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Sea urchin *Paracentrotus lividus* (Lamarck 1816) eggs and endotrophic larvae: Potential of their use as marine larval fish first-feeding.

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DOUTORAMENTO EM BIOLOGIA

Especialidade: Biologia Marinha e Aquacultura

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Tese orientada pelo Professor Auxiliar Doutor Orlando de Jesus Luís

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NOTA PRÉVIA

A dissertação que agora apresento enquadra-se num projecto desenvolvido no Laboratório Marítimo da Guia da Faculdade de Ciências da Universidade de Lisboa em colaboração com a Estação Piloto de Piscicultura do Centro Regional de Investigação Pesqueira do Sul do IPIMAR, com o objectivo de testar a potencialidade um novo alimento vivo a ser utilizado na primeira alimentação larvar de peixes marinhos produzidos comercialmente em aquacultura. O novo alimento vivo que foi testado ao longo deste trabalho consistiu na utilização de ovócitos e larvas endotróficas do ouriço-do-mar *Paracentrotus lividus*. Visto que as principais contrariedades no cultivo de peixes marinhos com interesse comercial prendem-se com a mortalidade ocorrida durante a sua fase larvar, o estudo de novas alternativas alimentares é essencial para tentar melhorar a elevada mortalidade que ocorre quando são utilizados os alimentos vivos tradicionais (o rotífero *Brachionus* spp. e os estados larvares do crustáceo *Artemia* spp.). A escolha de ovócitos e larvas endotróficas do ouriço-do-mar *Paracentrotus lividus* prendeu-se com o facto de ser conhecido que as larvas de ouriços-do-mar constituem um abundante alimento natural de larvas de peixe no plâncton marinho.

Pessoalmente, este trabalho foi parcialmente suportado por uma Bolsa de Doutoramento da Fundação para a Ciência e Tecnologia (SFRH / BD / 22747 / 2005), visto que durante o tempo de realização deste trabalho estive a exercer funções como docente Assistente da Escola Superior Agrária de Santarém.

A presente dissertação está dividida em 5 capítulos em que em alguns destes são apresentados trabalhos já publicados ou submetidos para publicação em revistas internacionais da especialidade, nos termos do nº 1 do Artigo 41º, Capítulo V, do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República – II série Nº 209 de 30 de Outubro de 2006. Neste contexto refiro que participei de forma integral em todos os trabalhos, desde o seu planeamento, concretização e redacção dos manuscritos resultantes.

O capítulo 1, designado de "*General Introduction*", apresenta sumariamente o estado da arte relativa à primeira alimentação larvar de peixes marinhos produzidos comercialmente, introduz a temática da investigação que foi realizada e apresenta os objectivos da presente tese de doutoramento.

No capítulo 2, "*Live Prey Availability and Production*", são apresentados os efeitos que as condições de cultivo a que estão sujeitos os ouriços adultos reprodutores (dieta, temperatura,

fotoperíodo, densidade e técnica de indução de postura) têm nas respectivas posturas, de forma a aferir o potencial quantitativo de produção de presas a serem utilizadas na alimentação de larvas de peixes marinhos. Este capítulo é constituído por 1 trabalho publicado na revista *Aquatic Living Resources* (2005), por 1 trabalho submetido à revista *Journal of Shellfish Research*, e por um trabalho submetido à revista *Aquaculture International*.

O capítulo 3, designado de "*Nutritional quality of Paracentrotus lividus eggs and endotrophic larvae for marine fish larvae first-feeding*", avalia a qualidade nutricional dos ovócitos e larvas endotróficas do ouriço-do-mar *Paracentrotus lividus*, para dois nutrientes essenciais na dieta das larvas de peixes marinhos (ácidos gordos e aminoácidos). Neste capítulo também é analisada a possibilidade de melhoramento nutricional destas presas vivas através da manipulação da composição da dieta dos ouriços adultos reprodutores. Este capítulo é constituído por 1 trabalho publicado na revista *Aquaculture Nutrition* (2009) e por um trabalho submetido na revista *Journal of Shellfish Research.*

No capítulo 4, "*Survival and growth of selected marine fish larvae first fed with eggs and endotrophic larvae of the sea urchin Paracentrotus lividus*", são apresentados os resultados dos ensaios realizados na Estação Piloto de Piscicultura do Centro Regional de Investigação Pesqueira do Sul do IPIMAR, onde foram avaliadas as taxas de ingestão, o crescimento e a sobrevivência de larvas de peixes marinhos quando alimentadas com ovócitos e larvas endotróficas do ouriço-do-mar *Paracentrotus lividus* durante os primeiros tempos de vida. Neste capítulo, os resultados obtidos para estas variáveis foram sempre comparados com aqueles que se obtiveram quando as larvas de peixes marinhos foram alimentadas com o tradicional primeiro alimento vivo (o rotífero *Brachionus* spp.). Este capítulo é constituído por 1 trabalho aceite para publicação na revista *Aquaculture Research.*

Finalmente no capítulo 5, denominado de "*Final Considerations*", é analisado e discutido o potencial geral que os ovócitos e larvas endotróficas do ouriço-do-mar *Paracentrotus lividus* podem ter como primeiro alimento vivo no cultivo larvar de peixes marinhos, assim como são sugeridas possíveis novas linhas de investigação dentro desta temática.

Lisboa, Dezembro de 2009

João Gago

AGRADECIMENTOS

A realização dos diversos trabalhos desenvolvidos, ao longo dos vários anos dispendidos na concretização da presente tese de doutoramento, teve a imprescindível colaboração de diversas pessoas e instituições. Ao nível institucional devo referir que quer o Laboratório Marítimo da Guia (Centro de Oceanografia da Faculdade de Ciências de Lisboa) quer a Estação Experimental de Piscicultura (Centro Regional de Investigação Pesqueira do Sul do Instituto Português de Investigação Marítima) me proporcionaram as necessárias condições materiais para a execução dos vários ensaios experimentais realizados.

A título pessoal devo referir que várias foram as pessoas que com carácter mais formal, laboral ou afectivo colaboraram na prossecução dos trabalhos desta tese, e que de uma forma mais ou menos presente me incentivaram ao longo deste percurso. A este nível devo salientar a orientação e o apoio constante do Professor Doutor Orlando de Jesus Luís e a disponibilidade demonstrada pelo Professor Doutor Pedro Pousão. Também, sem a ajuda do aluno de mestrado Tiago Martins, as experiências desenvolvidas na Estação Experimental de Piscicultura em Olhão poderiam ter ficado comprometidas. A eles e a todos os outros (“pessoal da Guia e do IPIMAR”) o meu muito obrigado.

Finalizo com um sentido apreço pela compreensão dos meus familiares, particularmente da minha mulher e da minha filha, pelo tempo que utilizei extra-laboralmente, na realização da presente tese de doutoramento.

ABSTRACT

Studies carried out both in natural environments and in laboratory indicate that sea urchin eggs and larvae are meroplanktonic preys available to marine fish larvae. Therefore, the potential of *Paracentrotus lividus* eggs and endotrophic larvae as first live feed in marine fish larviculture was evaluated and compared with the traditional ones used in hatcheries: the rotifer *Brachionus* spp. and the naupliar stages of the brine shrimp *Artemia* spp. Three main criteria were tested to determine the suitability of this organism as a mass produced source of zooplankton for marine fish larvae first-feeding: year-round availability of a large number of prey; nutritional quality; and prey acceptability.

The effects of several captive broodstock conditioning factors (captivity time, broodstock diet, density and spawning induction methods) on spawning performance and fertilization were assessed. Year-round availability of a large number of *P. lividus* eggs and larvae was obtained through a combination of factors like 3 months, simple diets, high densities, intra peristomial injection of 1 mL KCl 0.5M. Other spawning induction techniques were also evaluated and broodstock emersion for a period of 3 to 6 hours appeared to be a good substitute to KCL preventing broodsotck mortality.

P. lividus eggs and endotrophic larvae fatty and amino acids profiles were comparable to either *Brachionus* spp. and/or *Artemia* spp. Contrarily to protein composition, it was demonstrated the fatty acid enrichment possibilities of eggs and larvae trough manipulation of *P. lividus* broodsotck diet lipid content and composition.

Results obtained in the prey acceptability trials demonstrated that, in spite of being ingested by fish larvae, manipulated *P. lividus* eggs and endotrophic larvae showed lower ability to first feed marine fish larvae when compared to *Brachionus* spp.

No added value for marine fish larviculture was found in the use of *P. lividus* eggs and endotrophic larvae as live feed.

Keywords: *Paracentrotus lividus*, eggs, endotrophic larvae, live prey, fish larvae first-feeding

RESUMO

Os trabalhos apresentados nesta tese resultam concomitantemente de uma sugestão baseada em factos científicos previamente relatados e de uma hipótese que foi testada no sentido de uma nova aplicabilidade dada aos ovócitos e larvas endotróficas do ouriço-do-mar *Paracentrotus lividus* (Echinodermata: Echinoidea) (Lamarck, 1816).

A sugestão deriva da demonstração da importância das larvas de ouriço-do-mar na composição e biomassa do plâncton marinho realizada por alguns estudos ecológicos assim como na prova laboratorial de que estas larvas são consumidas por vários predadores zooplanctónicos. Ambos estes factos apontam para a existência de uma relação trófica existente entre as larvas de peixes e as larvas de ouriço-do-mar nos ecossistemas marinhos.

Desta forma, a hipótese formulada consistiu na seguinte questão: Dado que as larvas de ouriço-do-mar são alimentos naturais de larvas de peixes marinhos será que poderiam ser utilizadas massivamente como primeiras presas vivas em piscicultura marinha e desta forma poder ser uma alternativa/complemento às presas usadas tradicionalmente (o rotífero *Brachionus* spp. e o naúplio de *Artemia* spp.)? De realçar que esta hipótese reveste-se de especial importância visto que é precisamente no cultivo da fase larvar dos peixes explorados comercialmente que surgem as maiores dificuldades quer ao nível da sobrevivência, quer ao nível do crescimento, quer ao nível do aparecimento de várias malformações das larvas. Inclusivamente, o insucesso do cultivo nesta fase para algumas espécies de peixe condiciona a sua exploração comercial.

Consequentemente, optou-se por avaliar o potencial dos ovócitos e larvas endotróficas do ouriço-do-mar *Paracentrotus lividus* como primeiras presas vivas em cultivos larvares de peixes marinhos. A escolha desta espécie deveu-se ao facto de ser o equinóide mais abundante nas costas rochosas de Portugal Continental e a escolha dos ovócitos e estados larvares endotróficos está relacionada com a sua dimensão que se pensou ser adequada para a abertura bucal dos primeiros estados larvares de peixes marinhos.

Em concreto, a análise desta potencialidade como alimento vivo centrou-se em 3 questões fundamentais.

1) Primeiramente foi avaliado o efeito que diversos factores de condicionamento dos ouriços reprodutores ao cativeiro (tempo de cativeiro, dieta, densidade e técnica de indução da postura) tinham no desempenho das suas posturas e na capacidade de fertilização dos seus gâmetas, de forma a aferir qual o potencial quantitativo na produção de presas vivas. Como

principal resultado pode-se referir que o ouriço-do-mar *Paracentrotus lividus* se adapta muito bem às condições artificiais de cultivo, bastando um tempo reduzido de cativeiro (aproximadamente 3 meses), uma dieta simples (por exemplo uma mistura de grãos de milho com pedaços da macroalga *Laminaria ochroleuca*), para quando induzidos quimicamente com injecção intra-peristomial de cloreto de potássio (KCl) 0,5 M, libertarem uma elevada quantidade de gâmetas viáveis. Desta forma ficou provada a capacidade de obtenção de presas vivas durante todo o ano sem necessidade de recurso ao meio natural. O principal constrangimento encontrado foi a mortalidade associada à técnica de indução da postura, limitando a reutilização do mesmo "stock" de reprodutores. Contudo, foram avaliados outros métodos de indução da postura e, de acordo com os resultados, parece que a manutenção a seco durante um período de 3 a 6 horas poderá ser uma alternativa possível ao cloreto de potássio com grandes vantagens ao nível da sobrevivência dos ouriços reprodutores.

2) Seguidamente foi avaliada a qualidade nutricional destas presas vivas ao nível de dois nutrientes fundamentais para o desenvolvimento das larvas de peixes marinhos fazendo a sua comparação com as presas vivas usadas comummente em cultivos larvares marinhos: conteúdo lipídico e composição em ácidos gordos e conteúdo proteico e composição em aminoácidos. De uma forma geral pode-se dizer que os valores encontrados na concentração destes dois nutrientes nos ovócitos e larvas endotróficas de *P. lividus* são comparáveis aos existentes nas presas vivas comummente utilizadas. Todavia, esta comparação depende grandemente do tipo de emulsão comercial utilizada no enriquecimento nutricional dos *Brachionus* spp. e dos náuplios de *Artemia* spp. Porém, quando se testaram as capacidades de enriquecimento nutricional dos ovócitos e larvas endotróficas de *P. lividus* através da manipulação quer lipídica quer proteica da dieta dos seus progenitores, apenas para os ácidos gordos se obtiveram resultados positivos. De facto, dietas inertes usadas na alimentação dos ouriços em cativeiro, e formuladas com óleos com maior proporção de gorduras insaturadas promovem a incorporação de ácidos gordos polinsaturados essenciais para as larvas de peixes nos ovócitos e larvas endotróficas de *P. lividus*. Contrariamente, a manipulação da fonte proteica e da percentagem de proteína na dieta dos ouriços reprodutores tem apenas um efeito muito reduzido nos ovócitos e larvas de *P. lividus* resultantes.
3) Finalmente, os ovócitos e larvas endotróficas de *P. lividus* foram usados como alimento vivo em condições normais de funcionamento de uma maternidade de peixes marinhos. Nesta situação foi avaliada a sobrevivência e crescimento das larvas de dourada (*Sparus aurata*) e sargo (*Diplodus sargus*) desde a eclosão até aos quinze dias de vida, quando

alimentadas com estas presas vivas alternativas em diferentes tipos de protocolos alimentares. Os valores encontrados nestas variáveis demonstraram a menor qualidade dos ovócitos e larvas endotróficas de *P. lividus* como primeiro alimento vivo quando comparada à do rotífero *Brachionus* spp. Da mesma forma, quando se analisaram as taxas de ingestão em 24 horas para cinco diferentes espécies de larvas de peixe exploradas comercialmente e que iniciam a sua alimentação exógena, provou-se que as presas vivas alternativas testadas são consumidas, mas provavelmente a taxas mais reduzidas que *Brachionus* spp.

Como conclusão geral pode-se afirmar que a avaliação das presas vivas alternativas testadas como primeiro alimento vivo no cultivo larvar de peixes marinhos não apresenta nenhum valor acrescentado às presas vivas mais utilizadas na actualidade. Todavia, será aconselhável avaliar o potencial como alimento vivo dos ovócitos e larvas endotróficas de *P. lividus* em outras espécies de peixes, particularmente naquelas em que a elevada mortalidade larvar inviabiliza a sua exploração comercial, para poder retirar conclusões mais fundamentadas. Igualmente, antes de rejeitar esta hipótese de alimentação larvar, deverão ser analisadas outras linhas de investigação: o potencial destas presas alternativas no cultivo de larvas de crustáceos marinhos e a utilização da sua fase exotrófica onde, dado o comportamento filtrador dos equinopluteus, o potencial de enriquecimento nutricional é muito superior pois pode ser feito directamente pela adição de substâncias ao meio de cultivo.

Palavras-chave: *Paracentrotus lividus*, ovos, larvas endotróficas, presas vivas, primeira alimentação de larvas de peixes marinhos

CONTENTS

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

As seafood demand continues to grow, increasing demand is being satisfied from aquaculture sources in both developed and developing countries, and currently aquaculture accounts for 43 percent of global fish production used for human consumption and is expected to grow and compensate for the predicted global shortage of supply from capture fisheries and the demands of society (FAO 2009). Good quality food, produced under controlled conditions and in increasing quantities can be supplied by aquaculture, but like in the other traditional sectors of animal production, the development of aquaculture relies in the intensification of farming systems. In this type of intensive systems with very high fish densities there must be a high control level in water quality, as well as the fish farmer have the responsibility to provide all the food throughout the production cycle. These nutritional features are essential in aquaculture because the survival and growth of cultivated specimens depends largely on the quantity and quality of the food provided. Moreover, the highest production cost in intensive aquaculture is related with the price of the food given, so food formulation and distribution must be optimized to increase profitability.

Considering marine fish larvae hatcheries, in spite of recent advances with inert artificial feed (Cahu & Zambonino 2001, Curnow *et al.* 2006), live feed is still needed for marine fish larvae feeding, particularly during the early larval stages (Shields 2001, Støttrup & McEvoy 2003). Movement and small size of live prey, related with the predatory behaviour of fish larvae are the main reasons pointed out. Additionally, when marine fish larvae starts exogenous feeding the gut has very low digestion and assimilation capacities and therefore it is difficult to formulate an inert feed which meets the dietary requirements of fish larvae (Kvåle *et al*. 2007).

The choice of the species of phytoplankton and zooplankton used as live feed in aquaculture hatcheries depends crucially on its size, its nutritional value (chemical composition) and easiness of culture. Taking into account microalgae production, the methods are well known and currently several microalgae species are produced as direct larval food, or as the first link in the food chain in zooplankton production, or even as “green water” medium in rearing systems.

Considering zooplankton, the food given to carnivorous marine fish larvae, in spite of being a highly diverse group, only two taxa have been so far mass produced: the rotifer *Brachionus* spp. and the brine shrimp *Artemia* spp. Undemanding cultivation, quickness to obtain high densities all year-round and enrichment possibilities are the main reasons to use these zooplankton taxa in fish larvae feeding. However, it represents a situation of extreme vulnerability

that worldwide marine fish larviculture relies solely on the production of these two live feed items, so rather than interesting, it is critical to consider new possibilities. Furthermore, new live feeds may act as a complement and/or alternative on the fish larvae rearing and may represent the key to commercial produce other fish species where larvae mortality is still unsolved. For instance, in some marine fish species (*i.e.* siganids, groupers, snappers) very small zooplankton, such as trochophora larvae (~50 μm), need to be used as a starter feed, since the commonly used rotifers are still too big (Lavens & Sorgeloos 1996). Due to better nutritional quality, the class Copepoda has been presented as a good alternative live feed (Shields *et al.* 1999; Evjemo *et al.* 2003, Helland *et al.* 2003, van der Meeren *et al.* 2008), but yet no mass production has been achieved. Main limitations with the use of copepods are related with providing sufficient quantities, the disease risk from extensive culture methods, and the labour-intensive and costly intensive culture (Støttrup 2000; Helland *et al.* 2003). Similarly, nematodes have also been suggested as alternative live feed for first-feeding fish larvae and larval penaeid shrimps, to replace the traditionally used living organisms, but they have not yet been tested in marine fish larval species (Schlechtriem *et al.* 2004). In this context, the aim of this study was to determine the potential of a new live food for marine fish larval aquaculture composed by the eggs and the endotrophic larval forms of the sea urchin *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata: Echinoidea). Some previous reasons led to testing this hypothesis:

1) Several ecological studies have demonstrated the importance of echinoplutei in the composition and biomass of zooplankton communities (Rassoulzadegan & Fenaux 1979, Fransz *et al.*1984, Lindley *et al.*1995, López *et al.* 1998) suggesting their role not only as herbivores but also as prey of other plankton species (Rumrill 1990, McEdward & Miner 2001);

2) Laboratory studies have demonstrated the selective predation upon sea urchin embryos and larvae by several common zooplanktonic predators, including fish species (Rumrill *et al.* 1985, Pennington *et al.* 1986, Allen 2008). Despite this evidence, some caution must be taken when evaluating predation upon invertebrate larvae: under natural conditions, planktonic predation had been reported as being very low (Johnson and Shanks 1997, 2003); and the presence of chemical defence mechanisms were already suggested by Cowden *et al.* (1984) for echinoderm larvae.

3) The prey size was another good indicator to test the use of *P. lividus* eggs and endotrophic larvae in marine fish larviculture, because free-living endotrophic prey of different sizes (from approximately 90 μm diameter in eggs to 350 μm length in four armed plutei, 72 h after fertilization) (Fenaux *et al.* 1985) can be produced from the same *P. lividus* spawning within 3 days. This prey size range is wider than rotifer *Brachionus* spp. (123 to 292 μm lenght) (Snell & Carrillo 1984), and is smaller than *Artemia* spp. nauplius (420-475μm length) (Narciso 2000).

Therefore, if sea urchin eggs and larvae are suggested as fish larvae natural preys, they may also be used as a mass-produced source of live feed zooplankton in marine fish hatcheries. This idea was previously mentioned by Hubbard *et al.* (2003) for *Lytechinus variegatus* early developmental stages, and this same hypothesis for *P. lividus* eggs and endotrophic larvae was evaluated in the present study. First of all, *P. lividus* was the sea urchin species chosen because it is the dominant littoral sea urchin species along Portuguese rocky shores and its bio-ecology on Cascais coastal waters had been recently studied (Gago *et al.* 2003).

In spite of sea urchins have been exhaustively studied for several purposes and many milestones in biological sciences, such as the observation of fertilisation or the isolation of eukaryotic messenger RNA have been carried out using these organisms (Yokota 2002), the present study is the first approach to a new utilization of sea urchin larvae as live feed in marine fish larviculture. However, the knowledge obtained in the present study can be used to improve other sea urchin utilizations. In fact, many sea urchins are edible, and therefore studies on natural bio-ecological parameters like diet, growth, and reproduction cycles are needed for protection measures implementation on exploited wild populations. The continued demand for sea urchins coupled to the high economic value of their fishery has led to its overexploitation and decline in many countries (Andrews *et al.* 2002). Sea urchin roe is highly regarded as a luxury food item and according to Kelly (2004), around 100,000 tons of sea urchins are landed annually from the world's fisheries, with a value of over 0.5 billion Euros. Related with this fact, sea urchin culture (echinoculture) started to complement roe global demand, to reduce excessive dependence on natural stocks and enhance roe content and quality. Therefore, captivity techniques and methods have also been studied in order to increase the economic profitability of this culture. Two main goals are generally seek at the same time: suppress gametogenesis in order to produce good-quality gonads for human consumption; or promote gametogenesis for increased production of larvae. The present study is included in this second goal, but larvae would not be used for rearing but instead for feeding marine fish larvae.

Considering *Paracentrotus lividus*, in most of its geographical range, in past or present times and on a regular or occasional basis, its gonads have been appreciated as seafood and *P. lividus* has been intensely harvested. Nowadays, the consumption of *P. lividus* is mainly limited to France and Spain, and to a lesser extent to Italy and Greece, although harvesting occurs, or has occurred, over a much larger area (*e.g.* Ireland, Portugal and Croatia) for export (Boudoresque & Verlaque 2007 and the references therein). Taking into account *P. lividus* echinoculture, Grosjean *et al.* (1998) have already described an aquaculture pilot scale facility for the entire life cycle, and several studies (*e.g.* Fernandez & Pergent 1998, Spirlet *et al.* 2000, 2001, Shpigel *et al.* 2005,

Cook & Kelly 2007) had evaluated the effect of different general rearing conditions on stocking yields.

Additionally, sea urchin eggs and larvae of several species have been exhaustively used as live biological tools for embryological and toxicological studies, and to what *P. lividus* is concerned it's embryonic and larvae development has also already been evaluated for such purposes (*e.g.* Ozretic *et al.* 1998, His *et al.* 1999, Marin *et al.* 2000, Férnandez & Beiras, 2001, Pesando *et al.* 2003, Ghirardini *et al.* 2005, Aluigi *et al.* 2008).

Considering the new utilization proposed in this study for *P. lividus* eggs and endotrophic larvae, several main criteria must be evaluated in order to determine the potential of an organism as live prey in marine fish larviculture: year-round availability, large prey production, nutritional quality and prey acceptability by fish larvae. These criteria were evaluated in the present study and the main results are presented in this report.

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CHAPTER 2

Live Prey Availability and Production

CHAPTER 2.1.

Year-round captive spawning performance of the sea urchin *Paracentrotus lividus*: Relevance for the use of its larvae as live feed.

Orlando Luis, Filomena Delgado & João Gago (2005)

Aquatic Living Resources 18: 45-54

Aquat. Living Resour. 18, 45–54 (2005)

DOI: 10.1051/alr:2005004
www.edpsciences.org/alr

Aquatic Living Resources

Year-round captive spawning performance of the sea urchin *Paracentrotus lividus*: Relevance for the use of its larvae as live feed

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Received 30 June 2004; Accepted 12 January 2005

Abstract – Field studies describe echinoplutei not only as grazers but also as prey of naturally occurring fish and shellfish larvae. This finding suggests their potential as live feed in aquaculture. This paper reports on consistent spawnings of the captive sea urchin *Paracentrotus lividus* (Lamarck 1816) (Echinodermata: Echinoidea) throughout the year using diets of plant origin (yellow maize and/or dried seaweed) with fixed environmental conditions equivalent to field conditions during late spring (14 h of daily illumination and 18 °C of temperature). Broodstock maturation without unwanted spontaneous spawnings was achieved in two ways: extending the natural season of reproduction and inducing out-of-season wild specimens to mature. Controlled spawnings of captive sea urchins were induced every month of the year by KCl 0.5 M injections. The diet maize/seaweed combination gave the best results (79% of the tested urchins) in terms of consistent large spawnings throughout the year, followed by the pure maize diet (50%) and the pure seaweed diet (36%). When out-of-season wild sea urchins were induced to maturation, the majority (72%) of tested individuals required at least 60 days to spawn under KCl injection when fed the combination diet. The results demonstrate the feasibility of producing larval *P. lividus* in that high numbers of fertilized eggs (up to 5 million per female) can be obtained year round. The main limitation of exploiting *P. lividus* as planktonic feed seems to be the mortality of broodstock after injection with 1 ml KCl 0.5 M, which prevents reutilization. The 1-month post-injection survival rate was 30 ± 8% (mean ± SE). All surviving sea urchins spawned again after re-injection 1 month later, with a 1-month survival rate of 29%.

Key words: Spawning / Gonad maturation / Planktonic feed / Sea urchin / *Paracentrotus lividus*

Résumé – Performance de pontes de l'oursin, *Paracentrotus lividus*, tout au long de l'année : intérêt de ses larves en tant qu'aliment vivant. Des études in situ décrivent les larves d'échinodermes non seulement comme brouteuses mais aussi comme proies de larves de poissons, crustacés, mollusques. Ce qui laisse présager de leur potentialité en aquaculture, en tant qu'aliment vivant. Nous présentons ici la reproduction tout au long d'une année de l'oursin violet en captivité *Paracentrotus lividus* (Lamarck 1816) (Echinodermata : Echinoidea) nourri d'aliments d'origine végétale (maïs et/ou graines séchées) en conditions environnementales contrôlées équivalentes à celles observées in situ durant le printemps (14 h d'éclairage et une température de 18 °C). La maturation des géniteurs sans ponte spontanée a été obtenue selon 2 procédés : en étendant la saison de reproduction et en induisant des spécimens sauvages à atteindre leur maturité sexuelle hors saison. Des pontes contrôlées d'oursins ont été induites chaque mois de l'année par injections de KCl 0.5 M. Le régime alimentaire combinant maïs/algues a donné les meilleurs résultats (79 % des oursins), en terme de pontes importantes tout au long de l'année, suivi par un aliment constitué de maïs (50 % des oursins) et d'algues (36 % des oursins). Lorsque l'induction de la maturation d'oursins sauvages a été effectuée, la majorité des oursins (72 %) testés ont demandé 60 jours au moins pour pondre sous injection de KCl et nourris du régime alimentaire mixte. Les résultats démontrent la faisabilité de produire des larves de *P. lividus* d'un grand nombre d'œufs fertilisés (jusqu'à 5 millions par femelle) qui peuvent être obtenus tout au long de l'année. La principale limite à l'exploitation de *P. lividus* en tant que nourriture planctonique semble être la mortalité des oursins reproducteurs après l'injection de 1 ml KCl 0.5 M. Le taux de survie des oursins, un mois après injection, était de 30 ± 8 % (mean ± SE). Tous les oursins survivants ont pondu à nouveau après ré-injection un mois plus tard, avec un taux de survie de 29 %.

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1 Introduction

Since the early 20th century, many university laboratories have reared sea urchin larvae for embryological studies or practical classes. Embryos or pluteus of *Paracentrotus lividus* are currently being explored as bioassays for toxicological studies (Bougis et al. 1979; His et al. 1999; Radenac et al. 2001). However, *P. lividus* larvae have seldom been the main objects of rearing studies. Fenaux et al. (1985) established equations for larval growth, including gross biochemical composition, of *P. lividus* reared to metamorphosis. Studies of the exploitation of sea urchin larvae for feeding other fish and shellfish larvae are lacking, although a recent study pointed out their potential use (Hubbard et al. 2003).

Ecological studies have shown the importance of echinoplutei, at least seasonally, in the composition and biomass of zooplankton communities. Although copepods generally dominate, 1980s plankton surveys in the central North Sea (Fransz et al. 1984) showed that echinoplutei were just as numerous; subsequent plankton surveys through the early 1990s showed the reverse situation, in which echinopluteus and ophiopluteus became more abundant than any single holoplanktonic species (Lindley et al. 1995). Rassoulzadegan and Fenaux (1979) used particulate carbon consumption data to estimate that during periods of larval abundance, echinoplutei account for 3% of the phytoplankton biomass. A 1998 report of plankton samples from the Mediterranean (López et al. 1998) recorded peak *P. lividus* larval densities of up to 33 m^{-3}. Such high densities suggest that echinoplutei are important not only as grazers but as prey of naturally occurring fish and shellfish larvae. McEdward and Miner (2001) suggest that predation might be one of the major causes of echinoid larval mortality in the field and can range from 6% to 27% per day.

It follows that if echinoplutei are natural prey, they may also be useful in the growth of fish and shellfish larvae as a mass-produced source of zooplankton. To be suitable for such mass production, any species of zooplankton must meet most of the following criteria:

- Year-round availability;
- Large number of eggs and larvae;
- Short period of embryonic development;
- Herbivory quality;
- Nutritional quality.

Thus far, only two species have met these criteria: the rotifer *Brachionus plicatilis* (length, 123–292 μm) (Snell and Carrillo 1984) and the nauplii of *Artemia* brine shrimp (length, 420–475 μm). However, some fish and crustacean larvae do not accept these species as zooplankton food, which has prompted the search for alternative and reliable sources of living zooplankton. The class Copepoda (length, ~2000 μm) has been studied for several decades, but the reliability of copepods has yet to be proved, especially in terms of achieving the numbers required for nourishment of the early developmental stages of fish larvae (Stottrup 2000). During endotrophic development, *P. lividus* larvae are 125–350 μm in length, which is comparable with the dimensions of prey commonly used in aquaculture.

