MAYA MILLICENT GRACE LOGOSE

EVALUATING THE EFFECT OF TEMPERATURE STRESS ON *OCTOPUS VULGARIS* PARALARVAE

UNIVERSIDADE DO ALGARVE
Faculdade de Ciências e Tecnologias
2017

MAYA MILLICENT GRACE LOGOSE

EVALUATING THE EFFECT OF TEMPERATURE STRESS ON *OCTOPUS VULGARIS* PARALARVAE

Mestrado em Biologia Marinha

Trabalho efetuado sob a orientação de:

Dr. Francisco Javier Rocha Valdés (Universidade de Vigo)
Dr. José Pedro Andrade (Universidade do Algarve)

UNIVERSIDADE DO ALGARVE
Faculdade de Ciências e Tecnologias
2017

Declaração de autoria de trabalho

Evaluating the effect of temperature stress on *Octopus vulgaris* paralarvae

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

(Maya Millicent Grace Logose)

Copyright © 2017 Maya Logose

A Universidade do Algarve reserva para si o direito, em conformidade com o disposto no Código do Direito de Autor e dos Direitos Conexos, de arquivar, reproduzir e publicar a obra, independentemente do meio utilizado, bem como de a divulgar através de repositórios científicos e de admitir a sua cópia e distribuição para fins meramente educacionais ou de investigação e não comerciais, conquanto seja dado o devido crédito ao autor e editor respetivos.

FRANCISCO JAVIER ROCHA VALDÉS, DOCTOR EN BIOLOGÍA CON DNI 35.456.014E, PROFESOR TITULAR DE UNIVERSIDAD Y MIEMBRO DEL DEPARTAMENTO DE ECOLOGÍA Y BIOLOGÍA ANIMAL DE LA UNIVERSIDAD DE VIGO

CERTIFICA:

Que Doña **MAYA MILLICENT GRACE LOGOSE** ha realizado una estancia en el grupo de investigación BioCephALab, realizando sus labores en el laboratorio del que dispone el grupo en la Estación de Ciencias Marinas de la isla de Toralla (ECIMAT) de la Universidad de Vigo.

Durante tiempo, Maya Logose no ha manipulado animales (pulpos) en cautividad, realizando estudios sobre muestras previamente obtenidas.

Y para que conste a los efectos oportunos se expide el presente certificado en Vigo a 18 de diciembre del año dos mil diecisiete.

UNIVERSIDADE DE VIGO - DPTO. DE ECOLOXÍA E BIOLOXÍA ANIMAL -

Fd. Francisco Rocha Valdés

Departamento de Ecología y Biología Animal, Facultad de Ciencias del Mar
Campus as Lagoas – Marcosende, 36310 Vigo, España

Acknowledgements

I would like to thank my supervisor Dr Francisco Rocha for welcoming me into the BioCephALab research group and guiding me throughout my thesis work. I thank my UALG supervisor Dr José Pedro Andrade for assisting me throughout my thesis. I also would like to give thanks to Dr Leonardo Mantilla Aldana and Raquel Fernández Gago for assisting me throughout my thesis laboratory work and the ECIMAT Marine Station staff.

Lastly, I want to thank the friends I made in Portugal during my course, closest friends and family for always supporting and cheering me up through hard times throughout my studies.

UniversidadeVigo

Table of Contents

Abstract i
Resumo ii
Index of Figures vi
Index of Tables viii
Abbreviations, acronyms and symbols x

1. Introduction 1
1.1. *Octopus vulgaris* Cuvier, 1797 1
1.2. The state of *O. vulgaris* aquaculture 2
1.3. The state of *O. vulgaris* paralarval culture 3
1.3.1. The embryonic phase 7
1.3.2. The paralarval phase 9
1.3.3. Rearing conditions for paralarval survival 13
2. Objectives 17
3. Material and Methods 18
3.1. Paralarvae sample 18
3.2. Thermal shock cultivation experiment 18
3.3. Additional temperature experiments 19
3.3.1. Cultivation at different constant temperatures 20
3.3.2. Cultivation during increasing temperature 21
3.4. Specimens for analysis 22
3.5. Beak microstructure analysis 23
3.6. Dry weight and morphometrical analysis 28
3.7. Statistical analysis 29
4. Results 30
4.1. Thermal shock experiment 29
4.2. Additional temperature experiments 36
4.2.1. Cultivation at different constant temperatures 36
4.2.2. Cultivation during increasing temperature 38
5. Discussion 40
5.1. Effects of temperature on mortality and growth 40
5.2. Evaluating temperature stress from beak microstructures 41
5.2.1. Stress marks 40
5.2.2. Increment width 43
5.2.3. Beak mounting and ageing 44
5.3. Limitations 45
6. Conclusions 47
7. References 48
8. Annexes 55

Abstract

Paralarval survival of *Octopus vulgaris* can be limited by temperature via short, abrupt changes ("thermal shocks") above the natural, geographical temperature range. Thus, temperature stress can affect the growth of paralarvae. Beaks are a tool for stress, registering as darker increments ("stress marks") during cultivation. Due to minimal knowledge of temperature stress on *O. vulgaris* paralarval beaks, it must be researched for improved industrialised culture. To evaluate the effect of temperature stress on paralarvae, a group was subjected to induced thermal shocks (16+3°C) for 2 hours (laboratory located ECIMAT, Vigo, Spain). Additional temperature experiments (constant temperatures - 14, 16, 18, 21°C and increasing temperature - 16-21°C) were run to determine the general effect of temperature on paralarvae. Differences in dry weight, morphometrics, stress marks and mean increment width and age (via gelatine mounting microincrement analysis of the rostral sagittal surface), were assessed between groups in the rostral sagittal surface for the thermal shock experiment. Age and mean increment width (measured on the lateral wall surface) were determined for additional temperature experiments. Dry weight and morphometrics in thermally shocked paralarvae were not significantly different compared to the control group (and for morphometrics in additional temperature experiments). Stress marks were found in 2 of 3 thermally shocked paralarvae and corresponded to the thermal shock day, however, were not statistically different to the control group. 1 of 3 paralarvae was negative for stress marks, hinting adaptability to temperature stress. Marks were observed on alternative days, possibly being "post-stress marks" or confounding stress. Increment width was not significantly different on the thermal shock day and between groups (or with increasing temperature). Positively, gelatine mounting could be a novel technique for increment visualisation. Further research is needed to validate all outcomes of this study (removing confounding stressors and increasing the sample size analysed).

Keywords: *Octopus vulgaris, paralarvae, temperature, stress, thermal shock, beaks.*

Resumo

O polvo comum, *Octopus vulgaris* (Cuvier, 1797), é um cefalópode bentónico encontrado em águas costeiras de regiões temperadas, tropicais e subtropicais. O ciclo de vida completo de *O. vulgaris* dura entre 12 e 18 meses, durante o qual há a eclosão de paralarvas plantónicas, capazes de nadar livremente. Quando as paralarvas atingem o "tamanho crítico" (> 7.5 mm) assentam na zona betónica da coluna de água, onde desenvolvem as fases de juvenil e adulto. Comparando com outras espécies de cefalópodes, *O. vulgaris* apresenta elevado potencial para ser cultivada em aquacultura. As três principais características que justificam o seu uso como modelo experimental e o tornam candidato a aquacultura são o seu curto ciclo de vida, crescimento acelerado e rápido índex de conversão alimentar. Paralelamente, a sua elevada taxa de fecundidade, tamanho e alto valor comercial (adjacente ao considerável consumo global de polvo) culminaram num grande interesse em cultivar esta espécie a uma escala industrial. Contudo, a aquacultura desta espécie ainda não atingiu o nível industrial devido a complicacoes críticas no cultivo das paralarvas que permanecem ainda por resolver. De modo a removeras complicações, inúmeros autores têm tentado completar o ciclo de vida de *O. vulgaris* em cativeiro com altas taxas de sobrevivência e de assentamento. Todavia, atualmente ainda não existe um protocolo *standard* estabelecido para o cultivo em cativeiro de *O. vulgaris*.

A temperatura é um dos fatores determinantes em termos de regulação da sobrevivência e crescimento de larvas invertebradas, incluindo as de *O. vulgaris*. Nas paralarvas, a temperatura regula a eficiência da absorção do vitelo (alimentação exógena) durante os primeiros dias de vida. A temperatura influencia a duração da fase plantónica e o assentamento das paralarvas devido à correlação positiva com o metabolismo. Deste modo, a fase paralarval é considerada o "período crítico" no crescimento e desenvolvimento de *O. vulgaris*, sendo este comparável com o período crítico das larvas de peixe. Em estudos prévios, as temperaturas de cultivo utilizadas variaram entre 16 e 23°C. A utilização de uma temperatura fora destes limites poderá resultar num crescimento e desenvolvimento das paralarvas reduzido ou, em casos extremos, levar à mortalidade das mesmas.

Os efeitos do stress térmico podem ser observados diretamente no crescimento e morfologia das paralarvas (e.g. comprimento do manto e comprimento total) ou pelos

incrementos registados nas estruturas calcificadas. No caso do *O. vulgaris*, o bico (situado na massa bocal) é constituído por duas mandíbulas, superior e inferior, utilizadas para a ingestão de presas, em particular presas de concha dura. Estudos anteriores revelaram que a indução de aumentos de temperatura ("choques térmicos") pode causar a disrupção na deposição de linhas no bico, ficando registadas marcas escuras denominadas "marcas de stress". Assim, o bico constituí um potencial biomarcador para stress térmico. Adicionalmente, ainda nenhum autor tentou relacionar a largura dos incrementos com a temperatura de stress induzida. Se for encontrado sustento para esta correspondência, a largura dos incrementos nos bicos das paralarvas de *O. vulgaris* poderá ser utilizada como um meio quantitativo de análise de stress térmico.

Apesar do stress térmico ser evidentemente desfavorável para a criação bem sucedida de *O. vulgaris*, existe uma considerável falta de conhecimento relativamente ao impacto do mesmo na morfologia e microestruturas do bico (e de nova técnicas de preparação) dentro da área de investigação da aquacultura de *O. vulgaris*. O estudo deste tema irá melhorar a pratica da aquacultura de *O. vulgaris* e refinar o bem estar das paralarvas em condições de cultura.

De modo a analisar o stress térmico, um grupo de paralarvas foi exposto a um choque térmico (aumento da temperatura da água de 16 a 19°C) durante 2 horas no quinto dia dum cultivo de 10 dias (nas instalações localizadas na Estação Marinha ECIMAT, Vigo, Espanha). Adicionalmente, noutras duas experiências de temperatura, vários grupos de paralarvas foram submetidos a diferentes temperaturas constantes (14, 16, 18 e 21°C) durante 15 dias, e a gradientes de temperatura de 16 a 20°C do dia 6 ao dia 9 (com temperatura constante a 20°C a partir do dia 9), durante uma cultura de 25 dias. Foram também criados tanques controlo para todas as experiências, nos quais a temperatura foi estabilizada a 16°C. Os efeitos gerais da temperatura na mortalidade e crescimento das paralarvas submetidas a choques térmicos foi analisada através do peso seco e análises morfométricas (e.g. comprimento total, comprimento do manto, largura dorsal do manto e largura da cabeça – em mm). As paralarvas das experiências adicionais foram analisadas morfometricamente. O stress térmico em paralarvas previamente submetidas a um choque térmico foi avaliado através da análise das microestruturas do bico (envelhecimento, "marcas de stress" e largura do incremento a partir da secção sagital rostral - RSS) Em alternativa, a superfície da parede lateral foi estimada para paralarvas através de experiências de temperatura adicionais (temperatura constante – idade e largura de incremento,

aumento de temperatura – idade). Em termos de análise de precisão da leitura de idade, foi utilizado o coeficiente de variação (CV%), para determinar o potencial da gelatina como meio de montagem para preparações microscópicas utilizadas na observação de incrementos em bicos, e o seu uso em futuras análises de microincrementos. Os dados obtidos foram analisados estatisticamente para averiguar diferenças entre o controlo e os grupos experimentais.

A mortalidade em paralarvas sujeitas ao choque térmico foi superior à observada nos organismos de controlo, o que evidencia que aumentos abruptos de temperatura durante um curto período de tempo podem afetar diretamente a sobrevivência de paralarvas de *O. vulgaris*. Apesar de não se verificarem diferenças significativas, o peso seco obtido para as paralarvas sujeitas ao choque térmico foi menor que o obtido em organismos do grupo de controlo. Morfometricamente, todas as paralarvas dos grupos experimentais aumentaram as suas dimensões ao longo do tempo, porém não existiu um aumento significativo entre os mesmos e as paralarvas dos grupos de controlo correspondentes. A análise de precisão da leitura de idade revelou precisão (CV = 0%) para mais de 80% dos espécimes em todas as experiências, indicando o potencial da utilização da gelatina, em estudos futuros, como um novo meio de montagem em preparações para a visualização de incrementos em bicos. Ademais, confirmou-se uma correspondência entre todas as idades das paralarvas e o número de incrementos contados, validado assim uma taxa de deposição de incremento diária (1 incremento.dia^{-1}). Apesar das marcas de stress terem sido encontradas em quase todas as paralarvas submetidas a choques térmicos, não foram encontradas diferenças significativas ($p = 0.74$) quando comparadas com o grupo de controlo. Foram igualmente observadas marcas de stress nos dias não associados aos choques térmicos, o que pode ser explicado por fatores de stress perturbadores (e.g. perturbações nos tanques) ou como marcas de stress posteriores aos choques térmicos. Uma das paralarvas submetidas a choques térmicos não apresentou qualquer marca de stress, o que poderá indicar que alguns indivíduos possuem uma alta capacidade de adaptação ao stress térmico. A largura dos incrementos, na superfície da parede lateral, não foi significativamente maior nos dias associados a choque térmico (dia 6-10 – $p = 0.28$, dia 6 – $p = 0.16$) ou de aumento de temperatura (entre grupos de temperatura constante LWS – $p = 0.11$). No entanto, várias limitações foram encontradas durante este estudo, tal como o pequeno tamanho da amostra disponível para analisar nas experiências de temperatura. *Octopus vulgaris* apresenta alta variabilidade no crescimento

individual, característica a qual pode dificultar a clareza e interpretação dos resultados obtidos. Ademais, a aplicação temporal de marcas de stress e largura dos incrementos é desconhecida, uma vez que as paralarvas não foram analisadas durante mais do que 15 dias. Deste modo, a análise de microincrementos em paralarvas submetidas a stress térmico deverá ser repetida de novo num período mais avançado da fase plantónica.

Resumidamente, as marcas de stress podem ser induzidas nos bicos de *O. vulgaris* através de stress térmico, apesar de ser necessária a remoção de fatores de stress perturbadores para clarificar os resultados. Adicionalmente, tendo em conta o pequeno tamanho da amostra e da alta variabilidade no crescimento de *O. vulgaris*, será necessário repetir este estudo para validar os resultados aqui expostos. Este estudo revelou consideráveis lacunas na investigação relacionada com stress térmico e o quão vital é para o desenvolvimento das paralarvas de *O. vulgaris* a serem criadas.

Palavras-chave: *Octopus vulgaris, paralarva, temperatura, stress, choque térmico, bicos*.

