seawater (temperature and salinity), after each survival rate sampling procedure. For the species *L. seticaudata*, larviculture trials ended after first IX zoea stage, within all newly hatched *Artemia* spp. nauplii replicates, moulted into post larvae. *M. bracydactyla* and *P. marmoratus* larviculture trials ended when no survival was observed in the experimental treatments where *P. lividus* plutei was delivered as live preys.

2.9 Statistical analysis

Before statistical analysis, percentage data was transformed (arcsine and ln). When data normality and variance homogeneity was observed, one-way ANOVA was applied for statistical analysis. Data that did not comply with ANOVA assumptions (data normality and/or variance homogeneity) were analysed by ANOVA on ranks (Kruskal-Wallis). Tukey and Dunn testing were performed for multiple comparison analysis between different experimental treatments. Statistical analysis was performed using SigmaStat and SigmaPlot software (Systat Sofware Inc., Windows version 3.10).

3. Results

3.1 Crustacean larvae ingestion

The 24-hour ingestion for different crustacean decapod larvae are shown in figure 1. Live prey selectivity was higher for *Palaemon elegans* and *Xantho incisus*. *Lysmata seticaudata* was the only crustacean species that ingested, at a high or less extent, all kinds of

live prey tested. With the exception for *L. seticaudata*, no consumption of 6 and 8-arm plutei was observed for remaining predator species. Likewise for newly hatched nauplii and 24h *Artemia* metanauplii live feeds, no ingestion was observed by *P. marmoratus* and *X. incisus* larvae.

Palaemon elegans zoea showed a statistically significant higher ingestion of brine shrimp preys (0.95 and 1.06 *Artemia* spp. larvae^{-1}h^{-1}) in comparison to *P. lividus* oocytes (0.26 oocytes.larva^{-1}h^{-1}), while *P. marmoratus* zoea ingested significantly more gastrulas (0.68 live prey.larva^{-1}h^{-1}) in comparison to *P. lividus* oocytes and 4-arm plutei (0.15 oocytes.larva^{-1}h^{-1}; 0.16 and 0.17 plutei.larva^{-1}h^{-1}). *Xantho incisus* zoea ingestion rates were statistically different (ANOVA, Tukey test, $p<0.001$), comparing *P. lividus* oocytes (0.25 oocytes.larva^{-1}h^{-1}) *vs*. 3 DPF 4-arm plutei (0.06 plutei.larva^{-1}h^{-1}) and gastrulas (0,06 prey.larva^{-1} h^{-1}). Newly hatched *Artemia* spp. was the live prey with the highest ingestion rate for *L. seticaudata* zoea (1.14 nauplii.larva^{-1}h^{-1}) in comparison to 4-arm plutei with 3 DPF (0.70 plutei.larva^{-1} h^{-1}) and 24h *Artemia* spp. metanauplii (0.93 metanauplii.larvae^{-1} h^{-1}). Concerning remaining live preys, statistical differences (ANOVA, Tukey test, $p<0.05$) were found between 8-arm plutei larvae (0.20 plutei.larva^{-1}h^{-1}) *vs*. oocytes (0.70 oocytes.larva^{-1}h^{-1}), 3 DPF 4-arm plutei (0.70 plutei.larva^{-1}h^{-1}) and 6-arm plutei (0.68 plutei.larva^{-1}h^{-1}) and between 24h *Artemia* spp. metanauplii (0.20 plutei.larva^{-1}h^{-1}) *vs*. 8-arm plutei larvae and gastrulas (0.57 gastrulas.larva^{-1}h^{-1}) treatments. *Maja brachydactyla* zoea ingested more newly hatched *Artemia* spp. (0.77prey.larva^{-1}h^{-1}), in comparison to 3 DPF 4-arm plutei (0.33 prey.larva^{-1}h^{-1}), 24h *Artemia* spp. metanauplii (0.30 prey.larva^{-1}h^{-1}), *P. lividus* oocytes (0.27 prey.larva^{-1}h^{-1}) and 5 DPF 4-arm plutei (0.18 prey.larva^{-1}h^{-1}). *Paracentrotus lividus* oocytes were the only live feed, which was ingested by all decapod crustacean larvae species (0.15-0.69 oocytes.larva^{-1}h^{-1}). Within control replicates with only crustacean larvae, no mortality was observed. Where live prey controls were concerned, negligible differences were found, with mortality values always less than 2%.

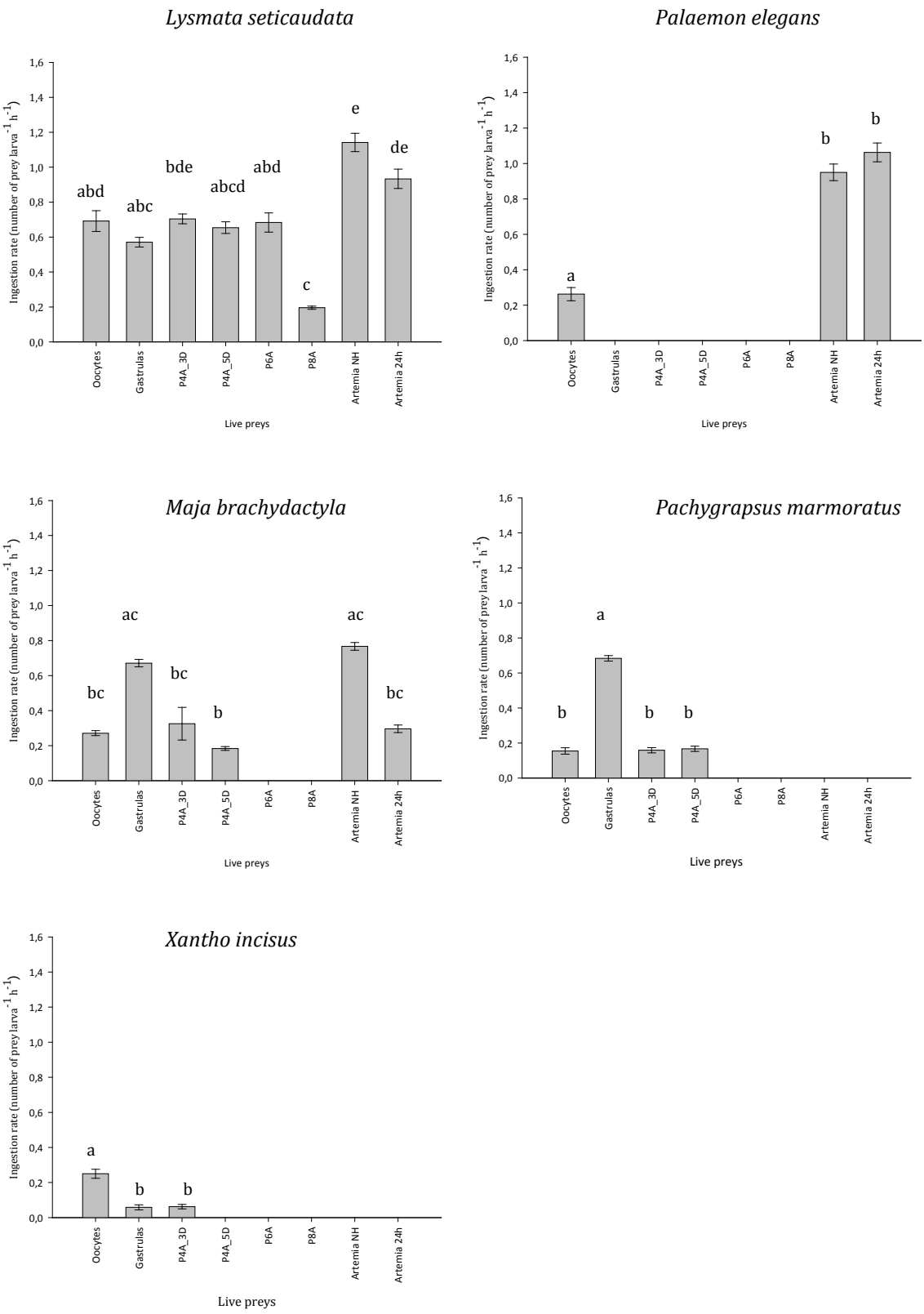

Figure 1 Mean ingestion rates (number of prey ingested.larva^{-1}.h^{-1}) ± standard error of 5 different decapod crustacean larvae over *P. lividus* early live stages (oocytes, gastrulas, 4, 6 and 8-arm plutei larvae) and *Artemia* spp. (newly hatched nauplii and 24h metanauplii) live preys. Different letters placed on the top of the bars represent statistical differences between live prey treatments, within the same crustacean species (ANOVA, Tukey test, $p<0.05$). P4D_3D: 4 arm plutei (3 DPF); P4D_5D: 4 arm plutei (5 DPF); P6A: 6 arm plutei (12 DPF); P8A: 8 arm plutei (18 DPF); Artemia NH: Newly hatched *Artemia* spp. nauplii; Artemia 24h: 24h *Artemia* spp. metanauplii.

3.2 Crustacean larviculture

3.2.1 *Lysmata seticaudata*

Results concerning mean larval survival of *L. seticaudata* larvae reared with different live preys are shown in table 2. Statistical differences (ANOVA, Tukey test, $p<0.001$) were denoted at 15 and 22 DAH, between *Artemia* NH and *Artemia* 24h *vs. P. lividus* 4-arm plutei experimental treatments. No larvae survived beyond 28 DAH, within treatments where *P. lividus* 4-arm plutei were fed as live preys. Between treatments with newly hatched and 24h *Artemia* spp. as live preys, statistical differences (ANOVA, Tukey test, $p<0.001$) were found at 15, 22, 29 and 31-34 DAH. Newly hatched brine shrimp seemed to be the best live prey provided to *L. seticaudata* larvae. The highest survival rate was recorded for this treatment with a mean value of 90.3%, at 31-34 DAH. Larvae survival results at 8, 15 and 22 DAH, concerning treatments with *P. lividus* 4-arm plutei live prey suggest that the employed 8 hour flow interruption (P3ID and P5ID) within larviculture tanks does not improve survival in comparison to P3D and P5D treatments.

Table 2 Mean survival (%) ± standard error of *Lysmata seticaudata* larvae reared with different live preys (*P. lividus* 4 arm-plutei and *Artemia* spp.). Different letters placed at the side, indicate statistical differences (ANOVA, Tukey test, $p<0.05$) between different treatments at the same sampling time period.