Any assessment of *P. lividus* larvae as a reliable source of planktonic feed must first prove that captive broodstock can produce large numbers of fertilized eggs year-round and for extended periods. There are reports of gametogenesis occurring throughout the year among captive broodstocks of *P. lividus* (Grosjean et al. 1998; Spirlet et al. 2000; Shpigel et al. 2004). However, we lack details concerning the organism's spawning performance over the course of a year, and most studies have centered on the promotion of vitelogenesis for human consumption or have been short-term. Our report of year-round controlled spawnings of *P. lividus* provides evidence that *P. lividus* larvae could be a reliable source of living zooplankton, a source that meets at least four of the abovementioned criteria.

2 Material and methods

All the sea urchins used in the present work were collected during full-moon low tides from pools on the central west coast of Portugal near Cascais (Lisbon). A previous study of this population (Gago et al. 2003) established the annual variability of gonad index, spawning periods, and influence of food availability on gonad size.

Evaluation of the spawning performance of captive *P. lividus* involved three experiments. The first tested the hypothesis that large numbers of fertilized eggs could be produced year round by broodstock held captive for extended periods (long-term experiment). The second experiment was devised as a backup in case the first yielded negative results: wild immature sea urchins also were evaluated to see how fast they could mature in captivity (short-term experiment: induction of maturation during out-of-season gametogenesis). The final experiment evaluated broodstock mortality following injections with a spawning trigger (short-term experiment: survival tests to KCl injections).

Since gonad production of *P. lividus* is seasonal and follows an annual cycle (Gago et al. 2003), control over the maturity of broodstock and spawnings involves, as with many other seasonally reproducing marine species, rigorous attention to three parameters: photoperiod, temperature, and diet.

All experiments used a fixed photoperiod of 14L:10D cycle to mirror the prevailing conditions of mid-spring, when most sea urchins are mature in the field. Daylight fluorescent tubes of 58 W provided artificial illumination over each tank or aquariumm, generating 700 lux as measured (Gossen Lunasix 3) at the water surface of the tanks. Sea water was kept at 18 ± 0.5 °C in all three experiments, which also reflects the conditions of late spring and early summer in the field. We expected that such a narrow range would favor gonad maturation but not spontaneous spawning in captivity, which could be easily checked by the presence of gametes over the aboral hemisphere of sea urchins.

P. lividus is basically an herbivorous echinoid (Verlaque and Nédelec 1983; Boudouresque and Verlaque 2001; Gago et al. 2003) and thus was fed appropriate diets of plant origin. Basuyaux and Blin (1998) established the suitability of maize-based feeds coupled with algae for the survival and somatic growth of *P. lividus*. Likewise, three convenient plant-derived diets were tested during the long-term spawning experiment. The first diet consisted of commercial yellow grains of the maize *Zea mays* (13.5% moisture). The second diet consisted of the commercial dried seaweed *Laminaria ochroleuca*

(Kombu, Algas de Galicia, Algamar, Spain; 13.4% moisture). The third diet was a combination of the two basic diets wherein the components were alternated. Maize was presented as dry grains that were randomly distributed by tank water current as they sank. Dried seaweed fronds were pre-cut into small pieces, wetted for 15 min, and then also randomly distributed. Both of these basic diets are easily purchased and rich in carbohydrates (69.8% and 52.1%, respectively). The literature describes the biochemical compositions (gross, minerals, vitamins, and amino acids) of these diets. Crampton and Harris (1969) tabulated maize composition, and Sáa (2002) analyzed the dried seaweed. Protein (8.4% for maize and 6.9% for seaweed) and lipid contents (4.3% and 1.1%, respectively) were not very dissimilar. Maize is devoid of vitamins B_{12} and C and of the amino acids tryptophan and cystine, but maize has carotene at a concentration of 1.8 mg kg^{-1}. Most fatty acids from total lipids are polyunsaturated in maize (linoleic acid, 45%) and monounsaturated in the seaweed (oleic acid, 30%).

Gonad indices (measured in grams of wet gonad weight/grams of whole animal weight × 100) were determined immediately after spawning and in the same individuals to ensure a close correspondence between gonad size and range of spawnings. This technique thus might have underestimated gonad indices. However, pre-tests with wild sea urchins revealed this method yielded gonad indices similar to the ones reported by Gago et al. (2003).

Spawning was induced by injecting 1 ml KCl 0.5 M through the peristomial membrane using a 0.9 mm (external diameter) × 50 mm (length) needle coupled to a 5 ml syringe; the main environmental trigger of spawning is not clearly known (López et al. 1998). Each sea urchin was placed for 30 min in individual plastic beakers filled with 2 L of aerated sea water at 18 °C, where oocytes or sperm were eventually emitted. Light microscopy (×40) was used to evaluate spawning. Results were measured qualitatively according to the area occupied by gametes and concurrently compared with electronic counts. The resultant semi-quantitative classification of spawning consisted of *no spawning* (−); *spawning up to 500 thousand oocytes or 200 million spermatozoa* (+); and *spawning greater then previous values* (++). Precise gamete counts and oocyte size measurement were obtained with a Coulter Counter (model ZM with 140 μm cell aperture) coupled to a multichannel particle analyzer (Channelyser 256) during the gametogenesis season (May to September), allowing the spawning performances of wild and captive sea urchins to be compared simultaneously.

Each batch of emitted oocytes was checked for maturity (lack of central nucleus) and viability (formation of a fertilization membrane after fecundation). Fertilization rates were not precisely measured, but the large majority of emitted oocytes (in both wild and captive urchins) were mature and displayed fertilization membranes.

2.1 Long-term experiment: Year-round production of fertilized eggs

The evaluation of controlled spawning performance of broodstock held captive for extended periods took place over 12 months (October 2001 to September 2002) in a recirculating rearing system with a total volume of 3480 L of unfiltered natural sea water whose salinity was kept at 34–35 ppt. Sea urchins were tested in eight cylindrical black fiberglass tanks that were 0.80 m (base diameter) × 0.80 m (depth), equivalent to 402 L. No UV water sterilization or chemical/mechanical filtration was used; sea water treatment was achieved through biological filtration and high-efficiency protein skimming (Tunze Aquarientechnik, Venturi type model 3160). Plastic media (spiny balls) were used as substrate for nitrifying bacteria. Water changes took place during feces removal (15% of monthly total volume). Each tank was stocked with an initial density of 47 sea urchins/tank, equivalent to 94 sea urchins m^{-2} (area of base) or 117 sea urchins m^{-3} with a test diameter of 47 ± 0.7 mm (mean and SE) and a weight of 29 ± 1 g (mean and SE). Each diet was given to two tanks while the urchins in the remaining two tanks served as unfed controls. According to the method described by Spirlet et al. (1998), sea urchins were fed discontinuously twice a week for a period of 12 months. Every month, seven urchins were sampled from each treatment and subjected to induced spawnings immediately followed by measurement of gonad indices.

A simple rearing method provided a check of larval development to early four-armed pluteus. Fertilized eggs from captive and wild sea urchins, taken from May to July 2002, were washed on a 30 μm mesh and then placed into 2 L plastic beakers filled with aerated sea water for 3 days (endotrophic phase) at a temperature of 18–20 °C and salinity of 35 ppt.

2.2 Short-term experiments

The short-term experiments, induction of maturation during out-of-season gametogenesis and survival tests to KCl injections, took place in stand-alone aquaria. Wild sea urchins for both short-term experiments were tested in five glass tanks (0.425 × 0.345 × 0.250 m) filled with 37 L of natural sea water kept at 34–35 ppt. Four tanks were allocated as replicates, and one tank served as a control. Sea water treatment was provided through biological, mechanical, and chemical filtration enclosed in a hang-on external wet/dry filter (Millenium 1000, Aquarium Systems). Protein skimming was also performed (Berlin Air-Lift 60, Red Sea Fish Pharm). Additional water currents were produced by a power-head (Eheim 1005, 300 L h^{-1}). Nutrient export was ensured by water changes during weekly tank siphoning to remove feces.

2.2.1 Induction of maturation during the out-of-season gametogenesis

The induction of maturation and spawning of sea urchins during out-of-season gametogenesis (October to April) took place over two trials of 38 days (from December 2001 to January 2002) and 61 days (from February to April 2002). Tested sea urchins were stocked at 15 wild sea urchins per tank (100 sea urchins m^{-2}). Except for the unfed control group, all sea urchins were fed the combination diet twice a week. At the end of each trial, sea urchins were sampled from each replicate and subjected to induced spawnings immediately followed by determination of gonad indices.

2.2.2 Survival tests to KCl injections

Survival tests to 1 ml KCl 0.5 M injections took place during four consecutive trials from April to August 2002, the first three with recently collected wild sea urchins (0, 5, and 10 days of previous captivity) and the last trial with long-term captive sea urchins. Apart from the control group, sea urchins were pre-subjected to injections of 1 ml KCl 0.5 M and their survival was checked over the following 30 days. The surviving sea urchins were again injected and followed for another 30 days. All sea urchins were fed the combination diet twice a week and stocked at densities of 33–67 sea urchins m^{-2}, depending on the number of surviving sea urchins. At the end of each trial, surviving sea urchins from each replicate were counted and again subjected to induced spawning. To elucidate the diverse survival patterns following KCl injections, internal water volumes of selected wild sea urchins were measured ($N = 19$, test diameter ≥38 mm, ≤60 mm). Internal water was extracted through the peristomial membrane with a hypodermic syringe equal to the one used to inject KCl. Linear regression analysis of volume on diameter showed that V (ml) $= -32.013 + 0.916 \times D$ (mm) ($R = 0.942$, $t = 11.617$, $p < 0.001$).

Data were tested for normality, and one-way ANOVA (with diet as the source of variation) was used to analyze both semi-quantitative (arcsine transformed) and quantitative spawning results. Variations among tanks (duplicates) were tested by ANOVA, Model II. Differences between two data means (wild vs. captive, males vs. females) were analyzed using Student's t-distribution for independent values. A t-test was used to determine the significance of the regression coefficients of weights on diameters (Bailey 1976).

3 Results

3.1 Long-term experiment: Year-round production of fertilized eggs

P. lividus is a species quite suited to long-term captivity. Over 12 months, none of the 376 initially stocked sea urchins died, including the unfed control group. Pooled data for captive fed urchins showed an increase in mean diameter from an estimated 47 ± 0.7 mm (mean and SE) at the beginning of the experiment to 51 ± 0.4 mm at the end of the experiment ($p < 0.001$) and in mean weight from 29.1 ± 0.9 g to 33.6 ± 0.7 g ($p > 0.05$). Differences in regression coefficients for the same test diameter showed that captive fed sea urchins weighed more than wild sea urchins ($p < 0.05$), a possible indication of suitability. No correlation was found between weight and gonad index in wild or captive sea urchins ($p > 0.05$).

Gonad indices did not differ significantly between the sexes ($p > 0.05$), as Guettaf and San Martin (1995) and Gago et al. (2003) had reported earlier. Pooled data always showed that gonad indices were higher in the captive fed groups (mean, 10.5%) than in the concurrent wild population (mean, 6.1%) during every month of out-of-season gametogenesis (October to April) ($p < 0.001$). Although significantly different, the gonad indices of the wild sampled sea urchins (6.1%) and the captive unfed group (5.7%) were very close. Gonad indices over 10% were easier to achieve using maize or the combination diet than dried seaweed alone ($p < 0.001$). There was no significant difference in gonad indices between maize and combination treatment. Variations among tanks (duplicates) were not significant ($p > 0.05$). Gonad indices reached as high as 24% in sea urchins fed the combination diet compared with a maximum of 15% with dried seaweed alone.

Large (++) and/or small (+) spawnings were registered all the year round for both male and female pooled captive fed sea urchins (Table 1). Overall analysis showed that when large and small spawnings are considered simultaneously, pooled male and female captive sea urchins performed better than wild sea urchins ($p < 0.05$). Captive sea urchins also seemed to perform better than wild ones when only large spawnings are considered, but the differences were not significant ($p > 0.05$). However, no large spawnings were obtained with the wild sea urchins between October and January.

When diet is used to compare the results of induced spawnings of captive sea urchins (Table 2), the combination diet (maize/seaweed) performs best ($p < 0.05$) in terms of consistent large spawnings (++) of pooled males and females over a year (79% of tested urchins), followed by the maize diet (50%). The same applies for large spawnings when only females are considered ($p < 0.01$). The seaweed diet was clearly less efficient ($p < 0.05$): only 36% of the tested sea urchins emitted large quantities of oocytes or spermatozoa.

Checks for completeness of larval development from May to July 2002 showed that, for both wild and captive urchins, fertilized eggs at 18–20 °C developed into endotrophic early larvae (free-living blastula, prism, and prepluteus with 200 μm) within 36 h and then to planktotrophic four-armed plutei (350 μm long) within 72 h.

Precise counts of oocytes and spermatozoa emitted by captive sea urchins during the gametogenesis season (May to September) and compared with concurrent wild samples are represented in Figure 1. Captive sea urchins performed better than the wild ones over the entire experimental period. Table 3 shows that the maize diet (mean sperm count, 311 million spermatozoa per male) was more favorable ($p < 0.05$) for sperm emission than the two other diets (276 and 270 million spermatozoa per male). Sperm counts from sea urchins fed any of the three diets were higher ($p < 0.05$) than counts from emissions of wild sea urchins. Table 3 also shows that the maize diet (mean oocyte count, 1788×10^3 per female) was also more favorable ($p < 0.01$) for oocyte emission than any other diet (1582×10^3 and 863×10^3 oocytes per female). Oocyte counts obtained from females fed the seaweed diet were not significantly different from those obtained from wild sea urchins.

Oocyte sizes, measured electronically as modal frequencies, were similar for all three diet groups and similar to those obtained from wild urchins. During the maturation season, wild and captive sea urchins emitted small (~65 μm in diameter) and large oocytes (~90 μm), except during June 2002, when only large oocytes were produced. Oocyte size distributions were sometimes bimodal or even trimodal regardless of the origin of sea urchins. Except with wild males, the number of emitted gametes did not correlate with test diameters for either captive or wild sea urchins ($p > 0.05$).

Table 1. Induced spawnings in wild and captive fed sea urchins. Each group included 21 sea urchins sampled each month. Results express % of total sea urchins in each sub-group.

	Wild								Captive fed							
	Males				Females				Males				Females			
	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)
October	12	58	42	0	9	56	44	0	13	31	23	46	8	12	38	50
November	10	90	10	0	11	100	0	0	9	0	78	22	12	58	42	0
December	7	100	0	0	14	100	0	0	10	60	20	20	11	18	55	27
January	8	62	38	0	13	100	0	0	11	0	82	18	10	30	50	20
February	8	38	62	0	13	54	38	8	11	0	73	27	10	0	50	50
March	11	0	82	18	10	0	90	10	10	0	80	20	11	9	73	18
April	7	0	29	71	14	0	50	50	13	0	15	85	8	0	50	50
May	9	56	11	33	12	25	58	17	9	0	33	67	12	0	8	92
June	10	0	0	100	11	0	0	100	7	0	43	57	14	0	7	93
July	7	0	0	100	14	0	7	93	11	0	18	82	10	0	30	70
August	13	0	0	100	8	0	50	50	10	0	10	90	11	0	64	36
September	10	0	20	80	11	0	100	0	8	0	13	87	13	0	54	46
	112				140				122				130			
Mean (%) ± se			17 ± 1	37 ± 4			30 ± 2	20 ± 3			40 ± 1	52 ± 1			42 ± 1	45 ± 1

Pooled males and females (+ and ++): wild = 25.5 ± 0.6%, captive = 44.6 ± 0.2%. t = 2.29 with df = 76, $p < 0.05$.

Pooled males and females (++): wild = 27.9 ± 1.5%, captive = 48.4 ± 0.5%. t = 1.51 with df = 37, $p > 0.05$.

(#) Number of sampled sea urchins.

(-) No spawning; (+) spawning up to 500 thousand oocytes or 200 million spermatozoa; (++) spawning greater than previous values.

Table 2. Comparative results of induced spawnings in captive sea urchins fed two basic diets and a combination. Each group included 7 sampled sea urchins each month during one year. Results express % of total sea urchins in each sub-group.

	Seaweed								Maize (yellow, grain)								Combination (maize/seaweed)								Control (unfed)							
	Males				Females				Males				Females				Males				Females				Males				Females			
	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)	(#)	(-)	(+)	(++)
Oct.	6	0	17	83	1	0	100	0	5	40	40	20	2	0	100	0	2	100	0	0	5	20	0	80	2	0	50	50	1	0	0	100
Nov.	3	0	100	0	4	75	25	0	2	0	50	50	5	60	40	0	4	0	75	25	3	33	67	0	2	100	0	0	5	60	40	0
Dec.	1	100	0	0	6	33	67	0	4	50	50	0	3	0	67	33	5	60	0	40	2	0	0	100	4	100	0	0	3	100	0	0
Jan.	2	0	100	0	5	20	60	20	4	0	100	0	3	67	33	0	5	0	60	40	2	0	50	50	4	100	0	0	3	100	0	0
Feb.	3	0	67	33	4	0	50	50	3	0	33	67	4	0	50	50	5	0	100	0	2	0	50	50	3	100	0	0	4	100	0	0
Mar.	3	0	100	0	4	25	75	0	3	0	100	0	4	0	100	0	4	0	50	50	3	0	33	67	2	100	0	0	5	100	0	0
Apr.	5	0	40	60	2	0	100	0	3	0	0	100	4	0	50	50	5	0	0	100	2	0	0	100	4	100	0	0	3	100	0	0
May	2	0	0	100	5	0	20	80	4	0	25	75	3	0	0	100	3	0	0	100	4	0	0	100								
Jun.	2	0	0	100	5	0	20	80	2	0	0	100	5	0	0	100	3	0	0	100	4	0	0	100								
Jul.	4	0	0	100	3	0	33	67	4	0	25	75	3	0	33	67	3	0	0	100	4	0	0	100								
Aug.	3	0	0	100	4	0	75	25	3	0	0	100	4	0	50	50	4	0	0	100	3	0	33	67								
Sep.	3	0	0	100	4	0	100	0	1	0	0	100	6	0	17	83	4	0	0	100	3	0	0	100								
	37				47				38				46				47				37				21				24			
Mean (%)			29	59			70	16			31	60			45	40			12	72			10	85								
± sem			4	4			2	2			3	3			2	3			3	3			1	2								
(after angular transformation)																																

F-tests: Females (++) = 6.51 with df = 2, 33 $p < 0.01$; Males (++) = 0.15 with df = 2, 33 $p > 0.05$;

Pooled females and males (++) = 3.41 with df = 2, 69 $p < 0.05$. Combination diet (78.5% ± 1.7) > maize diet (49.8% ± 1.5) > seaweed diet (36.1% ± 1.3).

Pooled females and males (+ and ++) =0.01 with df = 2, 141 $p > 0.05$. Combination diet (42.7% ± 0.9); maize diet (43.7% ± 0.7); seaweed diet (41.8% ± 0.8).

(#) Number of sampled sea urchins in each batch of 7 sea urchins.

(-) No spawning; (+) spawning up to 500 thousand oocytes or 200 million spermatozoa; (++) spawning greater than previous values.

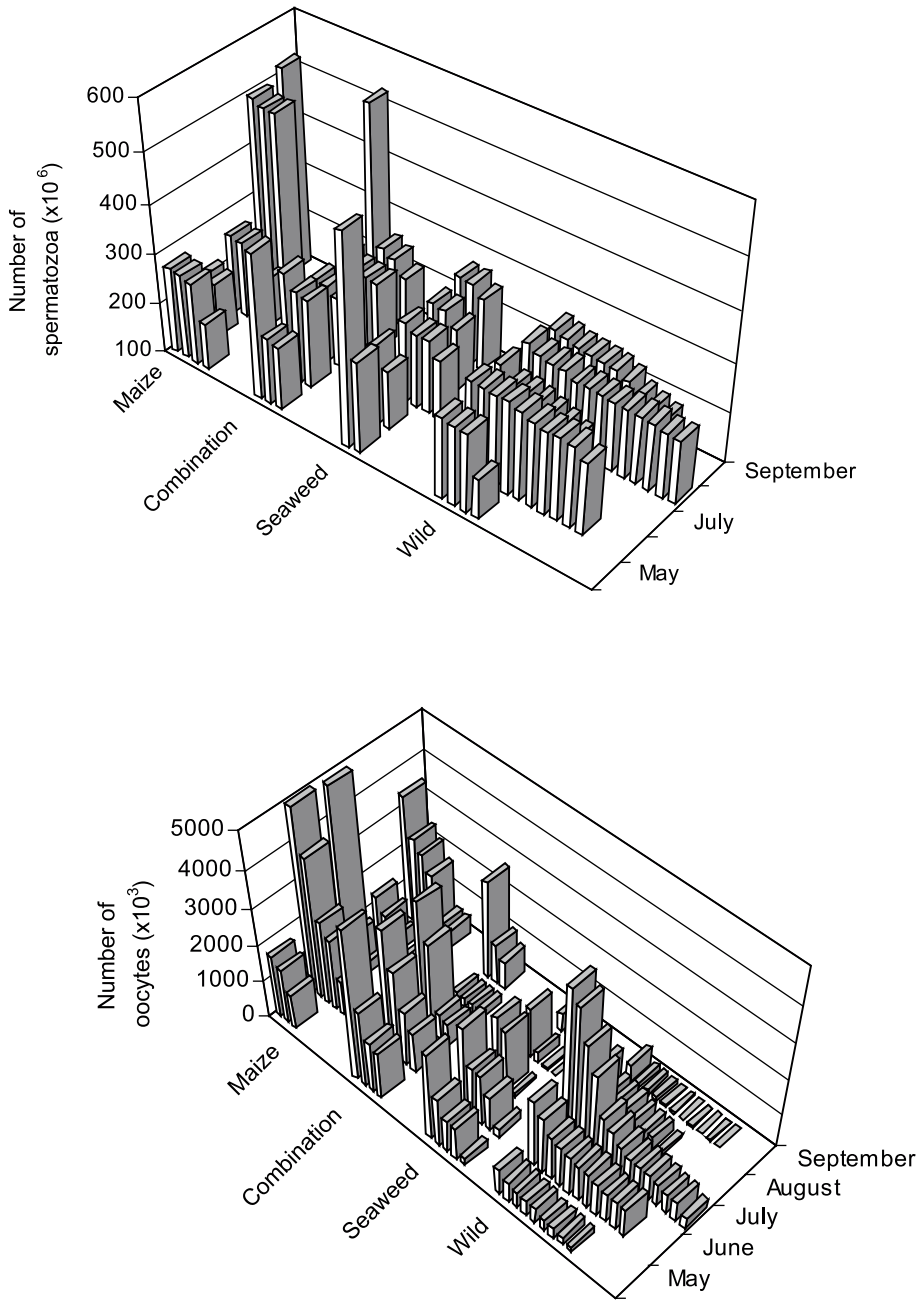

Fig. 1. Male (above) and female (below) sea urchin induced spawnings. Quantitative analysis during the gametogenesis season.

3.2 Short-term experiments

3.2.1 Induction of maturation during out-of-season gametogenesis

Gonad maturation can be induced in wild sea urchins during out-of-season gametogenesis (October to March), as shown in Table 4. Both trials showed that 20% of tested urchins spawned as quickly as 30 days, but most animals (72%) required at least 60 days to spawn when fed the combination diet. The gonad index decreased by a factor of 3 in unfed controls, which did not spawn. Once again, sea urchins were shown to adapt quite well to the experimental conditions of the stand-alone small aquaria (40 L of volume), given that no animals died and they readily consumed the combination diet.

3.2.2 Survival tests to KCl injections

Survival rates 1 month after injection varied between 17% and 44% over the three trials (30.1% ± 7.8%, mean and SE), whereas no mortality was registered for any sea urchins assigned to the control groups. Survival results from the first three trials seemed to suggest that the previous captive period (0, 5, and 10 days respectively) improved survival. However,

Table 3. Comparison of emitted number of spermatozoa and oocytes during the gametogenesis season by captive fed and wild sea urchins. # – number of sampled sea urchins.

	Male		Female	
Diet	Emitted spermatozoa ($x10^6$) (mean ± SE)	#	Emitted oocytes ($x10^3$) (mean ± SE)	#
Maize	311 ± 33	14	1788 ± 310	21
Combination	276 ± 19	17	1582 ± 279	19
Seaweed	270 ± 20	14	863 ± 176	21
Wild	244 ± 4	44	788 ± 119	53
	$F = 2.88$ $df = 3.67$; $p < 0.05$ All means are significantly different except the pair combination/seaweed		$F = 5.31$ $df = 3.92$; $p < 0.01$ All means are significantly different except the pair seaweed/wild	

Table 4. Induction to maturation of wild sea urchins during out-of-season gametogenesis. Trials took place in five stand-alone 40 L glass tanks. All sea urchins, except the unfed control group, were fed the combination diet and were stocked at 15 sea urchins per tank (100 sea urchins m^{-2}). First trial took place during December 12, 2001 to January 18, 2002 (38 days). The second trial took place from February 5 to April 6, 2002 (61 days) with two sampling dates.

	Sampled sea urchins			Mean test	Mean GI (%)		Induced spawning		
	Male	Female	Total	diameter (mm)	Initial	Final	Male	Female	Total
1st trial	24	15	39	43	6.1	6.7	1 (4%)	4 (27%)	5 (13%)
2nd trial									
Mar-08	14	11	25	47	4.7	5.4	4 (29%)	3 (27%)	7 (28%)
Control	3	2	5	48	4.7	4.4	0	0	0
Apr-06	15	10	25	49		4.4	10 (67%)	8 (80%)	18 (72%)
Control	3	2	5	49		1.6	0	0	0

mean survival rates were not significantly different ($p > 0.05$). In the fourth trial, exclusively carried out on long-term captive sea urchins, no animals survived. Surviving reinjected sea urchins spawned again, with a survival rate of 29% after 30 days.

Analysis of the mean test diameters and weights of all the surviving sea urchins from the first three trials ($n = 24$) revealed that they were larger (mean diameter, 60 mm vs. 50 mm; $p < 0.001$) and heavier (mean weight, 42.6 g vs. 30.7 g; $p < 0.001$) than the average captive sea urchin ($n = 244$). Therefore, although 1 ml KCl 0.5 M effectively induced spawning, the higher post-injection survival rate in these larger sea urchins could be due to the dilution of KCl. We estimated that 1 ml KCl 0.5 M injected into a sea urchin of 50 mm diameter (13.8 ml of measured internal volume) was equivalent to an internal KCl concentration of 0.27%. The same volume of KCl injected into urchins of 40 mm diameter (4.6 ml of internal volume) and 60 mm diameter (22.9 ml of internal volume) was equivalent to an internal KCl concentration of 0.81% and 0.16% KCl, respectively.

4 Discussion

Current research on the aquaculture of sea urchins seeks to learn how to *suppress* gametogenesis in order to produce good-quality gonads for consumption or how to *promote* gametogenesis for increased production of seed stock.

Our studies concern gametogenesis promotion, but only up to the stage of production of endotrophic plutei, disregarding metamorphosis-competent larvae. In the laboratory, common predators more readily consume embryos and early-stage larvae than late-stage larvae (Rumrill et al. 1985; Pennington et al. 1986). Our aim was to evaluate the feasibility of exploiting sea urchins in the near future as reliable sources of living zooplankton for rearing fish and shellfish larvae.

The results show that mass production of the early pluteus stage of the purple sea urchin *P. lividus* is feasible given that (1) high numbers of fertilized eggs (up to 5 million per female) can be obtained daily all the year round, (2) oocyte sizes are similar to those obtained from wild urchins, (3) fertilized eggs emitted by captive broodstock fully develop into endotrophic early larvae and then to planktotrophic four-armed plutei. Therefore, free-living endotrophic prey of different sizes (125−350 μm) can be produced from the same spawning within 3 days. Large-scale larvae production has also been reported for *Psammechinus miliaris* (Kelly et al. 2000) and *Echinus esculentus* (Jimmy et al. 2003), but such production has been for echinocultural purposes.

P. lividus broodstock are well suited to long-term captivity in that they showed no mortality and were easy to feed, readily consuming any of the tested diets. Tank cleaning is also relatively simple and not time-consuming in that water siphoning performed during water changes easily removes the abundant excreta produced by the urchins. Stocking densities could certainly be increased from the test numbers of about 100 sea urchins m^{-2} of floor space (3 kg m^{-2}) to the maximum density in the field of 234 urchins m^{-2} (Gago et al. 2003) or even up to 650 urchins m^{-2} (20 kg m^{-2}), probably without impairing survival or gonad index (Fournier 2001).

The gonad indices of wild sea urchins, determined immediately after induced spawnings and in the same individuals, were not dissimilar from the ones reported by Gago et al. (2003). It seems that full spawnings are rare given that even after large induced gamete releasing, gonad indices were always significant in captive and wild sea urchins. Spring and summer spawnings with more than one event have been reported for the present field population of *P. lividus* (Gago et al. 2003) and for another Atlantic population (Crapp and Willis 1975). The number of oocytes released per spawning can likely be improved further because it appears that egg production is far from realizing its potential with current procedures.

Throughout the year, all feeding treatments stimulated significantly higher gonad indices and larger induced spawnings in captive animals than with urchins concurrently sampled from the field. Urchins fed maize or maize/seaweed had significantly higher gonad indices (11.7 ± 0.5% and 11.5 ± 0.6%, respectively) than urchins given seaweed alone (8.2 ± 0.5%). The combination diet of maize/seaweed achieves dominance (78.5 ± 1.7% of tested sea urchins) in terms of the ultimate goal of this study: large spawnings throughout the year. In terms of cost and availability, maize alone (49.8 ± 1.5% tested urchins) is also a good diet if very large spawnings are not required, and during the maturation season (May to September) the maize diet was superior even to the combination diet in terms of large spawnings of oocytes and sperm.

In their natural environment, the studied *P. lividus* population feeds primarily on erect algae or encrusting algae, with the former leading to better somatic growth and gonad size (Gago et al. 2003). The Mediterranean purple sea urchin is also mostly herbivorous, with 86%–96% of gut contents being of algal origin (Phaeophyceae, 41%; Rhodophyceae, 19%) (Verlaque and Nédelec 1983). However, the role of consumed algal components in the promotion of gonad growth is not yet understood. Gonad production is similar when *Strongylocentrotus franciscanus* is fed algal or prepared diets (McBride et al. 1997, 2004) but lower when *Loxechinus albus* (Lawrence et al. 1997) or *Psammechinus miliaris* (Otero-Villanueva et al. 2004) is fed an algal diet compared with a prepared diet. Our results also show consistently lower gonad indices (8.2 ± 0.5%) and lower spawnings (36.1 ± 1.3%) with sea urchins fed the seaweed diet alone. Prepared feeds, including extruded feeds, have been used successfully for gonad production with *P. lividus* (Lawrence et al. 1989, 1992, 2001; Fernandez et al. 1996). On the other hand, Frantzsis and Grémare (1992) suggested similar qualitative nutrient requirements for somatic and gonadal growth of *P. lividus*. Algal diets seem to be superior to prepared diets when fed to juvenile *Strongylocentrotus droebachiensis*, suggesting that sea urchins may not require animal protein for growth (Kennedy et al. 1999).