Index of Figures

Figure 1.1. - Distribution of the *Octopus vulgaris* (*sensu stricto*, types I – IV). From Norman et al. (2016). 1

Figure 1.2. - Relationship between total length and days after hatching in *O. vulgaris* rearing (Itami et al., 1963). 4

Figure 1.3. - The embryonic development of *O. vulgaris* 18 ºC. a) Prior to first inversion (Naef IV-VI stage). B) Post first inversion (Naef VIII stage). C) Embryo differentiation (Naef XI stage). D) Naef XII-XIV stage. E) Chromatophores and internal yolk (Naef XV stage). F) Post second inversion (Naef XIX stage). Taken from Iglesias & Fuentes (2014). Photographs by M. Nande. .. 7

Figure 1.4. - A left lateral view of the optical section in Octopodidae. A, arm; AB, arm base; ADM, anterior margin of dorsal mantle; AVM, anterior margin of ventral mantle; DE, dorsal eye; DH, dorsal head; DM, dorsal mantle; F, funnel; PC, posterior cap; V, visceral; VH, ventral head; VM, ventral mantle. Diagram from Villanueva & Norman (2008) and adapted from Hochberg et al., (1992). 10

Figure 1.6. - An *O. vulgaris* paralarva at a) 0 days-old (1.5 mm DML, 3 suckers per arm) and b) 52 days old (5.75 mm DML, 21 suckers per arm). From Iglesias et al. (2007). 10

Figure 1.8. - A diagram of (a) the lower jaw (LJ) and upper jaw (UJ) of *O. vulgaris* beak (FAO, 2016) and (b) The UJ lateral wall surface (LWS) and the rostral hood containing the rostral sagittal section (RSS) – from Perales-Raya et al. (2014b). 11

Figure 3.1. - (a) A photo of an individual *O. vulgaris* paralarvae in distilled water for beak extraction (under the inverted microscope) and (b) the precision needles used to remove and clean the beak. 23

Figure 3.2. - (a) Gelatine stored in a tube for mounting *O. vulgaris* paralarvae beaks, (b) AOYUE 804 single hot-air gun to melt the gelatine on the glass slide and (c) mounted beaks of sample specimens ready for microscopic observation. 24

Figure 3.3. - Nikon Eclipse Fluorescence 90i Microscope and NIS Elements used to take digital pictures of paralarvae beaks for the microstructure analysis. 25

Figure 3.4. - Example of beak microincrement analysis of RSS (ageing and increment width analysis) of a paralarva 5 days after hatching for the thermal shock experiment (under x 40 magnification and Nomarski DIC). Inset: Increment width measured on Digimizer photo analysis software (grey circles). Dots - beak increments. 27

Figure 3.5. - A schematic diagram of the body dimensions of an *O. vulgaris* paralarva specimen (from a tank maintained at 14°C during the constant temperature experiment). *DML - dorsal mantle length, TL - total length, MW - mantle width and HW - head width*. 28

Figure 4.1. - The mortality (%) of *O. vulgaris* paralarvae subjected to control (CTRL) and thermal shock (TS) conditions. 30

Figure 4.2. - The dry weight (mg) of *O. vulgaris* paralarvae subjected to the control (CTRL) and thermal shock treatment (TS). *Note: due to missing data, the standard deviation for paralarvae 0 DAG could not be presented.* 31

Figure 4.3. - Mean body dimensions of *O. vulgaris* paralarvae 0, 5 and 10 DAH (days after hatching) subjected to control and thermal shock conditions. (a) total length, (b) mantle width, (c) dorsal mantle length and (d) head width. *Note: due missing data on for paralarvae 0 and 5 DAH, the standard deviation for these days were not presented in this figure*. 32

Figure 4.4. - Mean increment width (µm) for paralarvae 10 DAH (a) in thermal shock conditions before and after thermal stress (b) between control and thermal shock conditions on day 6-10. . 35

Figure 4.5. - Mean body dimensions against increasing temperature (from *O. vulgaris* paralarvae 5 DAH cultivated at selected temperatures – 14, 16, 18 and 21°C). (a) total length, (b) dorsal mantle width, (c) mantle length and (d) head width. *Note: due to missing data, the standard deviation could not be displayed in this figure.* 36

Index of Tables

Table 1.1. - Summary of various Spanish research groups rearing methodology and conditions of *Octopus vulgaris* paralarvae culture (adapted from Carrasco et al., 2006; Berger, 2010; Iglesias & Fuentes, 2014; Iglesias et al., 2004; Seixas et al., 2010 and Villanueva, 1995). *Note: TL, total length; l, light; Lx, Lux; n.p., not provided; ind.mL^{-1}/ind.L^{-1}, individuals per millilitre/litre; CEP, Centro de Experimentación Pesquera; IEO, Instituto Español de Oceanografia; ICM-CSIC, Instituto de Ciencias del Mar-Consejo Superior de Investigaciones Científicas and USC, Universidad de Santiago de Compostela.* 6

Table 3.1. - Experimental design for culture tanks containing *O. vulgaris* paralarvae in thermal shock experiment (a) and the tank conditions (b). *TSD* – thermal shock day. 18

Table 3.2. - Experimental design for culture tanks containing *O. vulgaris* paralarvae for the constant temperature experiment (a) and the tank conditions (b). 20

Table 3.3. - Experimental design for culture tanks containing *O. vulgaris* paralarvae for the increasing temperature experiment (a) and the tank conditions (b). 21

Table 3.4. - The collection regime and analysis of *O. vulgaris* paralarvae specimens in temperature cultivation experiments (thermal shock, constant and increasing temperature). *n* = number of specimens. *DAH* - days after hatching. 22

Table 4.1. - Mean body dimensions and significance of differences between *O. vulgaris* paralarvae 10 DAH cultivated in control and thermal shock conditions. 31

Table 4.2. - Age precision (CV) of *O. vulgaris* paralarvae specimens in all groups from the thermal shock experiment. *TS – thermal shock*. 33

Table 4.3. - Presence of stress marks on the day 6 (thermal shock) and additional stress marks in RSS of beaks from *O. vulgaris* paralarvae 10 DAH. 33

Table 4.4. - Mean increment widths in the RSS of *O. vulgaris* beaks 10 DAH (by group and specimen) reared for the thermal shock experiment. 34

Table 4.5. - Analysis of mean increment width between experimental groups of the thermal shock experiment (and differences within groups) for paralarvae 10 DAH. CTRL = control and TS = thermal shock. 34

Table 4.6. - Mean body dimensions and significance of differences between *O. vulgaris* paralarvae 5 DAH cultivated in selected constant temperature conditions.................................. 36

Table 4.7. - *O. vulgaris* specimens with age validity > 0% CV for cultivation at a constant temperature (counting region – LWS). 38

Table 4.8. - Mean increment width of LWS in the beaks of *O. vulgaris* paralarvae 5 DAH and subjected to different cultivation temperatures. 38

Table 4.9. - Mean body dimensions and significance of differences between *O. vulgaris* paralarvae (a) 5 DAH, (b) 10 DAH and (c) 15 DAH cultivated in control and increasing temperature (IT) conditions.. 39

Table 4.10. - *O. vulgaris* specimens with age validity > 0% CV for cultivation at an increasing temperature (counting region – LWS). 39

Abbreviations, acronyms and symbols

Abbreviations: CEP - Centro de Experimentación Pesquera, CIAC - Cephalopod International Advisory Council, ECIMAT - Estación de Ciencias Marinas de Toralla, FAO – Food Agricultural Organisation, IEO, Instituto Español de Oceanografia, ICM-CSIC, Instituto de Ciencias del Mar-Consejo Superior de Investigaciones Científicas, USC - Universidad de Santiago de Compostela.

Acronyms: A – arm, AB - arm base, ADM - anterior margin of dorsal mantle, AVM - anterior margin of ventral mantle, CTRL – control, CV – coefficient of variation, DAH – days after hatching, DE - dorsal eye, df – degrees of freedom, DH - dorsal head, DM - dorsal mantle, DML – dorsal mantle length, DNA - deoxyribonucleic acid, DW – Dry Weight, F – funnel, f – f-value, HW – head width, Hsp70 - Heat Shock Protein 70, IT – increasing temperature, ind.L^{-1} - individuals per litre, ind.mL^{-1} - individuals per millilitre, l – light, L – litre, LC 50 – lethal concentration 50, LJ – lower jaw, LWS – lateral wall surface, Lx – lux, µm – micrograms, Mean Sq. – mean squares, mg - milligrams, mm – millimetres, MW – mantle width, n.p. – not provided, N – number of specimens, p – p-value, PC, Posterior cap, ppm – parts per million, PUFAs - polyunsaturated fatty acids, PSU - partial salinity unit, RNA – ribonucleic acid, RSS - rostral sagittal section, Sum Sq. - sum of squares, t – t-statistic, TL - total length, TSD – thermal shock day, TS – thermal shock, UJ – upper jaw, ind.L^{-1} - individuals per litre, ind.mL^{-1} - individuals per millilitre, IT – increasing temperature, V- visceral, VH - ventral head, VM - ventral mantle, mg/L – milligrams per litre, L/h – litres per hour.

Symbols: α – alpha, °C – degrees Celsius, σ - standard deviation.

.

1. Introduction

1.1. *O. vulgaris* Cuvier, 1797

The common octopus, *Octopus vulgaris* Cuvier, 1797, is a "cosmopolitan" marine cephalopod species found in temperate, tropical and subtropical waters of all oceans. However, its distributional limits are unknown. The localities of *O. vulgaris* are the North Atlantic and the Mediterranean (*sensu stricto*), the Western Central Atlantic Ocean (type I), the Southwest Atlantic Ocean (type II), South Africa and the Southern Indian Ocean (type III) and East Asia (type IV) (Figure 1.1 – Norman et al., 2016).

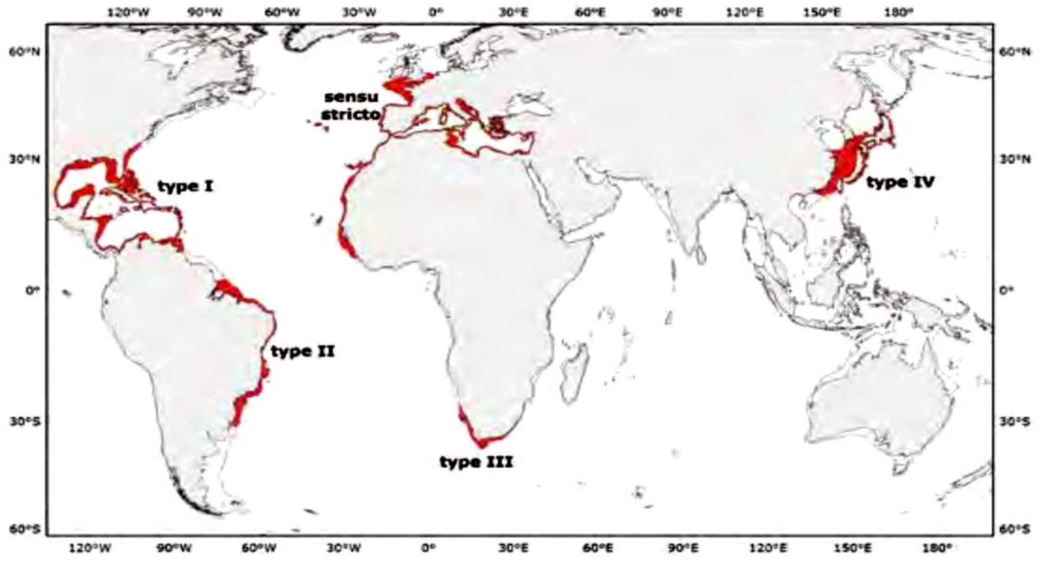


Figure 1.1. Distribution of the *Octopus vulgaris* (*sensu stricto*, types I – IV). From Norman et al. (2016).

O. vulgaris is a benthic dwelling organism and inhabits depths of 0 to 200 m in the sublittoral zone and occupies various types of habitats (e.g. rocks, coral reefs, seagrass beds) in coastal waters (Norman et al., 2016). As an incirrate octopod, it possesses the primary external characteristics (e.g. spherical bodies that lack fins, tubular funnel on the underside of mantle cavity, deep web sectors, suckers on arms, ink sac, makes with a modified arm tip, beak, and radula, etc. - Norman et al., 2016). The main features used to distinguish *O. vulgaris sensu stricto* from other types of this species are indicated in Annex I.

O. vulgaris can reach a maximum mantle length (ML) of 400 mm and commonly a total length (TL) of ~1.8m (males are ~1.3 m and females are ~1.2. m – Roper et al., 1984). It has a maximum weight of 20 kg, still, 3 kg is its common weight (Norman et al., 2016; Roper et al., 1984; Vidal et al., 2014). The diet of *O. vulgaris* comprises of prey such as crustaceans, fish, and molluscs as all octopods are carnivores. They capture prey via their arms and use their beak/radula to ingest. Like all cephalopods, *O. vulgaris* is a dioecious species, in which the male mates by transferring sperm with its shorter third right modified arm (i.e. hectocotylised and rounded at the extremity) into the internal cavity of the female. In wild populations, eggs of *Octopus vulgaris* are mainly laid in shallow waters by females (Mangold, 1983). Spawning female octopuses can produce from 100,000 to 500,000 eggs (< 2mm), bound in strings called festoons (Roper et al., 1984; Iglesias et al., 2000; Norman et al., 2016; Vaz-Pires et al., 2004; Mangold, 1983). It has been reported that brooding wild males and females together in suitable conditions (adequate food and shelters), approximately 100% of females mature n lay eggs. Being a semelparous species, females produce eggs once in their lifetime and die soon after eggs hatching (males will die after spawning). Eggs hatch into free-swimming planktonic paralarvae (in the water column) to continue their life cycle (from 12-18 months - Iglesias et al., 2007; Normal et al., 2016).

1.2. The state of *O. vulgaris* aquaculture

O. vulgaris has been considered an experimental model and its potential for aquaculture has slowly progressed over the decade with aims to reach an industrial scale (Vidal et al., 2014). Three main characteristics have influenced its consideration for aquaculture as a short life cycle, fast growth (up to 15% in body weight/day in subadults) and high food conversion rate (15-43% - dependant on the rearing temperature and diet). Additionally, their high fecundity (100-500 thousand eggs per female), size and seafood market price (average EU/kg grade T1[1] = 8.95 in the Spanish market – Josupeit et al., 2016), has allowed their potential for culturing to be recognised (Iglesias et al., 2007; Navarro & Villanueva, 2000; Vaz-Pires et al., 2004). They are greatly valued organisms of commercial interest in Spain and highly regarded for diversifying the aquaculture within the Mediterranean (Iglesias & Fuentes, 2014; Mancuso, 2014).

[1] Grade T1 is a whole octopus of ≥ 4 kg

Thus far, the only industrialised phase of *O. vulgaris*' life cycle is as a subadult (defined as "the life-history stage in which all diagnostic features used to define the species are attained and ends when sexual maturity is achieved" - Young & Harman, 1988). Subadults are caught from wild populations and grown in tanks or floating sea cages, to more advanced developmental stages. This process is known as rearing (Berger, 2010; Iglesias et al., 2000; Vaz-Pires et al., 2004). Iglesias et al. (1997) and Rama-Villar et al. (1997) published significant data on octopus growth rates of subadults, prompting the industrial production in Galicia (NW Spain) in the mid-1990's. Rearing is generally successful, however, variations in physical parameters for subadult growth in sea cages may interfere with survival (Boletzky & Hanlon, 1983; Vidal et al., 2014).

Like subadult rearing, numerous factors in culturing *O. vulgaris* paralarvae (and cephalopod culture in general) can cause major setbacks in completing the full life cycle, for example transportation, uncontrolled physical and chemical parameters, insufficient or inappropriate diet, disease etc. (Navarro, et al., 2014; Vidal et al., 2014; Villanueva et al., 2014). Hence, the primary focus of research within the past decade has been to remove rearing bottlenecks to complete the life cycle of *O. vulgaris*.

1.3. The state of *O. vulgaris* paralarval culture

According to Boletzky & Hanlon (1983), *culture* refers to the growth of an embryo, hatching to a complete life cycle (laying eggs that hatch into viable young). In the culture of cephalopods, the paralarval life stage ("a cephalopod post-hatching growth stage that is pelagic in near-surface waters during the day and a differing habitat from that of older conspecific individuals" – Young & Harman, 1988), is a critical period, especially for octopuses (Vidal et al., 2002). During this period, paralarvae (more than one paralarva – "planktonic young of Octopods that meet certain ecological, and morphological criteria" - Young & Harman, 1988) must initiate the consumption of a suitable diet to grow, requiring stable conditions for proper development (Iglesias & Fuentes, 2014; Iglesias et al., 2007). Consequently, this period of growth is the primary bottleneck in viable industrialised culture.

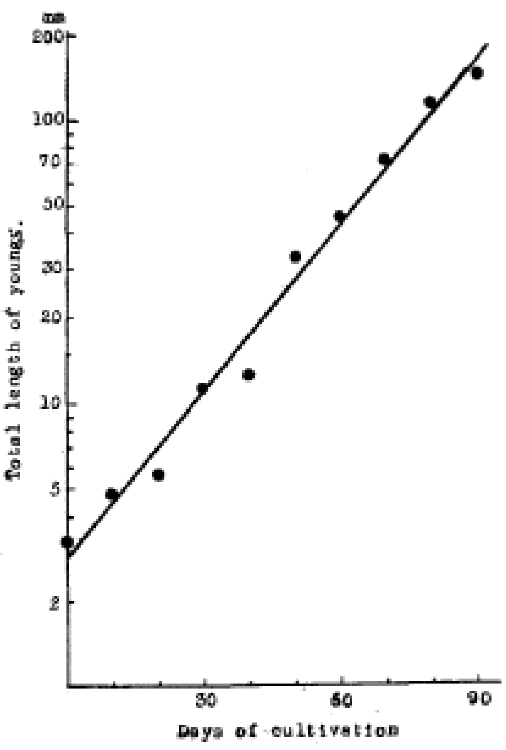


Figure 1.2. Relationship between total length and days after hatching in *O. vulgaris* rearing (Itami et al., 1963).

The first research in rearing *O. vulgaris* paralarvae started in the 1960's with Itami et al. (1963), accomplishing to rear hatchlings to benthic juveniles after 33 days (mean temperature of 24.7°C) with *Palaemon serrifer* shrimp zoeae in Japan. Paralarvae were found with 4~6 suckers on each arm at hatching and increased to 24 at settlement. The TL of octopus paralarvae examined also increased in size, being 11.8 mm at settlement (Figure 1.2). Nevertheless, the survival rate was low at 9% and the authors suggested that increasing the food supply during rearing would decrease mortalities.