Species	*Lysmata seticaudata*					
Live prey	*Artemia* NH	*Artemia* 24h	P3D	P5D	P3ID	P5ID
DAH						
8	**97.8**±1.36 a	**89.7**±1.39 ab	**90.6**±0.717 ab	**86.1**±1.47 b	**89.4**±1.47 ab	**86.7**±1.87 b
15	**93.9**±1.16 a	**82.5**±1.21 d	**38.1**±2.24 c	**25.8**±1.94 b	**39.7**±1.71 c	**34.2**±0.949 cb
22	**92.2**±1.20 a	**76.7**±1.01 b	**14.4**±1.04 c	**16.1**±1.19 c	**17.8**±1.06 c	**18.9**±0.786 c
29	**90.8**±1.23 a	**62.8**±1.73 b	----------	----------	----------	----------
31-34	**90.3**±1.05 a	**57.5**±0.833 b	----------	----------	----------	----------
Replicates (n)	4	4	4	4	4	4

Experimental treatments were as follows:
Artemia NH: Newly hatched *Artemia* spp.
Artemia 24h: 24h enriched *Artemia* spp.
P3D: Treatment with continuous water renewal within larviculture tanks and *P. lividus* 4-arm plutei with 3 DPF as live prey
P5D: Treatment with continuous water renewal within larviculture tanks and *P. lividus* 4-arm plutei with 5 DPF, as live prey
P3ID: Treatment with 8-hour/day water circulation stop within larviculture tanks and *P. lividus* 4-arm plutei with 3 DPF, as live prey
P5ID: Treatment with 8-hour/day water circulation stop within larviculture tanks and *P. lividus* 4-arm plutei with 5 DPF, as live prey

Results concerning mean values ± standard error for stage percentage of *L. seticaudata* larvae, at 15, 22 and 29 DAH are shown in figure 2.

Where experimental treatments with *P. lividus* 4-arm plutei as live prey are concerned, no development beyond zoea IV was observed, at 22 DAH. *Lysmata seticaudata* larvae in P3D and P5D treatments died between 24 and 26 DAH, respectively 24.7±0.479

and 25.0±0.408 DAH (mean ± standard error). For treatments P3ID and P5ID, 100% of crustacean larvae mortality was registered between 25 and 28 DAH (26.3±0.750 DAH for P3ID and 26.5±0.645 DAH for P5ID). At 15 and 22 DAH, *L. seticaudata* stage III and IV larvae percentage varied between 62.5-70.0 % and 13.3-26.7 %, for treatments P3D and P5ID respectively.

The DAH in which the first *L. seticaudata* postlarva settled was recorded in all 4 replicates of the treatment where newly hatched *Artemia* spp. was used as live prey (32.2±0.629 DAH, mean ± standard error) but no stage IX and post larva was recorded for 24h *Artemia* spp. metanauplii experimental trial.

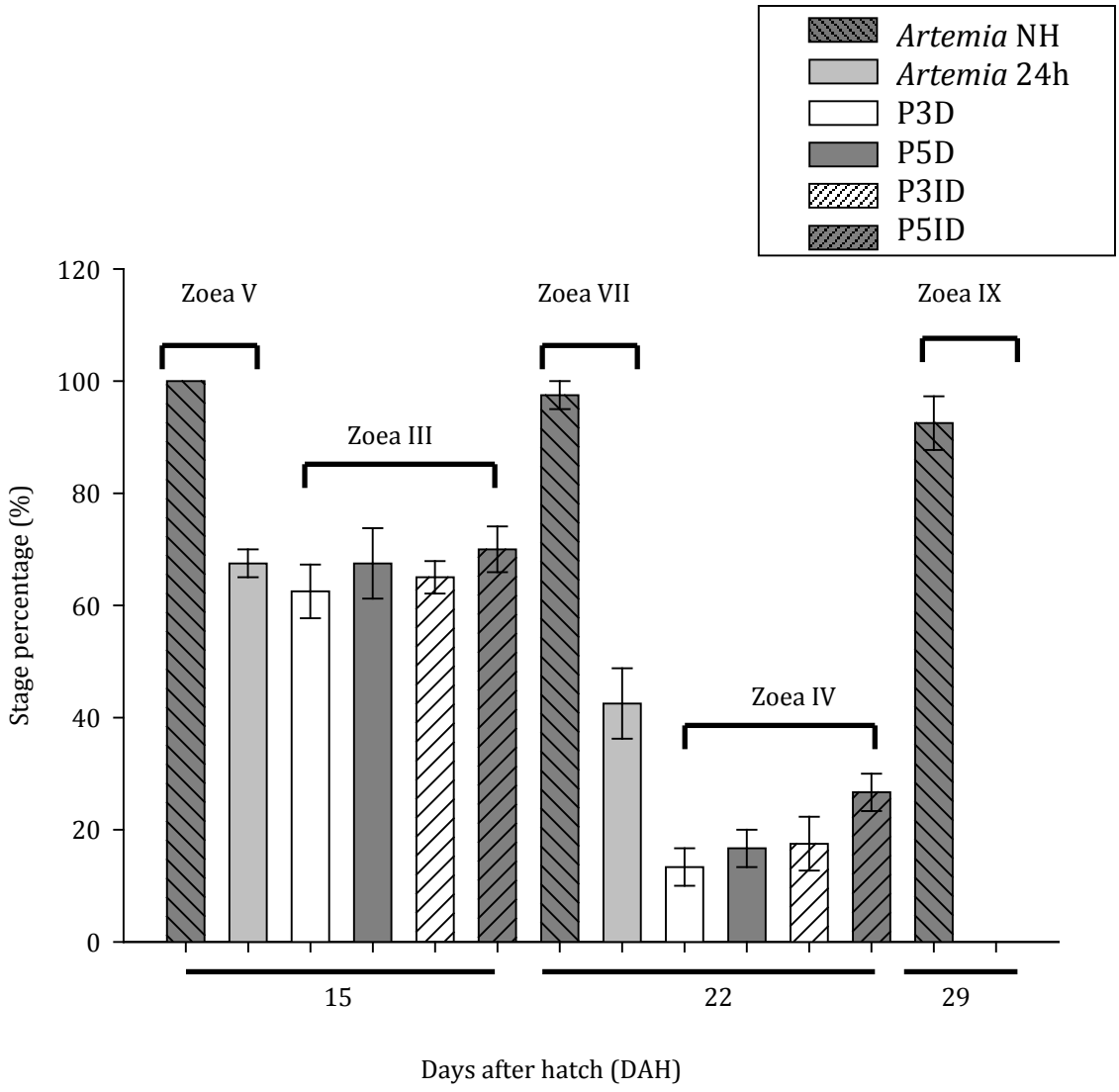

Figure 2 – Mean values ± standard error for stage percentage of *Lysmata seticaudata* larvae, at 15, 22 and 29 DAH. *Artemia* NH: Newly hatched *Artemia* spp.; Artemia 24h: 24h enriched *Artemia* spp.; P3D and P3ID: *P. lividus* 4-arm plutei (3 DPF); P5D and P5ID: *P. lividus* 4-arm plutei (5 DPF).

3.2.2 *Maja brachydactyla*

Mean larval survival of *Maja brachydactyla* larvae reared with different live preys, is shown in table 3. Statistical differences at 5 DAH (ANOVA, Tukey test, $p<0.05$) and 10 DAH (ANOVA, Dunn´s method, $p<0.05$) were found between treatments. The highest survival rates (66.9% and 55.3%), at 5 and 10 DAH, where obtained with the treatment where newly hatched *Artemia* spp. nauplii (Artemia NH) was used as live prey. Within *Artemia* NH and *Artemia* 24h treatments, first *M. brachydactyla* larvae moulted into megalopa stage between the 8 and 9 DAH, 8.25±0.250 and 8.75±0.250 (mean ± standard error) respectively. No *M. brachydactyla* larvae survival was recorded for treatments P3D, P5D, P3ID and P5ID beyond 12 DAH, where larvae died at 11.5±0.289, 11.0±0.000, 11.8±0.250 and 11,3±0,333 DAH (mean ± standard error), respectively. Results concerning *Maja brachydactyla* larvae survival at 5 and 10 DAH, with treatments with *P. lividus* 4-arm plutei as live prey suggests that the employed 8 hour flow interruption (P3ID and P5ID) does not improve substantially crustacean larvae survival in comparison to P3D and P5D treatments.

Table 3 Mean survival (%) ± standard error of *Maja brachydactyla* larvae reared with different live preys (*P. lividus* 4 arm-plutei and *Artemia* spp.). Different letters placed at the side, indicate statistical differences (ANOVA, Tukey test and Dunn´s method, $p<0.05$) between different treatments at the same sampling time period.

Species	*Maja brachydactyla*					
Live prey	*Artemia* NH	*Artemia* 24h	P3D	P5D	P3ID	P5ID
DAH						
5	**66.9**±1.53 a	**56.7**±1.01 d	**27.5**±0.949 c	**18.9**±1.70 b	**25.8**±1.99 cb	**21.9**±1.34 cb
10	**55.3**±2.85 a	**33.1**±1.23 b	**7.8**±0.79 c	**3.0**±0.37 d	**9.7**±0.95 c	**4.1**±0.39 d
Replicates (n)	4	4	4	n=4 (5DAH) n=3 (10 DAH)	4	n=4 (5DAH) n=3 (10 DAH)

Experimental treatments were as follows:
Artemia NH: Newly hatched *Artemia* spp.
Artemia 24h: 24h enriched *Artemia* spp.
P3D: Treatment with continuous water renewal within larviculture tanks and *P. lividus* 4-arm plutei with 3 DPF as live prey
P5D: Treatment with continuous water renewal within larviculture tanks and *P. lividus* 4-arm plutei with 5 DPF, as live prey
P3ID: Treatment with 8-hour/day water circulation stop within larviculture tanks and *P. lividus* 4-arm plutei with 3 DPF, as live prey
P5ID: Treatment with 8-hour/day water circulation stop within larviculture tanks and *P. lividus* 4-arm plutei with 5 DPF, as live prey

Where experimental treatments with *P. lividus* 4-arm plutei as live prey are concerned, zoea II *M. brachydactyla* larvae were observed only for treatments P3D and P3ID, at 10 DAH (figure 3). Percentage of megalopa stage larvae (mean ± standard error) were 64.4±2.24 % and 17.5±1.54 % for newly hatched *Artemia* spp. nauplii and 24h metanauplii treatments, while percentage of zoea II larvae in the P3D and P3ID treatments were 13.1±0.595 % and 17.6±2.82%, at 10 DAH.