Basuyaux and Blin (1998) proposed carbohydrate-rich compounds like maize as practical diets for rearing *P. lividus*. Maize, with 4804 cal g^{-1} (Crampton and Harris 1969), is as rich in energy as common prepared diets, but much lower in protein content (8.4% of dry matter compared with at least 20% of dry matter in prepared diets). Optimal gonad production with *P. lividus* may depend more on high energy than high protein, which may not be crucial. Frantzsis and Grémare (1992) also reported that somatic and gonadal growth depended more on the amount of ingested organic matter than on the amount of ingested protein. Therefore, with similar efficacy, maize is more economical than prepared feeds as a source of high energy and protein for *P. lividus*.

The adopted conditions of photoperiod (fixed cycle of 14L:10D) and temperature (18 ± 0.5 °C) seemed to work well for *P. lividus*. Broodstock was kept mature throughout the year without unwanted spontaneous spawnings, as no gametes were ever observed on the aboral hemisphere of captive sea urchins. Grosjean et al. (1998) were able to maintain the maturity of *P. lividus* broodstock throughout the year at 18–20 °C but in total darkness. More recently, Shpigel et al. (2004) presented evidence that temperatures of 18–22 °C enhanced gonad growth in *P. lividus* but that gametogenesis was controlled by photoperiod: long days reduced rates of gametogenesis and short days increased reproductive development. On the other hand, Spirlet et al. (2000) found temperature to be the main determinant of the reproductive cycle of *P. lividus*. Obviously, current data on the best combination of temperature and photoperiod to achieve continuous gonad growth and gametogenesis with *P. lividus* are ambiguous. It seems that any photoperiod works with captive *P. lividus* broodstock at the temperature range of 18–22 °C as long as diet is appropriate.

P. lividus was clearly able to produce mature gonads and spawnings in the relatively short period of at least 60 days.

With the same species, Fernandez et al. (1996) reported full gonad development in captivity using similar periods.

4.1 Limitations of method

The main drawback of the present method is the mortality of broodstock after injection of 1 ml KCl 0.5 M to induce spawning. Survival tests showed that only 30% of tested urchins could survive and spawn again after KCl injection, thus preventing extensive reuse of the same broodstock. Internal dilution of KCl in larger sea urchins can be an explanation to their survival to injections. A study reported full survival of juvenile green sea urchins at external water KCl concentrations less than or equal to 5%, but survival decreased to 0% at a 10% concentration (Hagen 2003a). Although the literature generally recommends injections of 1−2 ml of KCl 0.5 M, results suggest that lowering the dose of KCl improves smaller broodstock survival without impairing spawning. Electrostimulation is also common but is ineffective at inducing spawning, although survival rates (68.6%–91.4%) are better than with KCl (Hagen 2003b). Other inducers could be sought given that López et al. (1998) have proposed photoperiod, phytoplankton blooms, and turbulence as natural triggers of spawning. These authors also suggested that temperature is the main trigger of spawning episodes in field populations of *P. lividus*.

Another critical limitation may be the nutritional value of sea urchin larvae. While a full biochemical analysis of their food value is beyond the scope of the present study, the issue is already being addressed. However, at least in terms of lipid composition, prospects look good. Pantazis et al. (2000) and Bell et al. (2001) found that the gonads of *P. miliaris* can elongate-desaturate considerably from diets low in polyunsaturated fatty acids into highly unsaturated fatty acids, including 20:4n-6, 20:5n-3, but only vestigial amounts of 22:6n-3.

A final limitation could rest on the hypothesis that echinoderm larvae contain chemical defenses, as the larvae apparently have no structural defenses. Laboratory experiments with filter-feeding benthic predators showed that echinoderm larvae experienced the highest survival, which constitutes preliminary evidence of chemical defense mechanisms (Cowden et al. 1984). But experiments with zooplanktonic predators, including fishes and crustaceans, showed that predation rates differed with the stage of embryonic and larval development, with embryos and early larvae being more susceptible than later-stage echinoplutei (Rumrill et al. 1985; Pennington et al. 1986). Echinoplutei have several defenses that reduce predation (Rumrill 1990), such as the development of swimming behavior (Rumrill et al. 1985).

5 Conclusion

The combination of three factors—an appropriate diet composed of maize grains coupled with a dried seaweed, 18 °C sea water temperature, and a photoperiod of 14 h of artificial illumination—produced large numbers of viable *P. lividus* oocytes and spermatozoa over 1 year, including the period of out-of-season gametogenesis. However, broodstock survival following spawn induction must be improved before *P. lividus* can be fully considered as a suitable source of larval foods.

Our methods for obtaining the fertilized eggs and larvae of *P. lividus*, although targeted to aquaculturists and public aquarium professionals, might also aid toxicological studies since we have shown that embryos or larvae can be produced year round regardless of the availability of mature wild adults. In the same way, these results could help experimental classes in high schools and undergraduate courses in universities, which use sea urchin oocytes and sperm to introduce in vitro fertilization and embryology.

Acknowledgements. This study was supported by FCT through Financiamento Programatico and IMAR—Instituto do Mar. We would also like to express gratitude to the anonymous referees for their helpful comments on the manuscript.

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CHAPTER 2.2.

Stocking density and captive sea urchin *Paracentrotus lividus* (Lamarck, 1816) gamete production and fertilization.

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Submitted to *Journal of Shellfish Research*

Stocking density and captive sea urchin *Paracentrotus lividus* (Lamarck, 1816) gamete production and fertilization.

Abstract

Given that sea urchins eggs and larvae have been proposed as new live preys in marine larviculture, broodstock rearing conditions must be optimized for this purpose. In the present study, adult sea urchins *Paracentrotus lividus* were reared in 400 L tanks for five months under four stocking densities (100, 200, 300 and 400 sea urchins m^{-2}) in order to determine their effect on spawning performance and fertilization rate and on broodstock month relative growth (RG). For all stocking densities, more than 60% of sea urchins induced to spawn released a large number of gametes (more than 200 x 10^6 spermatozoa or more than 500 x 10^3 eggs), although the percentage of males with large emissions were higher at the 300 sea urchins m^{-2} density. Irrespective of broodstock stocking density, fertilization rate was always higher than 90%. Lower RG was recorded with 300 / 400 sea urchins m^{-2} density when compared with 100 m^{-2}. It is concluded that under the rearing conditions adopted in this study, captive *P. lividus* broodstock can be at least reared up to 400 m^{-2} without impairing reproductive performance.

Key words: *Paracentrotus lividus*; stocking density; spawning performance; gamete production; fertilization rate; broodstock growth.

Introduction

Sea urchins are known to be very prolific marine invertebrates, and females of some species, for example, can release as many as 100,000,000 eggs in a spawning (Randall *et al.* 2002). Therefore, at least seasonally, high densities of echinoderm larvae are found in plankton surveys (e.g. Franz *et al.* 1984; Lindley *et al.* 1995; López *et al.* 1998) and predation on these numerous echinoderm eggs and larvae seems to be a major cause of mortality (e.g. Rumrill 1990; McEdward and Minner 2001; Allen

2008). Although some caution must be taken when comparing invertebrate predation studies in the laboratory and under natural conditions (Johnson and Shanks 1997; 2003), that fact, coupled to the simple sea urchin broodstock rearing and maturation, spawning induction, fertilization and larvae rearing lead to the suggestion of using sea urchin eggs and larvae as another live feed in marine larviculture (Hubbard *et al.* 2003; Luis *et al.* 2005). For captive sea urchin *Paracentrotus lividus* in particular, Luis *et al.* (2005) obtained large number of eggs year-round and Gago *et al.* (2009) enhance *P. lividus* eggs and endotrophic larvae nutritional quality with essential fatty acids for marine fish larvae through manipulation of the lipid composition of the captive broodstock diet.

Consequently, if *P. lividus* eggs and larvae prove to be viable alternative sources of live feed to the commonly used live preys (the rotifer *Brachionus* spp. and the brine shrimp *Artemia* spp.), a large number of eggs and larvae will be necessary to sustain commercial marine fish hatcheries. As a result, *P. lividus* broodstock rearing conditions must be tested in order to optimize gamete and larval production without impairing broodstock survival and even growth. Therefore this study aims to analyse the effect of different stocking densities on gamete production and consequent fertilization rates. Secondarily, to evaluate general rearing conditions, captive *P. lividus* broodstock relative growth was also assessed.

Material and methods

Sea urchin collection and rearing

Adult sea urchins (test diameters above 30 mm and fresh weight above 20 g) were collected during full-moon low tides from pools on Cabo Raso (central west coast of Portugal near Cascais - Lisbon) during September 2004. Gago *et al.* (2003) have already studied the influence of habitat characteristics on several bio-ecological parameters of this population.

After collection, sea urchins were immediately transported to the Guia Marine Laboratory (5 km away from Cabo Raso) in water-filled containers. Then, sea urchins were randomly allocated to eight cylindrical black fibreglass 402 L tanks (0.80 m base diameter x 0.80 m depth), in a recirculation seawater rearing system with similar physical conditions (35 g L^{-1} salinity; 18 ± 0.5 ºC temperature; 14L:10D photoperiod and 700 lux overhead illumination) as described by Luis *et al.* (2005). Four stocking densities were used: 100, 200, 300 and 400 sea urchins m^{-2} (area of base),

which represents 50, 100, 150, 200 sea urchins per tank, respectively. Each density was tested in duplicate. Fifty sea urchins from each tank were measured for test diameter at the ambitus (measuring stick calliper to the nearest 1mm) and fresh weight (weighing scale to the nearest 0.01g, after 1 min drainage).

A two-week conditioning period was established during which sea urchins were unfed. Afterwards, sea urchins were fed twice a week a mixed diet consisted of, commercial yellow grains of the maize *Zea mays* (13.5% moisture) and of the commercial dried seaweed *Laminaria ochroleuca* (Kombu, Algas de Galicia, Algamar, Spain; 13.4% moisture). Only one component of the diet was given in each feeding day after tank cleaning by water siphoning in order to remove the abundant excreta produced by the sea urchins. Maize was presented as dry grains that were randomly distributed by tank water current as they sank and dried seaweed fronds were pre-cut into small pieces (~2 cm^2), wetted for 15 min, and then also randomly distributed. The number of maize grains or seaweed pieces given in each feeding was equal to the number of sea urchins captive in each rearing tank. This diet was chosen because both of the components are easily purchased and, according to Luis *et al.* (2005), led to captivity null mortality and good spawning performance (consistent large year-round spawning when sea urchins were induced to spawn). In order to maintain the stocking density constant throughout the study period, whenever a sea urchin died it was substituted by another one collected in the same above referred coast. However, mortality only occurred for a reduced number of sea urchins (14 out of 1000 individuals – 1.4%) and was restricted to the first 20 rearing days, probably related with injuries inflicted during collection.

Spawning induction, gamete counting and fertilization rate evaluation

After 5 months of captive period, and thus during out-of-season maturation period, 50 sea urchins from each tank were randomly removed and measured (test diameter and fresh weight). Relative growth (RG) was calculated on a monthly basis for each rearing tank separately, both in terms of weight (RG_w) and test diameter (RG_{td}) according to the formulas expressed in Castell and Tiews 1980.

After measuring, sea urchins were induced to spawn by intracoelomic injection of 1ml 0.5M KCl through the peristomial membrane, using a 0.9 mm (external diameter) x 50 mm (length) needle coupled to a 5 ml syringe. Each sea urchin was then placed for 30 min in an individual plastic beaker filled with 2 L of aerated filtered (1 μm mesh) sea water at 18 ± 0.5 C. Egg release was evaluated by

light microscopy (x40) and quantitative egg counts were obtained with a Coulter Counter (Coulter Corporation, Miami, USA) model ZM with 140 μm cell aperture. Sperm release was also evaluated by light microscopy (x100) and spermatozoa number was estimated semi quantitatively with modifications of the scale used by Luis *et al.* (2005): spawning up to 200 million spermatozoa; or spawning greater then previous value. To ensure gamete fertilization, 50 ml of sperm from the one apparent best individual emission (number and movement of spermatozoa) from spawned males from the same tank, was used to fertilize the 2 L egg volume during 30 minutes. Fertilization rate was calculated as the number of fertilized eggs (with fertilization membrane) per one hundred eggs observed in a microscopic concave glass and expressed as percentage.

Statistical analysis

The 'STATISTICA 8 for Windows' software package was used for statistical analyses. Except for the number of emitted eggs, arcsine transformations were calculated to normalize data prior to statistical analyses. The Levene statistic was used to test for homogeneity of variances for all data. Data were analysed using one-way ANOVA with Tukey's multiple comparisons to determine differences among independent factors (Sokal and Rohlf 1995; Zar 1999). The significance level used was $P < 0.05$. For population variables like RG, percentage of spawned sea urchins and percentage of males that released more than 200×10^6 spermatozoa the means and variances were obtained according to the results found for the two duplicate tanks for each broodstock stocking density ($n = 2$). In the other determined variables (number of emitted eggs and fertilization rate) mean and variance values were determined from all the sea urchins examined.

Results

Results obtained for the different variables followed in this study are represented in Table 1. For the variables "number of released eggs" and "fertilization rate", no significant differences were found between duplicate tanks (one-way ANOVA, $p>0.05$) and therefore comparisons were made among *P. lividus* broodstock stocking density.

After spawning induction with 0.5M KCl, no significant differences (one way ANOVA, $P>0.05$) were found in the percentage of spawned sea urchins according to the broodstock stocking

density. More than 60% of the sea urchins emitted gametes and a maximum of spawned sea urchins (70.8%) were found for 200 m^{-2} stocking density treatment. For all stocking densities, more than half of the spawning males released more than 200 x 10^6spermatozoa but a significant highest percentage (one-way ANOVA, $P<0.05$) was achieved with 300 m^{-2} stocking density, when compared with 100 and 400 sea urchins m^{-2}. Irrespective of the broodstock stocking density, mean number of released eggs was always higher than 500 x 10^3. The highest but no-significant value (one-way ANOVA, $P>0.05$) was found for 300 m^{-2} density.

No-significant differences (one-way ANOVA, $P>0.05$) were found for fertilization rates among broodstock stocking density and more than 90% fertilization rate were obtained for all stocking densities.

Considering RG_w values, significant differences (one-way ANOVA, $P<0.05$) were found in RG between 100 and 300 – 400 sea urchins m^{-2} broodstock stocking density. Taking into account RG_{td}, the same situation also occurred, but in this case significant differences were also found among 200 and 400 sea urchin m^{-2} density.

Table 1. Values of the several variables determined for *Paracentrotus lividus* captive population according to broodstock stocking density.

Variables (mean ± s.e.m.)	*Paracentrotus lividus* broodstock stocking density (sea urchins m^{-2})			
	100	200	300	400
1 Spawned sea urchins (%) [a]	60.8 ± 3.27	70.8 ± 7.63	62.2 ± 1.52	62.6 ± 1.15
	(n = 2)	(n = 2)	(n = 2)	(n = 2)
2 Female spawning (number of released eggs x 10^3) [a]	508 ± 118	562 ± 87.2	723 ± 193	574 ± 92.0
	(n = 25)	(n = 27)	(n = 27)	(n = 27)
3 Males that released more than 200 x 10^6 spermatozoa (%) [b]	55.9 ± 2.94 a	63.9 ± 0.7 ab	76.5 ± 1.39 b	54.3 ± 3.95 a
	(n = 2)	(n = 2)	(n = 2)	(n = 2)
4 Fertilization rate (%) [a]	95.1 ± 0.92	93.0 ± 0.79	92.0 ± 0.83	93.5 ± 0.52
	(n = 15)	(n = 20)	(n = 16)	(n = 15)
5 RG_w (% $month^{-1}$) [b]	0.70 ± 0.06 a	0.64 ± 0.01 ab	0.50 ± 0.01 b	0.53 ± 0.01 b
(initial population weight was 55.5 g ± 0.76 g, n=400)	(n = 2)	(n = 2)	(n = 2)	(n = 2)
6 RG_{td} (% $month^{-1}$) [b]	0.96 ± 0.03 a	0.86 ± 0.01 ab	0.76 ± 0.02 bc	0.76 ± 0.01 c
(initial population test diameter was 46.9 mm ± 0.27 mm, n=400)	(n = 2)	(n = 2)	(n = 2)	(n = 2)

Mean ± standard error of the mean for the variables **2** and **4** were calculated based on the results obtained from each sea urchin. In these variables the factor "tank" was not significant (one-way ANOVA, P>0.05) and therefore the results were pooled according to broodstock stocking density treatment. Mean ± standard error of the mean for the variables **1**, **3**, **5** and **6** were calculated based on the results obtained from the sea urchin stocked populations in the two duplicate tanks for each stocking density.

[a] No significant differences (one-way ANOVA, P>0.05) were found in variable**1**, **2** and **4** among broodstock stocking density.

[b] Mean values followed by different letters are significantly different (one-way ANOVA, P<0.05) among broodstock stocking density treatment for variables **3**, **5** and **6**.

Discussion

According to the results obtained in this study, *P. lividus* again proved to be a very suitable species for captivity (e.g. Gorsjean *et al.* 1996; Fernandez and Boudoresque 2000; Luis *et al.* 2005) because, irrespective of the stocking density, mortality was almost null and reduced to the initial rearing period, and also because broodstock fed on simple diets incremented both the mean size and mean weight on a short period of captivity.

Based on spawning results obtained for both males and females, the majority of the sea urchins released large number of eggs or spermatozoa, indicating completeness of gametogenesis. Natural spontaneous spawnings should not have occurred because it was never observed gametes over the aboral hemisphere of sea urchins, suggesting that sea urchins under constant captivity conditions are not stimulated to spawn (pers. obs.). Moreover, energy content is one of the limiting factors for gonad growth (Schlosser *et al.* 2005) and the diet given in this study had already been proved to be energetically sufficient to promote both gametogenesis and emission of a large number of gametes (Luis *et al.* 2005). The highest percentage found in males large spawnings were obtained with 200 / 300 m^{-2} broodstock stocking density. No precise explanations are forwarded to explain this result, however in the natural habitat were these sea urchins were collected, they are found in average densities of 234 sea urchins m^{-2} (Gago *et al.* 2003) which is an intermediate value between these two experimental densities tested.

In order to be used as larval food, production of a large number of eggs and larvae has also been reported for *Lytechinus variegatus* (Hubbard *et al.* 2003). With sea urchin aquaculture purposes George *et al.* (2000) for *Lytechinus variegatus*, Kelly *et al.* (2000) for *Psammechinus miliaris* and Jimmy *et al.* (2003) for *Echinus esculentus* also obtained large-scale larvae production.

Using similar rearing conditions but with 94 *P. lividus* m^{-2} density, Luis *et al.* (2005) referred a higher mean number of emitted eggs (1,582 x 10^3) when sea urchins were induced to spawn, but the rearing period was longer (12 months).

No density effect was detected on fertilization rate, and irrespective of the tested *P. lividus* broodstock stocking density more than 90% of the eggs were always fertilized. When evaluating the fertilization capability of *P. lividus* stored gametes to be used in bioassay tests, Lera and Pellegrini (2006) also referred high fertilization rates (80 to 90%) in the control groups. Successful fertilization (99 to 100% fertilization rate) had also been reported for *Lytechinus variegatus* (George *et al.* 2000). The secondarily assessed population growth of the broodstock allowed to attest for the general good rearing conditions adopted in the present study, where growth increments were always attained in every

tested treatment after the 5 month period. Significant lower RG percentages obtained with 300 / 400 sea urchins m^{-2} may be explained by higher intraspecific competition for both space and food. Density was already referred as an important factor negatively affecting sea urchin growth both in natural environments (Levitan 1988) and in captivity (Kelly 2002).

Comparing our RG results (monthly size increments ranging from 0.36 to 0.43 mm $month^{-1}$ for 400 and 100 m^{-2} densities, respectively, and monthly weight increments ranging from 0.29 to 0.35 g for 100 and 300 sea urchins m^{-2}, respectively) with studies that used *P. lividus* identical size classes, diverse monthly growth rates are reported. For *P. lividus* (40 to 45 mm) reared for 6 months with *Cymodocea nodosa* seaweed (Fernandez and Pergent 1998), lower growth rates of 0.10 to 0.32 mm $month^{-1}$ were obtained. The same situation occurred when *P. lividus* where fed a vegetable formulated feed (0.05 to 0.35 mm $month^{-1}$). With animal or mixed formulated feeds higher growths were attained, but the authors generally considered growth rate in this size class as being very low. When also testing different artificial food for 40 to 45 mm *P. lividus*, Fernandez and Boudoresque (2000) referred growth rates around 0.6 g $month^{-1}$ for sea urchins fed vegetable artificial food, but higher rates were attained with animal and mixed food. However no precise stocking density is referred in the studies referred above.

When rearing adult *P. lividus* with three stocking densities Mouzakitis (2006) obtained monthly growth rates of between 1 and 1.2 mm $month^{-1}$, which are values higher than the highest value obtained in this study for 100 m^{-2} sea urchin stocking density (0.43 mm $month^{-1}$). According to this author, *P. lividus* can be reared at 120 kg m^{-2} density, and if we assume an average weight of 50 g per sea urchin a 2,400 sea urchins m^{-2} density would be achieved. Major differences among this study and our results could be explained by different rearing systems. In fact, in our rearing system sea urchins mainly occupy the tank base corners (pers. obs.) and therefore, for the same water volume, with a rectangular and less deep tank shape as the one used by Mouzakitis (2006), stocking density could certainly increase, and additionally the food supply can be given more easily with better distribution among individuals. In fact, with the rearing system used in the present study, sea urchins located at a less deep position in the tank will get more food particles and with higher frequency than the ones below. In the same rearing system used in the present study, Luis *et al.* (2005) reared adult *P. lividus* in 94 sea urchin m^{-2} density for 12 months and obtained an average monthly growth of 0.33 mm $month^{-1}$ and 0.38 g $month^{-1}$, which are respectively a lower and higher growth rate than the ones obtained in the present study. Overall, the diverse growth rates obtained in the present and in the other studies analysed demonstrate that captive *P. lividus* growth may depend on factors such as quantity and quality of the food and general rearing conditions. Furthermore, sea urchin gonads act as both reproductive and nutrient reserve storage organ (Walker *et al.* 2001), and depending on environmental ecological factors

the energy budget among these two functions can be altered and consequently change the energy allocated for somatic or reproductive growth.

According to the results obtained in this study, captive *P. lividus* can be reared up to 400 m^{-2} stocking density without prejudice to broodstock survival, gametogenesis and fertilization rate. However, if faster and higher growths are the main goals, some caution must be taken when choosing the *P. lividus* stocking density. These conclusions are important to maximise production of *P. lividus* eggs and larvae to feed marine fish larvae, but also can be used to develop increasing sea urchin aquaculture for human consumption, both in terms of seed production and stock growth.

Acknowledgments

This research was financed by FCT (Fundação para a Ciência e Tecnologia) through IMAREDIS project.

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CHAPTER 2.3.

Comparison of spawning induction techniques on *Paracentrotus lividus* (Echinodermata: Echinoidea) broodstock. What can trigger its spawning?

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Submitted to *Aquaculture International*

Comparison of spawning induction techniques on *Paracentrotus lividus* (Echinodermata: Echinoidea) broodstock. What can trigger its spawning?

Abstract

The performance of different spawning induction methods were compared on both mature wild and captive *Paracentrotus lividus* populations (N = 20). Thermal, saline and mechanical shocks, emersion for different periods of time, addition of co-specific gametes and different KCl concentrations were assayed. Percentage of spawners, mean number of released eggs, percentage of males that released more than 200 million spermatozoa, and survival after 5 days were the variables analysed. Results indicate that both thermal and saline shocks were ineffective methods to trigger spawning. Mechanical shock and addition of co-specific gametes were able to promote spawning but with a reduced number of released gametes. Emersion for a period of 3h induced spawning with 100% broodstock survival but longer periods can cause significant broodstock mortality. An injection of 1 ml intra-peristomial KCl was an expedite method to obtain *P. lividus* gametes, but mortality is always associated and is related with excessive KCl concentration. When there is need for a small number of gametes the mechanical shock technique can be considered since led to 100% survival. When large spawnings are required the emersion can be a viable method but further investigation must be carried out to assess the best time period to obtain broodstock total survival.

Key words: *Paracentrotus lividus*; spawning; induction technique; gamete production; survival

Introduction

A correct knowledge on which factors control the sea urchin *Paracentrotus lividus* (Lamarck, 1816) gametogenesis and spawning is essential because there is an increasing demand for eggs and larvae. Emergent *P. lividus* culture requires a continuous supply of eggs and larvae (Grosjean *et al.* 1998, Cook & Kelly 2007); mass production of *P. lividus* eggs and larvae for marine fish larvae

feeding is an hypothesis recently proposed (Luis *et al.* 2005, Gago *et al.* 2009); and at a small scale, *P. lividus* eggs and larvae are also needed as biological tools for different studies such as in embryological and toxicological research (*e.g.* Ozretic & Krajnovic-Ozretic 1985, Warnau & Pagano 1994, Aluigi *et al.* 2008). Several factors like temperature, photoperiod, dietary resources availability and water turbulence are pointed out as controlling gametogenesis of wild *P. lividus* populations (Fenaux 1968, Crapp & Willis 1975, Régis 1979, Byrne 1990, Lozano *et al.* 1995), but taking spawning into account the main environmental trigger is not clearly known (López *et al.* 1998). Likewise, the factors that naturally initiate spawning in other sea urchin species are also relatively unknown (Cochran & Engelman 1975, 1976, Starr *et al.* 1990, 1992, Takahashi *et al.* 1990, 1991). Considering wild *P. lividus* populations, gametogenic cycle seems to be annual, and reproduction normally restricted to the spring and summer period, although some differences can occur among localities (Byrne 1990, Lozano *et al.* 1995, Spirlet *et al.* 1998, Gago *et al.* 2003, Martínez *et al.* 2003). For this reason, broodstock captivity is essential in order to get an all year-round supply of eggs and larvae. Captive *P. lividus* broodstock maturity has been achieved in a short period of time (Fernandez *et al.* 1996, Luis *et al.* 2005) by easily controlling the temperature, photoperiod and diet (Grosjean *et al.* 1998, Spirlet *et al.* 2000, Shpigel *et al.* 2004, Schlosser et al. 2005). One problematic issue is the spawning induction technique applied to mature sea urchins when large spawnings are needed, because with the most expedite method (intracoelomic injection of 0.5M KCl), a significant part of the broodstock normally die (Luis *et al.* 2005). Therefore, alternative viable methods must be evaluated in order to induce *P. lividus* to spawn without impairing broodstock survival and even reutilization. Therefore, this study is a preliminary survey which analyse the spawning performance and survival of captive and wild adult *P. lividus* subjected to different kinds of inductors (chemical, thermal, saline, mechanical and co-specific gamete introduction stimulus).

Material and methods

Two groups of sea urchins were tested: captive and wild. Captive sea urchins were cultivated for 5 months on a recirculation seawater rearing system with similar physical conditions (35 g L^{-1} salinity; 18±0.5 ºC temperature; 14L:10D photoperiod and 700 lux overhead illumination) as described by Luis *et al.* (2005) and Gago *et al.* (2009). A mix diet of yellow grains of maize *Zea mays*, with fragments of the commercial dried seaweed *Laminaria ochroleuca* (Kombu) was given

alternatively two times per week to these captive sea urchins. With this diet and in this rearing system, Luis *et al.* (2005) referred two months as the required time of captivity for most of broodstock to mature and release, after 1ml 0.5M KCl intracoelomic injection, a large number of eggs or spermatozoa. The wild sea urchins were collected in low tide pools on Cascais (Lisbon, Portugal) coastal waters during June 2006. They were immediately transported to the laboratory in water filled containers and subjected to spawning induction. For this population, Gago *et al.* (2003) referred this time period for natural spawning and Luis *et al.* (2005) found that all wild sea urchins collected in June and induced to spawn with 1 ml KCl 0.5 M intracoelomic injection release a large number of eggs or spermatozoa. According to the previous explanations it was considered that both captive and wild broodstock were mature at the time of spawning induction. The application of the several spawning induction methods occurred during a seven day period on June 2006. Twenty adult *P. lividus* (45 mm to 55 mm as maximum test diameter range) were randomly chosen to test each spawning induction method which is revised in Table 1.

The first approach was to subject sea urchins to different kinds of physiological stress. Temperature and salinity variations as well as mechanical shake-up and emersion for several periods of time were the methods chosen. The second approach was to test the effect of co-specific gamete addition on the spawning induction. Finally, different KCl concentrations were tested in order to evaluate the concentration effect on both spawning performance and survival.

Each spawning induction technique was evaluated individually and each sea urchin was placed in individual plastic beakers filled with 2 L of aerated sea water, where eggs or sperm were eventually emitted. Light microscopy (×40) was used to evaluate spawning. Precise egg counts were obtained with a Coulter Counter (model ZM with 140 μm cell aperture). Sperm release was also evaluated by light microscopy (x100) and spermatozoa number was estimated semi quantitatively with modifications of the scale used by Luis *et al.* (2005): small spawning (up to 200 million spermatozoa); or large spawning (more than 200 million spermatozoa). For all trials, a control treatment were performed where ten sea urchins were placed in similar individual plastic beakers filled with 2 L of aerated sea water at 18ºC for 3h were spawning were assessed. After performing the spawning induction trials, mortality was again individually followed during a five day period in plastic beakers filled with 2 L of aerated sea water, at the same conditions of captive broodstock (35 g L^{-1} salinity; 18±0.5 ºC temperature; 14L:10D photoperiod and 700 lux illumination). No tube feet and spine movement was the criteria to assess mortality.

Table 1. Description of the several techniques performed to *Paracentrotus lividus* in order to assess their spawning induction capacity.