Three decades later, Villanueva (1995) achieved benthic juveniles after 47 days for the first time in Europe (Spain) at a mean of 21.2°C and paralarvae were fed crab *Palaemon serrifer* zoeae (Table 1.1). In comparison to Itami's study, the survival (8.9%) and mean ML (6.4 mm) at settlement was lower. These differences may have been due to the lower temperature range in Villanueva's study (1995). To add, comparing results of *O. vulgaris sensu stricto* cultivation from the Mediterranean versus *O. vulgaris* type IV from North-west Pacific Ocean, were taken with caution due to the geographical temperature and no evidence of gene flow mechanisms (Norman et al., 2016). Nevertheless, the Villanueva's (1995) results confirmed that settlement

only occurs at a critical size and the duration of the planktonic stage is temperature-dependent, being a vital piece of knowledge used in *O. vulgaris* rearing today.

By the early 2000's, Moxica et al. (2002) carried this rearing procedure forward by using larger prey (*Artemia* and spider crab zoeae). This resulted in increased survival and dry weight of paralarvae at one month. However, settlement was not attained during this experiment. Yet ultimately, Iglesias et al. (2004) described the first successful completion of the life cycle with a considerably higher survival rate (31.5%) at settlement (day 40) compared to previous experiments. Paralarvae ready for settlement attained 23 suckers per arm and dry weight of 9.5 mg. This result was attained via feeding paralarvae with live *Artemia* and spider crab zoeae, at a mean temperature of 18°C.

Since Iglesias et al. (2004) breakthrough, culture studies have been carried out in many regions of Spain (examples of rearing can be seen in table 3, Garrido et al., 2016; Iglesias et al., 2007) and various other countries (e.g. Japan, Italy and Brazil – Iglesias & Fuentes, 2014; Iglesias et al., 2007). Thus far, a standardised culture system and a rearing protocol have never been established. This issue prompted an international workshop at the Cephalopod International Advisory Council (CIAC) Symposium of Cephalopod Culture in 2012 (Florianópolis, Brazil), aiming to define the status and research priorities of four cultured cephalopods (including *O. vulgaris*). This included the reproduction, comparison of rearing techniques and identification mortality sources in paralarval culture (Vidal et al., 2014).

The most determinant factor regulating development and growth of *O. vulgaris* paralarvae (during all life stages) is temperature (Mangold & Boletzky, 1973). If the temperature is not suitable, the growth of paralarvae is disrupted and can result in poor development or even high mortality. Consequently, it is a significant bottleneck in *O. vulgaris* aquaculture. Studying the effects of temperature stress on paralarvae and acquiring knowledge on how it influences survival during the larval phase, represents a crucial step towards the advancement of the aquaculture of *O. vulgaris*. The developmental stages, growth/morphology, survival, and physical/chemical conditions (regarding temperature) of *O. vulgaris* rearing are discussed further in the following sub-chapters.

Table 1.1. Summary of various Spanish research groups rearing methodology and conditions of *Octopus vulgaris* paralarvae culture (adapted from Carrasco et al., 2006; Berger, 2010; Iglesias & Fuentes, 2014; Iglesias et al., 2004; Seixas et al., 2010 and Villanueva, 1995). *Note: TL, total length; l, light; Lx, Lux; n.p., not provided; ind.mL^{-1}/ind.L^{-1}, individuals per millilitre/litre; CEP, Centro de Experimentación Pesquera; IEO, Instituto Español de Oceanografia; ICM-CSIC, Instituto de Ciencias del Mar-Consejo Superior de Investigaciones Científicas and USC, Universidad de Santiago de Compostela.*

	Iglesias et al., 2004 (Galicia IEO)	**Seixas et al. (2010) Iglesias (Galicia USC)**	**Villanueva, 1995 (Catalonia ICM-CSIC)**	**Carrasco et al., 2006 (Asturias CEP)**
Tank volume (L)/colour/shape	1,000, black, cylindrical	50, white, conical	25-50, black, cylindrical parabolic	30, white, parabolic
Water System	First week stagnant, the semi-open (3-4h = 100% day^{-1})	10 % day−1	Open (flow rate 120/l/h)	Open (recirculation)
Paralarval density (ind.L^{-1})	5	10	13-48	25
Prey type, density (ind.mL^{-1}), size [(TL) (mm)]	Zoeae *Maja* (0.01-0.1) (when available) + *Artemia* (0.05-0.1); Zoeae: 1 mm, *Artemia*: 2–3 mm	Artemia juveniles (0.05); *Artemia*: 1.5–2.8m	Zoeae (*Liocarcinus* and *Pagurus)* + nauplii *Artemia* (2–6) and *Artemia* biomass; Zoeae: 1.3–3.1 mm, *Artemia* nauplii: 1–3 mm *Artemia* biomass	Zoeae Maja (0.7–1) + *Artemia* (3 times week−1) (0.5–0.7); Zoeae: 1, *Artemia* retained in 300 µm sieve
***Artemia* enrichment**	Reared in commercial cereal flour, enriched with *Nannochloropsis* (5 × 106 cells mL−1)	Reared with *Rhodomonas lens* and *Isochrysis galbana* and then enriched with different procedures	DCSuperSelco, Methionine	Reared and enriched with *Tetraselmis*
Light (photoperiod)	24h, 2 fluorescents 36 W 2,000 Lx	14 h L–10 h D Fluorescent daylight lamp	24h, bulb 60 W 900 Lx	12h l – 1h -D, 1 florescent 40 W
Temperature (ºC)	20-22	9–20	19-23	20-22
Aeration	Yes, intermediate	n.p.	No	Yes, gentle
Clean/green water (algae added to tank)	Green*, (Isochrysis + Nannochloropsis)*	Clear	Clear	Clear
Cleaning	No bottom cleaning until day 30	n.p.	Daily tank bottom syphoning	Every 20 days changing tank by pipetting and checking the survival
Sampling	Every 7 days	Days 15, 25 and 35	Every 7–10 days	Every 10 days
Survival (%)	31.5 (day 40)	35–53 (day 15), 7–20 (day 25)	54 (20 day) w/ *Artemia* nauplii (with poor growth) and 0.8 (day 60) w/ zoeae	89.6–93.5 (day 20) and 3.4 (day 60)

1.3.1. The embryonic phase

Embryonic development of *O. vulgaris* initiates instantly after eggs are laid by the females. The first study of embryonic development in cephalopods by Naef (1928), presented that metamorphosis could be categorised into 28 stages (I – XX "Naef stages" – Figure 1.3). This includes the formation of head, eyes, gills, buccal cavity, olfactory organs and more importantly the yolk sac (the endogenous food source) during incubation. Under the right conditions (i.e. sufficient temperature, oxygen concentration, pH, salinity, and light), the embryos will hatch directly into free-swimming individuals (a success rate of 80% in *situ* – Vidal et al., 2014).

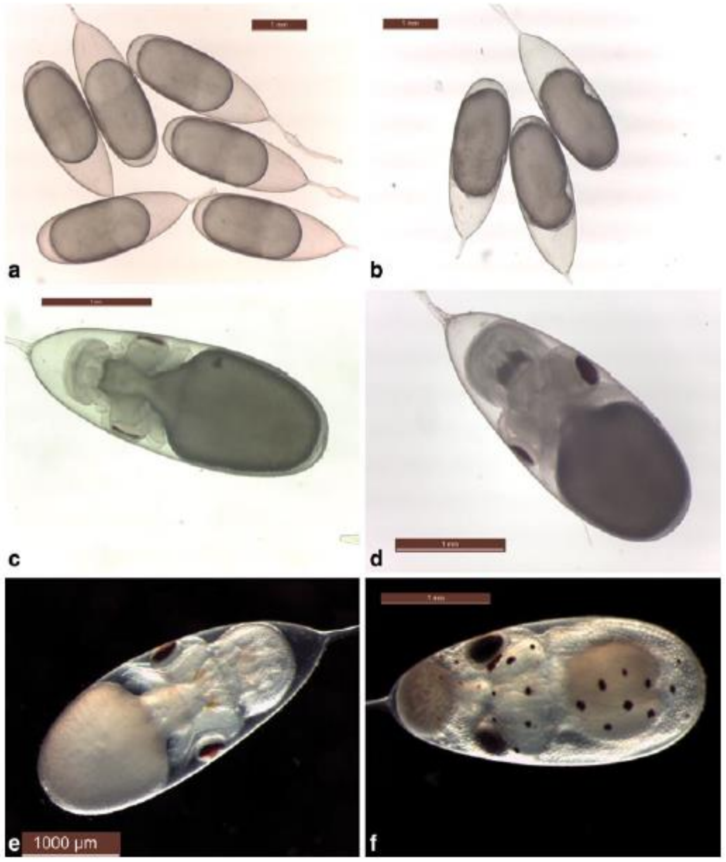


Figure 1.3. The embryonic development of *O. vulgaris* 18 ºC. a) Prior to first inversion (Naef IV–VI stage). b) Post first inversion (Naef VIII stage). c) Embryo differentiation (Naef XI stage). d) Naef XII–XIV stage. e) Chromatophores and internal yolk (Naef XV stage). f) Post second inversion (Naef XIX stage). Taken from Iglesias & Fuentes (2014). Photographs by M. Nande.

Transportation of eggs from the broodstock tanks (with the original female that spawned) should be performed with care before the second embryonic inversion occurs to the main incubation tanks (Naef XIX stage – f, Figure 1.3), as fluctuations in temperature will significantly affect embryonic development after this stage (Nande et al., 2016; Iglesias & Fuentes 2014; Vidal et al., 2014; Naef, 1928). If this is not executed, the eggs are in danger of premature hatching. Additionally, eggs should be incubated at a temperature in the range of the natural seawater temperature cycle of their geographical origin and maintained in plastic containers, filled with oxygen-supersaturated seawater (especially when the female is not present for maternal care - Iglesias & Fuentes, 2014; Vidal et al., 2014).

Temperature is one of the main factors influencing the embryonic development and regular hatching in cephalopods including in *O. vulgaris*. It controls the rate of metabolic processes and efficiency of yolk utilisation, which is directly linked to growth and weight during the hatching period (Vidal et al., 2014; Boletzky, 1987). Effectively, the embryonic duration time is temperature-dependent and thus, the period of hatching can be estimated if eggs are from a broodstock (Iglesias & Fuentes, 2014). According to Nande et al.'s (2016) study on the effects of temperature on energy demands, it is suggested that lower temperatures (i.e. 14°C) can be used during the first days (4 days) of embryonic development and during the last five Naef stages (XV-XX) as energy requirements are lower, demonstrated by low inner yolk utilisation (in controlled conditions). Additional factors such as the type of food fed to the broodstock, or the geographical location of species collection, may influence the accumulation of yolk reserves. Nevertheless, it is recommended that the temperature must be controlled for sufficient yolk reserves at the paralarval critical period, where the highest mortality rates occur (Uriarte et al., 2011; Villanueva, 1995).

Other physical parameters such as oxygen, light (i.e. intensity and photoperiod - influencing the hatching time), pH (indicating the production of ammonia or nitrate), salinity (controlling normal development and viable paralarvae) and nitrogenous waste (i.e. nitrite and ammonia, causing toxicity) must be monitored during organogenesis for a well-developed or non-premature culture of paralarvae. Prematurely hatched paralarvae can be identified by the large size of the inner yolk sac (reducing the total water volume entering the mantle), the arms within the outer yolk sac (should be absorbed by mature hatchlings) and the small body which all reduce their swimming performance. The latter will promote further problems during the

paralarval stage such as the abrasion of arms and tentacles against the walls of the tank (Vidal et al., 2002) and thus, reduce *O. vulgaris* paralarval survival (Vidal et al., 2014).

1.3.2. The paralarval phase

Growth and Morphometry. Monitoring the growth and morphometry of octopuses in their larval stage can be a good indicator of their welfare and survival both in wild and captive environments (Perales-Raya et al., 2014a). During the hatching period, paralarvae will actively swim into the water column and will require large quantities of prey and adequate nutritional quality to maintain their high metabolic rates. Higher growth rates will be promoted if young hatchlings are moved to cylindro-conical rearing tanks with volumes ranging from 500 to 1000L (Iglesias & Fuentes, 2014). Results from Sánchez et al. (2013) determined that tank size positively influences growth, attaining a higher dry weight in larger tanks (1000L = 1.73 ± 0.27 mg) than smaller tanks (100L = 1.44 ± 0.33 mg) of 1-month hatchlings, promoting higher growth rates. The dry weight of same-aged paralarvae in similar studies (Moxica et al., 2002; Viciano et al., 2011; Villanueva et al., 2002) displayed results akin to Sánchez et al. (2013). However, other variables in these experiments (i.e. water temperature fluctuations are greater in smaller tanks) may have promoted high survival rates, as hypothesised by De Wolf et al. (2011).

Changes in morphometry occur rapidly in *O. vulgaris* (and other octopods) due to the development of arms and growth of the mantle (Figure 1.5 and 1.6). However, net growth only occurs once all endogenous yolk reserves are depleted during the first days of planktonic life, as demonstrated in *Loligo opalescens* squid paralarvae (Vidal et al., 2002). A study on *O. vulgaris*' growth parameters (Nixon, 1971) highlighted the relationship between body weight and total length (TL)/dorsal mantle length (DML).

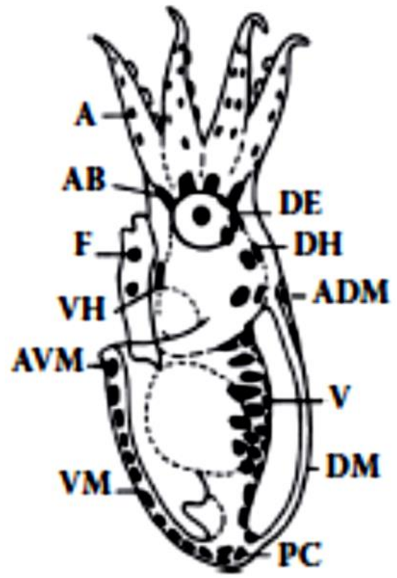
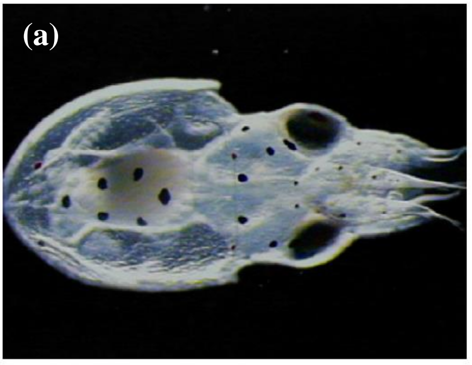


Figure 1.5. A left lateral view of the optical section in Octopodidae. A, arm; AB, arm base; ADM, anterior margin of dorsal mantle; AVM, anterior margin of ventral mantle; DE, dorsal eye; DH, dorsal head; DM, dorsal mantle; F, funnel; PC, posterior cap; V, visceral; VH, ventral head; VM, ventral mantle. Diagram from Villanueva & Norman (2008) and adapted from Hochberg et al. (1992).



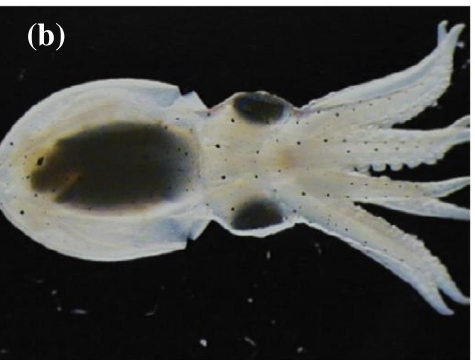


Figure 1.6. An *O. vulgaris* paralarva at (a) 0 days-old (1.5 mm DML, 3 suckers per arm) and (b) 52 days old (5.75 mm DML, 21 suckers per arm). From Iglesias et al. (2007).

It has been reported that the mantle length of newly hatched paralarvae ranges from 1.0 – 1.5 mm, 3 suckers per arm and a dry weight of 0.20 – 0.35 mg (Iglesias & Fuentes, 2014; Iglesias et al., 2007; Iglesias et al., 2004). As they are developing, their movement for foraging can still be limited as their skin is very delicate (a loose film layer) and sensitive to physical damage (Iglesias & Fuentes, 2014; Vidal et al., 2014; Villanueva & Norman, 2008).

Beak Structure. The beak of *Octopus vulgaris* is designed as a pair of sharp-edged chitinous jaws within the buccal cavity used in carnivorous feeding (Figure 1.7). From the embryonic stage, the beak of the individual grows by the secretion of tall columnar cells known as "beccublasts" in layers and expand from the rostral hood tip to the edges (Fernández-Gago et al., 2017; Dilly & Nixon, 1976; Perales-Raya et al., 2010).