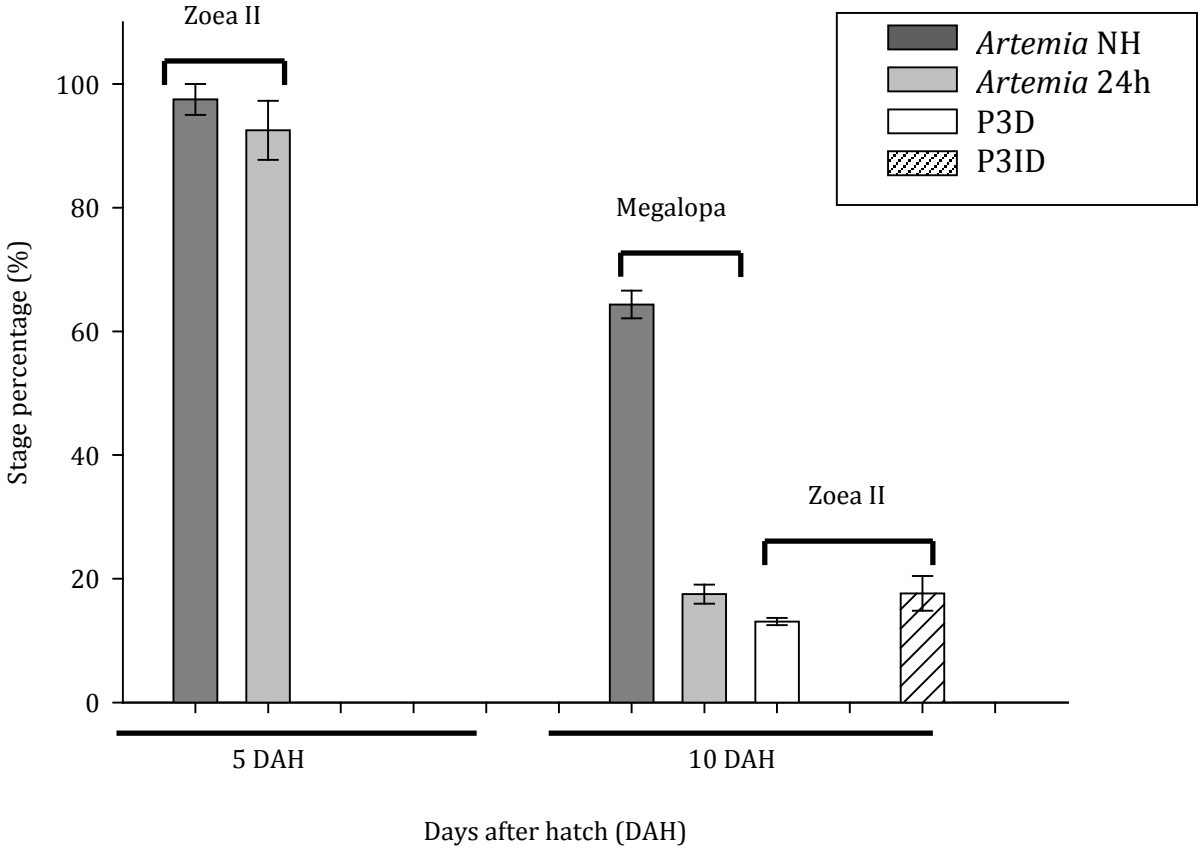

Figure 3 – Mean values ± standard error for stage percentage of *Maja brachydactyla* zoea II and megalopa, at 5 and 10 DAH. *Artemia* NH: Newly hatched *Artemia* spp.; Artemia 24h: 24h enriched *Artemia* spp.; P3D and P3ID: *P. lividus* 4-arm plutei (3 DPF).

3.2.3 *Pachygrapsus marmoratus*

Mean larval survival of *Pachygrapsus marmoratus* larvae reared with different live preys, is shown in table 4.

No statistical differences (ANOVA, Tukey test, $p<0.05$) were found at 5 DAH, where experimental treatments with *P. lividus* 4-arm plutei (3 and 5 DPF) as live prey are concerned. For the *Artemia* NH and *Artemia* 24h treatments, *P. marmoratus* larvae died between 3 and 4 DAH. For P3D, P5D, P3ID and P5ID treatments, *P. marmoratus* larvae died between 7 and 9 DAH: 7.75±0.479, 7.75±0.250, 8.25±0.439 and 8.00±0.408 DAH (mean ± standard error), respectively. No zoea II stage larvae were observed until 5 DAH, inclusively.

Table 4 Mean survival (%) ± standard error of *Pachygrapsus marmoratus* larvae reared with different live preys (*P. lividus* 4 arm-plutei and *Artemia* spp.). Different letters placed at the side, indicate statistical differences (ANOVA, Tukey test, p<0.05) between different treatments at the same sampling time period.

Species	*Pachygrapsus marmoratus*					
Live prey	*Artemia* NH	*Artemia* 24h	P3D	P5D	P3ID	P5ID
DAH 5	----------	----------	**20.3**±1.23	**17.2**±1.73	**23.1**±1.15	**18.7**±1.81
Replicates (n)	4	4	4	4	4	4

Experimental treatments were as follows:
Artemia NH - Newly hatched *Artemia* spp.
Artemia 24h - 24h enriched *Artemia* spp.
P3D: Treatment with continuous water renewal within larviculture tanks and *P. lividus* 4-arm plutei with 3 DPF as live prey
P5D: Treatment with continuous water renewal within larviculture tanks and *P. lividus* 4-arm plutei with 5 DPF, as live prey
P3ID: Treatment with 8-hour/day water circulation stop within larviculture tanks and *P. lividus* 4-arm plutei with 3 DPF, as live prey
P5ID: Treatment with 8-hour/day water circulation stop within larviculture tanks and *P. lividus* 4-arm plutei with 5 DPF, as live prey

3.3 Live prey fatty acid composition

Results concerning mean values of total lipid and fatty acid composition (mass percentage of total lipid fatty acid methyl esters, FAME) of *P. lividus* 4-arm plutei (3 and 5 DPF) and 24h *Artemia* spp. metanauplii, are shown in table 5.

Unsaturated fatty acids of live preys tested (4-arm plutei and 24h *Artemia* spp. metanauplii) had the highest percentage among analysed fatty acids and varied between 76,4% and 81,6%. Polyunsaturated fatty acids (PUFA) were the major component (61.8% and 62.1%) for plutei with 3 and 5 DPF, in comparison to monounsaturated fatty acids (MUFA) for 24h *Artemia* spp. metanauplii (46.0%). Within 4-arm plutei (3 and 5 DPF), the most represented fatty acid among analysed PUFA was C20:1n-9, with significantly (ANOVA, Tukey test, p<0.001) higher percentages (7.99% and 8.97%, respectively) in comparison to 24h *Artemia* spp. metanauplii (0.66 %). MUFA percentage was higher (ANOVA, Tukey test, p<0.001) for *Artemia* spp. metanauplii (46.0%) in comparison to *P. lividus* 4-arm plutei with 3 and 5 DPF (18.3% an 19.5%, respectively), with oleic (C18:1n-9) and palmitoleic (C16:1n-7) fatty acids representing over 68% of analysed MUFA. The palmitoleic (C16:1n-7; ANOVA, Tukey test,p<0.001), vaccenic (C18:1n-7; ANOVA, Tukey test, p<0.001) and linoleic (C18:2n-6; ANOVA, Tukey test, p<0.001) fatty acids had higher percentages in 24h *Artemia* spp. metanauplii in comparison to *P. lividus* 4-arm plutei. Eicosapantaenoic (EPA) percentage was similar between *P. lividus* 4-arm plutei (5 DPF) and 24h *Artemia* spp. metanauplii but statistically higher between *P. lividus* 4-arm plutei (3DPF) *vs*. *Artemia* spp. metanauplii (ANOVA, Tukey test, p<0.001) and between *P. lividus* plutei 3DPF *vs*. *P. lividus* plutei 5DPF

(ANOVA, Tukey test, p<0.01). The fatty acids C22:1n-9, C22:3n-3 and C22:6n-3 were not present in 24h *Artemia* spp. metanauplii. *Paracentrotus lividus* 4-arm plutei had statistically lower n-3/n-6 ratios (ANOVA, Tukey test, p<0.001) in comparison to 24h *Artemia* spp. metanauplii and statistical differences (ANOVA, Tukey test, p<0.001) were also found for n-3/n-6 ratios between 4-arm plutei 3DPF *vs*. 4-arm plutei 5 DPF.

Table 5 Mean values ± standard error of total lipid and fatty acid composition (mass percentage of total lipid fatty acid methyl esters) of *P. lividus* 4-arm plutei (3 and 5 days PF) and 24h *Artemia* spp. metanauplii live preys, fed with *D. tertiolecta*.

	P. lividus		*Artemia* spp.
	4-arm plutei (3 DPF)	4-arm plutei (5 DPF)	24h metanauplii
Total lipid (g.kg^{-1} dry mass)	**118.84**±14.149 a	**73.22**±19.41 b	**197.05**±28.342 a
Fatty acid % (FAME)			
C14:0	**4.60**±0.0978 a	**3.38**±0.0248 b	**0.99**±0.0587 c
C16:0	**10.10**±0.172	**9.99**±0.318	**10.8**±0.429
C16:1n-7	**2.17**±0.122 a	**2.49**±0.384 a	**10.0**±0.103 b
C16:3n-4	**4.00**±0.971	**3.20**±1.02	**2.66**±0.0490
C18:0	**4.07**±0.201 a	**3.77**±0.0559 a	**7.91**±0.0944 b
C18:1n-9	**1.72**±0.104 a	**3.32**±0.123 b	**21.3**±0.235 c
C18:1n-7	**2.15**±0.108 a	**1.86**±0.0602 a	**13.7**±0.101 b
C18:2n-6	**1.58**±0.0978 a	**1.93**±0.0879 a	**3.93**±0.0826 b
C18:3n-3	**8.71**±0.307	**8.13**±0.420	**8.92**±0.0816
C18:4n-3	**0.66**±0.0302	**0.54**±0.174	**1.07**±0.0141
C20:1n-9	**7.99**±0.197 a	8.**97**±0.812 a	**0.66**±0.0113 b
C20:1n-7	**2.51**±0.108 a	**1.47**±0.0229 ab	**0.34**±0.0051 b
C20:2 Δ5,11 NMID	**5.44**±0.220 a	**4.93**±0.151 a	**0.00**±0.00 b
C20:2n-6	**3.01**±0.0661 a	**3.66**±0.119 b	**0.19**±0.015 c
C20:4n-6 (ARA)	**14.0**±0.300 a	**16.2**±0.387 a	**1.24**±0.0327 b
C20:3n-3	**2.47**±0.0369 a	**1.86**±0.0761 b	**0.42**±0.13 c
C20:5n-3 (EPA)	**14.7**±0.376 b	**12.0**±0.243 a	**11.7**±0.0708 a
C22:1n-9	**1.78**±0.0317 a	**1.39**±0.0871 ab	**0.00**±0.00 b
C22:3n-3	**1.29**±0.00967 ab	**1.36**±0.120 a	**0.00**±0,00 b
C22:6n-3 (DHA)	**0.73**±0.0126 ab	**1.26**±0.248 a	**0.00**±0.00 b
ΣSFA	**19.2**±0.418 a	**17.7**±0.397 a	**22.4**±0.397 b
ΣMUFA	**18.3**±0.300 a	**19.5**±0.411 a	**46.0**±0.239 b
ΣPUFA	**61.8**±0.212 a	**62.1**±0.164 a	**30.4**±0.163 b
ΣHUFA (≥20C ≥ 3uns)	**34.2**±0.524 a	**32.8**±0.837 a	**13.3**±0.0838 b
n-3/n-6	**1.83**±0.0348 a	**1.32**±0.0329 b	**4.14**±0.0504 c
EPA/ARA	**1.05**±0.00428 a	**0.74**±0.069 b	**9.45**±0.215 c
DHA/EPA	**0.05**±0.01 ab	**0.11**±0.020 a	**0.00**±0.00 b

Mean values represent analyses of three samples. Mean values followed by different letters represent statistical differences (ANOVA, Tukey test, p<0.05) between live preys treatments. Totals (Σ) included all fatty acids that were identified.