Spawning induction technique	**Description**
Control	Sea urchins were placed for 3h at 18ºC.
Thermal shock	In all "thermal shock" trials, salinity was set at 35 g L^{-1}.
TS 1	Sea urchins were placed for 3h at 13ºC.
TS 2	Sea urchins subjected to technique TS 1 were then placed 3h at 23ºC.
TS 3	Sea urchins were placed for 3h at 23ºC.
TS 4	Sea urchins subjected to technique TS 3 were then placed 3h at 13ºC.
TS 5	Sea urchins were placed for 3h at 3ºC.
TS 6	Sea urchins subjected to technique TS 5 were then placed 3h at 18ºC.
Saline shock	In all "saline shock" trials, temperature was set at 18ºC.
SS 1	Sea urchins were placed for 30 min at 0 g L^{-1}.
SS 2	Sea urchins subjected to technique SS 1 were then placed 3h at 35 g L^{-1}.
SS 3	Sea urchins were placed for 30 min at 20 g L^{-1}.
SS 4	Sea urchins subjected to technique SS 3 were then placed 3h at 35 g L^{-1}.
SS 5	Sea urchins were placed for 30 min at 50 g L^{-1}.
SS 6	Sea urchins subjected to technique SS 5 were then placed 3h at 35 g L^{-1}.
SS 7	Sea urchins were placed for 30 min at 70 g L^{-1}.
SS 8	Sea urchins subjected to technique SS 5 were then placed 3h at 35 g L^{-1}.
Mechanical shock	In all "mechanical shock" trials, temperature was set at 18ºC and salinity at 35 g L^{-1}.
MS 1	Sea urchins were energetically shake-up for 1min out of water and then placed 3h in seawater.
MS 2	Sea urchins were energetically shake-up for 5 min out of water and then placed 3h in seawater.
Emersion	In all "emersion" trials, temperature was set at 18ºC and salinity at 35 g L^{-1}.
E 1	Sea urchins were placed out of water for 3h, covered with a wet towel, and then placed 3h in seawater.
E 2	Sea urchins were placed out of water for 6h, covered with a wet towel, and then placed 3h in seawater.
E 3	Sea urchins were placed out of water for 12h, covered with a wet towel, and then placed 3h in seawater.
E 4	Sea urchins were placed out of water for 24h, covered with a wet towel, and then placed 3h in seawater.
Gamete addition	In all "gamete addition" trials, temperature was set at 18ºC and salinity at 35 g L^{-1}.
GA 1	Sea urchins were placed 3h in seawater with 1 x 10^3eggs.
GA 2	Sea urchins were placed 3h in seawater with 10 x 10^3 eggs.
GA 3	Sea urchins were placed 3h in seawater with 1 x 10^6 spermatozoa.
GA 4	Sea urchins were placed 3h in seawater with 10 x 10^6 spermatozoa.
KCl concentration	In all "KCl concentration" trials, temperature was set at 18ºC and salinity at 35 g L^{-1}.
KCl 1	Sea urchins were injected with 1 ml KCl 0.1M through the peristoma and then placed 3h in seawater.
KCl 2	Sea urchins were injected with 1 ml KCl 0.25M through the peristoma and then placed 3h in seawater.
KCl 3	Sea urchins were injected with 1 ml KCl 0.5M through the peristoma and then placed 3h in seawater.
KCl control	Sea urchins were injected with 1 ml deionised water through the peristoma and then placed 3h in seawater.

Statistical analyses were only performed to the mean female spawning, because considering the other variables (percentage of spawned sea urchins, percentage of males that released more than 200 x 10^6 spermatozoa and survival), only one value was determined for each sea urchin group (N = 20) and no replicates were performed. In this case 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 two-way ANOVA (with broodstock origin: wild or captive *P. lividus*; and spawning induction method as factors) 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 $P<0.05$.

Results

The spawning performance (percentage of spawned sea urchins, number of released eggs and percentage of males that released more than 200 x 10^6 spermatozoa) and survival results for the spawning induction methods tested for both wild and captive sea urchins are showed on Table 2. No data is presented in Table 2 for the thermal and saline shocks spawning induction techniques because in all the trials performed with these methodologies no sea urchin spawned. However, considering mortality some differences were observed. All thermal shock trials led to null mortality. Equally, saline shocks SS 3, SS 4, SS 5, SS 6, SS 7 and SS 8 also led to null mortality, but when sea urchins were placed in a 0 g L^{-1} solution for three hours (SS 1) all of then died.

Considering the mechanical shock trials, it seems that the time of shaking had some effects on spawning performance for both wild and captive *P. lividus* populations. In fact, when sea urchins are disturbed only for one minute, the values obtained for the percentage of spawned sea urchins, the mean number of released eggs as well as the percentage of large male spawnings are higher than the ones achieved when sea urchins were shake for a longer period (5 minutes). For the female spawnings the differences are inclusively statistically significant for both wild and captive *P. lividus* (Tukey test, $P<0.05$). Independently of the induction period, the survival obtained with the mechanical shock stimulus was always 100%.

Table 2. Spawning performance (percentage of spawned sea urchins, mean number of released eggs and percentage of males that released more than 200 x 10^6 spermatozoa) and survival results for each induction method tested for both wild and captive sea urchins.

A – Mechanical shock (MS).

	Wild *P.lividus*		Captive *P. lividus*	
Spawning induction technique *	MS 1	MS 2	MS 1	MS 2
Variables:				
1. Spawned sea urchins (%).	60	20	40	15
2. Mean (± s.e.m.) female spawning (released eggs x 10^3). **	178 ± 59 a	23 ± 10 b	267 ± 21 a	72 ± 16 b
3. Males that released more than 200 x 10^6 spermatozoa (%).	25	0	35	0
4. Survival (%).	100	100	100	100

* MS 1 - Sea urchins were energetically shake-up for 1min out of water and then placed 3h in seawater.

MS 2 - Sea urchins were energetically shake-up for 5 min out of water and then placed 3h in seawater.

** Mean values of released eggs followed by different letters are significantly different among each sea urchin group ($P<0.05$)

B – Emersion (E).

	Wild *P.lividus*				Captive *P. lividus*			
Spawning induction technique *	E 1	E 2	E 3	E 4	E 1	E 2	E 3	E 4
Variables:								
1. Spawned sea urchins (%).	65	80	100	0	40	60	100	0
2. Mean (± s.e.m.) female spawning (released eggs x 10^3).	1983 ± 495	2154 ± 386	1889 ± 363	0	2316 ± 249	2212 ± 212	2165 ± 187	0
3. Males that released more than 200 x 10^6 spermatozoa (%).	0	11	20	0	0	16.7	33	0
4. Survival (%).	100	65	0	0	100	55	0	0

* E 1 - Sea urchins were placed out of water for 3h, covered with a wet towel, and then placed 3h in seawater.

E 2 - Sea urchins were placed out of water for 6h, covered with a wet towel, and then placed 3h in seawater.

E 3 - Sea urchins were placed out of water for 12h, covered with a wet towel, and then placed 3h in seawater.

E 4 - Sea urchins were placed out of water for 24h, covered with a wet towel, and then placed 3h in seawater.

C – Gamete addition (GA).

	Wild *P.lividus*				Captive *P. lividus*			
Spawning induction technique *	GA 1	GA 2	GA 3	GA 4	GA 1	GA 2	GA 3	GA 4
Variables:								
1. Spawned sea urchins (%).	0	15	0	25	0	10	0	20
2. Mean female spawning (released eggs x 10^3).	0	53 ± 14	0	25 ± 11	0	67 ± 16	0	57 ± 15
3. Males that released more than 200 x 10^6 spermatozoa (%).	0	0	0	33	0	0	0	50
4. Survival (%).	100	100	100	100	100	100	100	100

* GA 1 - Sea urchins were placed 3h in seawater with 1 x 10^3 eggs.

GA 2 - Sea urchins were placed 3h in seawater with 10 x 10^3 eggs.

GA 3 - Sea urchins were placed 3h in seawater with 1 x 10^6 spermatozoa.

GA 4 - Sea urchins were placed 3h in seawater with 10 x 10^6 spermatozoa.

D – Potassium chloride concentration (KCl)

	Wild *P.lividus*				Captive *P. lividus*			
Spawning induction technique *	KCl 1	KCl 2	KCl 3	KCl control	KCl 1	KCl 2	KCl 3	KCl control
Variables:								
1. Spawned sea urchins (%).	75	95	100	15	65	95	100	10
2. Mean female spawning (released eggs x 10^3). **	114 ± 48 a	512 ± 102 a	2226 ± 129 b	0	174 ± 13 a	707 ± 71 a	2738 ± 151 b	0
3. Males that released more than 200 x 10^6 spermatozoa (%).	10	44	100	0	25	67	100	0
4. Survival (%).	75	55	15	100	90	65	30	100

* KCl 1 - Sea urchins were injected with 1 ml KCl 0.1M through the peristoma and then placed 3h in seawater.

KCl 2 - Sea urchins were injected with 1 ml KCl 0.25M through the peristoma and then placed 3h in seawater.

KCl 3 - Sea urchins were injected with 1 ml KCl 0.5M through the peristoma and then placed 3h in seawater.

KCl control - Sea urchins were injected with 1 ml deionised water through the peristoma and then placed 3h in seawater.

** Mean values of released eggs followed by different letters are significantly different among each sea urchin group ($P<0.05$)

When wild and captive sea urchins are placed out of water for different periods of time an inverse relationship occurred among spawning performance and survival until the total emersion period of 12h. The longer emersion time tested (24h) led to both null spawning and null survival, but unviable gametes were observed in the aboral surface of the sea urchins test when the wet towel were removed, indicating that spawning stimulus had previously occurred. It seems that a 3h to 12h emersion period stimulate female spawning with a number of released eggs around 2 x 10^6 without impairing survival. No statistical differences (Tukey test, P>0.05) were found in female spawnings among E1, E2 and E3 induction method for both wild and captive *P. lividus*. However, large male spawnings were only achieved when sea urchins were placed out of water for 6h and 12h. Although, in these cases, survival decrease to values of 65% and 55% in a 6h emersion period for wild and captive *P. lividus*, respectively, while for the 12h period survival is null. Considering the 12h emersion period, some gametes were also observed in the aboral surface of the test when the towel were removed, but in these cases, contrarily to the 24h emersion period, when the sea urchins were placed in the water they continued to released eggs and spermatozoa. However, after the five day period used to assess survival these sea urchins were all dead.

When co-specific gametes were added to the water medium, spawning only occurred with the higher concentrations of both eggs and spermatozoa (10 x 10^3 and 10 x 10^6, respectively). In these cases it seemed that egg addition stimulated more the female spawning and spermatozoa addition stimulated more the male spawning. In fact, for both wild and captive *P. lividus*, large male spawnings were only obtained with addition of 10 x 10^6 spermatozoa. The mean number of released eggs was higher when 10 x 10^3 eggs were added to the sea water, but no significant differences (Tukey test, P>0.05) were found between GA2 and GA4 induction methods for both wild and captive *P. lividus*. Nevertheless, as expected, in all gamete addition trials, the survival was always 100%.

Taking into account the KCl concentrations, for both wild and captive *P. lividus* it was notorious the relationship: when KCl concentration increased, larger spawnings were obtained but on the other hand the broodstock survival decreased. The mean number of released eggs was statistical higher (multiple comparisons of mean ranks for all groups, P<0.05) when both wild and captive *P. lividus* were injected with KCl 0.5M (KCl 3 induction technique) when compared with the number of eggs obtained with both the KCl 1 (0.1 M) and KCl 2 (0.25 M) spawning induction technique. Only with the injection of 1mL deionised water (KCl control), 100% survival was obtained. In this case, some males spawned but with very few spermatozoa.

Considering the control treatment (sea urchins placed in individually plastic beakers 3h at 18ºC in seawater) neither spawning nor mortality had ever occurred for all the trials performed.

General comparison between wild and captive *P. lividus* results seemed to indicate that both populations were identically stimulated with the same spawning induction methods. However, the number of spawned captive sea urchins was smaller than the wild ones, but on the other hand, when captive *P. lividus* spawned the number of released gametes was often higher, although this difference was not statistically different considering the female spawnings (Tukey test and multiple comparisons of mean ranks for all groups, $P>0.05$).

Discussion

This study also corroborates the fact that the intra peristomial injection of 1ml KCl 0.5 M is a viable expedite method to obtain large number of spawners and consistent large spawnings. But as expected with this technique, *P. lividus* broodstock mortality occurs with significant values. As well, more reduced KCl concentrations increased survival (although it never achieved 100% values), but the number of released gametes was very much reduced. The toxic effect of KCl was already studied by Hagen (2003a) on *Strongylocentrotus droebachiensis*. This author obtained null survival when juveniles were reared with 10% KCl external water concentration. In order to increase *P. lividus* survival, Luis *et al.* (2005) indicated that KCl concentration used on the injections must be related with urchin size and its internal volume and therefore suggested to use lower concentrations on smaller sea urchins. Similarly, Leahy (1986) for *Strongylocentrotus purpuratus* indicated that the KCL 0.5M volume (around the mean of 0.5 ml) should vary with the size of the sea urchin. However, this author reported large spawnings with negligible *S. purpuratus* mortality and referred that the same broodstock can be reutilized several times.

Considering the alternative spawning induction methods tested, the thermal and saline shock induction must not be utilized since no spawning had occurred. In spite of Lopez *et al.* (1998) indicated temperature as the main trigger of spawning episodes in field populations of *P. lividus*, this effect was not detected in the present study. Additionally, Himmelman (2008) showed that *Strongylocentrotus droebachiensis* natural spawning coincided with the first major intrusion of warm surface into a region normally dominated by cold upwelling. However, in the present study, even when the sea urchins were suddenly placed for 3 h at 23ºC, no gamete release was detected. General different ecological conditions prevalent in natural populations where sea urchins are not isolated compared with the individual plastic beakers could be one probable explanation for this dissimilarity, or even the 3 h time period used is still insufficient to initiate spawning.

Saline shock also proved to be an unsuccessful method. Furthermore, null survival obtained with 0 g L^{-1} salinity also demonstrated the *P. lividus* susceptibility to low salinities. Fernandez *et al.* (2003) have already reported *P. lividus* mass mortality after exceptional rainfall and resultant low salinities as low as 7 g L^{-1}.

The mechanical shake up and co-specific gamete addition stimulus used in the present study promoted spawning but with a reduced number of gametes. However, since total survival was obtained with these two methodologies, they can be considered in cases of no need to have a large number of eggs and spermatozoa such are the cases of embryological and toxicological research as well as practical classes. The mechanical shake up stimulus proved to be more efficient when *P. lividus* were shaked for shorter periods (1 min). Maybe, increased time of shaking can instead act as a signal for stopping individual biological activities. In fact, when these sea urchins were placed in individual plastic beakers to analyse spawning, they were inactive for longer periods. Considering co-specific gamete addition stimulus seemed to be effective only when large quantities of gametes were used. Presence of co-specific spermatozoa was already suggested by Starr *et al.* (1990, 1994) as influencing *Strongylocentrotus droebachiensis* spawning, and inclusively, Gaudette *et al.* (2006), speculated for the same species that mass spawning is more likely to occur in large, dense populations where sperm concentrations reach high enough levels to trigger spawning. However, Wahle & Gilbert (2002) based on *S. droebachiensis* 50-day fertilization assays considered spawning as a gradual and continuous phenomenon without mass spawning events. Our experience on the wild *P. lividus* populations used in the present study also indicate a large spawning period (Gago *et al.* 2003) and inclusively, mature sea urchins are found in high ratio during all spring and summer period (Luis *et al.* 2005). Nevertheless, the co-specific gamete stimulus was detected in the present study but only promoted very few male and female large spawnings. Additionally, it seemed that the presence of eggs is also effective for inducing spawning on both males and females. However, this methodology is not practical since previous large spawnings, obtained by other techniques, are needed in order to have enough gametes to be used as inductors.

The emersion stimulus also promoted the emission of a large number of eggs but the time must be controlled. Reduce periods of time out of water (3h) were insufficient to induce large male spawning but a very large period (24h) can lead sea urchins to spawn out-of-water impairing the use of the gametes for fertilization. Also generalized mortality occurred on longer periods (12 to 24h) probably related with desiccation and/or hypoxia. In natural environments, the *P. lividus* intertidal populations only occupies the lower parts of the rocky shores, so even in spring tide periods they are only out-of-water for reduced periods of time. Furthermore, sea urchins occupy crevices which retain sea water at

low tide. Although, because it can be a viable method to obtain large spawnings preventing broodstock mortality, this technique should be further assessed and the results should be analysed considering shorter time period intervals and considering different air temperatures.

With the same purposes as the ones referred in the present study (large spawnings coupled to higher broodstock survival), Hagen (2003b) analysed the effects of electrostimulation. Although the survival rates obtained were better than with KCl injection, this method proved ineffective to induce the release of a large number of eggs and spermatozoa.

This preliminary approach to alternative *P. lividus* spawning induction techniques to KCl injections must only be seen as an introductory study to encourage new studies on this issue. Additionally, the description of the results obtained and the discussion performed around them must be interpreted with some caution because, except for the number of released eggs, they lack statistical validation.

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CHAPTER 3

Nutritional quality of *Paracentrotus lividus* eggs and endotrophic larvae for marine fish larvae first-feeding

CHAPTER 3.1.

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

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Aquaculture Nutrition 15: 379-389

Aquaculture Nutrition **2009 15;** 379–389 doi: 10.1111/j.1365-2095.2008.00602.x

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

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Abstract

Sea urchin eggs and larvae have been suggested as potential live prey for marine fish larval feeding. This study evaluated the fatty acid composition of *Paracentrotus lividus* eggs, prisms and four-armed plutei, obtained from wild and captive broodstocks fed on raw diets: maize, seaweed and a combination of maize and seaweed. Amounts of essential fatty acids (EFA) for marine fish larvae [arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA)] were determined in eggs and endotrophic larvae. ARA ranged from 3.93% in eggs from combination to 18.7% in plutei from maize diets. In any developmental stage, EPA amounts were always lower than 5% for the raw diets, and DHA showed null or trace amounts including the wild diet. Thus, broodstock-prepared diets had to be formulated based on different lipid sources (Algamac, linseed oil, cod liver oil and olive oil) in order to test eggs and larvae EFA enhancement. EFA improvement was possible for all tested prepared diets. Algamac diet lead to superior EFA enhancement mainly in DHA (7.24%, 4.92% and 6.09% for eggs, prisms and plutei, respectively) followed by cod liver oil diet. Only these two lipid sources should be considered for prepared broodstock diets in order to obtain suitable live prey for fish larval feeding.

KEY WORDS: broodstock diets, eggs, endotrophic larvae, essential fatty acids, marine fish larval nutrition, *Paracentrotus lividus*

Received 17 July 2007, accepted 14 May 2008

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Introduction

In the recent years, considerable progress has been achieved with inert artificial food (Cahu & Zambonino 2001; Robinson *et al.* 2005; Curnow *et al.* 2006), but the rearing of most marine larvae species still depends on phytoplankton and/or zooplankton as live feed (Støttrup & McEvoy 2003). Considering zooplankton only two taxa have been so far mass produced: the rotifer *Brachionus* spp. and the brine shrimp *Artemia* spp. Other taxa, like Copepoda, have been investigated in order to improve survival and growth of current- and future-targeted marine larval species (Lavens & Sorgeloos 1996; Cutts 2003; Drillet *et al.* 2006).

The potential use of eggs and larvae of sea urchins as live feed has also been suggested. Hubbard *et al.* (2003) developed cultivation techniques for the sea urchin *Lytechinus variegatus* and pointed out the potential use of its early developmental stages as larval food. Luis *et al.* (2005) hypothesized the same idea and reported consistent large year-round spawnings of the captive sea urchin *Paracentrotus lividus*. Both studies refer as main criteria the easy maintenance of broodstock, simple spawning induction with potassium chloride (KCl), large spawnings, simple larval rearing and the size similarity with rotifers and brine shrimp. However, in order to be considered an additional fish larval food, predator's acceptance and nutritional quality of sea urchin eggs and larvae must yet be analysed. Few predation studies have included sea urchin eggs and larvae. Two fish species (Pennington *et al.* 1986) and zoeae of the red crab *Cancer productus* (Rumrill *et al.* 1985) were reported as predators of embryos and larvae of the Pacific sand dollar *Dendraster excentricus*. Egg and/or larval proximate chemical composition have been studied for *Strongylocentrotus droebachiensis* (Thompson 1983), *Paracentrotus lividus* (Fenaux *et al.* 1985), *Strongylocentrotus purpuratus* and *Lytechinus pictus* (Shilling & Manahan 1990), *Arbacia lixula* (George *et al.* 1990), *Encope michelini* (George *et al.* 1997) and *S. purpuratus* (Meyer *et al.* 2007). Fatty acids profiles have only been recently studied for *D. excentricus* (Schiopu

et al. 2006) and *Paracentrotus lividus* exotrophic larvae (Liu *et al*. 2007) and were analysed according to the microalgae species given in the diet.

This paper presents the fatty acid profiles of *Paracentrotus lividus* eggs and two endotrophic larval stages and discusses their value as live feed for early marine fish larvae. Fatty acid profiles were differentially analysed according to different captive broodstock diets and compared with the respective eggs and larvae obtained from wild broodstock.

Materials and methods

Sea urchin collection and rearing

Sea urchins (test diameters above 40 mm) were collected during full-moon low tides from pools on the central west coast of Portugal near Cascais (Lisbon) in two distinct periods: September 2004 for raw diets tests and September 2005 for prepared diets tests. A previous study on the same population (Gago *et al*. 2003) established the annual variability of gonad index, spawning periods and the influence of habitat characteristics on energy partition for reproduction and growth.

After collection, sea urchins were immediately transported to the laboratory in water-filled containers, and randomly allocated to eight cylindrical black fibreglass 402 L tanks (0.80 m base diameter × 0.80 m depth), at 50 sea urchins m^{-2} density, in a recirculation seawater rearing system with similar physical conditions (35 gL^{-1} salinity; 18 ± 0.5 °C temperature; 14L:10D photoperiod and 700 lux overhead illumination) as described by Luis *et al*. (2005).

Broodstock diets

A two-week conditioning period was established during which sea urchins were unfed. Afterwards, sea urchins were fed twice a week one of the tested diets. Tanks were randomly allocated to each diet and food portions were distributed by tank water circulation as they sank. Feeding finished when the majority of sea urchins had captured at least one food portion.

Raw diets

We used the same diets previously used by Luis *et al*. (2005) for spawning performance quantification: (1) commercial whole yellow grains of maize *Zea mays*; (2) pieces of the commercial dried seaweed *Laminaria ochroleuca* (Kombu) and (3) combination of the previous diets, where components were alternately presented. These diets were again chosen because they are simple to utilize, cheap and promote *P. lividus* gametogenesis.

Prepared diets

As raw diets lead to insufficient essential fatty acids (EFA) amounts for feeding fish larvae (see Results), prepared diets had to be considered. The possibility to enhance eggs and endotrophic larvae highly unsaturated fatty acids (HUFA) content through broodstock diet was tested using five isocaloric diets based on equal parts of maize flour and wheat flour but different lipid sources (Table 1). The maize and wheat flour were chosen because they complement each other in terms of essential amino acids and vitamins (Crampton & Harris 1969), and also because they lead to minor percentages of four-armed pluteus malformations (Repolho, pers. comm.). Each dietary lipid source was chosen to test either HUFA transfer to eggs and endotrophic larvae or elongation/desaturation capability. These capabilities were already evidenced by Pantazis *et al*. (2000), Cook *et al*. (2000), Bell *et al*. (2001) and Castell *et al*. (2004) for sea urchin gonads and by Schiopu *et al*. (2006) and Liu *et al*. (2007) for sea urchin exotrophic larvae. Olive oil and linseed oil were mainly chosen to provide substrates for elongation/desaturation because both are rich in C18:1n-9 and C18:2n-6 and linseed oil is also rich in C18:3n-3. Cod liver oil and Algamac were mainly used to test HUFA, and particularly C22:6n-3 (docosahexanoic acid, DHA), transfer and accumulation in eggs. However, the metabolic explanations of such processes were out of scope, as the primary goal was to assess EFA enhancement of these potential live preys (*P. lividus* eggs and endotrophic larvae).

Table 1 Composition of the prepared diets

Ingredients	Dry mass ($g\ kg^{-1}$)
Integral maize flour ('DATERRA', Ignoramus – Produtos Naturais Lda, Portugal)	425
Integral wheat flour ('DATERRA', Ignoramus – Produtos Naturais Lda, Portugal)	425
Lipid source: Algamac ('2000™'); olive oil ('Azeite Galo'); linseed oil ('Emile Noël'); cod liver oil ('José M. Vaz Pereira, S.A.)	100
Vitamin mix ('S.N. PV 10/8', PREMIX – Especialidades Agrícolas e Pecuárias Lda, Portugal)	10
Alginic acid, sodium salt – powder (Sigma-Aldrich)	20
Sodium hexametaphosphate (Rhodia)	20

Water was added at a mass ratio of 1 : 1 (water/dry ingredients).

The ingredients were weighed and dry mixed. After water addition, the moist feed was transferred to an extruder where it was pressed to form 5-mm-diameter cylindrical rods. These rods were oven dried (48 h at 35 °C) and cut into pieces about 1 cm long.

Spawning induction, egg collection and larvae rearing

After 8 months of captive period, sea urchins were randomly removed from each tank and induced to spawn by injecting 1 mL 0.5 M KCl through the peristomial membrane, using a 0.9 mm (external diameter) × 50 mm (length) needle coupled to a 5-mL syringe. Each sea urchin was then placed for 30 min in an individual plastic beaker filled with 2 L of aerated filtered (1 μm) sea water at 18 ± 0.5 °C. Sperm or egg release was evaluated by light microscopy (40×) and quantitative egg counts were obtained with a Coulter Counter (model ZM with 140 μm cell aperture). Only female sea urchin spawnings over 1×10^6 eggs were selected. For egg collection, the 2 L volume was filtered onto a 30-μm nylon mesh and then washed with seawater into a 30-mL centrifuge tube. After centrifugation ($1500 \times g$, 1 min), the supernatant was removed by tube inversion and the precipitate resuspended with 1 mL deionized water and transferred to 2 mL eppendorf tubes. In this way, the eppendorf tubes contained no less than 1×10^6 and no more than 5.6×10^6 eggs. To obtain endotrophic larvae, 50 mL of sperm from the one apparent best individual emission (number and movement of spermatozoa) from the males fed the same diet, was used to fertilize the 2 L egg volume during 30 min. The fertilized eggs were then washed onto a 30-μm nylon mesh and placed into 2-L plastic beakers filled with aerated filtered sea water and reared with the same physical conditions of the captive broodstock. Beside eggs (~90 μm), two endotrophic stages were analysed: swimming prism (24 h after fertilization: ~120 μm) and four-armed pluteus (72 h after fertilization: ~370 μm). At the end of each rearing period, the larvae were filtered through 60 μm nylon mesh and collected like the eggs. The number of endotrophic larvae collected into each eppendorf tube was previously estimated by five 1-mL counts of the 2 L rearing volume. Thus, the number of prisms ranged from 9×10^5 to 3.2×10^6 while the number of plutei ranged from 4×10^5 to 1.9×10^6.

Egg and larval samples from wild broodstock were obtained from sea urchins collected in low tide pools at the above-referred coastal waters on July 2005.

All eggs and larvae samples were immediately freeze dried and stored under nitrogen at −20 °C for subsequent analysis.

Lipid extraction and fatty acid analysis

Lipids were extracted according to Bligh & Dyer (1959). Lipid extracts with added 100 μL of internal standard (C19:0) were subjected to transesterification with boron fluoride-methanol solution for 45 min at 100 °C under nitrogen (Metcalfe & Schmitz 1961). After solvent evaporation, fatty acid methyl esters (FAME) were recovered in 2 mL isooctane and then 1 μL was injected into a capillary column (30 m fused silica, 0.32 mm I.D.) coated with Omegawax (Supelco, Bellefonte, USA) installed in a Varian Star 3400CX GLC. Helium was used as carrier gas, at a flow rate of 1 mL min^{-1}; oven temperature was 180 °C for 7 min and then 210 °C (with a temperature gradient of 4 °C min^{-1}) over a period of 40 min. Both the injector port and the FID detector were set at 250 °C. Fatty acids were identified by co-chromatography with standards (Sigma-Aldrich, St. Louis, USA) and, in addition, the peaks of chromatograms were compared with those of the methyl esters prepared from cod liver oil reference standard. Peak areas were measured by a computer program (Star Chromatography Workstation) installed in a IBM PS/1.

Statistical analysis

The 'STATISTICA 7 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 test statistic followed by multiple comparisons of mean ranks for all groups (Sokal & Rohlf 1995; Zar 1999). The significance level used was $P < 0.05$. Except for ratios, arcsine transformations were calculated to normalize data prior to statistical analyses.

Results

Dietary fatty acids

Major fatty acid composition of the raw and prepared diets tested is shown in Table 2. Considering raw diets, C18:2n-6, C18:1n-9 and C16:0 were the three more abundant fatty acids for maize, while for seaweed, besides C16:0 and C18:1n-9, HUFA were also present with 9.84% and 7.76% for C20:4n-6 (arachidonic acid, ARA) and C20:5n-3 (eicosapentaenoic acid, EPA), respectively. Broodstock fed on combination diet should have ingested the same dietary fatty acids of maize and seaweed but on a different amount basis.

Table 2 Major fatty acid composition (mass percentage of total lipid fatty acid methyl esters, FAME) of the raw and prepared diets

Fatty acid (% FAME)	Raw diets		Prepared diets			
	Maize	Seaweed	Algamac	Linseed oil	Cod liver oil	Olive oil
C14:0		3.43	13.4		3.64	
C16:0	14.2	27.5	28.0	5.41	14.7	9.60
C16:1n-7		2.01	5.98		5.98	
C18:0	2.00	1.74		3.28	3.41	2.79
C18:1n-9	32.0	21.4	3.92	26.3	23.7	69.5
C18:1n-7			3.96		2.81	
C18:2n-6	47.5	6.25	8.46	29.0	12.3	13.9
C18:3n-3	1.08	3.57		30.1	1.05	
C18:4n-3		8.25				
C20:1n-9					7.07	
C20:4n-6 (ARA)		9.84				
C20:5n-3 (EPA)		7.76			2.92	
C22:1n-11					4.17	
C22:5n-6			6.88			
C22:6n-3 (DHA)			19.9		5.01	

Values are the mean from the analyses of three independent samples. For each diet only fatty acids present at >1% are included in the table.

Concurring with the lipid source used for each prepared diet, C16:0 and DHA were the major fatty acids for Algamac diet; linseed oil diet was rich in C18:1n-9, C18:2n-6 and C18:3n-3; cod liver oil diet presented high percentages for C16:0, C18:1n-9 and C18:2n-6, and moderate amounts of HUFA with 2.92% for EPA and 5.01% for DHA; olive oil diet was mainly rich in C18:1n-9.

Egg and endotrophic larva fatty acids – raw and wild diets

The proportion of broodstock fed on the pure seaweed diet that spawned was not only lower than broodstock fed on the other diets, but the number of eggs and spermatozoa emitted were also considerably lower (only four out of eight females emitted more than 1×10^6 eggs). Thus, only the nutritional value of eggs was considered for this diet given the insufficient number of eggs for larval rearing.

Table 3 presents the fatty acid composition of eggs, prisms and four-armed plutei obtained from broodstock fed on raw and wild diets. The saturated fatty acids (SFA), C14:0, C16:0 and C18:0, were the most abundant with C16:0 being the more representative, irrespectively of the developmental stage considered.

Eggs

Unsaturated fatty acids represented the majority of egg fatty acid profile irrespective of the broodstock diet considered (Table 3). Egg monounsaturated fatty acids (MUFA) represented around 30%; polyunsaturated fatty acids (PUFA) ranged from 27.9% on the combination to 38.1% on maize diets; and HUFA ranged from 6.25% on combination to 14.8% on seaweed diets. Egg MUFA mainly included C18:1n-9 with higher percentage on maize and combination diets compared with eggs from seaweed and wild diets ($P < 0.001$); C20:1n-9 accounted for 6.43% in eggs from wild diet, which was significantly higher than eggs from maize and combination diets ($P < 0.05$). Major egg PUFA included C18:2n-6, although represented only in trace amounts in eggs from wild diet; C20:2Δ5, 11 non-methylene-interrupted dienoic fatty acids (NMID) for eggs from all the diets with major significant percentage in eggs from wild diet ($P < 0.05$) compared with eggs from seaweed diet; and C20:2n-6 with significant higher percentage in eggs from maize diet compared with eggs from wild diet ($P < 0.05$). Considering HUFA, ARA was the most abundant in eggs from any of the diets; EPA was present with significant higher percentage in eggs from wild diet than in eggs from combination diet ($P < 0.05$) and DHA was detected at only minor percentages in eggs from maize and seaweed diets. Fatty acids of the n-6 series were represented at larger percentages than n-3 fatty acids in eggs from all the diets, but this fact was more evident in eggs from maize and combination diets as showed by n3/n6 ratios. The DHA/EPA ratios were very low or nil. The EPA/ARA ratios were significantly superior in eggs from seaweed and wild diets than in eggs from maize and combination diets ($P < 0.001$).