The main interest in beaks is owing to their use in directly estimating age and growth. Beaks can be easily extracted and preserved, maintaining their calcified structures during freezing and storage in distilled water (Perales-Raya et al., 2010). However, they show a poor relationship with length and weight (Hernández-López et al., 2001; Nixon, 1973; Perales-Raya et al., 2014a;

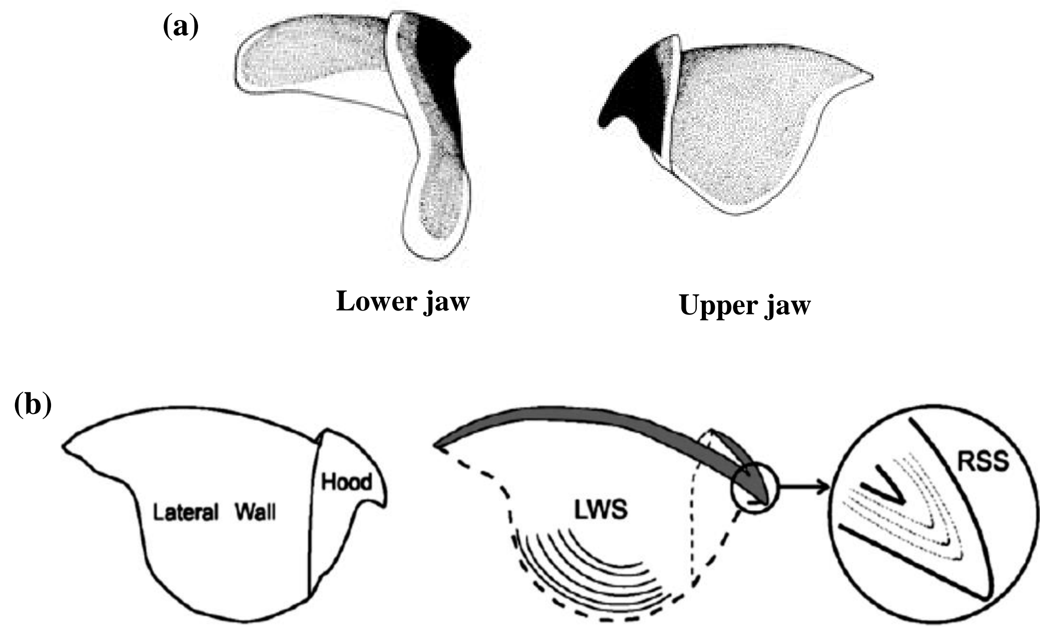


Figure 1.7. A diagram of (a) the lower jaw (LJ) and upper jaw (UJ) of *O. vulgaris* beak (FAO, 2016) and (b) The UJ lateral wall surface (LWS) and rostral hood containing the rostral sagittal section (RSS). From Perales-Raya et al. (2014b).

Perales-Raya et al., 2014b; Perales-Raya et al. 2010). Age can be observed in both the rostrum sagittal section (RSS) and lateral surface wall (LWS). It is also known that the first increment is deposited on the first day of hatching, with a 1 increment.day^{-1} deposition rate in paralarvae that are reared to 15 days (Franco-Santos et al., 2016). Furthermore, erosion can occur during the life of the individual, causing ageing bias during analysis (Perales-Raya et al., 2010).

Regarding the analysis of *O.* vulgaris paralarval beaks, the methodology for mounting has been homogenous, using water as an aqueous mounting medium, as well as cutting the UJ in half for improved mounting and clarity (Perales-Raya et al, 2010; Franco-Santos et al., 2016). Glycerol gelatine has not been considered as a slide mounting medium for *O. vulgaris,* although, it is used for histological slide mounting and available commercially (Olcott, 1960; Lyon, 1991; Spisni et al., 1998).

Moreover, beaks have been considered as biomarkers for stress in adult *O. vulgaris*, and more recently paralarvae. Stress (seen as "darker marks or checks") can be observed in the daily increments of RSS of the upper jaw (UJ) (Perales-Raya et al., 2014b; Franco-Santos et al., 2016). This may be comparable to stress reducing the brachial uptake of calcium in fish, which causes calcium-carbonate checks in the otolith structures. Campana (1983)'s study on salmon *Oncorhynchus kisutch* calcium deposition and otoliths checks in fish, supposed the intensity of the checks in were proportional to the strength and period of the stress. However, this hypothesis has only been supported in wild benthic *O. vulgaris* adults and cultured paralarvae up to 15 days old (Perales-Raya et al., 2014a). Previous studies have analysed the beaks of paralarvae submitted to various stress treatments (e.g. handling from tank transportation and siphoning, temperature, chemical etc.), with significant results of stress marks observed in the RSS via microstructure analysis (Franco-Santos et al., 2016; Perales-Raya et al., 2014a; Perales-Raya et al., 2014b). Beak microstructure increments can also be influenced by diet and light intensity (Hernández-López et al., 2001).

Environmental studies in other cephalopods have presented differences in increment width in alternate calcified structures. To add, various authors have displayed variability in increment width influenced by temperature and other variables that affect growth (Villanueva et al., 2003; Jackson & Moltschaniwskyj, 2001). Yet, no studies have endeavoured to correspond increment width with induced thermal shocks in *O. vulgaris,* which could potentially be used as a quantitative measure of temperature stress in paralarvae.

1.3.3. Rearing conditions for paralarval survival

Paralarval feeding and diet. For larval mortality, nutrition has been considered the highest priority for research in the development of *Octopus vulgaris* culture for the past decade (Iglesias et al., 2007; Villanueva et al., 2014). When an embryo hatches, the remaining endogenous yolk reserves are depleted and create space for the consumption of exogenous food sources (prey) to aid growth and development (Vidal et al., 2014; Uriarte et al., 2011). Vidal et al. (2002) suggest that this is parallel to the "critical period" in fish larvae, due to *O. vulgaris*' high metabolic rate. Even short periods of starvation or inadequate prey quantity and quality offered makes paralarvae susceptible to high mortality (Parra et al., 2000; Vidal et al., 2006).

Scarce information on the diet of *O. vulgaris* in the wild was available until the use of phylogenetics (i.e. PCR), which revealed they prey mainly on crustaceans (Iglesias & Fuentes, 2014; Roura et al., 2012). In the past seven years, extensive work on natural nutritional profiles of *O. vulgaris* ovaries, eggs and hatchlings during development has established the correct level of polyunsaturated fatty acids (PUFAs), amino acids, lipids, proteins, protein to lipid ratio, essential elements and vitamins for paralarvae diets (Villanueva et al., 2004; Villanueva & Bustamante, 2006; Berger, 2010; Reis et al., 2016). An adequate nutritional diet will permit high growth rates and survival, (Iglesias & Fuentes, 2014; Navarro and Villanueva, 2000; Prato et al., 2010; Vaz-Pires et al., 2004). Commonly, this is achieved by feeding paralarvae a mixture of enriched *Artemia* (fed on *Nannochloroposis* sp. and haptophyte *Isochrysis galbana*) and complimented with microalgae, live crustacean zoeae, copepods or low commercial fish flakes (Fuentes et al., 2011; Iglesias et al., 2007; Iglesias et al., 2004; Moxica et al., 2002; Villanueva et al., 2009). Still, crustacean larvae (i.e. *Maja squinado*, *P. serrifer* zoeae) are the prey preference for paralarvae which provide the correct balance of nourishment, but cannot be provided at a commercial level (Garrido et al. 2016; Iglesias et al., 2004).

Prey size. Prey size has been reported to influence paralarval growth and survival rates, which is supported by Fuentes et al. (2009) study on zooplankton and *Artemia* sp. diets. The paralarval growth rate was significantly higher based on a large (further developed) *Artemia* diet (1.5 mm total length - TL) fed in the second week of rearing experiment, compared to small *Artemia* diet (0.7 mm TL). This is due to the change in the nutrient composition of *Artemia* throughout their life stages, often meaning they must be enriched to be used as food for *O.*

vulgaris paralarvae (Seixas et al., 2008). From these findings, Iglesias & Fuentes, (2014) proposed that small (protein poor) *Artemia* between 0.5-0.7 mm length should be fed to paralarvae during the first 15 days and then increased in size to 1.5-2.0 mm length for experimental rearing. Additionally, a study on the effects of different feeding protocols by Iglesias et al. (2006) showed that *O. vulgaris* paralarvae have a feeding preference of large *Artemia* (from 1.4 ± 0.4 mm) over smaller *Artemia* (0.8 ± 0.1 mm). Accordingly, it would be assumed that as octopus paralarvae increase in size, so would their need to consume larger prey during their critical period. However, this depends on the nutritional composition of the prey being consumed and whether it is adequate for the paralarva. This study also advised that food should be given in multiple doses rather than in one big dose per day, as predatory behaviour is stimulated after first visual contact with prey (within the first 5 mins of feeding). This may be necessary as paralarvae display innate predatory behaviour and would allow all individuals to feed, thus, facilitates survival during culture.

Paralarval density. Densities of paralarvae must also be considered for determining rearing survival. Studies in Spain have used ranges from 5 to 48 individuals.L^{-1} (Iglesias et al., 2007, Carrasco et al., 2006; Villanueva, 1995), while Japanese studies have used paralarval densities as low as 3 ind.L^{-1} (Okumura et al., 2005). Yet, there is a lack of research into the effects paralarval density on growth and survival.

***Light*.** Light (i.e. type, intensity and photoperiod) influences egg hatching time. Natural and artificial light ranging in intensities from 300 to 2000 lux have been used in rearing studies, both contributing to settlement (Iglesias & Fuentes 2014; Iglesias et al., 2007). Octopus paralarvae present strong positive phototaxis to light in hatchlings, proposed to aid dispersal (due to surface water currents) or increase feeding on neustonic prey (i.e. crustacean zoeae) by Villanueva & Norman, 1998. This behaviour can be a tool for attracting individuals away from the walls and the bottom of the tanks, thus reducing mortality rates (Vidal et al., 2002). Still, higher surface light intensities and longer photoperiods are suggested for black tanks, in comparison to light-coloured tanks and black-walled/white-bottomed tanks (Iglesias & Fuentes, 2014; De Wolf et al., 2011).

***Water quality*.** Salinity, pH, oxygen (O_2), nitrite (NO_2), nitrate (NO_3) ammonia (NH_3) are the main chemical parameters influencing paralarvae culture. *O. vulgaris* is a strictly marine species,

living generally in salt concentrations between 32-40 PSU (Mangold, 1983). The effects of lower salinity are fatal, (Iglesias et al., 2016), where paralarvae ceased feeding at a PSU of 30 during the first day, while at 25 and 30 PSU food intake gradually reduced after 2 days. Paralarvae that survived recovered after salinity was stabilised to the optimum values. Accordingly, the recommended lower limit should be 20 PSU (Iglesias et al., 2016). Salinity is the only parameter to fluctuate in open culture systems, while in closed culture systems, all parameters can potentially fluctuate. pH changes can be kept constant with weekly water renewal in closed tank systems. Nevertheless, water is normally kept stagnant during the first week of culture, transitioning to an exchange of water for 4 $hr.L.day^{-1}$ (Iglesias & Fuentes, 2014; Iglesias et al., 2007; Viciano et al., 2011). Dissolved oxygen is a crucial parameter for survival and should be regulated to an optimal level of 6 – 8 $mg.L^{-1}$ (no less than 4 $mg.L^{-1}$). It is vital that enough oxygen is provided to paralarvae, as O_2 consumption increases two-fold after meal digestion (Cerezo Valverde & García García, 2004; Iglesias at al., 2014; Vaz-Pires, et al., 2004). Nitrite and ammonia levels must also be monitored, as accumulation can lead to lethal concentrations for newly hatched paralarvae. Feyjoo et al. (2011) identified after 24 hours the lethal concentration 50 (LC 50) that *O. vulgaris* paralarvae are less resistant to nitrate (LC 50 = 19.9 ppm) compared to fish larvae, yet resilient to free ammonia (LC 50 = 10.7 ppm). Conversely, low concentrations have been observed to impair paralarval chromatophore response and prey intake (Feyjoo et al., 2011).

Temperature. Temperature is considered the most important determinant of growth in all life stages of *Octopus vulgaris* (Iglesias & Fuentes, 2014; Mangold & Boletzky, 1973). *O. vulgaris* are poikilothermic organisms, adapting to the ambient temperature they are in (wild or captive) and so, their metabolism increases with rising temperature (Vidal et al. 2014). The planktonic period lasts around 47 – 54 days at 21.2°C and 30 – 35 days at 23°C, followed by settlement at the bottom of the water column for *O. vulgaris* paralarvae *sensu stricto* (Iglesias et al., 2007). Yet, the temperature determining the planktonic stages duration will change as *O. vulgaris* is cosmopolitan and its different geographical types (i.e. type I, II, III etc) are adapted to diverse temperature ranges depending on their location. Thus, it is important to select suitable rearing temperatures with this reason in mind.

As previously mentioned, temperature influences the hatching period. During the first days of a hatchlings life, temperature controls the efficiency of yolk absorption (decreasing

exponentially with increasing temperature - Vidal et al., 2014). Thus, transference of hatchlings from broodstock to paralarvae rearing tanks should gradually change in temperature (by +1ºC per day) and daily temperature fluctuations should be avoided. Furthermore, Villanueva (1995) proposed that temperature strongly influences paralarval settlement, which starts at a mantle length (ML) critical size of > 7.5 mm (regardless of age).

Various researchers have used different temperature ranges in paralarval rearing resulting in settlement (Berger 2010; Iglesias et al., 2004, Villanueva, 1995; Carrasco et al., 2003). Itami (1963) used a temperature range 23-26.7ºC (average 24.7°C) during its planktonic phase and reached settlement from day 35-45 (ML = 5.7 – 7mm). Whereas Villanueva (1995) used a culture temperature ranging from 19-23°C (average 21.2°C) to which settlement occurred from 47 to 52 days (ML = 6.55 – 7.5mm). Nevertheless, the survival rates of these studies were low: 8.9% survival at day 47 (Villanueva, 1995). Rearing temperatures of *O. vulgaris* (*sensu stricto*) above (> 23°C) have been shown to have limiting effects to paralarvae by disrupting feeding uptake efficiency in adults grown in captivity (Giménez & García, 2002). Still, this depends on the quality of the diet and food intake (i.e. smaller bogue-fed by individuals were more sensitive to temperature increases). Furthermore, laboratory studies on other cephalopods (*Sepia officianalis*, *O. bimaculoides, Loligo sp.*) have shown that temperature increases their growth considerably (Forsythe & Hanlon, 1988; Forsythe et al., 1994; Forsythe, 2004; Hatfield, 2000). Therefore, during rearing experiments, it is essential to select a natural temperature range to increase survival (Boyle, 1991; Forsythe, 2004). Iglesias & Fuentes (2014) recommend that a culture temperature between 20-22°C to obtain optimal growth and survival, however, reports of rearing temperatures as low as 16°C is plausible due to their natural temperature range in the sources populations physiological limits (Vidal et al, 2014). Vidal et al (2014), suggests if the purpose of culturing paralarvae is for human consumption, the temperature can be raised to the optimal range. This would shorten the growth period and therefore, enhance production.

2. Objectives

Revising the effect of temperature on paralarvae from previous studies, it is evident how detrimental temperature stress can be for *O. vulgaris* rearing success. Yet, there is a lack of knowledge on the effects of temperature stress in paralarvae (morphometry and beak microstructures), as only one study (Franco-Santos et al., 2016) has assessed transporation and siphoning stress in the rearing of this species for aquaculture. Additionally, alternative mounting techniques (besides the traditonal aqueous method – Perales-Raya et al., 2010) for microstructural analysis of beaks has not been discovered in approximately the past two decades.

Thus, it is necessary to investigate this topic; bridging the gaps in laboratory cultivation and advancing the practice of *O. vulgaris* aquaculture to commercial scale. Moreover, studying techniques and methods to assess temperature stress is vital to refining the welfare and fitness of this species in culture conditions.

Therefore, the objectives of this thesis (evaluating the effect of temperature stress on *O. vulgaris* paralarvae) are;

1. Appraise the general effect of various temperatures (14-21°C) and thermal shock (16 +3°C) on paralarvae via evaluating mortality and growth (via dry weight and morphometry).
2. Assess how temperature stress can be recognised in the calcified microstructures (via microincrement analysis of stress marks and increment width in the beak) of *O. vulgaris* paralarvae.
    - Increment width will be assessed in the RSS (or LWS) of beaks to determine if it corresponds with immediate temperature stress and its potential as a supplementary measure of paralarval rearing stress.

Furthermore, this study will review a new technique for observing beak increments (gelatine mounting) and its future use in microincrement analysis (i.e. ageing and assessing stress marks). The current study is a response to recent research trends indicated in Iglesias & Fuentes (2014) and Fiorito et al. (2015).

3. Materials and Methods

3.1. Paralarvae sample

O. vulgaris paralarvae were cultivated for this study between September and November 2015 in the facilities at ECIMAT Marine Station (Estación de Ciencias Marinas de Toralla), belonging to the University of Vigo (Vigo, Spain).

3.2. Thermal shock cultivation experiment

The experiment was performed in four small tanks (two replicate control tanks and two replicate thermal shock tanks - 50 L, 50 paralarvae per tank) containing seawater maintained at a standard temperature (16°C). After five days, the temperature of the thermal shock tanks was increased abruptly by three degrees (°C) for two hours on day 6. Subsequently, the temperature was reduced to the standard temperature by day 7 and maintained until the end of the experiment on day 10 (Table 3.1a). All parameters (nitrate, nitrite, oxygen, water renewal rate) were measured weekly and the temperature daily (Annex II). Mortality of paralarvae during cultivation was recorded (end-point = n.p.).

Table 3.1. Experimental design for thermal shock tanks containing *O. vulgaris* paralarvae in thermal shock experiment (a) and the tank conditions (b). *TSD* – thermal shock day.