Additionally, statistical differences (ANOVA, Tukey test, p<0.001) were found between *P. lividus* 4-arm plutei (3 and 5 DPF) vs. *Artemia* spp. metanauplii, for arachidonic fatty acid (C20:4n-6). A higher percentage of EPA/ARA ratio was found for 24h *Artemia* spp. metanauplii vs. *P. lividus* 4-arm plutei (3 and 5 DPF) treatments, with statistical differences (ANOVA, Tukey test, p<0.001) being denoted between *Artemia* spp. *vs*. 4-arm plutei (3 and 5

DPF) and between 4-arm plutei 3 DPF *vs*. plutei 5 DPF. Results also show low percentages values for DHA/EPA ratios for *P. lividus* 4-arm plutei with 3 and 5 DPF (0.05% and 0.11% respectively), in comparison to null for *Artemia* spp. metanauplii. Highly unsaturated fatty acids (HUFA) results show similar percentage values for 4-arm plutei treatments with 3 and 5 DPF, with statistical differences (ANOVA, Tukey test, $p<0.001$) being observed between 24h *Artemia* spp. metanauplii (13.3%) vs. *P. lividus* 4-arm plutei (3 and 5 DPF) treatments (34.2% and 32.8%, respectively). Additionally, saturated fatty acids (SFA) (ANOVA, Tukey test, $p<0,05$) and MUFA (ANOVA, Tukey test, $p<0.001$) percentages were statistically higher for 24h *Artemia* spp. metanauplii in comparison to *P. lividus* 4-arm plutei (3 and 5 DPF) treatments. Percentage of MUFA and PUFA increased, while SFA and HUFA percentages decreased, comparing *P. lividus* 4-arm plutei 3 DPF and 5 DPF treatments. Total lipids were significantly higher (ANOVA, Tukey test, $p<0.05$) for 24h *Artemia* spp. metanauplii in comparison to *P. lividus* 4-arm plutei (5 DPF).

4. Discussion

4.1 Crustacean larvae ingestion rates

Lysmata seticaudata was the only crustacean predator that was able to ingest all tested live preys. This species larva showed the highest feeding efficiency towards *Artemia* spp. nauplii, while 8-arm plutei was the less ingested live feed tested. During ingestion trials, *L. seticaudata* larvae were seen seizing and constantly rotating plutei in what appeared to be, in the first instance, an attempt to position live prey towards a more favourable feeding position (personal observation). This behaviour could also be related to the difficulty imposed by the plutei body and arm morphological structure, which to a certain extent could have acted as a defensive mechanism towards predation. These observations were more evident for *M. bracydactyla*. Our results show an ingestion rate decrease for 4-arm plutei with 3 DPF towards 5 DPF, with null ingestion for 6 and 8-arm *P. lividus* plutei. Also based on this species ingestion rates, a size dependent ingestion is to some extent evident, when comparing *Artemia* spp. nauplii *vs*. metanauplii and *P. lividus* 4-arm plutei with 3DPF *vs*. 5 DPF. Likewise, for remaining crustacean species, larvae were observed to have less capacity (*P. marmoratus* and *X. incisus*) or even inability (*P. elegans*) to prey upon *P. lividus* plutei.

Ingestion results of *P. marmoratus*, seem to indicate that the lower swimming capability of 4-arm *P. lividus* plutei in comparison to *Artemia* spp., could have positively

influenced the feeding efficiency of this species. Moreover, it was observed that *P. marmoratus* ingestion ability were null over *Artemia* spp., 6-arm and 8-arm *P. lividus* plutei, which are preys that possess higher swimming capability in comparison to 4-arm plutei. Although, the highest ingestion was observed for a fast swimming *P. lividus* development stage (gastrulas), this result probably is due to the fact that gastrulas develop into slow moving pre-plutei within a short time period, which in turn could have prompt ingestion rate outcome. Garcia and Rodrigues (1997) reared in laboratory *Pachygrapsus transverses* in order to study zoea morphology. These authors fed this species zoea stages with rotifers *(Brachionus* sp.), which are similar in size to gastrulas, during the first larviculture month. In another study, Cuesta and Rodríguez (2000), reared *Pachygrapsus marmoratus* zoeas I with *Chaetoceros gracilis,* followed by a mixed feed of *Chaetoceros gracilis* and *Brachionus plicatilis* (zoea II and III) and only *Artemia* sp. nauplii from stage IV to VI. It seems that for early zoea stages of *P. marmoratus*, a feeding preference towards smaller preys is evident. In this manner, feeding efficiency of *P. marmoratus* larvae, where live feed in concerned could be dependent upon prey swimming capability and size.

For *P. elegans*, ingestion rates show that this species larva had a live feed preference for *Artemia* spp., in comparison to remaining tested live preys. Concerning *P. lividus* live prey, null ingestion was observed, with the exception for oocytes. Yúfera and Rodríguez (1985) found that *P. serratus* ingest actively *Artemia* nauplii throughout the zoea stages indicating that probably in this case the feeding preferences are not directly related to an eventual constrain by prey size.

For *X. incisus*, a feed preference towards *P. lividus* oocytes was also observed in comparison to remaining live preys tested, where low ingestion rates values (*P. lividus* gastrulas and 4-arm plutei 3 DPF) or even null (remaining live preys), were observed. The results regarding *P. lividus* oocytes ingestion rates by *P. elegans* and *X. incisus* larvae could be due to possible absence of physical defences towards predation. Likewise, the null motility of this development stage could have potentially enhanced its predation by predator larvae.

In addition to morphological appendices with defensive function, the relation between the developmental stage of the predator and its prey is determinant in defining the prey accessibility. The progressive selection of larger prey with progressing the development has been clearly observed in crustaceans (Yúfera *et al*., 1984). Therefore, the predation upon a certain development stage can vary within different predator species (Iyengar and Harvell, 2001). In this context, the results obtained in this study, concerning ingestion rates of different crustacean larvae species, sustain this hypothesis.

Our work was based upon the hypothesis that since the tested adult decapod crustaceans share the same rocky shore with adult *P. lividus*, echinoplutei could probably be preyed by these species larval stages. Regarding this possibility, our team had previously performed an annual zooplankton survey in the rocky shore of Cabo Raso (West Coast, Portugal), during 2007-2008, in order to assess echinoplutei abundance. Interestingly, no plutei larvae were ever found, which could indicate that environmental factors, such as those regarding coastal hydrodynamics could enhance plutei dispersal towards offshore waters. Additionally, Silva *et al.* (2009) studied the genetic structure of the marbled rock crab *Pachygrapsus marmoratus* along the Portuguese coast, which was one of the species studied in our work. These authors suggest that their findings could be partially substantiated as a result of *P. marmoratus* larvae dispersal and drift towards open ocean waters.

Taking into consideration these authors suggestions and our findings, it is clear that although adult organisms of *P. lividus* and *P. marmoratus* share the same rocky shores, a trophic relation between their larval stages is restricted to a minimum level or even absent, within local rocky shores. These assumptions could partially explain the reduced echinoplutei ingestion by tested brachyuran zoea as a result of possible absence of natural occurring trophic interaction between these species.

4.2 Crustacean larviculture

Results concerning *L. seticaudata* and *M. bracydactyla* larviculture trials show that newly hatched *Artemia* spp., was the live prey tested which enabled the best results in terms of larvae survival, until post larva and megalopa stages, in opposition to *P. lividus* plutei.

Where *P. marmoratus* is concerned, we observed that *Artemia* spp. as live feed resulted in 100% mortality between 3 and 4 DAH, in opposition to *P. lividus* plutei, which sustained larvae survival until 9 DAH, although no stage II zoea were observed at 5 DAH.

Concerning *L. seticaudata* larviculture, our results show an average survival rate of 90.3%, until the settlement of the first post larva, only with newly hatched *Artemia* spp. treatments. For the same species, Calado *et al.*, (2005) obtained 94.8% survival rate when performing *L. seticaudata* larviculture, with newly hatched *Artemia* spp. as live prey (5 prey.mL^{-1}), with equivalent larvae stocking density (10 larvae.L^{-1}) and culture conditions (20±1^{o}C; 14:10 (L:D cycle); 34±1 PSU). In another study, the same authors obtained 94.3% of larvae survival reared at the same stocking density, salinity and live prey density, although some culture conditions, such as temperature and photoperiod, were different

from the ones used in our study and a surplus of minced squid feed was delivered beyond stage VII zoea (Calado *et al.*, 2008). With *L. seticaudata*, we have applied similar culture conditions as Calado *et al.*, (2005). Although values concerning *L. seticaudata* larvae survival rates were lower in comparison to those obtained by these authors, our results are consistent with these author findings, where newly hatched *Artemia* spp. as live prey is concerned.

Results concerning *P. lividus* live preys show that although *L. seticaudata* larvae survived until 28 DAH, this live feed was unable to sustain survival and stage development beyond zoea IV.

Likewise, *M. brachydactyla* survival was higher when newly hatched nauplii (55,3%) and 24h *Artemia* spp. metanauplii (33.1%) where used as live prey, in comparison to 4-arm plutei. Andrés *et al.* (2007) obtained larval survival of 92.0-95.6% to zoea I (3 DPH), 80.4-93.9% for zoea II (8 DPH), when *M. brachydactyla* was reared at initial stocking density of 10 and 50 larvae.L^{-1} respectively, with 60 prey.larva-^{1} of enriched *Artemia*. In the same study, survival rates of 30.2% and 25.3% to megalopa stage were obtained, with newly hatched and enriched *Artemia* as live preys, at 13 DPH.