Prisms

Unsaturated fatty acids, particularly PUFA, irrespective of diet, dominated the fatty acid profile of the prism stage (Table 3). MUFA percentages were significantly higher in

Table 3 Total lipid and fatty acid composition (mass percentage of total lipid fatty acid methyl esters, FAME) of *Paracentrotus lividus* eggs, prisms and four-armed plutei, according to raw and wild broodstock diet

	Eggs				Prisms			Four-armed plutei		
P. lividus	Maize	Seaweed	Combination	Wild	Maize	Combination	Wild	Maize	Combination	Wild
Total lipid (g kg^{-1}dry mass)	169 ± 8.75	124 ± 8.22	136 ± 17.4	127 ± 17.1	157 ± 17.8	132 ± 5.03	107 ± 8.19	78.6 ± 1.01 a	40.8 ± 2.07 b	30.8 ± 5.15 b
Fatty acid (% FAME)										
C14:0	8.29 ± 0.78	11.4 ± 2.70	11.3 ± 0.39	13.5 ± 0.44	6.18 ± 0.64 a	8.95 ± 0.53 b	9.03 ± 0.34 b	4.74 ± 0.47 a	5.47 ± 0.38 ab	6.49 ± 0.21 b
C16:0	17.6 ± 1.31	20.3 ± 3.74	23.1 ± 0.81	20.7 ± 0.58	15.1 ± 0.72 a	19.0 ± 1.07 b	13.5 ± 0.79 a	11.2 ± 0.63	12.6 ± 0.70	12.5 ± 0.48
C16:1n-7	2.91 ± 0.40	3.06 ± 0.70	3.13 ± 0.04	3.48 ± 0.20	2.42 ± 0.21	2.69 ± 0.31	2.11 ± 0.08	2.27 ± 0.40	2.12 ± 0.18	1.22 ± 0.12
C16:3n-4	0.84 ± 0.21	1.66 ± 0.50	0.71 ± 0.06	1.51 ± 0.07	0.40 ± 0.08 a	0.75 ± 0.04 a	1.63 ± 0.26 b	1.11 ± 0.32 a	1.38 ± 0.39 ab	2.97 ± 0.54 b
C18:0	3.24 ± 0.21	3.56 ± 0.39	3.44 ± 0.15	3.04 ± 0.24	3.21 ± 0.19 a	4.21 ± 0.35 b	3.10 ± 0.05 a	4.36 ± 0.32	4.35 ± 0.34	4.85 ± 0.18
C18:1n-9	7.48 ± 0.53 a	5.79 ± 0.60 b	8.15 ± 0.50 a	2.10 ± 0.11 c	6.65 ± 0.69 a	6.69 ± 0.62 a	2.17 ± 0.69 b	4.10 ± 0.38 a	5.63 ± 0.44 a	1.82 ± 0.28 b
C18:1n-7	1.92 ± 0.55	2.76 ± 0.36	2.31 ± 0.16	3.96 ± 0.16	1.86 ± 0.16 a	2.17 ± 0.11 a	2.86 ± 0.10 b	1.78 ± 0.11	1.82 ± 0.19	2.08 ± 0.12
C18:2n-6	11.6 ± 1.38 a	3.46 ± 1.41 ab	8.71 ± 0.77 ab	0.65 ± 0.09 b	16.7 ± 0.97 a	9.64 ± 1.26 b	1.70 ± 0.72 c	8.58 ± 0.63 a	8.82 ± 0.90 a	0.37 ± 0.04 b
C18:3n-3	0.38 ± 0.06 ab	2.29 ± 0.72 a	0.00 ± 0.00 b	0.67 ± 0.11 ab	0.00 ± 0.00	0.00 ± 0.00	1.16 ± 0.23	0.00 ± 0.00 a	0.34 ± 0.03 b	0.00 ± 0.00 a
C18:4n-3	1.21 ± 0.24 ab	2.43 ± 0.48 a	1.32 ± 0.13 ab	0.70 ± 0.32 b	0.60 ± 0.05	1.21 ± 0.43	1.46 ± 0.14	1.34 ± 0.27	1.82 ± 0.35	1.69 ± 0.20
C20:1n-9	3.52 ± 0.70 a	4.36 ± 0.35 ab	3.49 ± 0.57 a	6.43 ± 0.60 b	3.01 ± 0.22 a	4.23 ± 0.19 b	5.89 ± 0.39 c	5.21 ± 0.81 a	5.33 ± 0.68 a	16.8 ± 1.38 b
C20:1n-7	7.35 ± 0.49	6.60 ± 0.71	7.27 ± 0.13	5.38 ± 0.12	5.92 ± 0.27 a	6.90 ± 0.17 a	4.28 ± 0.43 b	5.37 ± 0.40 a	6.07 ± 0.11 a	3.36 ± 0.16 b
C20:2 Δ5,11 NMID	6.87 ± 0.39 ab	5.25 ± 0.76 a	6.52 ± 0.28 ab	7.88 ± 0.45 b	6.23 ± 0.46	7.00 ± 0.43	6.92 ± 0.28	7.40 ± 0.11	7.10 ± 0.43	8.74 ± 0.56
C20:2n-6	5.85 ± 0.60 a	4.40 ± 1.28 ab	3.65 ± 0.17 ab	2.06 ± 0.13 b	8.03 ± 0.45 a	6.04 ± 0.45 a	3.42 ± 0.54 b	9.27 ± 0.79 a	8.60 ± 0.49 a	2.75 ± 0.34
C20:3n-6	1.26 ± 0.34 a	0.89 ± 0.02 ab	0.66 ± 0.18 ab	0.51 ± 0.02 b	2.19 ± 0.17 a	1.14 ± 0.21 ab	0.69 ± 0.04 b	1.63 ± 0.12 a	1.31 ± 0.38 a	0.26 ± 0.01 b
C20:4n-6 (ARA)	6.36 ± 1.37	6.67 ± 2.80	3.93 ± 0.96	5.17 ± 0.62	10.3 ± 0.89 a	6.31 ± 0.82 b	11.2 ± 0.14 a	18.7 ± 1.35	14.2 ± 1.58	13.5 ± 1.04 b
C20:3n-3	0.23 ± 0.02 ab	1.20 ± 0.21 a	0.00 ± 0.00 b	0.99 ± 0.05 a	0.20 ± 0.02	0.20 ± 0.02	1.53 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.58 ± 0.06
C20:5n-3 (EPA)	1.76 ± 0.07 ab	4.70 ± 1.55 ab	1.23 ± 0.30 a	5.08 ± 0.96 b	1.80 ± 0.15 a	1.55 ± 0.22 a	12.4 ± 1.33 b	1.61 ± 0.26 a	1.89 ± 0.33 a	7.16 ± 0.21 b
C22:1n-9	2.96 ± 0.28	3.26 ± 0.73	3.45 ± 0.17	4.14 ± 0.29	2.31 ± 0.30 a	3.63 ± 0.30 b	3.14 ± 0.30 ab	3.00 ± 0.07	3.74 ± 0.45	3.54 ± 0.16
C22:3n-3	0.40 ± 0.04 ab	0.51 ± 0.19 ab	0.29 ± 0.03 a	1.60 ± 0.08 b	0.56 ± 0.03	0.61 ± 0.10	1.33 ± 0.49	0.80 ± 0.05 a	0.60 ± 0.20 a	2.47 ± 0.24 b
C22:6n-3 (DHA)	0.55 ± 0.12 a	0.27 ± 0.01 ab	0.00 ± 0.00 b	0.00 ± 0.00 b	0.22 ± 0.04 a	0.00 ± 0.00 b	0.39 ± 0.05 c	0.44 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
ΣSFA	31.5 ± 2.08	38.3 ± 6.74	40.0 ± 1.25	40.9 ± 1.21	26.8 ± 1.36 a	35.1 ± 1.61 b	29.4 ± 0.91 a	23.7 ± 0.87 a	26.3 ± 1.33 ab	28.3 ± 0.85 b
ΣMUFA	30.4 ± 2.51	29.4 ± 3.72	31.7 ± 0.90	29.7 ± 0.86	24.5 ± 1.30 a	29.4 ± 0.99 b	23.5 ± 1.07 a	24.2 ± 1.93	27.9 ± 1.95	30.7 ± 1.12
ΣPUFA	38.1 ± 3.51	34.7 ± 8.79	27.9 ± 2.12	28.8 ± 2.05	48.4 ± 1.76 a	35.3 ± 2.38 b	46.6 ± 1.28 a	52.1 ± 2.56 a	47.1 ± 2.46 ab	41.8 ± 1.11 b
ΣHUFA(≥20C ≥ 3uns)	10.8 ± 1.64	14.8 ± 4.75	6.25 ± 1.53	14.0 ± 1.63	15.4 ± 1.08 a	9.84 ± 1.00 b	28.7 ± 2.12 c	23.3 ± 1.26	18.3 ± 1.98	24.0 ± 0.89
n-3/n-6	0.22 ± 0.04	0.94 ± 0.17	0.20 ± 0.02	1.13 ± 0.05	0.11 ± 0.01 a	0.18 ± 0.03 a	1.10 ± 0.15 b	0.14 ± 0.02 a	0.16 ± 0.02 ab	0.71 ± 0.05 b
EPA/ARA	0.32 ± 0.08 a	0.90 ± 0.17 b	0.32 ± 0.04 a	0.96 ± 0.08 b	0.18 ± 0.02 a	0.25 ± 0.02 a	1.10 ± 0.11 b	0.09 ± 0.02 a	0.13 ± 0.02 a	0.54 ± 0.04 b
DHA/EPA	0.32 ± 0.08 a	0.09 ± 0.03 ab	0.00 ± 0.00 b	0.00 ± 0.00 b	0.12 ± 0.03 a	0.00 ± 0.00 b	0.03 ± 0.01 ab	0.29 ± 0.10	0.00 ± 0.00	0.00 ± 0.00

Values are the mean ± standard error of the mean from the analyses of four samples. Mean values followed by different letters are significantly different among diet treatment ($P < 0.05$) for each developmental stage separately.

Except for DHA (C22:6n-3), only fatty acids present at >1% for at least on diet and/or development stage are included in the table but totals (Σ) include all identified fatty acids.

prisms from combination diet than in prisms from the other diets ($P < 0.05$), mainly as a result of the significant higher percentages in C18:1n-9 ($P < 0.01$), C20:1n-7 ($P < 0.01$) and C22:1n-9 ($P < 0.05$). PUFA percentages were significantly more abundant in prisms from maize and wild diets ($P < 0.001$), with HUFA percentages significantly different among diets ($P < 0.001$). Thus, C18:2n-6 ($P < 0.001$), C20:2n-6 ($P < 0.001$) and C20:3n-6 ($P < 0.001$) were significantly more abundant in prisms from maize diet, whereas ARA ($P < 0.01$) and especially EPA ($P < 0.001$) were significantly more abundant in prisms from wild diet. The amount of DHA was also significantly higher in prisms from wild diet ($P < 0.001$), although only represented at trace amounts. The n3/n6 ratio was only greater than 1 in prisms from the wild diet, whereas in prisms from maize and combination diets, due to the majority of n-6 fatty acids, the ratio was around 0.1. Like eggs, prism DHA/EPA ratios were also reduced. The EPA/ARA ratio was significantly greater in prisms from wild than in prisms from maize and combination diets ($P < 0.001$).

Plutei

Plutei unsaturated fatty acids were still the dominant fatty acids with percentages around 75% for the three diets (Table 3), PUFA being the major component (52.1%, 47.1% and 41.8%, in plutei from maize, combination and wild diets, respectively). MUFA were more represented in plutei from wild diet mostly as a result of significant higher percentage for C20:1n-9 ($P < 0.001$). The fatty acids C18:2n-6 ($P < 0.05$), C20:2n-6 ($P < 0.001$) and C20:3n-6 ($P < 0.001$) had significantly higher percentages in plutei from maize and combination diets than in plutei from wild diet. HUFA percentages were similar in plutei from any of the diets (around 20%), being ARA and EPA (significantly higher in plutei from wild diet, $P < 0.001$) the more abundant. Plutei from wild diet had a significantly superior n3/n6 ratio ($P < 0.05$), when compared with plutei from maize diet, and EPA/ARA ratio ($P < 0.001$), when compared with the other two diets. Irrespective of diet, the DHA/EPA ratios were also much reduced.

Irrespective of diet, the lipid amounts decreased during the endotrophic phase, as noted by the significant total lipid minor percentage ($P < 0.05$) on the four-armed plutei stage compared with eggs and prisms. The fatty acid composition also presented a general evolution based on the decrease of SFA and MUFA percentages and an increase of PUFA. However, for wild diet, prisms had higher EPA percentages ($P < 0.01$), while plutei had higher C20:1n-9 percentages ($P < 0.001$) when compared with the other developmental stages.

Egg and endotrophic larva essential fatty acids – prepared diets

The proportion of broodstock fed on olive oil diet that spawned, as already occurred for seaweed raw diet, produced also an insufficient number of available eggs for sea urchin larval rearing (only four out of seven females emitted more than 1×10^6 eggs). Therefore, for this diet, only the nutritional value of eggs was evaluated.

Table 4 shows the fatty acid composition in the three EFA for fish larvae: ARA, EPA and DHA (Sargent *et al.* 1999), on sea urchin's three developmental stages from broodstock fed on prepared diets. There were no significant differences on egg total lipid and ARA resulting from broodstock fed on the tested prepared diets ($P > 0.05$). However, EPA had a significantly higher percentage in eggs from cod liver oil diet than in eggs from olive oil diet ($P < 0.05$). DHA amounts ($P < 0.001$) and DHA/EPA ratios ($P < 0.05$) showed always significantly higher values in eggs from Algamac diet, while the EPA/ARA ratios were superior in eggs from cod liver oil diet when compared with eggs from olive oil diet ($P < 0.05$).

Prism total lipid from cod liver oil diet was significantly higher than prism total lipid from Algamac diet ($P < 0.05$), while DHA amounts ($P < 0.05$) and DHA/EPA ratios ($P < 0.05$) were significantly higher in prisms from Algamac diet than in prisms from linseed oil diet.

Regarding plutei, significant differences were only recorded for the DHA contents resulting from Algamac and linseed oil diets ($P < 0.05$).

For all the prepared diets analysed, during the endotrophic phase, there was a significant decrease in total lipid up to four-armed plutei ($P < 0.01$), and a general EFA increase. In spite of DHA and EPA percentages decrease in prisms from Algamac diet, values were not significantly different when compared with egg EPA percentages and with eggs and plutei DHA percentages ($P > 0.05$).

Comparison of eggs and endotrophic larvae fatty acid composition based on raw and prepared diets

Comparing eggs and endotrophic larvae fatty acid composition based on raw diets (Table 3) and prepared diets (Table 4), it seems clear that their EFA enhancement was possible through broodstock prepared diet (Fig. 1). Raw diets resulted in poor EPA contents of eggs and endotrophic larvae, except eggs from wild and seaweed diets. Prepared

Table 4 Total lipid and essential fatty acid composition (mass percentage of total lipid fatty acid methyl esters, FAME) of *Paracentrotus lividus* eggs, prisms and four-armed plutei, according to prepared broodstock diet

	Eggs				Prisms			Four-armed plutei		
P. lividus	Algamac	Linseed oil	Cod liver oil	Olive oil	Algamac	Linseed oil	Cod liver oil	Algamac	Linseed oil	Cod liver oil
Total lipid (g kg^{-1}dry mass)	132 ± 3.68	149 ± 9.43	167 ± 16.4	184 ± 15.7	109 ± 15.4 a	117 ± 5.28 ab	161 ± 14.1 b	49.0 ± 12.3	62.0 ± 12.9	55.5 ± 7.65
Fatty acid (% FAME)										
C20:4n-6 (ARA)	11.0 ± 0.27	10.5 ± 0.16	9.74 ± 0.43	9.89 ± 0.60	12.7 ± 1.63	12.7 ± 1.07	11.8 ± 0.66	15.5 ± 2.82	19.9 ± 0.54	15.6 ± 0.65
C20:5n-3 (EPA)	6.08 ± 0.15 ab	5.87 ± 0.38 ab	6.43 ± 0.08 a	4.03 ± 0.52 b	5.90 ± 0.37	6.66 ± 0.54	7.00 ± 0.38	8.87 ± 0.10	8.71 ± 0.12	8.68 ± 0.47
C22:6n-3 (DHA)	7.24 ± 0.96 a	0.74 ± 0.31 b	2.88 ± 0.46 b	1.84 ± 0.98 b	4.92 ± 0.87 a	1.60 ± 0.46 b	3.82 ± 0.99 ab	6.09 ± 1.28 a	2.57 ± 0.42 b	3.58 ± 0.67 ab
EPA/ARA	0.55 ± 0.02 ab	0.56 ± 0.03 ab	0.66 ± 0.04 a	0.42 ± 0.07 b	0.48 ± 0.04	0.53 ± 0.05	0.60 ± 0.04	0.66 ± 0.16	0.44 ± 0.02	0.56 ± 0.02
DHA/EPA	1.19 ± 0.05 a	0.14 ± 0.07 b	0.45 ± 0.07 b	0.39 ± 0.17 b	0.83 ± 0.13 a	0.23 ± 0.06 b	0.56 ± 0.16 ab	0.69 ± 0.15	0.29 ± 0.04	0.41 ± 0.07

Values are the mean ± standard error of the mean from the analyses of four samples. Mean values followed by different letters are significantly different among diet treatment ($P < 0.05$) for each developmental stage separately.

diets increased EPA amounts to more than 5.80%, except for eggs from olive oil diet. Irrespective of the raw diet analysed and the developmental stage considered, DHA percentage was always lower than 1% but with prepared diets this value reached a maximum of 7.24% in eggs from Algamac diet. Olive and linseed oil diets resulted in lower DHA enrichment, while cod liver oil and especially Algamac diets led to DHA percentages several times higher than raw diets for any developmental stage. Regarding ARA, a general slight increase can be obtained with prepared diets, but this EFA was already present in considerable amounts in all sea urchins' developmental stages even with raw diets. Therefore, for all developmental stages, DHA/EPA ratio was always enhanced with prepared diets, especially Algamac and cod liver oil diets. The EPA/ARA ratio was also enhanced in eggs, prisms and plutei from prepared diets, when compared with maize and combination diets but not when compared with wild and seaweed diets.

Discussion

Spwanings

Two months are generally considered the required time of captivity for most of broodstock to mature and spawn under KCl injection (Fernandez *et al*. 1996; Luis *et al*. 2005). Thus, the 8-month captive period used in the present study was largely sufficient to promote broodstock gametogenesis. However, as also found by Luis *et al*. (2005), the raw diet based on the seaweed *Laminaria ochroleuca* proved to be inefficient in terms of consistent large spawning. Less energy from seaweed diet may be a reason for fewer emitted eggs: estimated caloric yield from its proximate analysis give a gross energy content of 14.6 kJ g^{-1}, while for maize the estimated value is 18.8 kJ g^{-1}. Likewise, olive oil prepared diet also prevented the emission of sufficient number of eggs for sea urchin larvae rearing. Since broodstock fed on olive oil diet took much more time to ingest the diet pieces (pers. obs.), organoleptic features associated to olive oil may have played a role on the few spwaning observed. Therefore, if the objective is to produce large numbers of eggs and larvae to feed marine fish larvae, both these captive broodstock diets should be avoided.

Lipid content and fatty acids

Considering *P. lividus* endotrophic development from all diets analysed, there was a general tendency for total lipid content and SFA and MUFA percentages to decrease

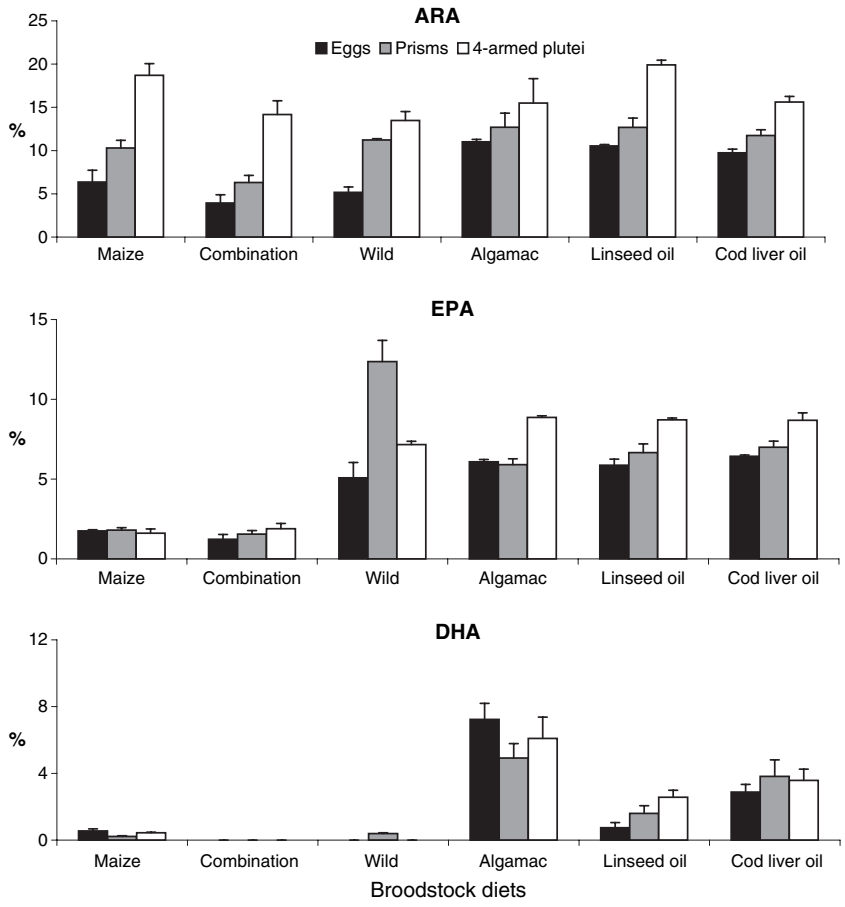

Figure 1 Mean percentages (±SE) of total lipid essential fatty acids: arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), according to broodstock diet and developmental stage considered. Seaweed and olive oil diets were not included since no data were obtained for prisms and four-armed plutei.

certainly related with energy consumption, while the more structural PUFA tended to increase. Sewell (2005) for *Evechinus chloroticus* and Meyer *et al*. (2007) for *Strongylocentrotus purpuratus* also noted an exponential decrease in lipid content during embryogenesis to fuel metabolism. However, some exceptions occurred when wild diet was taken into consideration: prisms from wild diet had higher EPA, while plutei from wild diet had higher C20:1n-9 percentages when compared with other developmental stages. No certain explanation was found for these results. Firstly, there is no precise information about the wild diet. Secondly, each egg and larva wild samples were obtained from different wild males and females. Therefore, untested factors like the precise fatty acid composition of the food ingested by the wild broodstock could be one of the reasons for this variation. In addition, the lowest percentage for total lipid and FAME was found in plutei from wild diet, resulting in a pronounced percentage increase variation among the more and less abundant fatty acids.

Unusual marine fatty acids like NMID were also found in the present work on *P. lividus* eggs and endotrophic larvae from all the tested diets analysed, being C20:2Δ5, 11 NMID the most abundant. The same fatty acids were previously found by Takagi *et al*. (1980), Cook *et al*. (2000) and Castell *et al*. (2004) for sea urchin gonads, and by Schiopu *et al*. (2006) and Liu *et al*. (2007) for sea urchin exotrophic larvae. As these fatty acids were absent from any of captive broodstock diets, we also concur that *P. lividus* is capable of *de novo* synthesis of these NMID fatty acids and their transfer to the eggs.

EFA in eggs and endotrophic larvae derived from raw and wild diets

Given the high growth rates of marine fish larvae, it is essential for efficient production that their nutritional requirements be fully met, both qualitatively and quantitatively (Sargent *et al*. 1997). The content of EFA in live feeds has long been considered as a major factor in determining their dietary value (Watanabe *et al*. 1983). Several studies with marine fish larvae (Koven *et al*. 1990; Watanabe 1993; Reitan *et al*. 1994; Rodríguez *et al*. 1997) have demonstrated

the importance of DHA and EPA as essential for fish larval survival and growth. Regarding these two fatty acids, the tested raw and wild diets results showed EPA percentages lower than 5% (but significantly higher in eggs, prisms and plutei from the wild broodstock), whereas DHA, when present, was always detected in trace amounts.

As ARA, like EPA, has an essential function in producing eicosanoids, it has been more recently considered an additional EFA for fish larval nutrition (Sargent *et al*. 1997, 1999; Bessonart *et al*. 1999; Estévez *et al*. 1999). Considering raw and wild diets, *P. lividus* eggs showed ARA percentages around 5% that increased in the prism and pluteus stages reaching 18.7% in plutei from maize diet. Therefore, it seems that no deficiencies are found in this EFA in *P. lividus* eggs and endotrophic larvae.

The rotifer *Brachionus plicatilis* fed on baker's yeast and unenriched *Artemia* spp nauplii (newly hatched) have reduced amounts of the three EFA. Rodríguez *et al*. (1997) found for rotifers fed on baker's yeast, less than 0.05%, 2.15% and 1.08% for ARA, EPA and DHA respectively, while Estévez *et al*. (1999) found 1.2%, 5.3% and 0.0% for the same fatty acids in unenriched *Artemia* spp nauplii. Comparing the tested raw and wild diets results with the EFA profile of these two live preys, *P. lividus* eggs and endotrophic larvae had higher amounts of ARA. Depending on the diet and developmental stage considered, EPA content may be lower, similar or higher than rotifers fed on baker's yeast and unenriched *Artemia* spp nauplii. Likewise, low DHA percentages were also found in *P. lividus* eggs and endotrophic larvae.

Because of competitive interactions in fish larval metabolism, Sargent *et al*. (1999) suggested that DHA, EPA and ARA cannot be considered in isolation, rather it is necessary to consider larvae requirements in DHA:EPA:ARA ratios. In this context, the tested raw diets results showed reduced DHA/EPA ratios like *Brachionus plicatilis* fed on baker's yeast and unenriched *Artemia* spp nauplii (Bell *et al*. 2003), due mainly to absence or trace amounts of DHA. The EPA/ARA ratio in eggs from seaweed and wild diets and in prisms from wild broodstock was about 1.0 which was considered as an optimal dietary ratio for sea bass larvae, but as insufficient for turbot and halibut larvae that need 10:1 or greater (Sargent *et al*. 1999).

EFA in eggs and endotrophic larvae derived from prepared diets

Regarding captive broodstock fed on raw diets as well as wild broodstock, it seems, therefore, quite obvious that *P. lividus* eggs and endotrophic larvae, like unenriched rotifer or *Artemia* spp nauplii, are deficient in EFA, particularly DHA, in order to be selected as a marine fish larval feeding. However, these eggs and larvae can also be enriched in EFA through prepared broodstock diets, or like rotifers and *Artemia* spp. nauplii, by using microalgae or commercial emulsions during the exotrophic phase. The first issue was positively tested in the present study as demonstrated by increased EFA contents in eggs and endotrophic larvae from prepared broodstock diets.

Sea urchin parental nutritional condition seems to have a reduced effect on larval growth parameters (Bertram & Strathmann 1988; Meidel *et al*. 1999), but as shown by the present study, lipid composition of the broodstock diet is strongly reflected on eggs and larvae lipid composition.

Palmtag *et al*. (2006) compared EFA composition of the standard live feeds for larviculture, *Brachionus plicatilis* and *Artemia* spp nauplii, reared under seven different enrichments. Considering only the prepared diets results of this study, ARA content was higher on *P. lividus* eggs and endotrophic larvae when compared with any of the determinations of Palmtag *et al*. (2006). Eggs, prisms and plutei from prepared diets (except olive oil) show always higher EPA percentages than *Artemia* spp nauplii. Comparing with rotifers, depending on the enrichment used by Palmtag *et al*. (2006), *P. lividus* eggs and endotrophic larvae EPA percentages were higher than one, similar to three, or lower than three of the enrichments. DHA enhancement on eggs, prisms and plutei from Algamac and cod liver oil diets showed higher percentages than *Artemia* spp nauplii obtained from four of the enrichments. Comparing with rotifers, eggs, prisms and plutei from Algamac diet had also higher DHA percentages than four of the enrichments. According to Palmtag *et al*. (2006), Algamac was the enrichment that led to superior DHA percentages on rotifers (21.8%) and was the second best for *Artemia* spp nauplii (8.9%). Our results also showed higher DHA percentages in eggs, prisms and plutei from broodstock fed on the prepared feed based on this enrichment media. On the contrary, EPA/ARA ratios from both enriched rotifers and *Artemia* spp are always higher than all *P. lividus* eggs and endotrophic larvae analysed, mainly because of their higher ARA percentages. Regarding DHA/EPA ratio, except on eggs from Algamac diet, values were always lower than 1, like in four of the treatments used by Palmtag *et al*. (2006) for rotifers and *Artemia* spp nauplii.

Liu *et al*. (2007) for *P. lividus* exotrophic larvae (21 days), fed with microencapsulated formulated feeds (larvae length > 500 μm), found for ARA, EPA and DHA percentages around 17%, 11% and 7%, respectively. Direct

comparison with these exotrophic larvae show values only slightly higher to the mean percentages found in eggs and endotrophic larvae from captive *P. lividus* fed on prepared diets. Depending on the microalgae used in the diet, Schiopu *et al.* (2006) determined for 4, 6 and 8-arm *Dendraster excentricus* larvae ARA, EPA and DHA percentages ranging from 0.72–3.87, 2.86–11.5 and 1.45–6.89, respectively. *P. lividus* eggs and endotrophic larvae ARA and EPA percentages for raw, prepared and wild diets fit in these intervals, while for DHA, occurs the same situation but only when results from prepared diets are regarded.

Copepods are considered as live preys with better EFA profile than both enriched rotifer and *Artemia* spp (McEvoy *et al.* 1998; Nanton & Castell 1999; Sargent *et al.* 1999; Shields *et al.* 1999; Evjemo *et al.* 2003), but its use is still limited in marine fish larvae culture mainly due to problems with providing sufficient quantities, as well as increased disease risk from extensive culture methods (Støttrup 2000).