(a)

Days	Temperature (experimental tanks)
Culture experiment initiated (08/09/2015)	
1-5	16°C
6 (TSD)	Risen three degrees (19°C) for 2 hours → returned to standard temperature
7-10	16°C
Culture experiment completed (02/10/15)	

(b)

	Rearing conditions
Diet/Feed	*Ad libitum* – *Artemia* sp. with *Rhodomonas*-enriched (0.5 per/ml) twice a day (12:00 & 19:00). Density: ± 500 *Artemia*/tank ± 100 mL.
Enrichment	*Rhodomonas* and *Isochrysis galbana* T-iso. ± 100 mL/day (50 mL in the morning and 50 mL in the afternoon).
Photoperiod	12:12 (light:dark hours)

3.3. Additional temperature experiments

To supplement the thermal shock experiment, two experiments were conducted with paralarvae cultured at increasing temperature and constant temperatures (see 3.3.1 and 3.3.2). As with the thermal shock experiment, all parameters (nitrate, nitrite, oxygen, water renewal rate) were measured weekly and the temperature was measured daily (Annex IV, V).

3.3.1. Cultivation at different constant temperatures

O. vulgaris eggs were collected from Ria de Vigo on 3rd October 2015 and incubated in ECIMAT from 6th October 2015. ~30 festoons of eggs were distributed between three seawater tanks at 13°C (with constant aeration) and hatched on 5th November 2015. For the constant temperature culture (cultivation protocol in Annex III), two replicate tanks (150 L, 300 paralarvae per tank) per temperature condition were maintained at 14, 16 18, 21°C for 15 days (Table 3.2a). Additionally, to the standard tank parameters, each tank's central mesh and phytoplankton drum were cleaned every three days.

Table 3.2. Experimental design for culture tanks containing *O. vulgaris* paralarvae for the constant temperature experiment (a) and the tank conditions (b).

(a)

Days	Temperature (Experimental Tanks)	No. of tanks
Culture experiment initiated (05/11/15)		
1-15[1]	14ºC	2
	16ºC*	2
	18ºC	2
	21ºC*	2
Culture experiment completed on Day 15 (20/11/15)		

[1]Heater strips for maintaining each temperature were located in a poor area within the tanks.
*On day 5 of the experiment the water intake was changed and the machine was removed for tanks at 16°C and 21°C. These tanks were connected to hot water intake (18ºC).

(b)

Rearing conditions	
Diet/Feed	*Ad libitum* – *Artemia* sp. with *Rhodomonas*-enriched two twice a day (12:00 & 18:00). Density: ± 0.1/mL
Enrichment	*Rhodomonas* and *Isochrysis galbana* T-iso added in two intakes (in the morning and afternoon). ± 16 L/day (or 1 million cells/mL)
Photoperiod	12:12 (light:dark hours)
Tank circuit type	Open – 4 renewals per day

3.3.2. Cultivation during increasing temperature

Four control tanks and four experimental tanks (50 L, 600 paralarvae per tank) were initially maintained at 16°C. On the sixth day after hatching, experimental tanks were subjected to a 1°C increase each day until day 9 and maintained at 20°C until the end of the experiment (Table 3.3).

Table 3.3. Experimental design for culture tanks containing *O. vulgaris* paralarvae for the increasing temperature experiment (a) and the tank conditions (b).

(a)

Days	Temperature (experimental tanks)
Culture experiment initiated (14/09/15)	
1-5	16ºC
6	17ºC
7	18ºC
8	19ºC
9-25	20ºC
Culture experiment completed on Day 25 (02/10/15)	

(b)

Rearing Conditions	
Diet/Feed	*Ad libitum – Artemia* sp. with *Rhodomonas*-enriched twice a day (12:00 & 19:00). Density: ± 0.2 *Artemia*/mL
Enrichment	*Rhodomonas* and *Isochrysis galbana* T-iso added in two intakes (in the morning and afternoon). ± 20L/day
Photoperiod	12:12 (light:dark hours)

3.4. Specimens for analysis

During the cultivation, paralarva specimens were collected for the analysis of dry weight, mortality, morphometry and beak microstructures (age, stress marks and beak increment width). Specimens were collected in 2015 and samples were misplaced/lost during the course of storage, thus fewer samples were available to analyse for the microincrement analysis (in 2017). As morphometry data was missing for some of the sampling days, full datasets for species (per day of collection) were used only, indicated in Table 3.4.

Table 3.4. The collection regime and analysis of *O. vulgaris* paralarvae specimens in temperature cultivation experiments (thermal shock, constant and increasing temperature). *n* = number of specimens used in total. *DAH* = days after hatching.

	Temperature Experiment		
	Thermal Shock	**Constant temperature**	**Increasing temperature**
Days of collection	0, 5, 10	0, 5, 10, 15	0, 5, 10, 15, 20
No. of specimens collected (per tank)	20	12	10

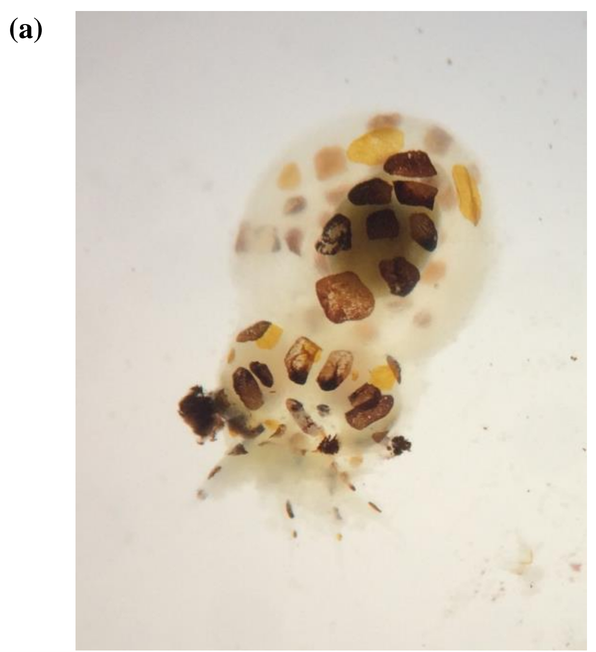
Analysis*	• Mortality (n = 40 – 20 per control or thermal shock condition])
	• Dry weight (n = 11)
	• Morphometry (paralarvae 10 DAH, n = 12 - 4 control and 8 thermal shock)
	• Beak microstructure analysis
	  ○ Age (n = 7)
	  ○ Beak increment width (n = 5)
		• Morphometry (paralarvae 5 DAH, n = 24)
		• Beak microstructure analysis
		  ○ Age (n = 27)
		  ○ Beak increment width (n = 21)
			• Morphometry (paralarvae 5, 10 & 15 DAH, n = 196)
			• Beak microstructure analysis (n = 53)
			  ○ Age

*All specimens were stored at -80°C in 0.5mL Eppendorf tubes (apart from live paralarva used for the dry weight analysis – anaesthetised using one drop of 70% alcohol in 10ml of water).

3.5. Beak microstructure analysis

All paralarvae specimens selected were thawed out (on ice), individually placed into a petri dish with distilled water and placed under an inverted microscope (Figure 3.1a). Using a thin precision needle and 0.15 mm forceps (Figure 3.1b), the upper jaw (of the beak) was extracted and cleaned in distilled water to remove any mucus.

(a)



(b)

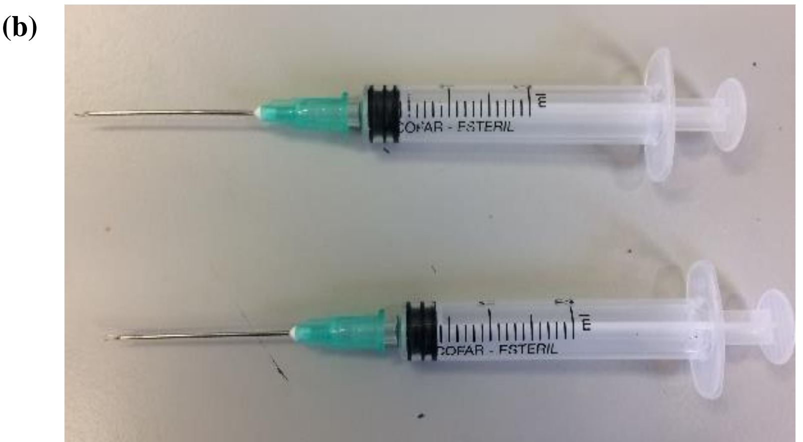


Figure 3.1. (a) A photo of an individual *O. vulgaris* paralarvae in distilled water for beak extraction (under the inverted microscope) and (b) the precision needles used to remove and clean the beak.

Due to the lack of high precision sectioning equipment, the UJs were not sectioned in half as described by Perales-Raya et al. (2014b, 2010). UJs of individuals were mounted for analysis by placing a small drop of gelatine (Figure 3.2a, protocol in Annex VI) on a glass slide and briefly melted using a hot-air gun (Figure 3.2b AOYUE 804 single hot-air gun heat, air flow – 6, heat - 4) beneath it. The beak was placed in the liquefied gelatine and gently pressed down with a glass coverslip and left for 5 minutes to set and secure the UJ (Figure 3.2c).

Figure 3.2. (a) Gelatine stored in a tube for mounting *O. vulgaris* paralarvae beaks, (b) AOYUE 804 single hot-air gun to melt the gelatine on the glass slide and (c) mounted beaks of sample specimens ready for microscopic observation.

Beaks were observed under a digital microscope (Nikon Eclipse Fluorescence 90i – Figure 3.3) with Nomarski Differential Interference Contrast (DIC) and photos were taken at the highest magnification possible for DIC (x40), using the Nikon NIS-Elements Basic Research 4.5 Microscope Imaging Software. Furthermore, photos of beaks that were not clear were recorded under a green filter to clarify the presence of increments.

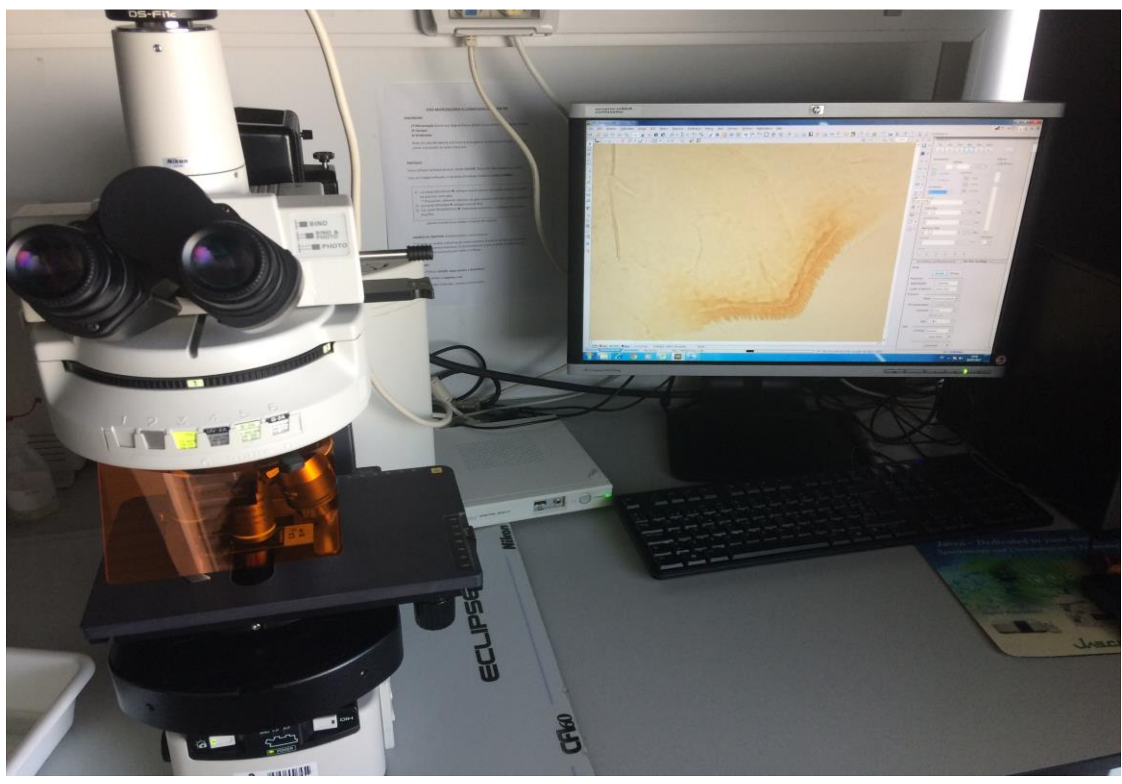


Figure 3.3. Nikon Eclipse Fluorescence 90i Microscope and NIS Elements used to take digital pictures of paralarvae beaks for the microstructure analysis.

Starting from the "last tooth" in the rostrum hood of the UJ (RSS) and at the anterior edge, increments were counted and recorded (Figure 3.4). For beaks of paralarvae specimens in the constant temperature and increasing temperature experiments, the LWS was counted (using a standard ageing methodology in Perales-Raya et al., 2010). Each beak was counted for each specimen on both halves of the RSS or LWS by the same reader. If the increments were unclear on one side, the reading was carried out twice on the clearer side for the coefficient of variation analysis. The coefficient of variation (CV) was calculated to determine the precision of reading the age of paralarvae (and the gelatine mounting technique) using the formula (Campana, 2001):

$$CV\ (\%) = \frac{100 \times \sqrt{(R1 - R)^2} + (R2 - R)^2}{R}$$

in which *R1* and *R2* are the first and second reading and *R* representing the mean number of increments. If the CV > 7.60% (recommended by Campana, 2001), individuals were discarded for the beak increment width analysis. Using the age count, the increment for the thermal shock day (day 6) was estimated and recorded for the presence of a stress mark (presented as darker lines compared to the majority). Other stress marks were also recorded to see if confounding stressors occurred.

Beaks of samples from the thermal shock and constant temperature were measured twice per increment for average increment width (µm) using Digimizer (Version 4.6.1) image analysis software (in Figure 3.4.).

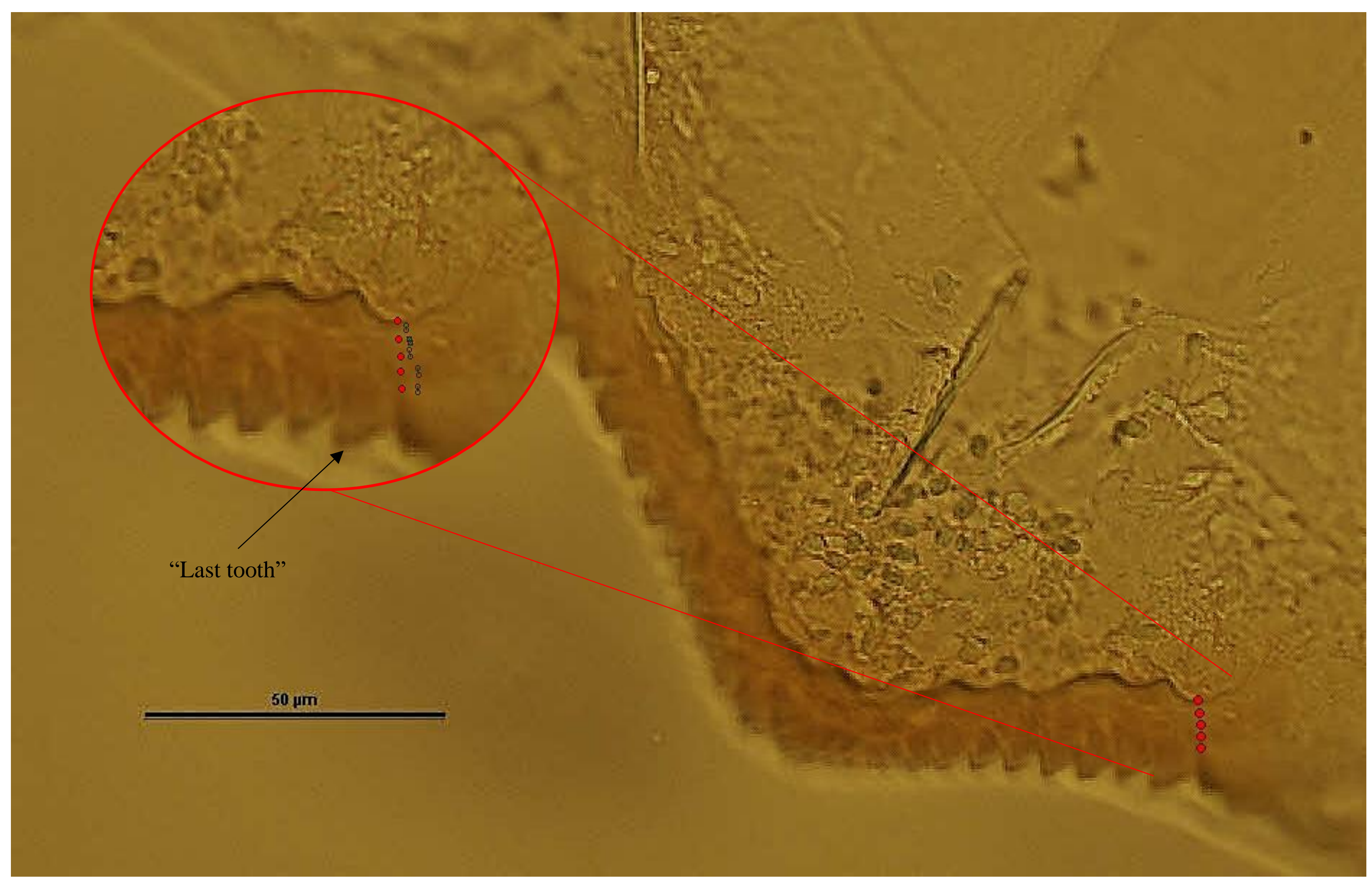


Figure 3.4. Example of beak microincrement analysis of RSS (ageing and increment width analysis) of a paralarva 5 days after hatching for the thermal shock experiment (under x 40 magnification and Nomarski DIC). Inset: Increment width measured on Digimizer photo analysis software (grey circles). Dots = beak increments.