Palma *et al.* (2008) for the species *Maja squinado*, obtained a survival rate of 32.1% to megalopa stage, when larvae were stocked at an initial density of 10 larva.L^{-1} and live feed (live *Artemia* spp. nauplii and metanauplii) were delivered at 5 prey.mL^{-1}.

Decapod crustaceans larvae survival and feeding efficiency is strongly dependent upon the ability to encounter live prey, which makes live feed concentration a key culture condition. Once, most brachyuran crab larvae are not selective feeders, live prey concentration is of extreme importance in order to sustain larval survival (Andrés *et al.*, 2007). Likewise, culture parameters such as larviculture apparatus and temperature can influence intermoult period (Matsuda and Yamakawa, 1997; Liddy *et al.*, 2004), larvae development (Calado *et al.*, 2005) and survival (Matsuda and Takenouchi, 2005; Matsuda and Takenouchi, 2007; Calado *et al.*, 2008) in decapod crustaceans. In our work we have obtained higher survival rates for *M. brachydactyla*, with zoea II larvae reaching megalopa stage earlier, than previously described. One factor that could explain these results could be the culture temperature (20^{o}C±1) used in our work, which apparently enhanced larvae development, by decreasing intermoult period. Additionally, since the prey:larva ratio used in our study (500:1) was higher than the one (60:1) used by Andrés *et al.* (2007), feeding encounters may have increased and consequently enhanced larvae development and survival.

For *P. marmoratus* larviculture, null survival at 3 and 4 DAH was obtained when *Artemia* spp. was used as live feed, which agrees with this species ingestion rates results. For *P. lividus* live preys, 4-arm plutei sustained P*. marmoratus* survival until 9 DAH, although no zoea II was observed until 5 DAH. As previously stated, it appears that this species has a feed preference towards low motility as well as smaller sized life preys.

Thus mortality observed in the larviculture trials performed, cannot be explained due to feed rejection, once ingestion rate results clearly show *P. lividus* 4-arm plutei ingestion by crustacean larvae, which indicates that larvae were able to obtain sufficient energy, that enabled, at a certain extent, survival (*L. seticaudata*, *M. brachydactyla* and *P. marmoratus*) and stage moulting (*L. seticaudata* and *M. brachydactyla*). Lindquist and Hay (1992) have experimentally proved that larvae of ascidian species (*Trididemnum solidum* and *Sigillina* cf. *signifera*) were unpalatable to reef fishes, due to the presence of chemical defenses within this development stage. Likewise and for echinoderms, McCiintock and Vernon (1990) found the presence of chemical deterrents in eggs of the sea star *Perknaster fuscus* and *Porania antarctica* and embryos of *Diplasterias brucei* and *Notasterias armata*, although no significant ichthyonoxicity was found for the remaining 11 species analyzed, of which three were sea urchins.

Cowden *et al*. (1984) studied predation by the ascidian *Styela gibbsii* and the mussel *Mytilus edulis* over invertebrate larvae. This author concluded that echinopluteus larvae had the highest survival, because *Styela gibbsii* siphon closure was more frequent, when faced by echinopluteus larvae homogenates, in comparison to seawater controls. These observations lead the author to conclude that these results could indicate chemical defensive mechanisms by this prey larva. Metaxas and Coutts (2006) have also shown that 4- and 6-arm plutei of *Strongylocentrotus droebachiensis* had a strong behavior response towards predation avoidance, through a lower spatial distribution in comparison to predator (ctenophore *Bolinopsis infundibulum*) positioning in the water column.

Allen (2008) analyzed the relation of *Dendraster excentricus* larvae size and age with predation by several predator species, including *Cancer magister* zoea and megalopa. The author observed for this predator species, that selective predation occurred towards more half-sized prey larvae and younger larvae, evidencing that age and size of *Dendraster excentricus* prey strongly influenced predation rates.

Thus given that prey capture by decapod crustaceans is primarily olfactory and mechanically driven (Hindley, 1975; Ache and Derby, 1985; Carr *et al.*, 1986) and the possibility that *P. lividus* plutei could possess chemical, behavioural and/or structural

defences against predation, could have provoked minimization of predation and consequently a negative impact in terms of larvae development and survival of *L. seticaudata*, *M. brachydactyla* and *P. marmoratus* larvae fed upon these live prey.

It is well known that in order to successfully perform, under controlled conditions, the larviculture of a particular crustacean species, it is imperative that certain culture conditions are meet and optimised, according to target species and development stage (Matsuda and Takenouchi, 2005; Smith and Ritar, 2006; Matsuda and Takenouchi, 2007; Calado *et al.*, 2008). Larvae feed, nutritional quality, biometry, composition and digestibility are key factors which enable, to a more or less extent, larval development and survival of decapod crustaceans (Jones *et al.*, 1997; Tong *et al.*, 2000; Ritar *et al.*, 2002; Robinson *et al.*, 2005; Sui *et al.*, 2007; Andrés *et al.*, 2010; Andrés *et al.*, 2011).

During larviculture trials, where *P. lividus* 4-arm plutei were used as live preys and water renewal of larvae culture tanks was constant, a small aggregation of plutei larvae in the outlet sieve mesh was still observed before filter changing procedure was daily performed. This fact could also partially explain the lower results in terms of crustacean larval survival within treatments P3D *vs*. P3ID and P5D *vs*. P5ID. Likewise, it was observed, within treatments where *P. lividus* plutei was used as live prey, that predator larvae distribution was constantly seen at a lower tank level in comparison to *Artemia* spp. treatments. This observation could also indicate an effect of water flow or its absence within larviculture tank zoea distribution, which in turn could have negatively influenced predator/prey encounter events and consequently larval development and survival.

4.3 Fatty acids of live preys

Live prey nutritional quality is of crucial importance in terms of larvae development and survival success. In this context, highly unsaturated fatty acids (HUFA), with special emphasis to eicosapentaenoic acid (C20:5n-3; EPA) and docosahaexaenoic acid (C22:6n-3; DHA), have been regarded to be essential nutrients for larvae of many marine organisms, such as bivalves (Nevejan *et al.*, 2003), sea urchins (Carboni et al., 2012) and crustaceans (Nelson *et al.*, 2004; Takeuchi and Murakami, 2007).

Microalgae represent the main source of both energy and organic matter within marine environments, being a source of essential fatty acids and food in general, for a variety of marine animals (Zhukova and Aizdaicher, 1995). In order to rear *P. lividus* 4-arm plutei as live prey to be used in this study, we used the microalgae *Dunaliella tertiolecta* as plutei feed.

Additionally, we supplied as brine shrimp feed, the same microalgae to 24h *Artemia* spp. metanauplii.

In our study, the fatty acid profile of *P. lividus* live preys used show low percentages of C22:6n-3 for 3 and 5 DPF 4-arm plutei in comparison to the absence for 24h *Artemia* spp. metanauplii. When considering C20:4n-6 (ARA), those percentages were higher for 4-arm plutei 5 DPF, followed by 3 DPF plutei and *Artemia* spp. metanauplii. EPA values varied between 14.7% and 12.0% for 4-arm plutei (3 and 5 DPF), and 11.7% for *Artemia* spp.. These fatty acids percentages were translated into low (4-arm plutei) or null (24h *Artemia* spp. metanauplii) DHA/EPA ratios, in comparison to higher levels of EPA/ARA, with special emphasis to 24h brine shrimp live prey.

Since *D. tertiolecta* has low or null C22:6n-3 content (Zhukova and Aizdaicher, 1995; Payne and Rippingale, 2000; Nevejan *et al.*, 2003; George *et al.*, 2008; Carboni *et al.*, 2012), it would be expectable either an absence or decline in DHA levels between 4-arm plutei with 3 DPF to 5 DPF (Carboni *et al.*, 2012) and absence for *Artemia* spp. But that was not the case when DHA percentage of *P. lividus* echinoplutei live prey was analysed. These results could indicate that EPA could have acted, at a limited level, as precursor of DHA biosynthesis. Such phenomenon was already hypothesized by Pantazanis *et al*. (2000) and Bell *et al.* (2001) for adult *Psammechinus miliaris.*

Artemia metabolizes highly unsaturated fatty acids and do not synthesize DHA (Sui *et al.*, 2007). This fact can possibly explain the lower HUFA and DHA absence observed for *Artemia* spp. metanauplii in comparison to 4-arm plutei live preys.

Since ARA, EPA (4-arm plutei 3DPF) and DHA (4-arm plutei 5 DPF) percentages were significantly higher in comparison to 24h *Artemia* spp. metanauplii, it would be expectable that *P. lividus* live preys could out perform brine shrimp as live feeds. However that was not the case. The only logical explanation, although not experimentally proved is based upon the hypothesis that due to possible innate plutei natural defenses (chemical, behavioral and/or structural), crustacean larvae feeding upon these live preys was not as effective as with brine shrimp preys.

The fatty acid profile of 24h *Artemia* spp. metanauplii also showed a higher SFA and MUFA percentage in relation to *P. lividus* live prey. Since SFA and MUFA are preferred over HUFA, in terms of catabolic sources for energy production (Sargent *et al.*, 1997) and 24h *Artemia* spp. metanauplii had higher percentage of these FA in comparison to *P. lividus* larvae, it would also be expectable a better performance, in terms of crustacean larvae development and survival fed brine shrimp, which eventually occurred for *L. seticaudata* and

M. brachydactyla. Additionally, the decreasing order of survival observed for crustacean larvae fed *Artemia* spp., 3 DPF and 5 DPF 4-arm plutei, could also be linked with the decreasing nutritional quality, in terms of lipid quantity present in these live preys.

To date, no previous studies are known to the authors, regarding the application of *P. lividus* exotrophic larvae stages as live preys in marine crustacean decapods. Only Gago *et al.*, (2009), studied the potential of *P. lividus* endotrophic life stages as live preys for commercially important marine fish species (*Sparus aurata*, *Diplodus sargus*), although results showed that this developmental stages could not out compete *Brachionus* spp.

5. Conclusions

Our working hypothesis was based on the fact that since the tested crustacean species cohabit with *P. lividus* in the same ecosystem, their larvae could possibility represent potential predators of *P. lividus* early development stages.

The results presented in this study show that although *P. lividus* 4-arm plutei are ingested by 4 out of 5 tested decapod crustacean larvae, they cannot out compete *Artemia* spp. nauplii and 24h metanauplii potential as live preys, in terms of larvae development and survival of *L. seticaudata* and *M. brachydactyla* species.