Conclusion

In conclusion, given that *P. lividus* broodstock raw diets led to eggs and endotrophic larvae with fatty acid poor nutritional value (mainly in DHA), and wild broodstock just spawn during the summer (Gago *et al.* 2003), prepared diets must be considered in order to obtain enhanced nutritional quality live preys year-round. From the results obtained in this study, it seems clear that the best EFA composition is achieved with the Algamac prepared diet. Because EFA composition of endotrophic larvae from cod liver oil diet did not significantly differ from the Algamac diet, the former enrichment oil should also be elected as a lower cost alternative for enhanced broodstock prepared diets.

The full implications of these findings for marine fish production await evaluation of survival and growth performance outcomes of fish larvae reared on these improved live feeds. This question will also assess, for selected fish larval species, the differential acceptability between *P. lividus* eggs and endotrophic larvae. This issue is currently being investigated by our research team. Others subjects like free amino acid profile of *P. lividus* eggs and endotrophic larvae are also being currently investigated in order to better ascertain the nutritional value of this proposed potential live prey for marine fish larvae.

Acknowledgements

The authors would like to thank Ana Pêgo and Filipa Faleiro for technical support, and also would like to thank two anonymous referees for their helpful comments on the manuscript. This research was financed by FCT through IMAREDIS project.

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CHAPTER 3.2.

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

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Submitted to *Journal of Shellfish Research*

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

Abstract

Eggs, prisms and pre-plutei total and soluble protein content and eggs amino acid (AA) composition of *Paracentrotus lividus* were determined according to different broodstock prepared diets used in adult rearing and compared with the one obtained from wild broodstock. Prepared diets differed on protein source (fish meal or textured soy protein) and on protein content (10, 20, 30 and 40 percent of dry weight (DW) diets). No major differences for all the above parameters analysed were found between the resultant alternative live feeds, suggesting that AA composition can not be enhanced through broodstock diet manipulation. Total and soluble protein determined for *P. lividus* eggs and larvae were always higher than 400 g Kg^{-1} and 200 g Kg^{-1} respectively. Indispensable AA (IAA) percentage of the protein-bound fraction (PAA) was approximately 50%. Eggs free AA (FAA) weight ranged from 59.5 to 96.3 μg mg DW^{-1}. IAA percentage of FAA ranged from 10% to 20%. Glycine was the most abundant FAA with more than 75% of FAA concentration. Similarities were found between *P. lividus* eggs and endotrophic larvae AA nutritional quality and rotifer *Brachinonus* spp. and for this reason, dietary AA deficiencies for marine fish larvae first feeding are considered equivalent.

Keywords: *Paracentrotus lividus*, eggs, endotrophic larvae, broodstock diets, protein, protein bound amino acids, free amino acids, *Brachionus* spp., marine fish larval nutrition.

Introduction

Studies carried out both in natural environments and in laboratory indicate that sea urchin eggs and larvae are prey for marine larvae fish (McEdward & Miner 2001, Allen 2008). For this reason, these natural marine zooplankton species have already been suggested as a potential live feed for marine larviculture (Hubbard *et al.* 2003). For *Paracentrotus lividus,* Luis *et al.* (2005)

reported undemanding maintenance of broodstock, simple spawning induction, large spawnings and simple larval rearing as main criteria for considering their eggs and endotrophic larvae as potentially viable live preys. Another criterion is the size range because it goes from approximately 90 μm in eggs to approximately 370 μm in pluteus (72 h after hatching). Therefore in a three day period it can be obtained a live prey which can compete with the rotifer (123-292 μm, Snell & Carrillo 1994) for marine fish larvae first feeding. Considering nutritional quality, Gago *et al.* (2009) studied the *P. lividus* eggs and endotrophic larvae fatty acid profile and demonstrated that manipulating the lipid composition of the captive broodstock diet, the eggs and endotrophic larvae can be readily improved on essential fatty acids for marine fish larvae. However, no published data was available for protein composition and amino acid profile of these potential alternative live feeds.

Nutritional requirements of marine fish larvae must be fulfilled in larviculture both in terms of quantity and quality of the diets provided. In this context, fish larvae amino acid requirements and utilization (Rønnestad *et al.* 1999, 2003; Conceição *et al.* 2003; Aragão *et al.* 2004a; Saavedra *et al.* 2006; Kvåle *et al.* 2007), concomitantly with the protein composition of marine fish larvae feeds, particularly free and protein bound amino acids profiles (Helland *et al.* 2000, 2003; Aragão *et al.* 2004b; Srivastava *et al.* 2006), are becoming increasingly studied. This fact is related with the importance of these nutrients since they are the major components of fish larvae dry weight and function both as structural molecules and as the main sources of energy (Fyhn 1989; Rønnestad *et al.* 2003). Also due to rapid growth of marine fish larvae (Kamler 1992; Conceição *et al.* 1997; Otterlei *et al.* 1999) the demand for dietary amino acids for protein accretion and catabolism is especially high. Therefore, the supply of all amino acids, both indispensable and dispensable, may become critical for sustaining fish larvae optimal survival and growth (van der Meeren *et al.* 2008).

Although recent advances have been achieved with inert artificial food (Cahu & Zambonino 2001; Curnow *et al.* 2006), the greatest successes in marine fish production so far have resulted from the use of live feeds as the initial form of nutrition after the fish larvae convert to exogenous feeding (Shields 2001; Støttrup & McEvoy 2003). The most commonly used live feeds in marine fish larvae feeding are the rotifer *Brachionus* spp. and the brine shrimp *Artemia* spp. The amino acid profiles of these live preys have been studied according to the enrichments, based on microalgae species or commercial products, used in order to improve their nutritional quality (Helland *et al.* 2000; Aragão *et al.* 2004b; Srivastava *et al.* 2006). *Artemia* spp. can not always be used as first feeding because its nauplius is too large for several marine fish larvae mouth opening, so rotifers are usually used as the sole live preys during the first days of feeding in small mouth fish. Due to good protein quality and amino acid composition (Helland *et al.* 2003;

Drillet *et al.* 2006; van der Meeren *et al.* 2008) the taxa Copepoda, particularly their naupliar stages, has also been suggested as an alternative live prey for marine fish larvae. Main limitations with the use of copepods are related with providing sufficient quantities, the disease risk from extensive culture methods, and the labour-intensive and costly intensive culture (Støttrup 2000; Helland *et al.* 2003).

Therefore this study aims to 1) evaluate the protein and amino acid nutritional quality of *Paracentrotus lividus* eggs and endotrophic larvae obtained from captive broodstock fed with artificial diets and compared with wild broodstock, 2) study improvement possibilities through the manipulation of the broodstock diets protein source and content 3) compare *P. lividus* eggs and endotrophic larvae nutritional value as live feed with the currently used live prey during first feeding in production hatcheries (*Brachionus* spp.) and 4) evaluate the potential protein and amino acid deficiencies of these alternative live preys by analysing the published values found for these nutrients in selected marine fish larvae.

Materials and methods

Sea urchin collection and rearing

Sea urchins (test diameters above 40 mm) were collected during full-moon low tides from pools on the central west coast of Portugal near Cascais (Lisbon) during March 2008. A previous study on the same population (Gago *et al.* 2003) established the annual variability of gonad index, spawning periods, and the influence of habitat characteristics on energy partition for reproduction and growth. After collection, sea urchins were immediately transported to the laboratory in water-filled containers. They were randomly allocated to 16 rectangular dark blue plastic containers (50 cm length x 30 cm width x 25 cm depth) placed in the surface of a seawater recirculating rearing system consisting of 8 cylindrical black fibreglass 402 L tanks (0.80 m base diameter x 0.80 m depth; 35 g L^{-1} salinity; 18±0.5 ºC temperature; 14L:10D photoperiod and 700 lux overhead illumination) as described by Luis *et al.* (2005). In each plastic container 25 individuals (approximately 166 sea urchins m^{-2}) were placed, and there were two containers in each cylindrical tank of the recirculating system. To maximize water circulation in the plastic containers, they were perforated with multiple 1 cm diameter holes.

Broodstock diets

A two week conditioning period was established during which sea urchins were unfed. Afterwards sea urchins were fed three times a week one of the 8 tested diets. Each diet was randomly allocated to two of the sixteen plastic containers (2 replicates for each diet). Food portions were distributed by hand. Feeding finished when all sea urchins had captured at least one food portion.

The diets differed in the percentage of protein (10%, 20%, 30% and 40%) and in the protein source ingredient (fish meal – FM, or textured soy protein – TSP). The carbohydrate source was wheat starch type I – unmodified S-5127 (Sigma-Aldrich, St. Louis, USA). The lipid percentage was 10% for all the diets and the main lipid source was cod liver oil. A vitamin mix ('S.N. PV 10/8', PREMIX – Especialidades Agrícolas e Pecuárias Lda, Viana do Castelo, Portugal) was incorporated in order to get 1% dry mass in all the diets. Alginic acid (sodium salt powder, Sigma-Aldrich, St. Louis, USA) and Sodium hexametaphosphate (Rhodia, Paris, France) were also used as binders in all diets and each one represented 2% of their dry mass. In order to obtain the desired protein percentage for each diet, matrix calculation was performed with Winmat software in order to obtain the dry mass of the ingredients (Table 1).

The ingredients were weighed and dry mixed. The textured soy protein was previously minced. After water addition, the moist feed was transferred to an extruder where it was pressed to form 5 mm diameter cylindrical rods. These rods were oven dried (48 hours at 35 ºC) and cut into pieces about 1cm long.

Table 1. Composition of the 8 broodsotck diets tested

	Protein Source							
	Fish meal (FM)				Textured soy protein (TSP)			
Ingredients (dry mass – g Kg^{-1})	10%	20%	30%	40%	10%	20%	30%	40%
Fish meal ('Narciso Dias', Peniche Portugal)	161	323	484	645	-	-	-	-
Textured soy ('Protisoja', Salutem, A. Centazzi, Lda, Lisboa, Portugal)	-	-	-	-	200	400	600	800
Wheat starch type I – unmodified S-5127 (Sigma-Aldrich, St. Louis, USA)	702	553	405	256	652	454	256	58
Cod liver oil ('José M. Vaz Pereira, S.A.', Lisboa, Portugal))	87	74	61	49	98	96	94	92

Spawning induction, egg collection and larvae rearing

After 5 months of captive period, all sea urchins were induced to spawn by injecting 1ml 0.5M KCl through the peristomial membrane, using a 0.9 mm (external diameter) x 50 mm (length) needle coupled to a 5 ml syringe. Each sea urchin was then placed for 30 min in an individual plastic beaker filled with 2 L of aerated filtered (1 μm mesh) sea water at 18 ± 0.5 ºC. Sperm or egg release was evaluated by light microscopy (x40) and quantitative egg counts were obtained with a Coulter Counter (Coulter Corporation, Miami, USA) model ZM with 140 μm cell aperture. Spermatozoa number was evaluated semi quantitatively according to the scale used by Luis *et al.* (2005). For egg collection the 2 L volume was filtered onto a 30 μm nylon mesh and then washed with seawater into a 30 ml centrifuge tube. After centrifugation (3.000 rpm, 1min) the salty supernatant was removed by tube inversion and the precipitate resuspended with 1 ml deionised water and transferred to 2 ml Eppendorf tubes. To obtain endotrophic larvae, 50 ml of sperm from the one apparent best individual emission (number and movement of spermatozoa) from the males fed the same diet, was used to fertilize the 2 L egg volume during 30 minutes. The fertilized eggs were then washed trough a 30 μm nylon mesh to get rid of excess spermatozoa, and placed into 2 L plastic beakers filled with aerated filtered sea water and reared with the same physical conditions of the captive broodstock. Beside eggs (~90 μm), two endotrophic stages were analysed: swimming prism (24h after fertilization: ~120 μm) and pre-pluteus (48h after fertilization: ~250 μm). At the end of each rearing period the larvae were filtered through 60 μm nylon mesh and collected like the eggs. The number of endotrophic larvae collected into each eppendorf tube was previously estimated by five 1ml counts of the 2L rearing volume. There were also obtained egg and larval samples from wild sea urchins collected in low tide pools at the above referred coastal waters on July 2008, using the same procedure as referred above. All eggs and larvae samples were immediately freeze-dried and stored under nitrogen at -20 ºC for subsequent analysis.

The number of eggs, prisms and pre-plutei samples collected into the eppendorf tubes ranged from 1 x 10^6 to 9 x 10^6 eggs; 5 x 10^5 to 5 x 10^6 prisms; and 4 x 10^5 to 3 x 10^6 pre-plutei. Two egg, prism and pre-pluteus samples from each diet replicate (plastic container) were used for total protein determination and protein-bound and free amino acid analysis, while for soluble protein, three samples were used.

Protein and amino acid analysis

Total protein was determined using LECO instrument Model FP-528 nitrogen analyzer (St. Joseph, Miami, USA). This instrument uses the Dumas method which consists of (a) converting all the forms of N into gaseous nitrogen oxides (NO_x) by complete combustion in an induction furnace, (b) reducing the NO_x gases to N_2 and (c) quantifying N_2 by thermal conductivity (Sweeney & Rexroad 1987; Jones 1991). Total protein was estimated by the usual N x 6.25 multiplication factor.

For soluble protein determination and amino acid (AA) analysis, sub-samples (2-10 mg) of the freeze-dried samples were extracted in Eppendorf tubes in 1 ml 6% tri-chloro-acetic acid (TCA) under rotation (VWR, Pennsylvania, USA, model VV 3) for 24 h at 4ºC. After centrifugation (15,000xg, 10 min, 4ºC) the supernatant was used for free amino acid (FAA) analysis. The precipitate was dissolved in 1 ml of 0.5M NaOH by rotation for 48h at room temperature. An aliquot (25 µl) of this solution were used for total soluble protein determination with "Bio-Rad RC DC Protein Assay Kit II" (Bio-Rad Laboratories, California, USA) based on Lowry *et al.* (1951) with bovine serum albumin in 0.5M NaOH as standard and 0.5M NaOH as blank. The sample absorbance was read on a Pye Unicam SP6, Model 550 spectrophotometer (Pye Unicam Ltd, Cambridge, England) at 750 nm. Another aliquot (20µl) of this solution was used for protein-bound amino acids (PAA) determination. This analysis was performed in an Alliance reverse-phase HPLC System – Waters (Waters Corporation, Massachussets, USA) at 254 nm after pre-column derivatization with phenylisothiocyanate (PITC). Hydrolysis and pre-column derivatization of samples were performed in a Waters Pico-Tag Workstation (Waters Corporation, Massachussets, USA), which has vacuum and nitrogen connections for drying and sealing the samples, as well as a thermostatically controlled oven. Four replicates of each sample were weighed in glass tubes containing Norleucine as internal standard. For cysteine quantification, 2 tubes were subject to performic acid oxidation before acid hydrolysis (vacuum, HCl 6N, 1% phenol, 24h at 110ºC). For the other amino acids there was no need for the previous performic acid oxidation. During hydrolysis with HCl asparagine and aspartic acid are converted into a single species (ASX), as well as glutamine and glutamic acid which are converted to GLX. Since tryptophan is completely destroyed during hydrolysis it was not determined in PAA analysis. The same procedure was used for FAA determination but without previous acid hydrolysis. PAA and FAA analysis were just done for *P. lividus* eggs since it was considered that if differences occur, along egg and larval development, between the several broodstock diets tested, they will appear right in the egg stage. Additionally, given that soluble protein values obtained in *P. lividus* eggs derived from the different broodstock diets studied did not showed significant differences among

them (see below), it was just chosen 3 diet treatments (Wild, FM 30% and TSP 30%) for *P. lividus* eggs PAA qualitative evaluation.

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 $P<0.05$. Arcsine transformations were used to normalise total and soluble protein percentage data prior to statistical analyses. Since no differences were found between diet replicates (two plastic containers for each diet) for all the variables studied (spawning, total protein, soluble protein, PAA and FAA), the results presented subsequently are compared according to the *P. lividus* broodstock diets analysed.

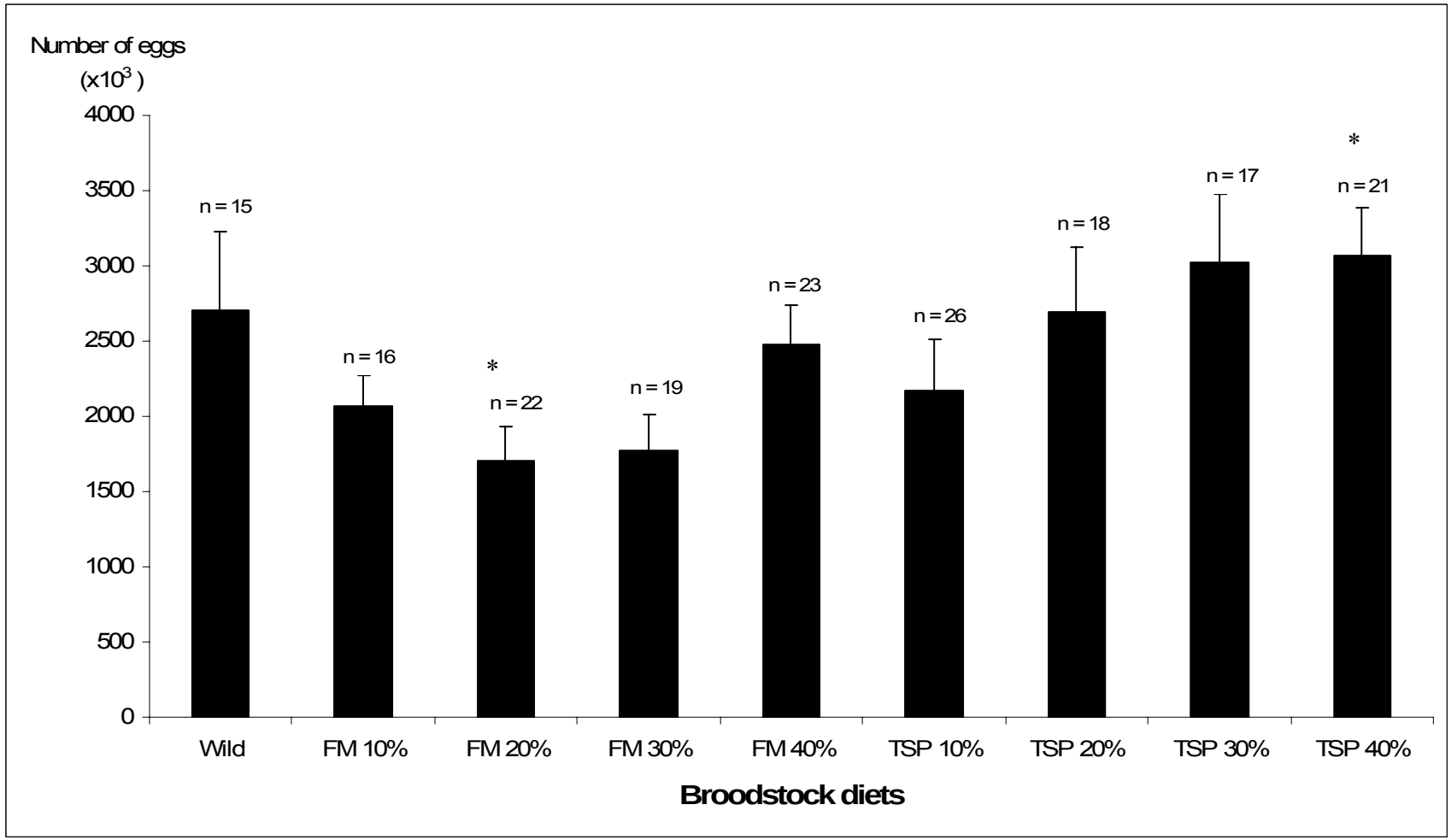
Results

Spawnings

The average number of emitted eggs of the females that were fed the different diets is represented in figure 1. Irrespective of diet (either considering protein source or protein percentage) all mean spawnings were higher than $1{,}500 \times 10^3$. The highest mean female spawning was obtained when they were fed the TSP 40% diet and the lowest when they were fed FM 20% diet. These two diets were the only ones to present significant differences on the number of emitted eggs (Kruskal-Wallis, $P<0.05$).

Regarding males, 82% of all the spawnings were classified in the highest class of the semi-quantitative scale (more than 200 million spermatozoa), while the rest were classified in the preceding class (up to 200 million spermatozoa).

Figure 1. Quantitative female sea urchin spawnings (mean + standard error of the mean) according to broodstock diet.
FM – fish meal
TSP – textured soy protein



* Only between FM 20% and TSP 40% broodstock diet treatments were found significant differences (Kruskal-Wallis, $P<0.05$) on the number of emitted eggs.

Total protein

Total protein values of eggs, prisms and pre-plutei obtained from sea urchins that were fed with the different tested diets are presented in Figure 2. As shown for the majority of the diets, there is a general trend for a slight decrease of total protein percentage along larval development. Exceptions were observed for TSP 10%, FM 20% and FM 30% where pre-plutei values were higher than the prisms, but significant differences between these two stages were only found for FM 30% (one way ANOVA, $P<0.01$). Overall, eggs presented a significant higher (one-way ANOVA and Kruskal-Wallis, $P<0.01$) total protein mean value than prims and pre-plutei when analysing either all diets together or each diet separately (except for wild diet). Total protein mean percentage for eggs obtained from all the broodstock diets studied is always higher than 530 g Kg^{-1} DW and reach a maximum of 593 g Kg^{-1} DW for TSP 40% diet. For prism and pre-plutei stage the lowest values in total protein mean values (409 and 441 g Kg^{-1} DW) were obtained from broodstock fed FM 30% and FM 10% diets respectively, while the highest values (543 and 507 g Kg^{-1} DW) derived from Wild and TSP 30% diets respectively. Analysing the *P. lividus* developmental stages independently it was not found significant differences (Kruskal-Wallis, $P>0.05$) between diets for eggs, and for pre-plutei larvae just TSP 30% and FM 10% diets presented significant differences (Kruskal-Wallis, $P<0.01$). Only for prism stage there was a considerable variation in total protein content between diets. No significant differences (one-way ANOVA, $P>0.05$) were only found among FM 10% – TSP 20% – TSP 30% – TSP 40%; TSP 10% – FM 20% – FM 40%; and Wild – TSP 40% diet groups.

Soluble protein

Soluble protein values of eggs, prisms and pre-plutei obtained from sea urchins fed with the different tested diets are presented in Figure 3. Unlike total protein no trend for decrease along larval development was clear, however when comparing all diets together eggs presented significant higher mean soluble protein percentage than prisms and prisms presented significant higher mean percentages than pre-plutei (Kruskal-Wallis, $P<0.01$). Considering each diet separately only eggs obtained from broodstock fed TSP 20% and TSP 40% diets had significant higher mean values (261 and 282 g Kg^{-1} DW, respectively) than pre-plutei (one-way ANOVA, $P<0.05$). For all the other diets no significant differences in mean soluble protein percentage were found among *P. lividus* developmental stages (one-way ANOVA and Kruskal-Wallis, $P>0.05$). Highest mean value was found for eggs obtained from wild broodstock (316 g Kg^{-1} DW), while pre-plutei obtained from TSP 40% diet presented the lowest mean value (228 g Kg^{-1} DW).

Regarding each development stage independently there were not found significant differences (one-way ANOVA and Kruskal-Wallis, P>0.05) among broodstock diets.

Soluble protein fraction of total protein in eggs represents 46.4% (using FM 30% diet) to 57.8% (wild diet) while in prisms and pre-plutei this fraction ranged from 46.3% (wild diet) to 69.0% (FM 30% diet) and 46.9% (TSP 40% diet) to 61.9% (FM 30% diet) of total protein, respectively.

Protein-bound amino acids

The content of protein-bound amino acids (PAA) in *P. lividus* eggs obtained from Wild, FM 30% and TSP 30% broodstock diet treatments is shown in Table 2. For all the parameters analysed, non significant differences (one-way ANOVA, P>0.05) were found among broodstock diet treatments. The sum of all PAA determined in weight (∑PAAw) represents approximately 27% of the eggs dry weight (DW). The indispensable amino acid (IAA) fraction, both considering it in weight and concentration (IAAw and IAAc), represents approximately 50%, for all the diets analysed. Hystidine (HYS) and Methionine (MET) are the less abundant IAA comparing with the more abundant IAA Leucine (LEU), Valine (VAL) and Isoleucine (ILE). *P. lividus* eggs dispensable amino acids (DAA) determination showed GLX (Glutamic acid + Glutamine) and ASX (Aspartic acid + Asparagine) as the more abundant DAA, and Tyrosine (TYR) as the less abundant DAA.

Free amino acids

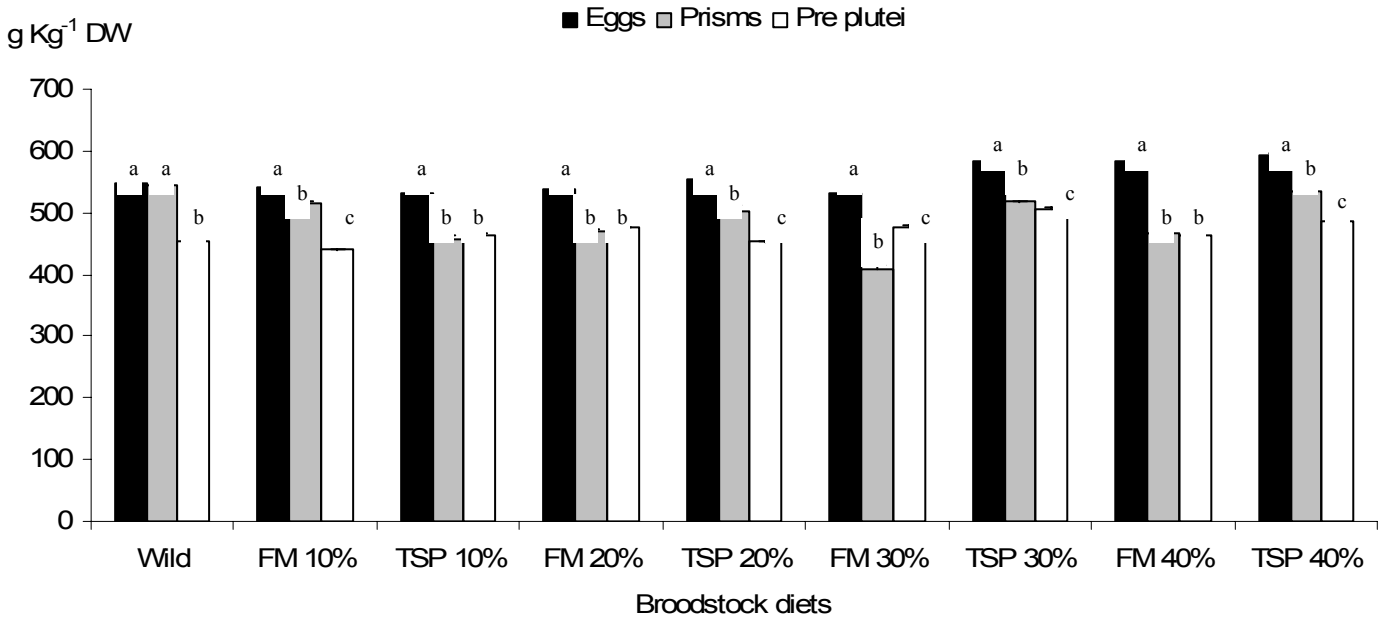
In Table 3 is represented the content of free amino acids (FAA) determined in *P. lividus* eggs obtained from all the broodstock diet treatments analysed. Non significant differences (one-way ANOVA and Kruskal-Wallis, P>0.05) were found among broodstock diet treatments for all the variables analysed. Considering FAA weight percentage it ranged from 5.95% (in TSP 30% diet) to 9.63% (in FM 40% diet) of eggs DW. The IAA percentage of FAA weight ranged from 10.3% to 20.2% in eggs obtained from *P. lividus* fed the FM 20% and FM 10% diets respectively, while when considering IAA percentage in concentration the highest value was also found in eggs derived from FM 10% broodstock diet (11.6%), but the lowest values was found in eggs derived from TSP 40% broodstock diet (5.20%). Low concentrations were determined for all IAA (less than 30 nmol $mgDW^{-1}$), especially for Phenylalanine (PHE) and Tryptophan (TRP). Arginine (ARG) was the most abundant IAA found in *P. lividus* eggs FAA fraction. Small amounts were also observed for DAA, except for Glycine (GLY) that was by far the more abundant FAA with

concentrations superior to 597 nmol mgDW^{-1} and reaching a maximum of 928 nmol mg DW^{-1} in *P. lividus* eggs obtained from broodstock fed the FM 20% diet.

Figure 2. Total protein (g Kg^{-1} DW) of *Paracentrotus lividus* eggs, prisms and pre-plutei, according to broodstock diet. Values are the mean ± standard error of the mean from the analyses of 4 samples (2 samples from each replicate).

FM – fish meal

TSP – textured soy protein

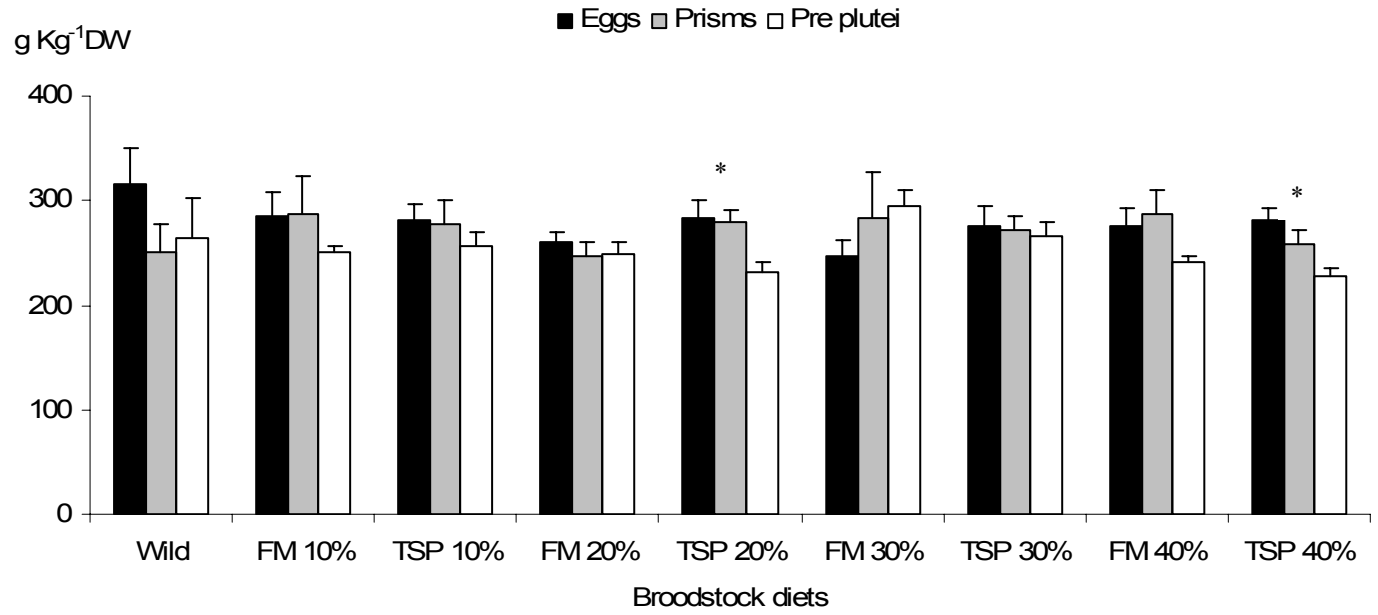


Different letters placed on the top of each bar represent significant differences (one-way ANOVA or Kruskal-Wallis, $P < 0.05$) on total protein content between *P.lividus* developmental stages within each broodstock diet treatment separately.