3.6. Dry weight and morphometrical analysis

Dry weight. The dry weight of specimens in the thermal shock experiment (three specimens per condition per day[2]) was calculated by weighing aluminium cuvettes (for each specimen) three times after three desiccations in an oven at 110°C and recording the weights before and after (in mg). Next, each paralarva was anesthetised in 10mL of water with one drop of 70% alcohol and desiccated in an oven at 110°C for 30 minutes in a previously weighed aluminium cuvette (refer to Annex VII). All specimens were weighed three times and the weight of the paralarva was calculated as follows,

$$DW = \text{TW (PW + AW)} - \text{AW}$$

where *PW* is the weight of the paralarvae, *AW* is the weight of the aluminium paper, *TW* is the total weight and *DW* is the dry weight. An average dry weight (mg) was calculated for each experimental condition (for each sample collection day).

Morphological analysis. Paralarvae were measured for total length (TL – from to the anterior arms to the posterior), dorsal mantle length (DML – from the anterior of the mantle to the posterior), mantle width (MW) and head width (HW), seen in Figure 3.5.

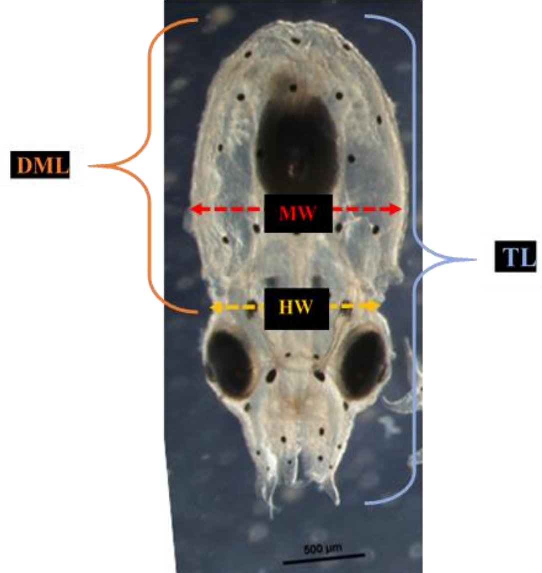


Figure 3.5. A schematic diagram of the body dimensions of an *O. vulgaris* paralarva specimen (from a tank maintained at 14°C during the constant temperature experiment). *DML - dorsal mantle length, TL – total length, MW - mantle width and HW- head width.*

[2] Apart from two specimens for thermally shocked paralarvae 10 DAH

3.7. Statistical analysis

RStudio (0.97.551) statistical software (with RCommander 2.3-0 statistical package) was used to analyse all experiments statistically ($\alpha = 0.05$). Data produced from all experiments were analysed for a normal distribution and homogeneity for variances (using Shapiro-Wilks test and Bartlett's/Levene's test – Shapiro et al., 1965; Bartlett, 1937; Levene, 1960).

For the thermal shock experiment the data for mortality, dry weight (due to missing data day 0 could not be statistically analysed), thermal shocks and mean increment width (on day 1–5, day 6 and day 6-10) were statistically analysed for differences between experimental groups via a t-test (or nonparametric Wilcoxon test - Kim, 2015; Wilcoxon, 1945). Due to a small sample size of data for paralarvae 5 DAH, only paralarvae 10 DAH were statistically analysed, comparing the control and thermal shock condition for mean increment width by a t-test (or ANOVA/ or nonparametric Kruskal-Wallis test for differences between days within an experimental condition - McDonald, 2014; Daniel, 1990).

Furthermore, for constant temperature experiment the data for morphometry and mean increment width were analysed using an ANOVA (or nonparametric Kruskal-Wallis test). If appropriate a post-hoc test was applied to the data to make pairwise comparisons between temperatures (Tukey's or Nemenyi's – Tukey, 1949; Nemenyi, 1963). Due to missing data on days 0, 10 and 15, paralarvae of this age could not be statistically analysed, comparing body dimensions between temperatures and days.

For increasing temperature experiment, data for morphometry was analysed using a t-test (or nonparametric Wilcoxon test) comparing body dimensions between the control and increasing temperature condition.

4. Results

4.1. Thermal shock experiment

Mortality. The mortality of paralarvae subjected to thermal shock (Figure 4.1) was significantly higher (*p = 0.03*) in paralarvae subjected thermal shock condition compared to the control group. A standard deviation for all days was not established due to missing data.

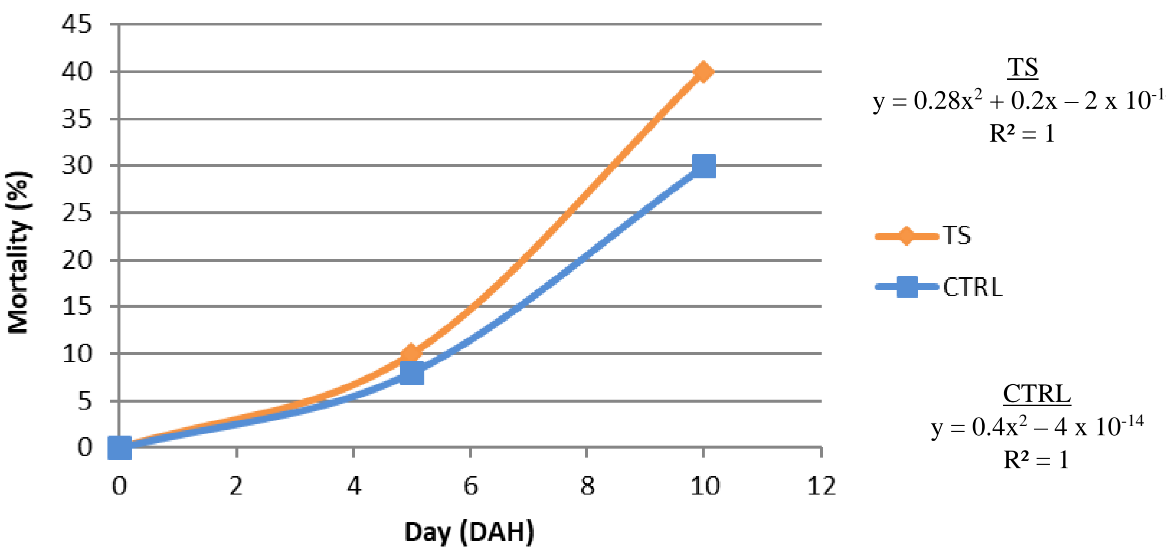


Figure 4.1. The mortality (%) of *O. vulgaris* paralarvae subjected to control (CTRL) and thermal shock (TS) conditions.

Dry weight and morphometrical analysis. The mean dry weight at hatching of both experimental groups was ~0.44 mg ± 0.30. The mean dry weight of paralarvae (5 and 10 DAH – days after hatching) subjected to the thermal shock treatment were not significantly higher than thermal shock conditions compared to the control group (5 DAH – *p = 0.40*, 10 DAH – *p = 0.12*).

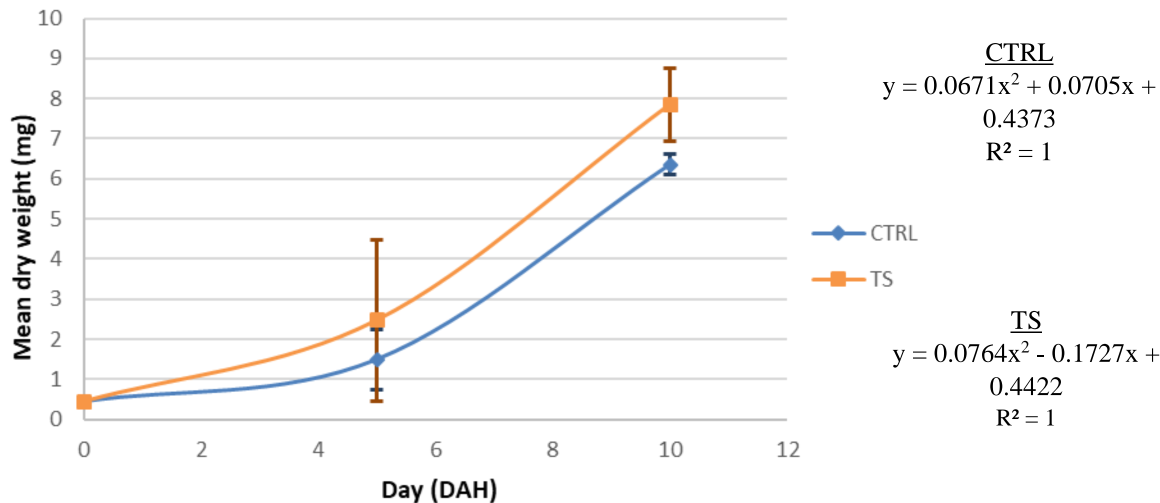
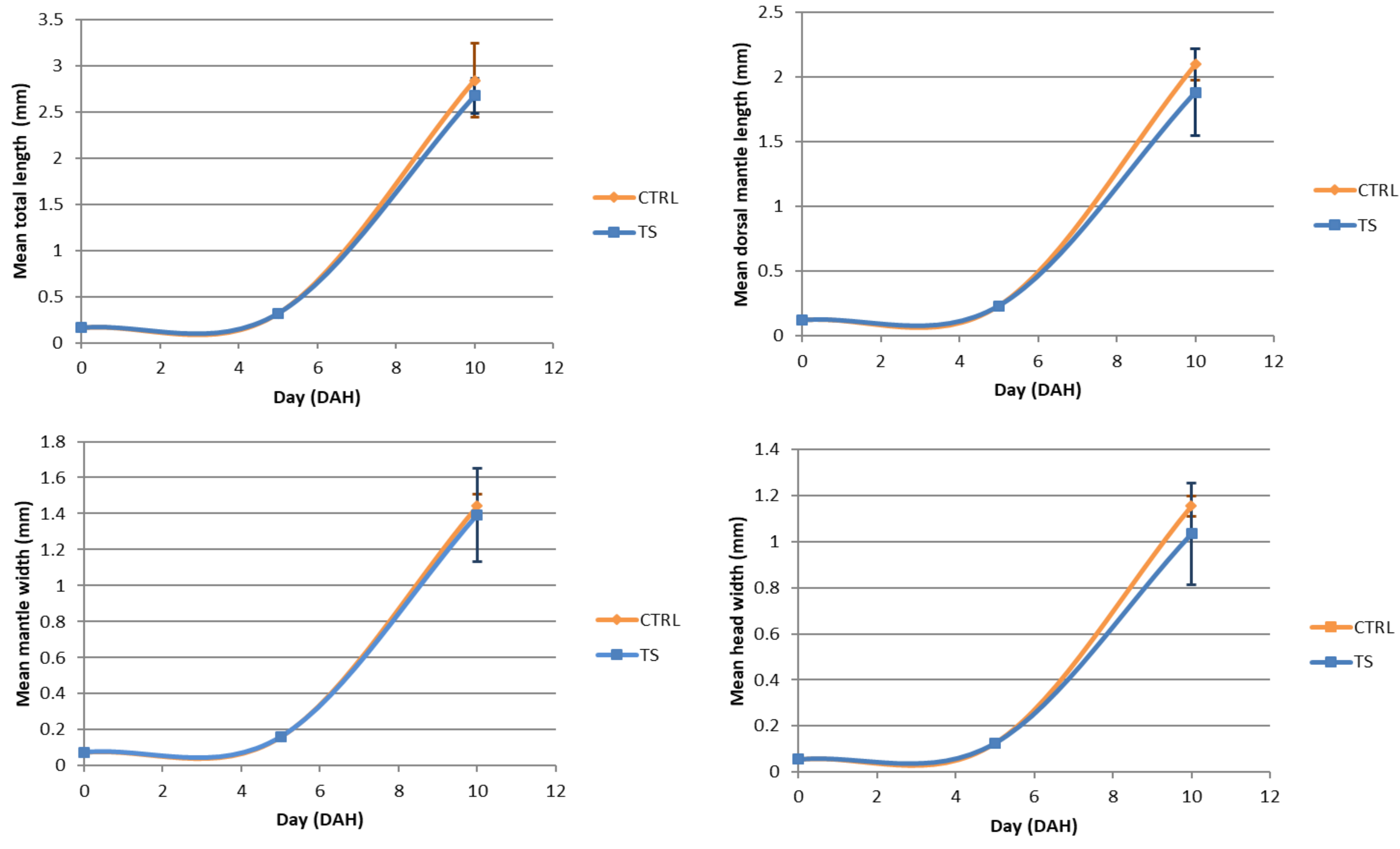


Figure 4.2. The mean dry weight (mg) of *O. vulgaris* paralarvae subjected to the control (CTRL) and thermal shock treatment (TS). *Note: due to missing data, the standard deviation for paralarvae 0 DAH could not be presented.*

Morphometrically, paralarvae 10 DAH in the control condition had a maximum TL of 3.10 mm and minimum of 2.52 mm, while paralarvae 10 DAH in the thermal shock condition had a maximum TL of 3.11 mm and a minimum of 2.15 mm. All body dimensions were not significantly higher for paralarvae 10 DAH subjected to the thermal shock treatment (seen in Table 4.1). Nevertheless, there was a general increase in all body dimensions along the course of the culture for both treatments (Figure 4.3).

Table 4.1. Mean body dimensions and significance of differences between *O. vulgaris* paralarvae 10 DAH cultivated in control and thermal shock conditions.

Mean body dimensions (mm)	CONTROL	TS	*p-value* ($\alpha = 0.05$)
TL	2.84 ± 0.19	2.67 ± 0.40	0.34
DML	2.10 ± 0.12	1.88 ± 0.35	0.28
MW	1.39 ± 0.07	1.44 ± 0.26	0.74
HW	1.15 ± 0.04	1.04 ± 0.22	0.57



<u>Figure 4.3.</u> Mean body dimensions of *O. vulgaris* paralarvae 0, 5 and 10 DAH (days after hatching) subjected to control and thermal shock conditions. (a) Total length, (b) dorsal mantle length, (c) mantle width and (d) head width in mm. *Note: due missing data on for paralarvae 0 and 5 DAH, the standard deviation for these days were not presented in this figure.*

Age (and age validation), stress marks and beak increment width. 85.71% of samples n = 7) were aged accurately from the RSS, apart from one control paralarvae 10 DAH (TS-2, Table 4.2). Mean CV was 1.06% ± 2.81% and the CV < 7.60%. No specimens paralarvae (10 DAH) were discarded for the beak increment width analysis. Day 6 stress marks were positive in the RSS of two specimens (10 DAH) from the thermal shock group and in one control sample, however, this result was not significant between groups ($p = 0.74$ - Table 4.3).

Table 4.2. Age precision (CV) of *O. vulgaris* paralarvae specimens in all groups from the thermal shock experiment. *TS – thermal shock*

Experimental Group	Real Age (DAH)	First Reading (R1)	Second Reading (R2)	Mean Increments (R)	CV (%)
Control-a	5	5	5	5	0
TS-b	5	5	5	5	0
Control-1	10	10	10	10	0
Control-2	10	10	10	10	0
TS-1	10	10	10	10	0
TS-2	10	9	10	10	7.44
TS-3	10	10	10	10	0

Table 4.3. Presence of stress marks on the day 6 (thermal shock) and additional stress marks in RSS of beaks from *O. vulgaris* paralarvae 10 DAH.

Group/Specimen	Thermal Shock Stress Mark on Day 6 (+/-)	Additional stress marks	Max/min increment width (µm)
Control-1	-	Increment 7 & 10	0.64 ± 0.22
Control-2	+	Increment 4 & 10	0.52 ± 0.13
TS-1	+	Day 10	0.81 ± 0.23
TS-2	+	Increment 7 &10	0.94 ± 0.25
TS-3	-	Day 10	0.70 ± 0.17

The mean of increment width of paralarvae in the control group was 0.58 ± 0.19 µm, while mean increment width of thermally shocked paralarvae 10 DAH was 0.82 ± 0.24 µm. Statistically, increment widths of paralarvae 10 DAH subjected to thermal shock were significantly different ($p = 3.4 \times 10^{-5}$) as well as for increments widths days 1-5 ($p = 0.0005 \times 10^{-5}$ Table 4.4. and 4.5.). Conversely, thermally shocked paralarvae 10 DAH mean increment widths between days 6-10 (Figure 4.4.) were not significantly higher ($p = 0.28$) as well as for

increment width of day 6 (*p = 0.16*) between thermal shock specimens and control specimens (Table 4.4 and 4.5.). Within groups, increment widths were not significantly different between increment days (*Control – p = 0.29, TS – p = 0.20*), thus, day 6 increment width was not significantly higher than increments width of other deposition days.