As live preys, *Paracentrotus lividus* exothrophic 4-arm plutei where able to sustain *L. seticaudata* larvae survival to 28 DAH and development until zoea IV. *Maja brachydactyla* larvae survived until 12 DAH but were unable to develop beyond zoea II, when fed the same live preys. *Pachygrapsus marmoratus* larvae were unable to reach zoea II stage and survival was null at 9 DAH, with plutei as live prey. Fatty acid profile of *P. lividus* 4-arm plutei and probable chemical and/or structural defences against predation are suggested as possible factors which could affect feed acceptance by crustacean larvae towards this live prey.

Future research concerning the usage of *P. lividus* exothrophic larvae as live prey in crustacean larviculture, should be directed towards several key aspects, particularly the nutritional quality improvement by means of alternative enrichment methodologies, either using other microalgae (*Cricosphaera elongata*) or other commercial available products, as plutei feed and the improvement of delivery techniques of *P. lividus* live preys to target species, within larviculture apparatus.

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CHAPTER 6

Can sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) spines be used as a tool for sex identification of adult individuals?

Can sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) spines be used as a tool for sex identification of adult individuals?

Abstract

This study investigated the possibility to use an external morphological characteristic, present in sea urchins spines, as a tool for sex identification between adult *Paracentrotus lividus*. Our findings show that 3 independent observers, correctly identified 63,1% of the females and 39,8% of the males sea urchins. However, 100% accuracy among independent observer readings was only achieved for 40.3% and 14.6% of the positively identified organisms, within females (FABT) and males (MABT) with test width between 4.0 and 5.9 cm treatments. Test height/width ratios between female and male sea urchins were analysed but no relation between this parameter and sea urchin sex was found. Based on these results, the morphological characteristic assessed in the present study, does not represent an accurate and feasible methodology for sex identification between *P. lividus* adults. Likewise, previous assumptions relating height/width ratios with sea urchin sex identification were not confirmed.

Keywords: *Paracentrotus lividus*; echinoderm; sea urchin; sex identification; test H/W ratio.

1. Introduction

Over-exploitation of wild stocks (Lawrence *et al.*, 2001; Pais *et al.*, 2007), mainly driven by increasing market demand for its gonads (roe), have led to research and development of echinoculture, during the last 2 decades (Fernandez, 1997; Basuyaux and Blin, 1998; Grosjean *et al.*, 1998; George *et al.*, 2000; George *et al.*, 2001; Lawrence *et al.*, 2001; Pearce *et al.*, 2004; Shpigel *et al.*, 2005; Cook and Kelly, 2007; Pantazis, 2009; Carboni *et al.*, 2012). Sea urchin research though is not restricted to fields of work directly related with aquaculture or even ecology but goes along other fields of biology science. As biological models, sea urchin gametes, fertilization and early life stages are studied in a variety of research fields from development, cellular and molecular biology (Berdyshev *et al.*, 1995; Dubois and Ameye, 2001; Thorndyke *et al.*, 2001; Voronina and Wessel, 2001; Thurber and Epel, 2007; Aluigi *et al.*, 2008; Agnello and Roccheri, 2010) to aquatic toxicology (Ozretic *et*

al., 1998; His *et al.*, 1999; Fernández and Beiras, 2001; Ghirardini and Novelli, 2001; Beiras, 2002; Ghirardini *et al.*, 2005; Soualili *et al.*, 2008; Marín *et al.*, 2010; Carballeira *et al.*, 2011).

One common feature to all scientific areas that use and study sea urchins early life stages as biological models is the need to obtain viable gametes from adult individuals. In this context, two main techniques are used: Ripping gonads from adult organisms or inject them with a 0.5M KCL solution, to induce spawning and collect gametes. While the first technique is 100% lethal, the second causes significant mortality (Luís *et al.*, 2005). Besides the use of alternative techniques such as electric stimulus (Marin *et al.*, 2007), KCL intra-peristomial injection still is the most commonly used methodology.

Gago and Luís (2011) studied the effect of several spawning inducing techniques over survival and spawning events in mature *P. lividus*. These authors concluded that both thermal and saline shocks were ineffective methods, mechanical shocks produced small gamete release but 100% survival of adult organisms and that KCL intra-peristomial injection although an expedite method that induced large spawnings, always caused high mortalities, among injected sea urchins.

One of the major biological milestones for those who work with sea urchins is sex identification among individuals. Once juvenile and adult sea urchins do not possess sexual dimorphism, one of the major enigmas, within sea urchin scientific community, is whenever gametes are needed and spawning have to be performed, which adult individual is male and which one is female. More recently, Zhao *et al.* (2010) addressed this issue in cultured *Strongylocentrotus intermedius* and concluded that female sea urchins were flatter than males, when test height-diameter ratios of both sexes were analyzed. Although and as previously stated, techniques that allow sea urchin sexual identification through gamete release are available, the problem still persists whenever immature individuals are to be identified. Furthermore, the development of a technique that allows juvenile, adult, mature or immature individuals sex identification would represent an economical saving, in terms of their echinoculture. Since female gonads reach higher market value in comparison to male reproductive organs, early stage sexual identification would be economically beneficial to this commercial activity, by allowing early female from male sea urchin identification at the beginning of the fattening process, targeting the production cycle to a higher economic revenue product. From the research point of view, this new target methodology, would allow a reduction in the number of organisms being used in experimental trials, with special emphasis to scientific areas where the application of certain spawning inducing techniques are associated with high levels of mortality, such as KCL injection.

The aim of this work was to investigate to what extent an external morphological characteristic of sea urchin spines between two different test width cohorts of adult individuals, would allow sex identification between wild adult *P. lividus* individuals. Additionally we also have analyzed test height/width ratio between female and male sea urchins, in order to assess the same objective. We analyzed a cohort of 214 wild collected sea urchins, within two categories of test width intervals (4.0-4.9cm; 5.0-5.9cm). Findings of the present study are presented and main results are discussed.

2. Material and methods

2.1 Collection of adult sea urchins from natural habitat

Paracentrotus lividus were collected from the wild, between July and October 2011, at Cabo Raso (38°42´30”N/9°29´11”W), located on the Western Coast of Portugal. After collection, sea urchins were transported and allocated to a recirculating aquaculture system (RAS), described elsewhere in detail (Luís *et al.*, 2005)

2.2 Adult captive stocking system

Adult *P. lividus* were stocked (±150 individuals.m^{-2}) in two 400L cylindrical black fibreglass tanks (0.4 m^3 in volume and 2.51 m^2 of total submerged area, per tank), linked in a recirculating aquaculture system. Water filtration was performed through mechanical (Glass Wool), physical (Protein skimmer Jetskim 200, Schuran Gbr – Germany) and UV sterilization (Aqua-UV, Model AN-55, De Bary Aquaristik, Deizisau - Germany) and biological (ouriço®, Fernando Ribeiro Lda, Portugal) filters. System aeration was maintained through an air compressor (Hiblow air pump HP-80, Techno Takatsuki Co. Ltd, Japan) and water temperature was kept through automatic thermoregulation by means of water heaters (1000W - STIU1, Electrifor SA., Barcelona, Spain) and a chilling system (Frimar, Fernando Ribeiro Lda, Portugal). A magnetic waterpump (Iwaki MX-70VM-13, Iwaki co, Ltd, Japan) ensured water circulation within the RAS, with a water flow rate for each culture tank of 8.5$Lmin^{-1}$ (55% renewal rate.$hour^{-1}$). Salinity was maintained at 35±1 PSU and water temperature at 18±1°C. Photoperiod of 14:10 (L:D) and illuminance of 700 Lux were kept through overhead fluorescent lighting (Digilamp FL-40SS/36 Daylight, 6400K, 2450 lumen).

Culture parameters were daily checked. Adult sea urchins were kept, within the culture system, without food supply, until experimental assays were performed.

2.3 Spawning induction

Adult sea urchins were injected with 0,5 mL (TW<4.9 cm) or 1 mL (TW>5.0 cm) of a KCl solution (0.5M), through the peristomial membrane, in order to induce spawning and determine sex. A total of 214 adult *P. lividus* were stocked into four separate tanks, within the same RAS. Sea urchins were separated according to test diameter (4.0-4.9 cm; 5.0-5.9 cm) and sex (Males *vs*. females). Wet weight, test height and width, as well as spine length and individual sex were recorded (table 1).

Table 1 Mean values for wet weight, test width, test height and spine length ± standard error of *P. lividus* adults, according to test width interval and sex. WW: wet weight; TW: test width; TH: test height; SL: spine length; n: number of sampled individuals. First column indicates test width interval (TWI).

TWI	Sex	n	WW	TW	TH	SL
[4.0; 4.9]	Female	54	44.8±1.04	4.5±0.035	2.1±0.024	1.7±0.024
	Male	55	45.6±1.00	4.5±0.039	2.1±0.027	1.7±0.023
[5.0; 5.9]	Female	57	60.0±1.36	5.1±0.022	2.3±0.026	1.8±0.026
	Male	48	55.4±1.78	5.1±0.030	2.2±0.026	1.9 ±0.024

2.4 Sea urchin spine sampling

After sex identification, one spine was removed from each individual. Afterwards this spine was placed individually in a randomly numbered box and completely immersed with 10% sodium hipochloride solution for 24h, to remove remaining animal tissues attached to the mineral matrix. Subsequently they were rinsed with freshwater and inserted into extruded polystyrene foam plates, with spine basal section facing outwards.

2.5 Spine observation

Observations of spine basal sections were performed under 40-50x stereoscope (Stemi 2000-C, Carl Zeiss, Gottingen, Germany). Female and male individuals were identified according to a hypothesized external morphological characteristic of their spines, as follows (figure 1): a) female spines present a straight slope without a pronounced curve, with a curved edge, completely surrounding the spine; b) males spines present a curved slope,

followed by a plateau with a pointed edge, totally or partially around the basal area of the spine. Three independent observers performed observations, in order to identify each spine respective sex, determined through the criteria described above.