Figure 3. Soluble protein (g Kg^{-1} DW) of *Paracentrotus lividus* eggs, prisms and pre-plutei, according to broodstock diet. Values are the mean ± standard error of the mean from the analyses of 6 samples (3 samples from each replicate).

FM – fish meal

TSP – textured soy protein



* Only on TSP 20% and TSP 40% broodstock diet treatments significant differences (one-way ANOVA, $P<0.05$) were found between eggs and pre-plutei soluble protein content.

Table 2
Content of protein-bound amino acids (PAA) in *P. lividus* eggs obtained from Wild, FM 30% and TSP 30% broodstock diet treatments.
FM - fish meal; TSP - textured soy protein

P. lividus egss	Abbreviations	*P. lividus* broodstock diets		
		Wild	FM 30%	TSP 30%
PAA in weight[a] (µg mg DW^{-1})	∑PAAw	280 ± 24.9	286 ± 23.3	258 ± 17.3
Indispensable amino acids (µg mg DW^{-1})	∑IAAw	154 ± 13.2	154 ± 13.4	138 ± 9.46
Indispensable amino acids (%)	IAAw/PAAw	55.1 ± 0.58	53.6 ± 0.40	53.8 ± 0.18
Indispensable to dispensable ratio	IAAw/DAAw	1.23 ± 0.03	1.16 ± 0.02	1.16 ± 0.01
PAA concentration (nmol mg DW^{-1})	∑PAAc	2.17 ± 0.19	2.26 ± 0.17	1.96 ± 0.13
Indispensable amino acids (nmol mg DW^{-1})	∑IAAc	1.10 ± 0.09	1.12 ± 0.09	0.97 ± 0.06
Indispensable amino acids (%)	IAAc/PAAc	51.0 ± 0.60	49.8 ± 0.15	49.3 ± 0.38
Indispensable to dispensable ratio	IAAc/DAAc	1.04 ± 0.02	0.99 ± 0.01	0.97 ± 0.01
Indispensable amino acids (nmol mg DW^{-1})	IAA			
Leucine	LEU	219 ± 16.2	218 ± 17.3	187 ± 12.6
Valine	VAL	167 ± 11.9	172 ± 12.4	148 ± 10.2
Lysine	LYS	127 ± 16.9	146 ± 11.6	113 ± 10.9
Isoleucine	ILE	158 ± 9.8	154 ± 8.74	139 ± 9.69
Arginine	ARG	109 ± 11.4	118 ± 13.3	100 ± 6.71
Phenylalanine	PHE	117 ± 7.54	108 ± 7.14	99.4 ± 7.46
Threonine	THR	87.1 ± 9.69	86.9 ± 5.47	78.3 ± 7.84
Methionine	MET	67.5 ± 6.53	69.9 ± 6.03	62.0 ± 4.15
Histidine	HIS	48.4 ± 6.52	51.2 ± 6.44	41.8 ± 2.99
Dispensable amino acids (nmol mg DW^{-1})	DAA			
Glutamic acid + Glutamine	GLX (GLU + GLN)	214 ± 22.2	230 ± 16.9	203 ± 11.2
Aspartic acid + Asparagine	ASX (ASP + ASN)	195 ± 18.6	219 ± 11.3	190 ± 10.0
Alanine	ALA	159 ± 15.1	166 ± 14.0	138 ± 10.0
Glycine	GLY	173 ± 16.2	189 ± 16.5	167 ± 12.07
Serine	SER	113 ± 10.2	124 ± 11.6	110 ± 10.1
Proline	PRO	133 ± 13.5	122 ± 5.54	115 ± 9.02
Tyrosine	TYR	78.7 ± 7.45	82.1 ± 7.08	72.1 ± 5.26

Values are the mean ± standard error of the mean from the analyses of four samples (two samples from each replicate).
Non significant differences (one-way ANOVA, P>0.05) were found among *P. lividus* broodstock diet treatment for all the factors analysed.
Tryptophan was not determined since it is destroyed during acid hydrolisis.

[a] PAA in weight were calculated as the sum of all amino acids weigth.

Table 3

Content of free amino acids (FAA) in *P. lividus* eggs obtained from Wild and captive broodstock fed the different tested diets .

FM - fish meal; TSP - textured soy protein

P. lividus egss	Abbreviations	*P. lividus* broodstock diets								
		Wild	FM 10%	TSP 10%	FM 20%	TSP 20%	FM 30%	TSP 30%	FM 40%	TSP 40%
FAA in weight[a] (µg mg DW^{-1})	∑FAAw	74.5 ± 9.69	77.6 ± 13.9	63.6 ± 11.3	88.3 ± 9.23	75.3 ± 4.07	87.9 ± 7.38	59.5 ± 3.41	96.3 ± 7.17	84.3 ± 4.45
Indispensable amino acids (µg mg DW^{-1})	∑IAAw	13.5 ± 5.19	14.1 ± 1.99	8.78 ± 1.41	8.81 ± 1.67	10.2 ± 2.42	10.5 ± 1.56	6.94 ± 1.14	14.5 ± 4.65	9.18 ± 0.54
Indispensable amino acids (%)	IAAw/FAAw	17.0 ± 4.25	20.2 ± 5.11	14.8 ± 3.50	10.3 ± 2.00	13.5 ± 2.76	11.8 ± 1.15	11.6 ± 1.70	14.5 ± 3.90	10.9 ± 0.38
Indispensable to dispensable ratio	IAAw/DAAw	0.22 ± 0.07	0.27 ± 0.09	0.18 ± 0.05	0.12 ± 0.02	0.16 ± 0.04	0.13 ± 0.01	0.13 ± 0.02	0.18 ± 0.06	0.12 ± 0.00
FAA concentration (nmol mg DW^{-1})	∑FAAc	845 ± 98.2	886 ± 181	749 ± 144	1070 ± 123	871 ± 51.1	1049 ± 79.4	701 ± 39.0	1107 ± 79.0	997 ± 49.1
Indispensable amino acids (nmol mg DW^{-1})	∑IAAc	93.3 ± 36.7	87.5 ± 10.2	59.1 ± 13.6	56.3 ± 8.82	61.1 ± 15.5	66.4 ± 9.98	41.2 ± 7.74	86.5 ± 27.7	51.9 ± 3.32
Indispensable amino acids (%)	IAAc/FAAc	10.4 ± 3.13	11.6 ± 3.49	9.03 ± 3.39	5.49 ± 1.04	7.08 ± 1.73	6.24 ± 0.66	5.82 ± 0.98	7.64 ± 2.24	5.20 ± 0.19
Indispensable to dispensable ratio	IAAc/DAAc	0.12 ± 0.04	0.14 ± 0.05	0.10 ± 0.04	0.06 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.03	0.05 ± 0.00
Indispensable amino acids (nmol mg DW^{-1})	IAA									
Leucine	LEU	18.0 ± 9.12	13.4 ± 2.69	7.05 ± 1.53	9.04 ± 0.73	6.78 ± 2.21	9.20 ± 2.60	4.60 ± 1.52	9.59 ± 3.12	3.54 ± 0.42
Valine	VAL	17.3 ± 8.60	12.7 ± 3.06	6.08 ± 1.76	8.46 ± 0.58	5.50 ± 2.02	8.71 ± 1.78	3.98 ± 1.49	8.36 ± 2.93	2.85 ± 1.05
Lysine	LYS	6.83 ± 2.83	22.9 ± 7.18	4.39 ± 1.48	6.58 ± 5.17	15.1 ± 5.51	8.62 ± 3.95	8.10 ± 2.51	16.7 ± 9.91	12.4 ± 1.72
Isoleucine	ILE	12.0 ± 6.27	7.96 ± 1.68	3.44 ± 1.32	5.64 ± 0.37	3.30 ± 1.51	5.84 ± 1.34	1.95 ± 1.21	5.00 ± 2.23	1.55 ± 0.57
Arginine	ARG	14.5 ± 1.99	16.2 ± 10.3	19.0 ± 2.74	15.0 ± 4.49	18.1 ± 3.16	17.8 ± 2.71	13.0 ± 1.67	29.0 ± 7.39	21.2 ± 1.28
Phenylalanine	PHE	0.00 ± 0.00	0.33 ± 0.33	0.70 ± 0.32	0.00 ± 0.00	1.03 ± 0.41	0.64 ± 0.37	1.13 ± 0.08	1.94 ± 0.72	0.94 ± 0.40
Threonine	THR	18.4 ± 8.56	6.33 ± 0.51	7.74 ± 1.86	8.56 ± 1.72	8.00 ± 2.55	10.7 ± 1.86	5.69 ± 1.75	11.5 ± 2.22	6.61 ± 0.71
Methionine	MET	4.06 ± 2.48	3.18 ± 1.37	2.12 ± 0.71	1.89 ± 0.70	1.63 ± 0.96	2.86 ± 0.33	0.80 ± 0.80	2.80 ± 1.11	1.20 ± 0.40
Histidine	HIS	1.99 ± 1.50	1.74 ± 0.65	2.12 ± 0.46	1.10 ± 0.38	1.14 ± 0.39	1.32 ± 0.44	1.84 ± 0.12	1.29 ± 0.45	1.61 ± 0.61
Tryptophan	TRP	0.20 ± 0.20	2.77 ± 2.51	0.39 ± 0.22	0.00 ± 0.00	0.46 ± 0.27	0.62 ± 0.62	0.18 ± 0.18	0.36 ± 0.21	0.00 ± 0.00

Table 3 (Cont.)

P. lividus egss	Abbreviations	*P. lividus* broodstock diets								
		Wild	FM 10%	TSP 10%	FM 20%	TSP 20%	FM 30%	TSP 30%	FM 40%	TSP 40%
Dispensable amino acids (nmol mg DW^{-1})	DAA									
Glycine	GLY	646 ± 64.3	712 ± 174	615 ± 147	928 ± 130	700 ± 62.1	891 ± 53.1	598 ± 30.1	886 ± 80.0	839 ± 37.4
Taurine	TAU	2.62 ± 1.27	1.86 ± 0.29	1.55 ± 0.63	2.57 ± 0.59	2.91 ± 0.41	1.99 ± 0.11	2.03 ± 0.36	2.67 ± 0.77	2.93 ± 0.26
Alanine	ALA	39.2 ± 11.8	25.8 ± 8.34	22.9 ± 4.11	29.1 ± 4.81	25.7 ± 8.00	26.3 ± 6.49	14.4 ± 2.40	43.1 ± 4.62	25.7 ± 2.87
Glutamine	GLN	10.7 ± 3.60	10.6 ± 3.61	11.7 ± 1.20	15.3 ± 3.19	14.5 ± 6.42	17.3 ± 6.31	11.8 ± 4.55	24.1 ± 4.40	12.2 ± 4.09
Proline	PRO	11.8 ± 6.52	7.99 ± 1.70	3.70 ± 1.45	6.12 ± 0.44	3.13 ± 1.43	6.13 ± 0.82	2.31 ± 0.88	5.92 ± 2.26	1.66 ± 0.67
Asparagine	ASN	12.1 ± 4.53	11.2 ± 4.22	7.00 ± 1.46	8.66 ± 3.30	12.3 ± 3.60	7.67 ± 1.83	6.11 ± 0.64	12.4 ± 3.72	10.7 ± 1.05
Serine	SER	10.6 ± 4.32	10.3 ± 5.57	19.4 ± 2.36	17.5 ± 10.2	42.3 ± 10.6	22.6 ± 6.70	15.7 ± 1.89	35.2 ± 11.3	44.1 ± 7.70
Gamma-amino butyric acid	GABA	5.62 ± 2.44	5.17 ± 0.95	3.33 ± 0.52	3.82 ± 0.32	4.02 ± 1.13	3.95 ± 0.55	2.50 ± 0.39	4.46 ± 0.68	3.12 ± 0.39
Tyrosine	TYR	1.07 ± 0.71	1.80 ± 1.17	2.36 ± 0.85	2.06 ± 1.19	3.24 ± 0.99	2.84 ± 1.02	2.46 ± 0.56	4.25 ± 1.23	1.90 ± 0.64
Cysteine	CYS	4.01 ± 1.90	1.35 ± 1.35	1.39 ± 0.80	0.57 ± 0.57	0.65 ± 0.65	0.00 ± 0.00	2.81 ± 0.26	0.71 ± 0.71	3.08 ± 2.45
Ornithine	ORN	10.2 ± 6.28	10.3 ± 4.55	1.59 ± 0.59	0.79 ± 0.79	1.02 ± 0.38	2.42 ± 1.65	2.00 ± 1.35	1.41 ± 0.18	0.43 ± 0.25

Values are the mean ± standard error of the mean from the analyses of four samples (two samples from each replicate).

Non significant differences (one-way ANOVA and Kruskal-Wallis, P>0.05) were found among *P. lividus* broodstock diet treatment for all the factors analysed.

Due to high interferences in the cromathogram area, Glutamic acid and Aspartic acid were not detected and quantified in FAA composition.

[a] FAA in weight were calculated as the sum of all amino acids weigth.

Discussion

Spawnings

Because wild *Paracentrotus lividus* just spawn during spring and summer at Cascais coastal waters (Gago *et al.* 2003), broodstock must be kept in captivity in order to get all year-round spawnings (Luis *et al.* 2005), and few months of captivity are sufficient for broodstock maturation (Fernandez *et al.* 1996). In these conditions, artificial diets are presently generally used due to their durability, year-round availability, biochemical formulation and independency of natural food collection (George *et al.* 2000).

Fish meal and textured soy protein are ingredients that can be used in order to obtain high protein concentrations for animal and vegetable base artificial diets respectively. In comparison to fish meal, soy bean has lower protein content, therefore in this study the maximum protein percentage used in the diets was limited to 40% that is approximately the maximum possible value using soy bean as protein source. The different protein concentrations used for the artificial diets tested tried to evaluate the possible gains in eggs and endotrophic larvae protein nutritional value without impairing the spawning performance which seems to be essentially related with energy content of the diet (Luis *et al.* 2005; Schlosser *et al.* 2005). Considering spawning performance, all the tested diets proved to be adequate to either promote *P.lividus* gametogenesis and to release high number of gametes when both males and females were induced to spawn. Therefore it seems that spawning performance is not related with dietary protein concentration, at least for the two protein sources used and for the dietary protein percentage range tested. In spite of Luis *et al.* (2005) obtained greater spawnings in *P. lividus* fed maize than fed seaweed *Laminaria ochroleuca*, the dietary protein concentrations are not so different (84 and 69 g Kg^{-1} DW, respectively), and dissimilarities in the other nutrients of the diets may have played a more important role. However, when considering other biological parameters than spawning better performances are achieved when *P. lividus* are fed artificial diets with higher protein content based on fish meal or soy bean: Fernandez (1997) concluded that the use of artificial feed containing fish meal favours storage of reserves in *P. lividus* gonad, gut and even in the test in the form of lipids and/or carbohydrates; Fernandez & Pergent (1998) obtained higher growth for captive *P. lividus* when fed formulated feed with fish meal than when fed plant meal or natural feed; Fernandez & Boudoresque (2000) obtained higher absorption value, assimilation efficiency, digestibility and growth in *P. lividus* fed a fish meal based diet when compared with lower protein content diets; Spirlet *et al.* (2001) and Schlosser *et al.* (2005) reported *P. lividus* enhanced gonadal growth for mixed fish meal and soy bean artificial diet. Therefore it is considered that captive *P. lividus*

spawning is not influenced by dietary protein content in artificial diets. Another possible explanation for the inexistence of significant differences, in spawnings among diets found in this study (except between FM 20% and TSP 40%), might have be the non limiting quantity of the food ingested, since sea urchins were fed quite often and always captured at least one food portion.

Total and soluble protein

Total protein results obtained in present study for *P. lividus* eggs ranged from 593 g Kg^{-1} DW (eggs obtained from TSP 40% diet) to 531 g Kg^{-1} DW (eggs obtained from TSP 10% diet). These values are lower than the ones obtained by Fernandez (1997) for *P. lividus* gonads (approximately 720 g Kg^{-1} DW for *P. lividus* captured in August), which might indicate that other gonad tissues than oocytes presents higher protein content and/or the sea urchins analysed were at different stage of gametogenesis. The general tendency for protein content slight decrease along development is concurrent with the results obtained by Gago *et al.* (2009) for lipids. However, for lipids there is a sharp decrease considered to fuel development, whereas proteins may play a more structural role. Similar results were found for the tropical echinoid *Tripneustes gratilla* (Byrne *et al.* 2008). In spite of some significant differences were found among diet treatments (especially for the prism stage), it seems that *P. lividus* eggs and endotrophic larvae total protein content do not considerable varied according to either the protein source and the protein content of the broodstock diet. This fact might underline that protein inputs in the oocyte during gametogenesis is more genetically determined than dependent on environmental factors.
When considering early fish larvae nutrition, soluble protein might be a more reliable indicator of nutritional quality than total protein, since it has been showed that soluble proteins are more available for fish larval digestion and absorption than are insoluble proteins (Carvalho *et al.*, 2004, Tonhein *et al.* 2007).

Likely to total protein, soluble protein values found for *P. lividus* eggs (ranging from 247 to 316 g Kg^{-1} DW) are lower than the ones found for gonads (approximately 430 g Kg^{-1} DW; Fernandez 1997), which is also suggestive of higher soluble protein content of other gonad tissues than oocytes and/or the sea urchins analysed were at different stage of gametogenesis. The decrease of soluble protein along development was not so evident but it might occur since statistical differences were found between developmental stages when considering all tested diets. Even more evidently than insoluble protein, was the null effect found for protein source and protein content of the broodstock diet on the soluble protein content of *P. lividus* eggs, prisms and pre-plutei. These results also emphasize the genetic control of individual protein content.

The protein content in the common used live prey during first feeding of marine larvae fish (*Brachionus* spp.) has been reported in several studies (Lubzens *et al.* 1989; Lie *et al.* 1997; Øie *et al.* 1997; Aragão *et al.* 2004b; Srivastava *et al.* 2006) but the determined values present a wide variation. Srivastava *et al.* (2006) reported that this great variation in reported levels is more likely the reflection of the different methodologies used rather than true variations in *Brachionus* spp. protein content. However, comparisons can be made with studies that used identical analytical procedures with the ones used in this study. Lie *et al.* (1997), based on nitrogen content, reported 570 g Kg^{-1} DW as the total protein content for *Brachionus plicatilis*, which is a value only slightly higher than the ones found in the present study for *P. lividus* prisms and pre-plutei, but similar to the ones found for eggs. For *Brachionus rotundiformis*, Aragão *et al.* (2004b) based on Lowry method, found amounts of soluble protein ranging from 280 to 371 g Kg^{-1} DW according to the rotifer's diet treatment, which are values slightly superior to the ones obtained for *P. lividus* eggs, prisms and pre-plutei. When evaluating both protein fractions Shrivastava *et al.* (2006) reported that soluble protein constituted 50.6% of total *B. plicatilis* protein content. This value is in the range found for *P. lividus* eggs and endotrophic larvae. Considering marine fish eggs, their total AA content is in range of 40-60% (Ketola 1982, Fyhn 1989, Rønnestad & Fyhn 1993, Rønnestad *et al.* 1999) and therefore it is comparable to total AA content found either in the traditional live feed used during first feeding (*Brachionus* spp.), either in the potential alternative live feeds (*P. lividus* eggs and endotrophic larvae).

Amino acids

The PAA and FAA composition determined in *P. lividus* eggs did not show statistical differences according to the broodstock diet given in adult rearing. Such result must highlight the genetic control of individual protein and AA composition. In fact, protein synthesis inside the cells is the result of DNA transcription and RNA translation which are molecules genetically inherited. Additionally, likely to every cell, the genetically determined integral proteins present at the plasma membrane of oogonia and oocytes, control both the active and passive transport of substances through the cell, which can not pass by diffusion. Therefore it seems evident in this study that *P. lividus* eggs and endotrophic larvae can not be enhanced in protein content and AA composition through manipulation of the broodstock diet. These results seem to differ from the ones obtained by Gago *et al.* (2009) for *P. lividus* eggs and endotrophic larvae lipid content and fatty acid composition. However, these molecules can pass through cell membrane by diffusion and therefore can be accumulated in cells during oogenesis. For *Brachionus* spp., the nutritional enhancement is done using different commercial enrichment media or microalgae species. In this

context, Aragão *et al.* (2004b) determined *Brachionus rotundiformis* PAA and FAA composition according to different enrichments and found that IAA percentage in the PAA fraction expressed in weight varied between 45% and 50%, which are values slightly lower than the ones obtained in this study for *P. lividus* eggs. Lower IAA percentages in *Brachionus plicatilis* were found by Srivastava *et al.* (2006) and van der Meeren *et al.* (2008) (47.5% and 48.4%, respectively), but with calculations based on Øie *et al.* (1997) data, 55.3% to 56.1% is the *B. plicatilis* IAA percentage range obtained. Considering PAA composition, the more and less abundant IAA and DAA found in *P. lividus* eggs are somehow the same found for *Brachionus* spp.: HIS and MET and TRP (when determined) were found to be the less abundant IAA (Øie *et al.* 1997, Aragão *et al.* 2004b, Srivastava *et al.* 2006, van der Meeren *et al.* 2008); while LEU, VAL, and ILE were considered the more abundant IAA (Aragão *et al.* 2004b, van der Meeren *et al.* 2008). Contrarily to *P. lividus* eggs ARG (Øie *et al.* 1997) and LYS (Srivastava *et al.* 2006) were also found to be ones of the most abundant IAA in *B. plicatilis*; GLX (GLU + GLN) and ASX (ASP +ASN) were also found to be the more abundant DAA and TYR the less abundant DAA in *B. plicatilis* by Srivastava *et al.* (2006) and van der Meeren *et al.* (2008); for Aragão *et al.* (2004b), CYS and PRO were also found to be the less abundant DAA for *B. rotundiformis*; and for Øie *et al.* (1997), GLY is also one of the less abundant DAA for *B. plicatilis*.

In spite of the major contribution to dietary AA profile came from PAA, FAA are vital nutrients for first feeding of the stomach less and low intestinal proteolytic and absorptive capacities marine fish larvae. FAA have considerably higher retention efficiencies and are faster and more absorbed than proteins (Rønnestad *et al.* 2000, Tonhein *et al.* 2005), and also function as substrate for energy production (Fyhn 1989). A high FAA content is typically found in marine invertebrates and other planktonic organisms (Yancey *et al.* 1982, Fyhn *et al.* 1993) which are natural preys of fish larvae, and in marine fish eggs (Rønnestad *et al.* 1999). In this context, the FAA weight found in *P.lividus* eggs range from 59.5 to 96.3 g Kg^{-1} of the egg's dry weight. A similar amount found for rotifers, in the literature analysed, was only determined by Aragão *et al.* (2004b) for *B. rotundiformis* fed *Tetraselmis chui* microalgae, while when fed other enrichments the FAA DW percentage was always lower than 4.2%. For *B. plicatilis*, 1.14% to 1.90%, 2.2% to 5% and 1.66%, are FAA DW determinations found by Øie *et al.* (1997), Srivastava *et al.* (2006) and van der Meeren *et al.* (2008), respectively. Regarding the IAA percentage of the FAA fraction, higher values were found for *B. plicatilis* than the ones found in this study for *P.lividus* eggs: Srivastava *et al.* (2006) reported 48.7% and van der Meeren *et al.* (2008) reported 34.7% and 30.6% for AA expressed in weight and concentration, respectively. Aragão *et al.* (2004b) also found higher concentrations of IAA for *B. rotundiformis* FAA fraction. Considering FAA composition GLY was found to be the most concentrated FAA in *P. lividus* eggs, which alone

correspond to more than 75% of FAA concentration. This situation was not found for *Brachionus* spp. in the literature analysed and only Aragão *et al.* (2004b), refer GLY as the second more abundant DAA in FAA fraction, but with much lower concentrations (less than 100 nmol $mgDW^{-1}$) than the ones found for *P. lividus* eggs. GLY is an AA implicated in purine synthesis and osmoregulation and in the free form has been shown to stimulate feeding behaviour in seabream larvae (Kolkovski *et al.* 1997) and in turbot larvae (Knutsen 1992). Similarities were found between *Brachionus* spp. and *P. lividus* eggs IAA composition of the FAA fraction. ARG was also the most abundant IAA found in rotifers, while MET and TRP were the less abundant IAA as well (Øie *et al.* 1997, Aragão *et al.* 2004b, Srivastava *et al.* 2006, van der Meeren *et al.* 2008). Considering the DAA of FAA fraction in *Brachionus* spp., ALA always appeared as one of the most abundant DAA (Øie *et al.* 1997, Aragão *et al.* 2004b, Srivastava *et al.* 2006, van der Meeren *et al.* 2008), but others DAA are also reported as abundant (*e.g.* GLU, ASP and ALA). Taurine (TAU) has been suggested as an essential nutrient for larval and juvenile marine fish (Conceição *et al.* 1997, Takeuchi *et al.* 2001), but very small amounts were found in *P. lividus* eggs and endotrophic larvae. Comparing with *Brachionus rotundiformis*, Aragão *et al.* (2004b), found higher TAU concentrations, particularly with *Tetraselmis chui* enrichment.

Ornithine (ORN) was present in the FAA fraction of *P .lividus* eggs, but in the literature revised, was not detected in *Brachionus* spp.. This AA is not incorporated into protein but is generally important as an intermediate in the reactions of the urea cycle and in ARG synthesis.

Considering marine fish larvae IAA requirements, major IAA deficiencies found for rotifers seems to be related with THR, LEU, ARG, MET, LYS and HIS (Conceição *et al.* 1997, 2003, Aragão *et al.* 2004a, Saavedra *et al.* 2006, Saavedra *et al.* 2007). Comparing the amounts of these IAA determined for *P. lividus* eggs and endotrophic larvae with the values reported in the above mentioned studies for *Brachionus* spp. it seems that, in spite of some minor differences, only for THR the weight percentage is lower. However this AA was considered the first limiting AA for sea bream larvae (Conceição *et al.* 2003) and when directly compare *P. lividus* eggs and endotrophic IAA profile with IAA profile of both *Diplodus sargus* (Saavedra *et al.* 2006) and *Diplodus puntazzo* larvae (Saavedra *et al.* 2007) all of these IAA are deficient in the studied alternative preys. Considering IAA profile of eggs and young larvae of Asian seabass *Lates calcarifer* (Dayal *et al.* 2003), potential deficiencies in *P. lividus* eggs and endotrophic larvae are minimized and are mainly found for THR.

Conclusion

It is concluded in this study that, in spite of being very hard to enhance the AA profile of *P. lividus* eggs and endotrophic larvae through manipulation of broodstock diet, resultant alternative live feeds are comparable to rotifer *Brachionus* spp. in what protein content and AA composition is concerned. Only the IAA percentage of the FAA fraction could possibly be not favourable to *P. lividus* eggs and endotrophic larvae, but the amount of FAA in these alternative live feeds appears to be higher than in *Brachionus* spp. However, like *Brachionus* spp., these alternative live feeds may present several AA nutritional imbalances for marine fish larvae first feeding and due to the more economic production of *Brachionus* spp. they do not bring any AA nutritional advantage for marine fish larvae rearing. In fact, *Brachionus* spp. use as live feed in production larviculture is more due to their ability to grow and reproduce in high density cultures rather than being nutritional superior to other live feed organisms (Srivastava *et al.* 2006). Therefore, since both rotifers and the studied alternative live feeds seem to be not nutritionally adequate for marine fish larvae feeding, it is important to continue to investigate the improvement of AA quality of feeds (live and artificial) and supplements given to commercial marine fish larvae species. Finally, to really determine the suitability of *P. lividus* eggs and endotrophic larvae for marine fish larvae first feeding and compare it to rotifers, it is necessary to evaluate the survival and growth performance outcomes of selected fish larvae reared on these potential alternative live feeds. This question is presently being addressed by our research team.

Acknowledgements

The authors would like to thank the Analytical Services Unit of ITQB-UNL (Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa) for amino acids analysis, to IPIMAR (Instituto Português de Investigação Marítima) for total protein analysis, and Ana Pêgo for technical support. This research was financed by FCT (Fundação para a Ciência e Tecnologia) through IMAREDIS project. The authors would also like to thank two anonymous referees for their helpful comments on the manuscript.

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CHAPTER 4

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

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Accepted for publication in *Aquaculture Research*

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

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-hour 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, however they cannot 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 starts the 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 & 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 seems 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 & Baca (2003), and the same utilization was preconized by Luis, Delgado & Gago (2005) and Gago, Repolho & 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 for fish larvae. But to fully determine the potential of these alternative live prey 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 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 prey. These two species were chosen because *S. aurata* continues to be a major produced species in southern Europe and *D. sargus* is considered to be a promising new species with high market price and demand (Pousão-Ferreira, Dores, Morais & Narciso 2001; Ozorio, Valente, Pousão-Ferreira & Oliva-Teles 2006). Additionally, in order to more precisely clarify general prey acceptability, 24h 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., *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

1. Paracentrotus lividus broodstock rearing

Sea urchin collection took place in September from 2007 to 2009 period, on the central west coast of Portugal near Cascais (Lisbon). After collection, sea urchins were transported to "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 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 took place.

For the first fish larvae experiments (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 which have cod liver oil as the main lipid source, as described by Gago *et al.* (2009). This prepared diet was chosen because resultant *P. lividus* eggs and endotrophic larvae are enriched with essential fatty acids (EFA) for marine fish larvae (Gago *et al.* 2009).

2. 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 1ml KCl 0.5M 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 2L egg volume during 30 minutes after which this volume was filtered using a 30 μm mesh in order to remove sperm excess. Besides eggs (~90 μm in diameter), two endotrophic stages were used: swimming prism (24h after fertilization: ~120 μm in diameter) and pre-pluteus (48h after fertilization: ~250 μm in length) that were reared in 7 L cylindro conical plastic beakers. The precise number of *P. lividus* eggs and endotrophic larvae given to feed fish larvae was estimated by 1 mL counts of the rearing volume, using a glass pipette observed through a binocular lens.

3. 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 *Diplodus sargus* and *Sparus aurata* larvae until 15 days after hatching (DAH). The second approach consisted in testing larvae's 24h ingestion rates for 5 different fish species. Thus, for both approaches, 4 different live feed items were used: *Brachionus* spp. (the standard live feed: lorica diameter of 167 µm ± 3.83 µm, mean ± s.e.m., 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.

3. 1. Rearing of Diplodus sargus and Sparus aurata larvae

After collected from the egg incubators, newly hatched larvae were transferred to 20L 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 ºC 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 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 2 times per day to the rearing tanks in order to obtain a total concentration of approximately 150 x 10^3 *N. oculata* cells mL^{-1} and 50 x 10^3 *I. galbana* cells mL^{-1}.