Table 4.4. Mean increment widths in the RSS of *O. vulgaris* beaks 10 DAH (by group and specimen) reared for the thermal shock experiment.

Group/Specimen	Mean Increment Width (µm)
Control-1	0.26 ± 0.15
Control-2	0.60 ± 0.09
TS-1	1.00 ± 0.49
TS-2	1.38 ± 0.21
TS-3	0.86 ± 0.09

Table 4.5. Analysis of mean increment width between experimental groups of the thermal shock experiment (and differences within groups) for paralarvae 10 DAH. *CTRL = control and TS = thermal shock.*

Analysis (Difference between groups /within groups)	Data Normality/Equal Variance ($\alpha = 0.05$)	Statistical Test	p-value ($\alpha = 0.05$)
CTRL vs. TS in mean increment width (paralarvae 10 DAH) for days 1-10	0.65/0.50	T-Test	3.4×10^{-5} (*t = -4.5417, df = 48*)
CTRL vs. TS in mean increment width (paralarvae 10 DAH) for days 1-5	0.20/0.51	T-Test	5.0×10^{-4} (*t = -4.0782, df = 23*)
CTRL vs. TS in mean increment width (10 DAH) for day 6	0.42/0.20	T-Test	0.16 (*t = -1.9411, df = 3*) SD =
CTRL vs. TS in mean increment width (10 DAH) for day 6-10	0.39/0.53	T-Test	0.28 (*t = -1.1013, df = 23*)
Mean increment width paralarvae (10 DAH) within CTRL conditions	0.90/0.88	ANOVA	0.29 (*Df = 9, Sum Sq. = 0.47, Mean Sq. = 0.053, f = 1.44*)
Mean increment width for paralarvae (10 DAH) within TS conditions	0.08/0.21	ANOVA	0.20 (*Df = 9, Sum Sq. = 0.62, Mean Sq. = 0.069, f = 1.64*)

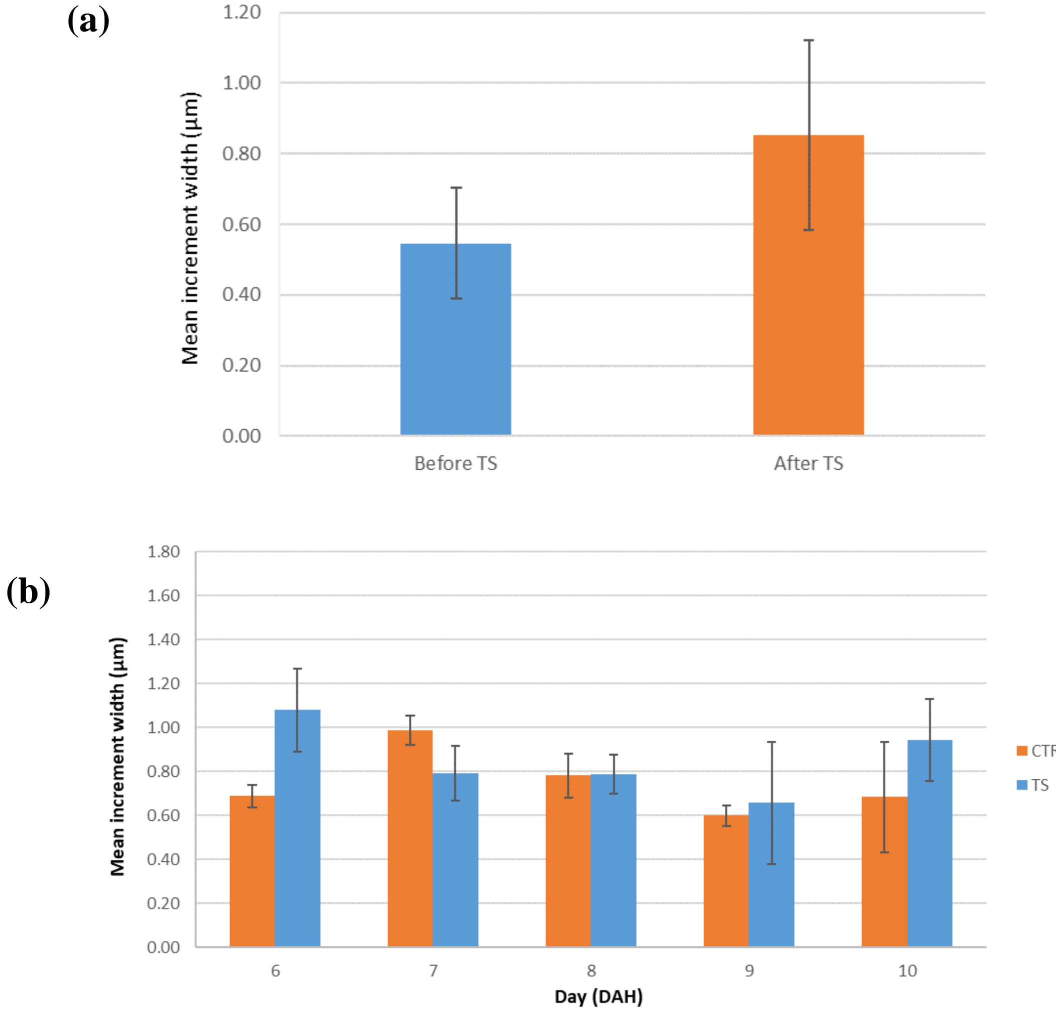


Figure 4.4. Mean increment width (µm) for paralarvae 10 DAH (a) in thermal shock conditions before and after thermal stress (b) between control and thermal shock conditions on day 6-10.

4.2. Additional temperature experiments

4.2.1. Cultivation at different constant temperatures

Morphometrical analysis. The max TL of paralarvae on the day of hatching was 3.01 mm and maximum ML was 1.50 mm. Mean TL was the highest for paralarvae cultivated at 16 (5 DAH), while mean DML. MW and HW were highest for paralarvae cultivated at 18 (5 DAH) (Table 4.6, Figure 4.5). Yet, the mean body dimensions for paralarvae 5 DAH were not significantly different between individuals in all experimental temperature groups (*p-values* in Table 4.6).

Table 4.6. Mean body dimensions and significance of differences between *O. vulgaris* paralarvae 5 DAH cultivated at different constant temperatures. *Note: due to missing data, the standard deviation of 14°C could not be displayed in this table.*

Experimental Temperature (°C)	**Mean body dimensions (mm)**			
	TL	**DML**	**MW**	**HW**
14°C	1.75	1.34	0.83	0.67
16°C*	2.94 ± 0.25	1.91 ± 0.19	1.30 ± 0.11	1.14 ± 0.07
18°C	2.87 ± 0.42	2.16 ± 0.40	1.46 ± 0.25	1.16 ± 0.13
21°C*	2.78 ± 0.39	2.10 ± 0.38	1.39 ± 0.25	1.12 ± 0.12
***Kruskal Wallis p-value* (*α = 0.05*)**	0.36	0.28	0.19	0.31

*Subjected to extraneous variables on day 5.

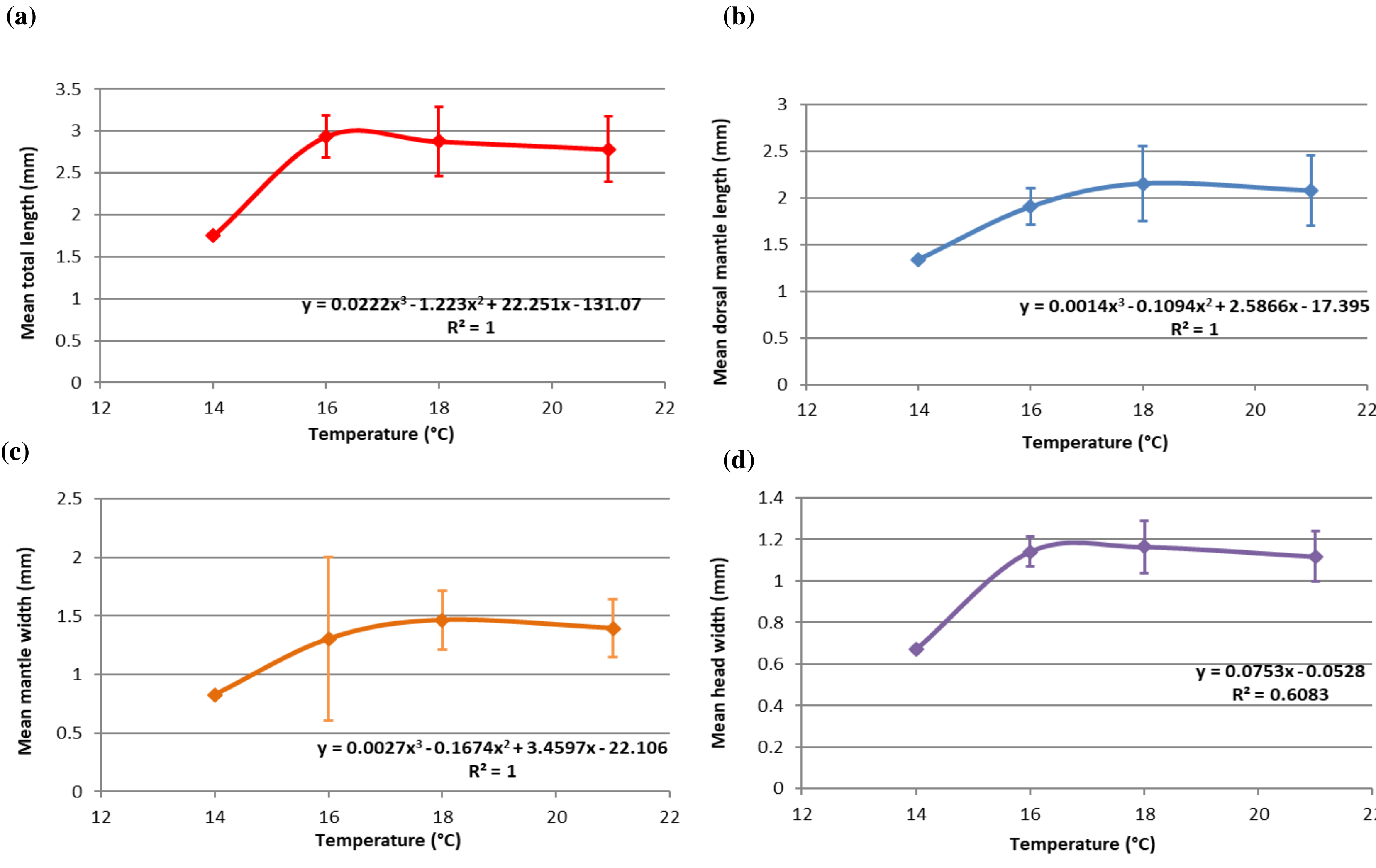


Figure 4.5. Mean body dimensions against increasing temperature (from *O. vulgaris* paralarvae 5 DAH cultivated at selected temperatures – 14, 16, 18 and 21°C). (a) total length, (b) dorsal mantle width, (c) mantle length and (d) head width. *Note: due to missing data, the standard deviation could not be displayed in this figure.*

Age and beak increment width. 81.48% were precisely aged from the LWS and a mean CV of 2.80% (σ = 6.01). Samples with a CV > 7.60% (Table 4.7) were discarded for the beak increment width analysis. No significant differences in mean increment width (Table 4.8) were found between experimental groups at constant temperatures ($p = 0.11$).

Table 4.7. *O. vulgaris* specimens with age validity > 7.60% CV for cultivation at a constant temperature (counting region – LWS) and discarded from the beak increment width analysis.

Experimental temperature (°C) /sample	Real Age (DAH)	First Reading (R1)	Second Reading (R2)	Mean Increments (R)	CV (%)
14-5	5	4	5	5	15.7
14-6	5	4	5	5	15.7
16-6	5	4	5	5	15.7
18-1	5	4	5	5	15.7
21-4	5	4	5	5	15.7

Table 4.8. Mean increment width of LWS in the beaks of *O. vulgaris* paralarvae 5 DAH and subjected to different cultivation temperatures.

Experimental Temperature (°C)	Mean Increment Width (µm)
14	0.53 ± 0.19
16	0.55 ± 0.19
18	0.62 ± 0.19
21	0.58 ± 0.18

4.2.2. Cultivation during increasing temperature

Morphometrical analysis. The max TL of paralarvae on the day of hatching was 3.07 mm and maximum ML was 1.19 mm. Mean TL and HW were the highest for paralarvae (10 DAH) cultivated in control conditions, while mean DML and HW were equal between paralarvae in the control and increasing temperature culture. Nevertheless, all mean body dimensions were not significantly different for paralarvae 15 DAH between experimental groups, observed in Table 4.9.

<u>Table 4.9.</u> Mean body dimensions and significance of differences between *O. vulgaris* paralarvae (a) 5 DAH, (b) 10 DAH and (c) 15 DAH cultivated in control and increasing temperature (IT) conditions.

(a)

Mean body dimensions (mm)	CONTROL	IT	*p-value (α = 0.05)*
TL	2.77 ± 0.22	2.60 ± 0.18	0.0003
DML	1.81 ± 0.20	1.63 ± 0.21	0.0002
MW	1.34 ± 0.08	1.18 ± 0.31	2.51×10^{-14}
HW	1.10 ± 0.11	0.98 ± 0.09	2.53×10^{-6}

(b)

Mean body dimensions (mm)	CONTROL	IT	*p-value (α = 0.05)*
TL	2.79 ± 0.20	3.32 ± 0.95	0.38
DML	1.87 ± 0.16	2.36 ± 0.66	0.68
MW	1.33 ± 0.09	1.57± 0.48	0.75
HW	1.13 ± 0.09	1.27± 0.40	0.18

(c)

Mean body dimensions (mm)	CONTROL	IT	*p-value (α = 0.05)*
TL	3.41 ± 0.16	3.36 ± 0.28	0.74
DML	2.36 ± 0.18	2.36 ± 0.21	0.99
MW	1.50 ± 0.02	1.26 ± 0.08	0.26
HW	1.32 ± 0.04	1.50 ± 0.09	0.87

<u>Age precision</u>. 94.34% of beaks were aged with the highest precision (CV = 0%, apart from specimens in table 4.10), with a mean CV of 0.52%.

<u>Table 4.10.</u> *O. vulgaris* specimens with age validity > 0% CV for cultivation at an increasing temperature (counting region – LWS)

Experimental Group	Real Age (DAH)	First Reading (R1)	Second Reading (R2)	Mean Increments (R)	CV (%)
Control	5	5	4	5	15.71
Control	20	18	20	19	7.44
Control	20	19	20	20	3.63

5. Discussion

5.1. Effects of temperature on mortality & growth

Mortality. As temperature is a central factor influencing growth, consequently affects the mortality of paralarvae during *O. vulgaris* aquaculture (Villanueva & Norman, 2008; Vaz-Pires et al., 2004, Vidal et al., 2002). In the current experiment, paralarvae subjected to the thermal shock (from 16°C to 19°C on day 6 for 2 hours) resulted in higher mortality, 10% more than the control group (16°C). This outcome suggests that abrupt increases in temperature for a short period could have mortal effects on *O. vulgaris* culture during this critical period in their life cycle, established by previous authors (Iglesias & Fuentes, 2014; Uriarte et al., 2011; Vidal et al., 2014). However, this also may be due to confounding variables during the rearing experiment (i.e. poor nutrition/diet), leading to low survival. Cultivation of paralarvae at 16°C is to some extent low in comparison to previous cultivation experiments with high survival rates (Carrasco et al., 2006; Iglesias et al., 2004; Villanueva, 1995 – see table 1.1). However, for this experiment, 16℃ as the control temperature was suitable for reducing mortality, as rearing temperatures should be kept close to that of the population's natural location (Atlantic Sea, NW Spain - 12-16℃, Vidal et al, 2014). Repeating the thermal shock cultivation at a higher temperature and subjected to an intense shock (more than a 3℃ increase) with controlled confounding variables, could result in a higher survival rate for paralarvae reared in the control conditions and increased mortality for paralarvae subjected to thermal shock.

Dry weight and morphometry. Dry weight (in the thermal shock experiment) and morphometry in this study represent the physical changes during the growth of paralarvae in all temperature experiments. In the case of the current study, the difference in mean dry weight between groups was not significant, potentially attributed to the small sample size or *O. vulgaris* proclivity as a species to have high individual variability in growth (Cuccu et al., 2013; Vidal et al., 2014). Alternatively, the nutritional composition of the diet fed to the thermal shock group could have been poor for individuals, thus, possessing a lower dry weight. Nonetheless, higher cultivation temperatures cause increased yolk absorption, food intake and metabolism, hence, growth (Mangold and Boletzky; 1973; Vidal et al., 2014). Sudden temperature changes, in theory, reduce food intake sufficient for development, influencing growth paralarvae. The weight at hatching for both the control and thermal shock group were ~56% below the expected weight

of *O. vulgaris* paralarvae at this age (1.0 – 1.4 mg - Nixon & Mangold, 1998). However, other factors prior to hatching can influence hatching size and growth (maternal body size, premature hatching – Vidal et al., 2014; Villanueva, 1995). By 5 DAH, both groups exceeded the expected dry weight, yet, this may be due to the method to obtain the dry weight of the paralarvae (refer to chapter 3.6.).