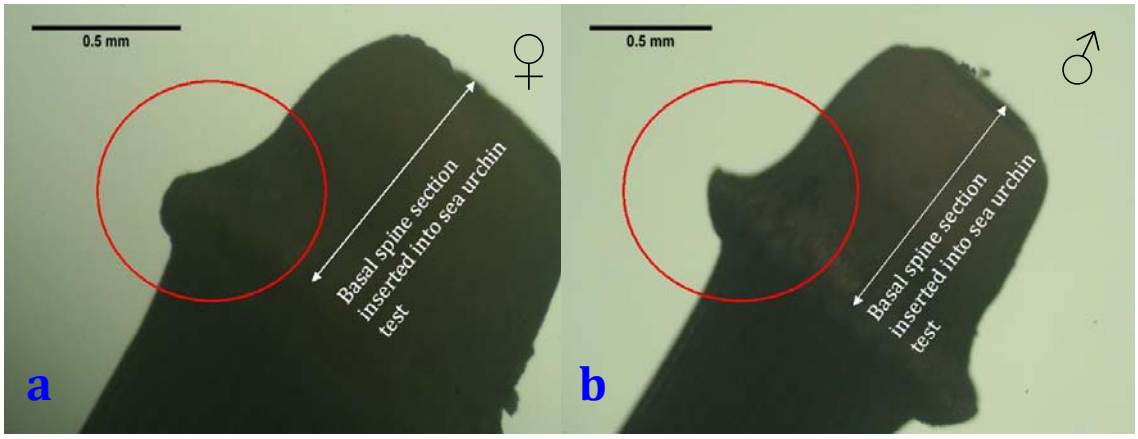

Figure 1 Criteria used to distinguish *P. lividus* female (a) and males (b), in this study. Photographs were taken to one female and one male adult *P. lividus*.

2.6 Test Height/Width ratio

Test H/W ratios were measured for all sampled sea urchins (n=214) between different sex individuals (female *vs*. male) and test width intervals ([4.0;4.9], [5.0;5.9] and [4.0;5.9]). H/W ratio was calculated, according to the formula:

Test **H/W ratio** = (test height (cm)/test width (cm))

2.7 Observer accuracy

Observer accuracy was determined as the percentage derived from positively identified *P. lividus* adults of FABT (Females with test width interval (cm) of [4.0; 5.9]) and MABT (Males with test width interval (cm) of [4.0; 5.9]) treatments, whenever independent observers identifications had all positively coincide.

2.8 Statistical analysis

Percentage data was normalised by arcsine transformation, prior to statistical analysis. One-way ANOVA analysis was performed followed by multiple comparison analysis

(Tukey test). The SigmaStat and SigmaPlot software (Systat Sofware Inc., Windows version 3.10) was used for statistical analysis of results.

3. Results

3.1 Percent sex identification

Results of mean values for percent sex identification of *P. lividus adults* are shown in figure 1. Statistical differences (ANOVA, Tukey test, $p<0.05$) were found between treatment FA (females with test width [4.0;4.9]) *vs*. treatments MA (males with test width [4.0;4.9]) and MB (males with test width [5.0;5.9]). Mean values for percentage of positively sex identification of adults, varied between 55.7% for females and males with test width between 4.0 and 4.9 cm (FMAT) and 47.9% for females and males with test width between 5.0 and 5.9cm interval (FMBT).

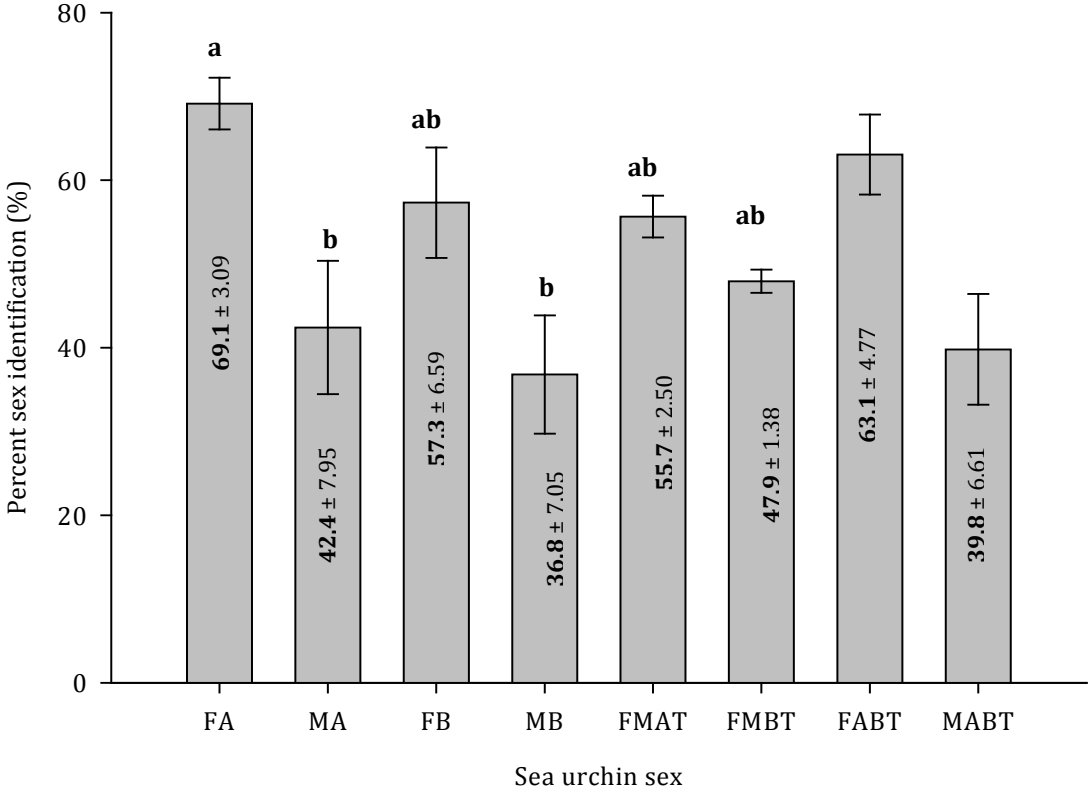

Figure 2 Percent sex identification (%) ± standard error of *P. lividus* adults. Different letters placed at the top of the bars indicate statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments. FMAT, FMBT, FABT and MABT represent pooled data. FA: Females with test width interval (cm) of [4.0;4.9]; MA: Males with test width interval (cm) of [4.0;4.9]; FB: Females with test width interval (cm) of [5.0;5.9]; MB: Males with test width interval (cm) of [5.0;5.9]; FMAT: Females and males with test width interval (cm) of [4.0;4.9]; FMBT: Females and males test width interval (cm) of [5.0;5.9]; FABT: Females with test width interval (cm) of [4.0;5.9]; MABT: Males with test width interval (cm) of [4.0;5.9].

3.2 Test H/W ratio

Mean values concerning test H/W ratio, of wild *P. lividus* adults, are shown in figure 3. Statistical differences (ANOVA, Tukey test, $p<0.05$) were found for males with [5.0;5.9] test width *vs*. females with [4.0;4.9] test width, males with [4.0;4.9] test width and females with [4.0;5.9] test width treatments.

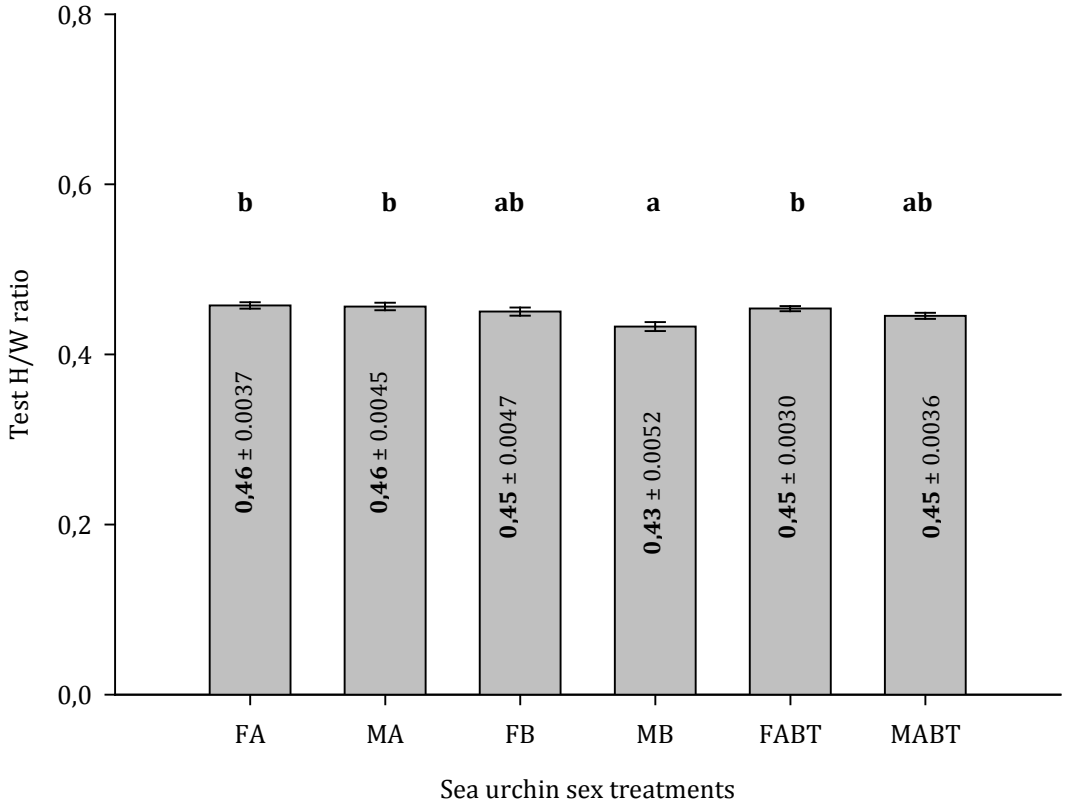

Figure 3 Test H/W ratio ± standard error of *P. lividus* adults. Different letters placed at the top of the bars indicate statistical differences (ANOVA, Tukey test, $p<0.05$) between treatments. FABT and MABT represent pooled data. FA: Females with test width interval (cm) of [4.0;4.9]; MA: Males with test width interval (cm) of [4.0;4.9]; FB: Females with test width interval (cm) of [5.0;5.9]; MB: Males with test width interval (cm) of [5.0;5.9]; FABT: Females with test width interval (cm) of [4.0;5.9]; MABT: Males with test width interval (cm) of [4.0;5.9];

3.3 Observer accuracy

Mean values regarding accuracy between observer's positive readings of sea urchin sex, concerning females (FABT) and males (MABT) treatments with [4.0;5.9] test width interval, are shown in figure 3. From initial correct identified female (FABT, 63.0%) and male (MABT, 39.8%) individuals, a percentage of 40.2% for FABT and 14.6% for MABT

treatments was obtained, when 100% accuracy between observer positive identifications was checked.