3.1.1. 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 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 ingested the alternative prey or these prey are nutritional poor. Therefore ingestion rates were evaluated (see point 3.2.) and other set of experiments were done 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 only fed one of the live prey that were tested independently. Since *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 Figure 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 only first fed with *Brachionus* spp. and then the potential live prey (*P. lividus* eggs, prisms and pre-plutei obtained from the broodstock fed the prepared diet) were presented since the 8th DAH.

For all the experiments, *Brachionus* spp. was used as larvae feed comparative treatment. *Brachionus* spp. were enriched for 24 h with DHA Protein Selco (INVE Aquaculture, Belgium), and for all food items each feeding was given at a 5 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.

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**	***P. lividus* broodstock 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 **4** - *P. lividus* pre-plutei **5** – Starvation	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		
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 Figure 1 (PP) **Feeding treatment 5**: 5 preys mL^{-1} (co-feeding 1:1), two times a day (CF) (total of 5.000 *Brachiounus* spp. and 5.000 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 8th DAH *D. sargus* larvae were fed *Brachionus* spp.

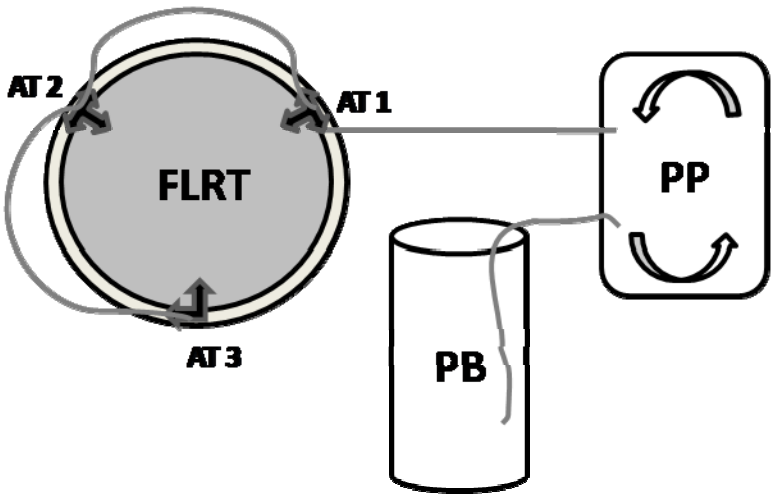


Figure 1. Schematic functioning of feeding treatment 4 used in Experiment 3: 200,000 *P. lividus* eggs were stocked in an aerated 7 L plastic beaker (PB) and pulled by a peristaltic pump (PP) through a capillary (3mm internal diameter) and then distributed in the fish larvae rearing tank (FLRT) by 3 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}.

3.2. First-feeding fish larvae ingestion rates

Fish larvae's 24h ingestion rates were determined for 5 different commercially important fish species: *Diplodus sargus*, *Diplodus vulgaris, Sparus aurata*, *Solea senegalensis* and *Dicentrarchus 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 previous determined *S. aurata* and *D. sargus* survival and growth data. Gut analysis was previously tested 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 two 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 *et al.* (1993), *S. aurata* larvae do no start to eat until 4 or 5 DAH. No aeration was provided to the beakers. Four to five prey per mL were then given and the beakers were placed on an acclimated room with 19º C temperature and a 14h light: 10 h dark photoperiod for a 24 hour 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 to 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 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 prey was also used to verify if the prey did not disrupt during the 24 hour 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.

4. 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 normalise data prior to statistical analyses.

Results

1 Rearing of Diplodus sargus and Sparus aurata larvae

In Experiment 1 (sea urchin prey from broodstock on raw feed) all the larvae of *Diplodus sargus* and *Sparus 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 to 9 DAH, 10 to 11 DAH, 12 to 13 DAH and 14 to 15 DAH for the starvation, pre-plutei, prisms and eggs feeding treatment, respectively. Mean growth determined in Experiment 1 was 2.60 mm 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), similarly to the results obtained in Experiment 1, the starvation treatment led to null survival between the 8th and 9th DAH for both species. However null survival for pre-plutei treatment occurred later (13 to 14 DAH) for both species. For the remaining treatments survivals are represented in Figure 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 Figure 2 and was 1.07 mm 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 mm 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. Due to the reduced number of surviving fish larvae obtained with prisms 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 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 Figure 3. *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 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 3 times a day treatment (3T); 1.50% and 1.31% with the *P. lividus* eggs given 2 times a day treatment (2T); 1.30% and 1.02% with the *P. lividus* eggs given by a peristaltic pump treatment (PP). Equally, all fish larvae in the starvation treatment, in Experiment 3, died between the 8th and 9th 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 treatment separately. Mean growth determined in Experiment 3 for both larvae species is represented in Figure 3 and was 1.27 mm, 1.71 mm 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 mm, 1.62 mm 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. Due to 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) (Figure 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* prisms treatment was significantly different (P<0.05) from all the other treatments and had an intermediate value. In Figure 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).

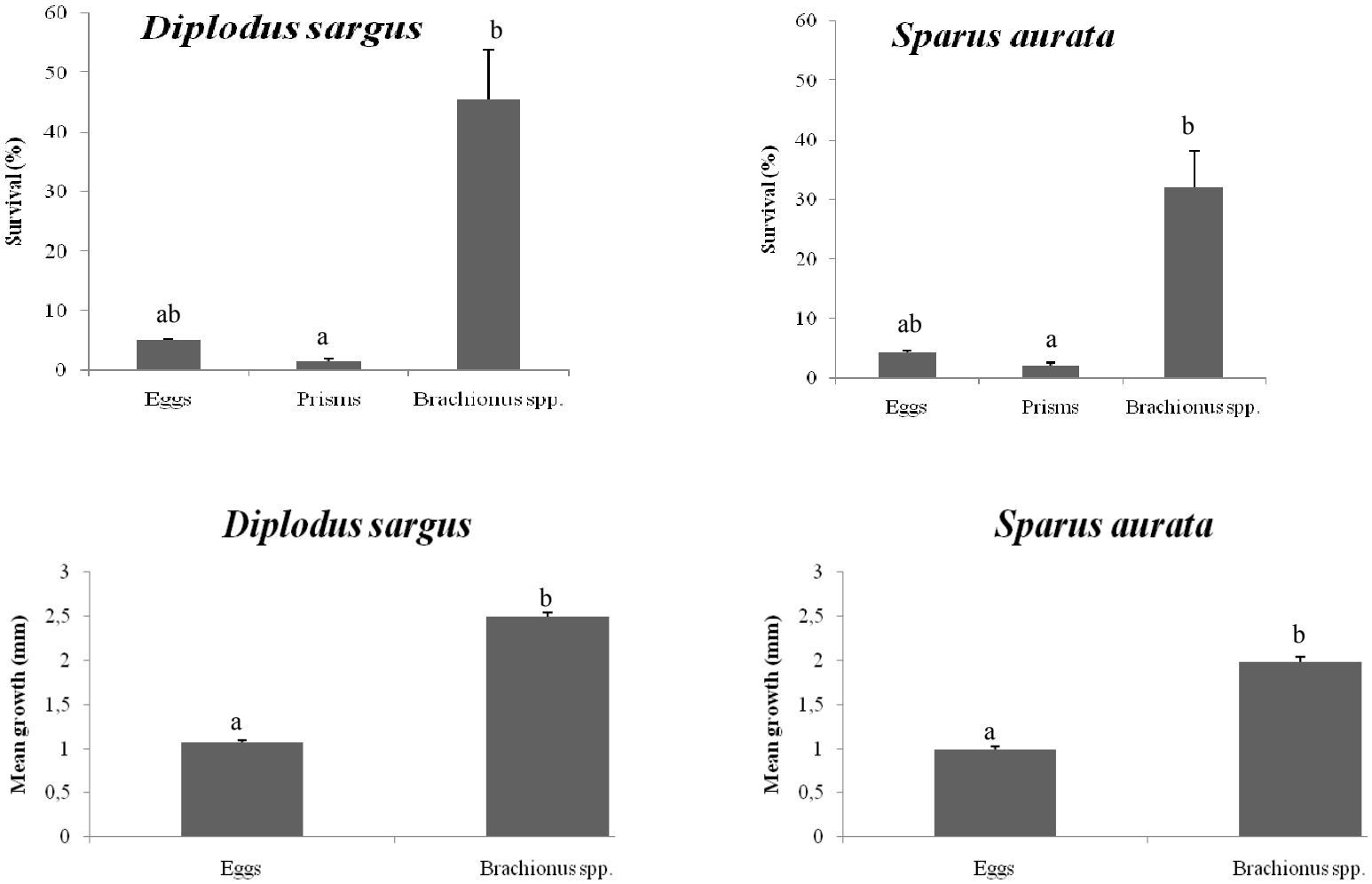


Figure 2. Mean survival (above) and mean growth (below) ± standard error of the mean of *Sparus aurata* and *Diplodus sargus* fish larvae reared from mouth opening to 15 DAH with different live preys (*P. lividus* eggs, prisms and *Brachionus* spp) – Experiment 2. No data is presented for pre-plutei and starvation due to null survival obtained with these feeding treatments. No growth data is presented for prisms feeding treatment due to 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, $P < 0.05$).

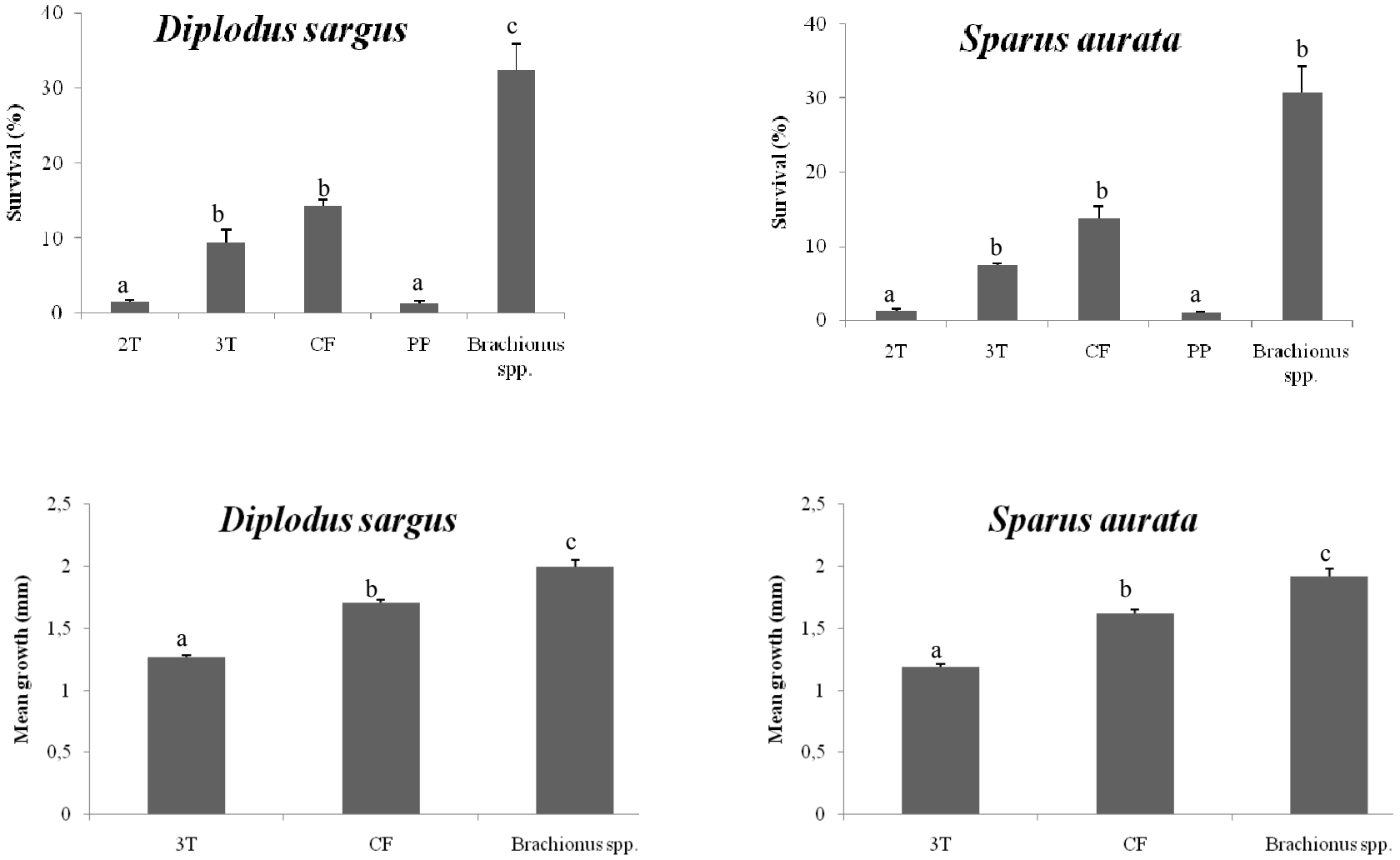
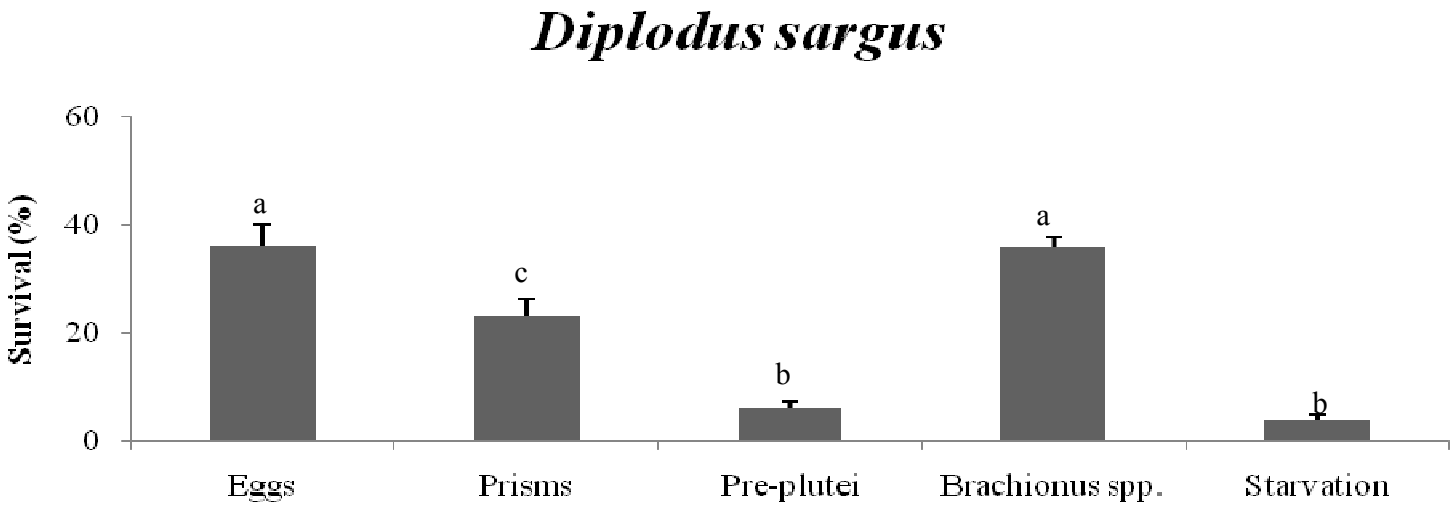


Figure 3. Mean survival (above) and mean growth (below) ± standard error of the mean of *Sparus aurata* and *Diplodus sargus* fish larvae reared from mouth opening to 15 DAH with different live preys (2T, 3T, CF, PP and *Brachionus* spp (see Table 1 and Figure 1)) – Experiment 3 . No growth data is presented 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-preys given two times a day; 3T-preys given three times a day; CF-co-feeding (*Brachionus* spp. + *P. lividus* eggs); PP-continuous feeding with peristaltic pump.



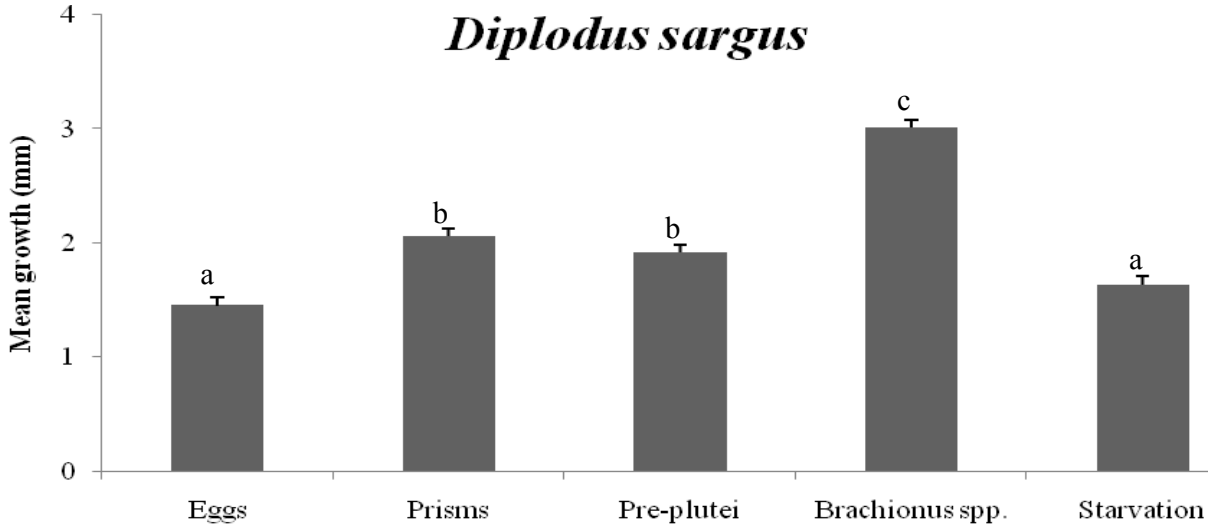


Figure 4. Mean survival (above) and mean growth (below) ± standard error of the mean of *Diplodus sargus* fish larvae reared from 8 DAH to 15 DAH with different live preys (*P. lividus* eggs, prisms and pre-plutei, *Brachionus* spp.) and one where the fish larvae were in starvation – Experiment 4. From mouth opening until the 8th 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, $P < 0.05$).

First-feeding fish larvae ingestion rates

In Figure 5 are represented the 24 hour ingestion rates for the several fish larvae species analysed. Considering first feeding (FF), except for *D. labrax* there was a 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 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 hours the number of rotifers was always higher than in the beginning of the experiment, both in 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 hour 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 (error always less than 3%).

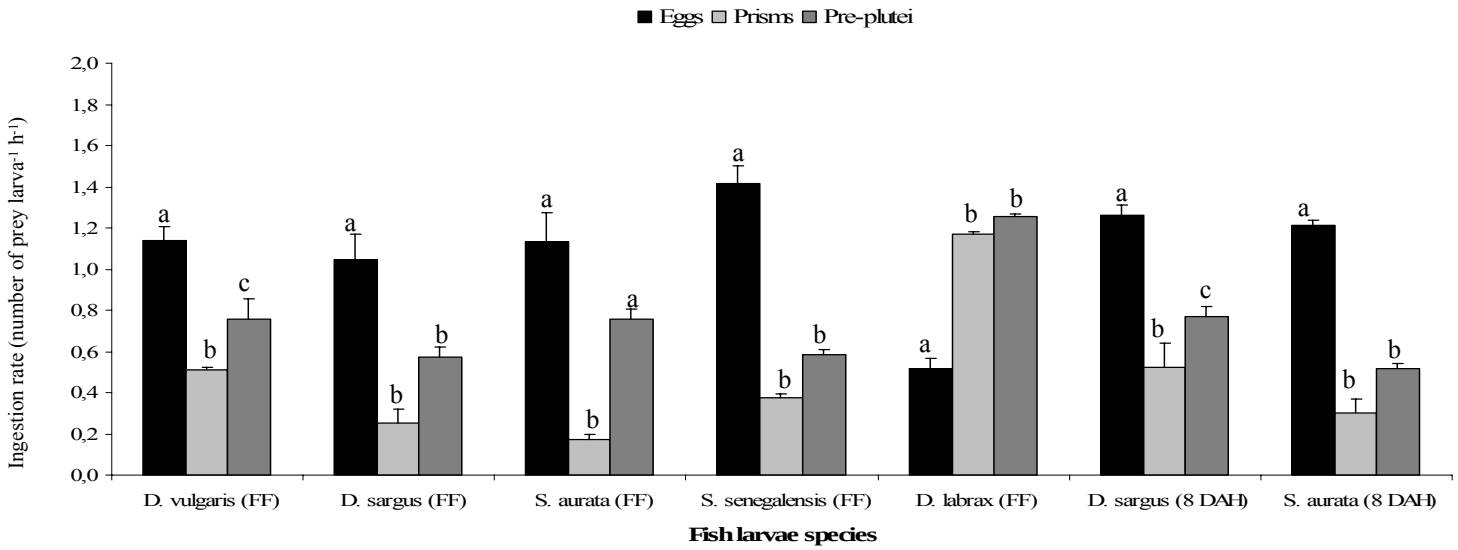


Figure 5. Mean ingestion rate (number of prey ingested larva^{-1} h^{-1}) ± standard error of the mean for 5 different fish larvae species using *P. lividus* eggs, prisms and pre-plutei as live prey (FF – first-feeding; DAH – Days After Hatching). Different letters placed on the top of each bar represent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

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 & 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 (2009a) and Saavedra, Barr, Pousão-Ferreira, Helland, Yúfera, Dinis & Conceição (2009b) referred respectively, 6.2% and 8.6% survival. For *S. aurata* larvae, Parra & Yúfera (2001) referred 15-20% survival rate at the end of the first month of culture. Higher survival values were obtained by Parra & 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 & Parra (2000) where *S. aurata* were fed 10 rotifers mL^{-1}. Testing the effect of rearing salinity, Tandler, Anav & 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 & Kentouri (2002) obtained 44% survival at 20 DAH. In the study of Pousão-Ferreira, Santos, Carvalho, Morais & 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 marine fish larvae survival high variability 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 8^{th} and 9^{th} DAH. Yúfera, Polo & Pascual (1993) also obtained a peak of mortality by starvation around the 9^{th} 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 9^{th} DAH when yolk reserves are exhausted.

When using the alternative live prey (*P. lividus* eggs, prisms and pre-plutei) it is notorious the difference in survivals obtained in Experiment 1 and 2. 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 prey consequence of the *P. lividus* broodstock diet (grains of maize). Gago *et al.* (2009) found little amounts of essential fatty acids (EFA) for fish larvae in *P.*

lividus eggs and endotrophic larvae resulting from broodstock fed this diet, particularly docosahexanoic acid (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 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 eggs feeding treatment which could reflect a nutritional quality increasing order also found by Gago *et al.* (2009) when considering lipid quantity which 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 to Experiment 1, particularly for eggs, but values are lower than the ones obtained with *Brachionus* spp. For pre-plutei 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 non parametric 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 had 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 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 higher consumption of these more numerous, but still of poor quality live prey. Survival obtained with co-feeding (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., since 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 which have 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 DHA/EPA ratio (as by manufacturer specifications, INVE Aquaculture, Belgium).

When considering mean larvae growth, in the 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 co-feeding treatment in Experiment 3. The same reasons used for survival rates can be used to explain these results and those reasons are mainly the poorer nutritional quality of *P. lividus* eggs and larvae when compared to enriched *Brachionus* spp. Besides nutritional quality, the capability of rotifers to reproduce may also explain the differences obtained both in survival and growth since 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 rearing conditions. In those 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 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 pre-plutei feeding treatment. Since rotifers have a high reproductive capability they were observed in all the tanks at the end of the experiment. Aragão, Conceição, Fyhn & 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, it was not found growth values expressed as increases in total length, and in the few studies analysed (Saavedra *et al.* 2006, Saavedra *et al.* 2009a, Saavedra *et al.* 2009b) dry weight was the variable determined, making the direct comparison impossible.

When analyzing first feeding 24 hour ingestion rates, except for *D. labrax*, there was higher *P. lividus* egg consumption than prisms and pre-plutei. Egg slow downfall coupled with its brown-orange color 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 seems that the previous explanations may also apply to *S. aurata* and *D. sargus* larvae with 8 DAH which might indicate that feeding behavior 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 behavior 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 type and size of prey, and moreover, this prey selectivity may play an important role during early larval development (Fyhn 1989). Rumrill, Pennington & 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 & 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-Dias, 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 analyzing 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. Since Yúfera *et al.* (1995) considered that microcapsules are ingested at similar rates to commonly used living prey, it is supposed that the alternative live prey tested are ingested at lower rates than *Brachionus* spp. Additionally for 6 DAH *S. aurata* larvae, Parra & 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 0.16µg (the *Brachionus plicatilis* weight found by Theilaker & McMaster, 1971) it is obtained a feeding rate of approximately 5 rotifers larva^{-1} h^{-1}. This value is also indicative of a higher *Brachionus* spp. ingestion as compared with the calculated best 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 since 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 low quantity of indispensable amino acids in the free form which seems 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 prey, they showed no advantage to be used at least in marine fish first feeding.

Acknowledgments

The authors would like to thank the IPIMAR group who helped with the live food chain and also would like to thank two anonymous referees for their helpful comments on the manuscript. This research was financed by FCT (Fundação para a Ciência e Tecnologia) through IMAREDIS project.

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CHAPTER 5

Final Considerations

FINAL CONSIDERATIONS

Three main criteria (1 – live prey availability and production; 2 – nutritional quality; 3 – survival and growth performance outcomes of fish larvae during first-feeding) were studied to assess the potential to use *Paracentrotus lividus* eggs and endotrophic larvae as marine fish larvae live prey during first-feeding.

Considering the first criteria, examined in Chapter 2 ("Live Prey Availability and Production"), it was concluded that a combination of three factors used on captive *P. lividus* broodstock conditioning (an appropriate diet, 18 º C sea water temperature and a photoperiod of 14 h of artificial illumination) is suitable to promote *P. lividus* gametogenesis on a short period of time and to obtain a large number of viable eggs and spermatozoa after spawning induction. Moreover, it was proven that *P. lividus* captive broodstock can be reared up to 400 m^{-2} stocking density without prejudice to broodstock survival, gametogenesis, spawning performance and fertilization rate. Therefore, it was demonstrated that a large number of *P. lividus* eggs and endotrophic larvae can be obtained year-round without need for repeated sea urchin collection on natural habitats, and thus demonstrating, for this criteria, their feasibility as live preys. The main drawback detected on this evaluation was *P. lividus* broodstock mortality (mean of 30%) after spawning induction with potassium chloride intra-peristomial injection, preventing broodstock reutilization. A first suggestion made to overcome this problem was to reduce potassium chloride internal concentration on smaller *P. lividus* individuals. Nevertheless, other alternative *P. lividus* spawning induction techniques were preliminary tested and it seemed that other inductor signals, particularly the *P. lividus* emersion for 3 to 6 hours, can replace potassium chloride injections, with gains on broodstock survival. Other methods like the mechanical stimulus and the addition of co-specific gametes also proved to induce spawning without broodstock mortality, but with small number of released gametes.

In all the experiments reported in Chapter 2, basic diets composed by grains of maize *Zea mays*, and/or fragments of the seaweed *Laminaria ochroleuca* were used. These raw diets proved, through the studies carried out in Chapter 3 ("Nutritional quality of *Paracentrotus lividus* eggs and endotrophic larvae for marine fish larvae first-feeding"), to be nutritional inferior to prepared diets, considering the profile in essential fatty acids for marine fish larvae existent in resultant *P. lividus* eggs and endotrophic larvae. Additionally, the first trials performed in Chapter 4 ("Survival and growth of selected marine fish larvae first fed with eggs and endotrophic larvae of the sea

urchin *Paracentrotus lividus*"), revealed the unsuitability of *P. lividus* eggs and larvae obtained from broodstock fed these basic raw diets, on rearing gilthead sea bream *Sparus aurata* and white seabream *Diplodus sargus* larvae. Therefore, enrichment of the potential alternative live feeds had to be evaluated through manipulation of broodstock diet composition due to the endotrophic character of both *P. lividus* eggs and larvae phases chosen to be used in the present dissertation. In this context, the effect of the lipid and protein source used in *P. lividus* broodstock prepared diets was studied on the resultant eggs and endotrophic larvae fatty acid and amino acid profile. These profiles were also compared with the commonly used live preys during marine fish larvae first-feeding (the rotifer *Brachionus* spp. and the naupliar stages of the brine shrimp *Artemia* spp.). Main results indicate that fatty acid enrichment is viable trough the utilization of specific lipid sources ("Algamac", cod liver oil) with high percentage of polyunsaturated fatty acids, on *P. lividus* broodstock diet preparation. On the other hand, *P. lividus* eggs and endotrophic larvae protein and amino acid enhancement through prepared diet protein composition proved to be ineffective. Comparing the resultant *P. lividus* eggs and endotrophic larvae fatty acid and amino acid profile with *Brachionus* spp. and/or *Artemia* spp., it was demonstrated the similitude among them. Nevertheless, in some nutritional characteristics like the docosahexanoic acid (C22:6n-3) / eicosapentanoic acid (C20:5n-3) ratio, and the percentage of indispensable amino acids on the free form, the enriched traditional live feeds seems to be better fish larvae feeding. However, the amount of amino acids in the free form on *P. lividus* eggs was found to be higher than in *Brachionus* spp.

In the later experiments performed in Chapter 4, *P. lividus* eggs and endotrophic larvae used as first-feed to evaluate the growth, survival and ingestion of selected marine fish larvae, were already enhanced in their fatty acid profile. In spite of these manipulated live feeds were readily ingested by young marine fish larvae, the growth, survival and ingestion rates results indicate their minor suitability as live first-feed when compared with *Brachionus* spp.

Considering the three main criteria used to evaluate the potential of an organism as a live prey in marine fish larviculture, it can be concluded that *P. lividus* eggs and endotrophic larvae fail partially in the second criteria (nutritional quality) and fail almost totally in the third (survival and growth performance outcomes of fish larvae during first-feeding). Therefore, as main conclusion of this dissertation it can be said that no added value for marine fish larviculture was found in the use of *P. lividus* eggs and endotrophic larvae as live feed. However, before discard this hypothesis, further investigations should be carried out. For instance, the survival and growth performance outcomes could be evaluate on other marine fish larvae species such as groupers and snappers, as well as crustacean species such as spiny lobster phyllosoma, where larval phases mortality is still unsolved. Similarly, the potential to use the *P. lividus* exotrophic larvae phase as live feed on fish

and crustacean larvae with wider mouth opening should also be analysed, since in this case the nutritional enrichment can be done through the rearing water media as done for *Brachionus* spp. and *Artemia* spp.

As a final remark, it must be said that in spite of the general negative result obtained in the present dissertation (potential of *P. lividus* eggs and endotrophic larvae as marine larval fish first-feeding), the knowledge obtained can be used for several other purposes where sea urchin larvae are needed. For instance, maximization of sea urchin eggs and larvae production can be used to develop increasing sea urchin aquaculture for human consumption and to restocking programmes. At a small scale, sea urchin eggs and larvae production is also needed for embryological and toxicological studies and as biological tools in practical classes of several courses.