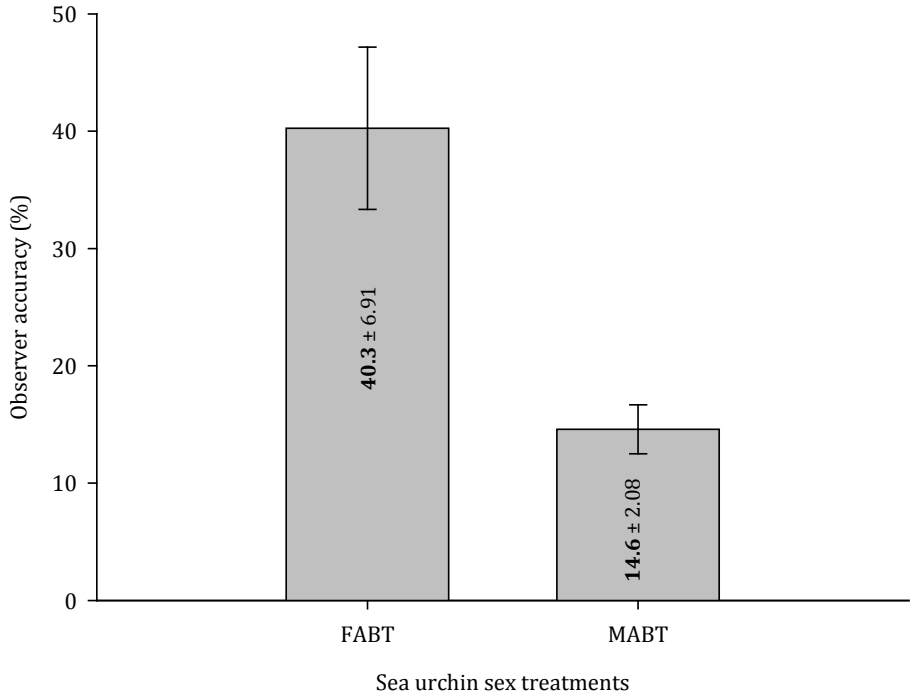

Figure 3 Observer accuracy ± standard error of *P. lividus* sex identification. FABT and MABT represent data concerning 100% accuracy observed between independent observer readings in relation to percent sex identification results. FABT: Females with test width interval (cm) of [4.0;5.9]; MABT: Males with test width interval (cm) of [4.0;5.9].

4. Discussion

For those studies performed under laboratory conditions where gametes have to be obtained from adult individuals, spawning is performed through the application of physical or chemical induction techniques, which at a more or less extent, can result in sea urchin mortality (Gago and Luís, 2011). In our study we aimed to investigate if an external morphological characteristic observable in sea urchin spines, would allow sex identification and consequent identification of adult *P. lividus*. Furthermore we investigated if test height/width ratio would also allow an insight over sex identification, as previously suggested for *Strongylocentrotus intermedius* (Zhao *et al.,* 2010). Our results show that percentage of correctly identified individuals, females and males of different test size cohorts varied between 55,6% and 47,9%, which by itself is close to the mean probability of correctly identifying sea urchin sex, taking into account, two possible hypotheses. However if 100% accuracy between independent observer readings was taken into consideration, initial

percentage of correctly identified females (63.1%) and males (39.8%), concerning treatments with test width interval between 4.0 and 5.9 cm (FABT for females and MABT for males), would decrease to mean values of 40.3% and 14.6%, respectively. As for test H/W ratios, values varied between 0.45-0.46 and 0.43-0.45 for female and male adult individuals, respectively. Since statistical differences, concerning test H/W ratio, were found between females *vs*. males from the same test size cohort or when polled data concerning treatments with females with test width interval of [4.0; 5.9] and males with test width interval of [4.0; 5.9] were compared, we did not found evidence that could lead us to sustain the hypothesis that male sea urchin have a higher test H/W ratio in comparison to females, as suggested by Zhao *et al.* (2010). However, the findings of these authors were based upon measurements performed on a different species (*Strongylocentrotus intermedius*) and captive breed organisms, unlike ours that were wild caught.

5. Conclusions

Our work was aimed to assess the ability to use a spine morphological characteristic as a non-invasive sex identification tool, in order to identify female from male adult *P. lividus*. The spine morphological characteristic tested in this work did not show itself as an accurate and feasible technique for sex identification between *P. lividus* adults. Additionally, no statistically significant evidence that could sustain assumptions that male sea urchin have a higher test H/W ratio in comparison to females, was found. Further effort should be employed in order to develop a non-invasive and cost effective method that allows sex identification of female and male *P. lividus*.

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CHAPTER 7

Final Considerations

FINAL CONSIDERATIONS

In order to evaluate *Paracentrotus lividus* exotrophic plutei as a potential live prey for marine decapod crustacean larviculture, we have studied several key point criteria to test our hypothesis: 1) broodstock diet manipulation in relation to larvae development and nutritional quality; 2) the effect of sperm:egg ratios and egg concentration on *P. lividus* percent egg fertilization, early stage segmentation and larvae development; 3) plutei mass-production and nutritional quality improvement; 4) live prey acceptability and marine decapod crustacean larval development and survival. Additionally, we also assessed the possibility to use an external morphological characteristic of adult sea urchins spines as a sex identification tool.

Considering experiments where broodstock diet manipulation was performed (Chapter 2), the effect of natural *vs*. artificial inert diets on total egg amino acid content and fatty acids profile of *P. lividus* eggs, prisms and endotrophic larvae, was analysed. We concluded that when an inert diet, based on maize and wheat flour mixture as dietary protein source, was provided to *P. lividus* captive broodstock, high percent egg fertilization (>99%) and similar percent normal development of 4-arm endotrophic plutei were achievable in comparison to wild *P. lividus* organisms. Likewise we concluded that total egg amino acid composition, derived from broodstock fed this inert diet, was similar in comparison to wild caught *P. lividus* adults but different from remaining captive fed treatments. These findings demonstrate that under captive conditions and as long as an appropriate inert diet is provided the quantitative and qualitative year round production of 4-arm echinoplutei is possible, free from environmental and *P. lividus* reproductive seasonal constraints. Within chapter 2.2 ("Fatty acid nutritional quality of sea urchin *Paracentrotus lividus* (Lamarck 1816) eggs and endotrophic larvae) the best nutritional improvement in terms of fatty acid profile, with special emphasis to essential fatty acids (EFA) and lipid content of 4-arm plutei was obtained by manipulation of *P. lividus* broodstock diet, through the inclusion of commercial available products (Algamac 2000, cod liver oil) as lipid sources. We therefore demonstrated that the inclusion of Algamac or cod liver oil as lipid sources, led to higher EFA content, in comparison to remaining treatments (linseed and olive oils) and therefore should be primarily considered for broodstock diet manufacture in order to

produce 4-arm plutei as standard live preys for marine larviculture, with a more suitable fatty acid profile.

Taking into consideration criteria 2 (Chapter 3), we have concluded that increasing sperm:egg ratios positively influenced percent egg fertilization but had a negative impact on normal early stage segmentation development, until 90 minutes after fertilization. Larvae total length was not influenced by either egg concentration or sperm:egg ratios and post-oral arm length and normal percent plutei development were found to be possibly dependent upon embryo/larvae culture densities, but not sperm:egg ratios. We therefore concluded that, sperm:egg ratios above 300:1, seem to induce deleterious effects on *P. lividus* percent segmentation stage development but not on analyzed larvae biometry parameters or normal development.

Considering criteria 3 (Chapter 4), culture water renewal (every 2 days) during *P. lividus* exotrophic larviculture, was found to be an essential condition in order to sustain plutei development and survival until 18 days post-fertilization (DPF). *Dunaliella tertiolecta* outperformed remaining tested microdiets (single or mixed) in terms of plutei development and survival outcome to 18 DPF. Experimental trials performed in order to analyse the effect of plutei stocking densities on their survival, proved that increasing culture densities had deleterious effects on *P. lividus* larvae survival, during an 18 days rearing period. Additionally, we demonstrated that mass production of *P. lividus* plutei at stocking densities higher than 1.5 plutei.mL^{-1} is possible but with a higher mortality rate associated. Nevertheless, if the aim is to mass produce plutei, a final survival of 1500 plutei.L^{-1} at 18 DPF starting with an initial culture densities of 5 plutei.mL^{-1}. Additionally, we concluded that a 12h Algamac enrichment period, using different concentrations, had deleterious effects over exotrophic plutei survival.

Concerning criteria 4 (Chapter 5), regarding foraging behaviour of decapod crustacean larvae predating on different developmental stages of sea urchin, we found that *P. lividus* oocytes were ingested by all crustacean species and *L. seticaudata* was the only crustacean predator whose ingestion on all live prey tested was observed. *Lysmata seticaudata*, *Maja brachydactyla*, *Pachygrapsus marmoratus* and *Xantho incisus* were the crustacean species where ingestion rates on *P. lividus* exothrophic plutei was recorded. Live prey size, swimming capability and plutei defensive mechanisms towards predation, alongside with crustacean larvae ability to seize live preys, are possible factors that could have influenced *P. lividus* live prey ingestion rates by crustacean larvae. Although *P. lividus* exotrophic plutei were able to sustain, to a certain extent, zoea survival of *L. seticaudata*, *M.*

brachydactyla and *P. marmoratus*, these live preys were unable to sustain complete larvae development and survival to settlement of the targeted crustacean species, in comparison to *Artemia* spp. Complete larvae development and survival until metamorphosis was only achieved for *L. seticaudata* when fed newly hatched *Artemia* spp. nauplii, while *M. brachydactyla* development until megalopa stage was achieved with both newly hatched nauplii and 24h *D. tertiolecta* fed *Artemia* spp. metanauplii, as live preys. Fatty acid profile of *P. lividus* 4-arm plutei, total lipid content and possible chemical or structural defences mechanisms towards predation could have acted as possible factors which might have affected result outcomes in what crustacean larviculture is concerned. In the last experiment of this study, we concluded that the chosen external morphological characteristic of adult *P. lividus* spines, did not allow accurate results while attempting to use it as a sex identification method.

As main conclusions of the present dissertation, *P. lividus* 4-arm exotrophic plutei as live prey failed to sustain development and survival to metamorphosis of the reared three marine decapod crustacean larvae tested. Nutritional improvement of *P. lividus* 4-arm plutei, by means of nutritionally superior microalgae, other enrichment products or even more readily acceptable and palatable microdiets, could improve development and survival outcome of targeted species. Moreover, further developments concerning plutei mass-production rearing techniques, could improve plutei larviculture development and survival results as well as optimisation of live prey delivery techniques, could eventually improve larvae development and survival of crustacean species aimed to be cultured.

Despite the overall negative results concerning our main objective ("Evaluation of *Paracentrotus lividus* (Lamarck, 1816) exotrophic larvae as live feed for marine decapod crustacean larvae"), the information derived from this dissertation is of valuable importance, not only for echinoculture development, commercially aimed or for restocking purposes, but also for certain research areas, such as environmental assessment, whereas *P. lividus* fertilization and early life stages are used as biological models.