Body dimensions of paralarvae 10 DAH from the control and thermal shock rearing and paralarvae 5 DAH of the increasing temperature experiment, increased in size over the course of the culture. As for paralarvae cultivated at selected constant temperatures (5 DAH – Table 10, Figure 4.7.), the body dimensions increased over the course of rearing and higher for the control group compared to the increasing temperature conditions (15 DAH – Table 4.10). Yet, the difference between paralarvae in control and experimental groups for all temperature experiments were not significant. Generally, it is known small increases in temperature can exceedingly rise somatic growth in cephalopods, especially in *O. vulgari*s (Villanueva, 1995). Accordingly, this outcome may be attributed to confounding stressors influencing the growth of paralarvae (i.e. in tanks at 16°C and 21°C for the constant temperature experiment and increment tanks for the increasing temperature), or as mentioned, the small sample size analysed. *O. vulgaris* as a species is known to be small at hatching (TL = 2.9 mm and ML = 2 mm - Boletzky, 1987; Nixon & Mangold, 1998). The TL and ML of paralarvae at hatching in the thermal shock, constant and increasing temperature experiments were above the expected size. This supports that notion that paralarvae were exempt from any major incubation stress during the embryonic stage and have not hatched prematurely evading complications in growth and development (Iglesias & Fuentes, 2016).

5.2. Evaluating temperature stress from beak microstructures

5.2.1. Stress marks

To determine the location of the induced stress mark, the daily increment deposition rate must be determined. The age of paralarvae from the thermal shock experiment corresponded to the number of increments counted and thus a 1 increment.day^{-1} deposition rate has been presented. Previous studies analysing the beaks of known-age specimens (particularly adults) have confirmed the same deposition rate for *O. vulgaris* regardless of stress and validated for

their full ontogenetic range (Hernández-López et al., 2001; Perales-Raya et al., 2014b). Franco-Santos et al., (2016)'s study on handling stress in paralarvae observed the first increment coincided with the first day of the experiment, thus validating the daily increment deposition rate. Collectively, these authors outcomes support the same case in the current study, as a one daily increment deposition rate coincided with stress marks made on day 6 for two (of three) positive specimens in the thermal shock conditions. Regarding temperature stress, Perales-Raya et al., (2014a) corresponded dates of high ΔT with the location of stress marks in wild specimens subjected to abrupt temperature fluctuations in the Central East Atlantic. Similarly, Hamasaki & Morioka (2002)'s study of temperature effects on *O. vulgaris* (type IV) paralarvae presented a 1 increment.day^{-1} deposition rate at the optimal temperature for growth, 21°C, and at < 1 increment.day^{-1} at the colder temperature of 14°C. Temperature stress above (≥) 16°C presumably does not affect the daily increment deposition rate in *O. vulgaris* paralarvae (as observed in Franco-Santos et al., 2016). As the current study's thermal shock experiment occurred at 16°C and thermal shock administered was 19°C (and ≥ 16°C for constant and increasing temperature experiments), can presume the daily increment rate would not be affected.

Stress marks were positive in two (out of three) of the UJ of beaks extracted from paralarvae subjected to thermal shock on day 6 of the culture, but not significant compared to the control beaks, resembling results presented in Franco-Santos et al. (2016) study. Stress marks were also spotted on other days during the culture, particularly day 10 (the final day of the experiment and removal of paralarvae from the culture tanks). Thus, confounding variables (uncontrolled stressors) could have induced the additional stress marks present (e.g. tank removal stress, switching from endogenous to exogenous feeding, etc. – Perales-Raya et al., 2014b). On another note, Franco-Santos et al., (2016) observed that stress mark deposition may not occur precisely at the instant of stress (in this case thermal shock). Rather they can deposit after the thermal shock "post-stress marks", i.e. increment 7 was presented in one paralarva (out of three) subjected to the thermal shock (underlined in Table 4.3.). The effects of water quality parameters (i.e. salinity, nitrates and nitrites) on hard structures are unknown in cephalopods. Still, since they were controlled to the expected range in water of NW Spain (Annex II), it is believed they would not affect the increment deposition or induce stress marks (Franco-Santos et al., 2016; Villanueva et al., 2003).

The paralarvae specimen with no day 6 stress mark present suggests that some paralarvae may be able to adapt to environmental changes and manage stress (in this case, abrupt temperature increases) better than other individuals. This also occurs in other benthic marine invertebrates (Pechenik, 1999). The high individual variability in growth and development for *O. vulgaris* (Cuccu et al., 2013; Vidal et al., 2014) includes the daily increment deposition and the stress marks recorded, influenced by temperature. The result resembles previous studies by Canali et al. (2011) and Perales-Raya et al. (2014a). Canali et al. (2011)'s study on the assessment of induced thermal markings in *O. vulgaris* beaks found 79% of specimens were not positive for stress marks in their beaks. The reasoning was unknown but was conceived to be either variance in response to thermal shock within the population, the difference in the body size between individuals in the experiment groups or experimental methods. The outcome of this current study stresses the importance of removing other variables (i.e. diet, human disturbance etc.) during rearing. Moreover, validating these findings would require analysing a larger sample size to determine the individual variability in stress response to sudden temperature increases.

5.2.2. Increment Width

Generally, mean increment width was expressively higher in the thermally shocked paralarvae 10 DAH in comparison to the control group as for comparing increments corresponding to days between 1-5. The paralarval groups possessing different mean increment widths at the beginning of cultivation could be attributed to the small sample size analysed, causing coincidental differences (from individual variation in growth and development - Canali et al., 2011; Cuccu et al., 2013; Vidal et al., 2014).

The opposite result was discovered for day 6 (thermal sock day) and days 6-10 increments (parallel to the thermal shock day and post-thermal shock days), in which the effect could be recorded in the beaks. The non-significant result in this study, could potentially be a domino effect of the small sample size used to analyse increment width as previously stated. Alternatively, this could be due to low variation in increment width in the RSS during the growth of the first 20 increments (observed in Perales-Raya et al., 2010) or that paralarvae were in a state of starvation due to the quality of diet fed (Vidal et al., 2006).

Therefore, it cannot be established that temperature stress can cause a general increase in increment width between 6-10 and day 6 increment width at this point. If a suitable sample size

was compiled and analysed, a positive result of significantly higher mean increment width for day 6, only then could increment width be considered as a quantitive tool for measuring temperature stress. Moreover, the outcome of the constant temperature experiment presented no significant differences in mean increment widths between temperatures. As mentioned before, high variability in this sample, low sample size or starvation from an insufficient diet may have attributed to this result. Nonetheless, it is necessary to repeat these experiments to compile concise results on temperature stress regarding increment width.

The relationship between beak increment width and the number of increments has been investigated in *O. vulgaris* adults from natural populations in Mauritian waters, presenting an average width of ~20 µm (LWS) in the first 10 increments (Raya & Hernández-González, 1998). Meanwhile, Perales et al. (2010) showed averages of ~85 µm LWS and counting along the dorsal region of the RSS resulted in a positive increase in increment width, with a mean of 6 µm observed in the first 10 increments. The current study did not analyse post-settlement and adult octopus to create a correlation between the number of increments and increment width. Still, maximum increment width in the RSS (1.4 µm) and LWS (1 µm) for paralarvae at constant temperatures were relatively low compared to previous studies on counting increment widths in the LWS and RSS. This result may be since the first stages are vital for the growth and development of *O. vulgaris* individuals. If the diet fed to paralarvae is of low nutritional quality no growth will occur, including the deposition of the beak (Iglesias & Fuentes, 2014; Iglesias et al., 2007; Navarro et al., 2014; Navarro & Villanueva et al., 2000). On the other hand, this difference may be owed to the size of *O. vulgaris* beaks during the paralarval stage in contrast to the authors analysing well-developed beaks of wild adult specimens.

5.2.3. Beak mounting and ageing

During the preparation of slides, splitting the UJ of beaks in half was not possible due to the lack of high precision equipment. Gelatine is viscous when liquefied, allowing the beak to be stationary on glass slides compared to water. Consequently, using gelatine as a medium allowed flattening of the UJ to be viewed clearly on the microscope and readjustment of beaks if needed by gentle reheating. Since beaks are very delicate, this technique can be used by inexperienced readers and permits mounting of beaks with minimal damage. Hence, this could be a novel technique for beak increment visualisation in *O. vulgaris* in the future.

The RSS of paralarvae beaks from the thermal shock experiment were successfully aged, with only one beak owning a CV > 0% (lower than the CV discard percentage – Campana, 2001). Aged beaks of paralarvae from the constant temperature and increasing temperature experiments (via the LWS) also possessed an average CV lower than the CV discard percentage. This presents the potential of gelatine as an aqueous mounting medium. However, for this to be a validated, this technique must be tried on known-age specimen beaks, including paralarvae more than 10 DAH. Additionally, the beaks of paralarvae could be blind tested by a reader to increase precision.

From the findings of this study, the best choice for age, stress mark and increment width analysis for evaluating temperature stress is via the RSS. If a stress mark analysis is not required, the LWS can be an alternative counting region of choice (Perales-Raya et al., 2010).

5.3. Limitations

Mortality and sample size. A major recurrent issue in this study is the size of sample available to analyse temperature effects. Temperature cultivation experiments of *O. vulgaris* paralarvae are limited in numerous ways. One reason being that rearing paralarvae in captivity are still susceptible to mortality, not triggered by thermal shocks or the rearing temperature at which the paralarvae are cultivated. Survival of paralarvae was lower than expected for the constant experiment (particularly in tanks of 16°C and 21°C) and therefore the sample size for analysis was less than expected. The consequence of mortality is the reduction of experimental specimens that can be collected for analysis. To extrapolate these findings for a whole species would be implausible, as *O. vulgaris* is known for high plasticity and intra-species variability in growth and development (Canali et al., 2011). In future, it is essential that this study is repeated to collate a database for counts of increments, positive stress marks and increment widths in the RSS. Yet, this can be time-consuming and is reliant on a large quantity and number eggs retrieved from the wild population for reduced, though, not impossible (Vidal et al., 2014). This will enable reputable results to be produced for a clearer understanding of temperature stress in this species.

Temporal application of stress mark and increment width analysis in paralarvae. *The* planktonic stage of *O. vulgaris* starts from the day of hatching and ends at settlement (between 65

– 75 days at 20°C and < 65 days below 20°C depending on the temperature – Iglesias & Fuentes, 2014). The effects of thermal shock and temperature stress in the later period of the paralarvae stage can still disrupt growth and development. Yet, only < 30% of the paralarval stage has been accounted for stress marks as the present study analysed paralarvae 10 DAH, whereas Franco-Santos et al. (2016) analysed paralarvae 15 DAH. Former studies have presented positive stress marks in adult beaks and statoliths of *O. vulgaris* and other cephalopods (Arkhipkin, 1995; Perales-Raya et al., 2014a), thus, thermal stress is recorded in the later stages of *Octopus vulgaris* life. As for increment width, further temperature experiments must be carried out on the RSS and LWS on paralarvae > 10 DAH after hatching to comprehend the temporal range of this analysis. Nevertheless, it can be supposed that beaks can be used as biomarkers for temperature stress of *O. vulgaris* in the paralarvae and benthic adult stage of their life cycle.

Confounding variable (stressors) in tanks and individual growth variability. Numerous authors have mentioned that high sensitivity of *O. vulgaris* (and other cephalopods) to temperature stress during the early stages of development, in addition to handling, cleaning of tanks and water parameters (Arkhipkin, 1995; Iglesias & Fuentes, 2014; Uriarte et al., 2012; Vaz-Pires at al., 2004; Vidal et al., 2002). During the constant temperature experiments, tanks for cultivation at 16°C and 21ºC were subjected to multiple stressors, for example, problems with the tank heater and the movement of equipment in tanks. For future studies, it is advisable that successful temperature stress experiments should be clear of major confounding stress to avoid discrepancies in the results.

Increment visualisation. Increments in the LWS and RSS were not visualised with ease, as beaks of *O. vulgaris* paralarvae are not as well developed at the developmental stage. There was difficulty in focusing on all increments simultaneously and the undissolved mounting gel caused issues with clarity during the readings. To solve this issue, numerous photos were taken at different depths of field and filters (green channel) to contrast increments and increase age precision. However, it is advisable to prepare beaks with delicacy to enable high-precision ageing in *O. vulgaris* paralarvae.

6. Conclusions

Increment deposition was recognised to be daily in the RSS and LWS, enabling thermal shocks to correspond with the age/increment count. The precision of counting was above the recommended CV%, indicating that gelatine beak mounting has the potential to be a novel method for visualising increments in the RSS (and LWS). Stress marks were present in paralarvae from both conditions in thermal shock experiment, however, stress marks were not significant in thermally shocked paralarvae. Then again, not all paralarvae may register a stress mark due to individual variability, high adaptability to stress or confounding stressors during paralarval or embryonic development. Furthermore, stress marks may not be registered at the precise time of thermal shock, rather, hypothetically recording as “post-stress marks”. A confident outcome presenting increment width increases in the RSS from induced thermal shocks and LWS with increasing temperature has not been established at this point. Thus, it is necessary to repeat this study by means of removing confounding variables and compiling further data with a larger sample size. Only then can increment width can be considered as a quantitative measure of temperature stress in *O. vulgaris* rearing.

In conclusion, evaluating temperature stress is a required process for improving the rearing success and welfare of *O. vulgaris* paralarvae. This study has highlighted the major drawbacks in rearing experiments, gaps in research methods and fractional knowledge on using beaks as tools for thermal stress in *O. vulgaris* culture. To progress the development of *O. vulgaris* paralarvae aquaculture for commercial circumstances, standard temperature stress assessments (i.e. in calcified microstructures and morphometric analysis) in combination with biomarkers of growth and physiological stress. This approach would require examining heat shock protein concentrations (HSP70 in *O. vulgaris*) and determining the RNA/DNA ratio to assess growth and the scale of coping with thermal stress in *O. vulgaris* (Garrido et al., 2017; Ramos et al., 2015; Zhang et al. 2012). Moreover, *O. vulgaris* paralarvae behavioural response to temperature stress (i.e. motility, movement activity and feeding behaviour) should be evaluated for potential as a rapid assessment tool of rearing stress.

7. References

Arkhipkin, A. (1995). Age, Growth and Maturation of the European Squid *Loligo vulgaris* (Myopsida, Loliginidae) on the West Saharan Shelf. *Journal of the Marine Biological Association of the United Kingdom*, *75*(3), 593–604.

Bartlett, M. S. (1937). Properties of Sufficiency and Statistical Tests. *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences*, *160*(901), 268–282. https://doi.org/10.1098/rspa.1937.0109

Berger, E. (2010). Aquaculture of Octopus species: present status, problems and perspectives. *The Plymouth Student Scientist*, *4*(1), 384–399.

Boidron-Métairon, I. F. (1995). Larval nutrition. In L. McEdward (Ed.), *Ecology of Marine Invertebrate Larvae* (pp. 223–248). CRC Press, Boca Raton, FL.

Boletzky, S., & Hanlon, R. T. (1983). A review of the laboratory maintenance, rearing and culture of cephalopod molluscs. *Memoirs of the National Museum Victoria*, *44*, 147–187.

Boletzky, S. V. (1987). Embryonic phase (cephalopods). In P. R. Boyle (Ed.), *Cephalopod Life Cycles. Volume 2. Comparative Studies* (Vol. 31, pp. 5–31).

Boyle, P. R. (1991). *The UFAW handbook on the care and management of cephalopods in the laboratory.* Potters Bar: Universities Federation for Animal Welfare.

Campana, S. (2001). Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *Journal of Fish Biology*, *59*(2), 197–242.

Campana, S. E. (1983). Calcium deposition and otolith check formation during periods of stress in coho salmon, *Oncorhynchus kisutch*. *Comparative Biochemistry and Physiology Part A: Physiology*, *75*(2), 215–220.

Canali, E., Ponte, G., Belcari, P., Rocha, F., & Fiorito, G. (2011). Evaluating age in Octopus vulgaris: Estimation, validation and seasonal differences. *Marine Ecology Progress Series*, *441*, 141–149.

Carrasco, J. F., Arronte, J. C., & Rodríguez, C. (2006). Paralarval rearing of the common octopus, *Octopus vulgaris* (Cuvier). *Aquaculture Research*, *37*(15), 1601–1605.

Carrasco, J. F., Rodríguez, C., & Rodríguez, M. (2003). Cultivo intensivo de paralarvas de pulpo (*Octopus vulgaris*, Cuvier 1797) utilizando como base de la alimentación zoeas vivas de crustáceos. In *IX Congreso Nacional de Acuicultura, Cádiz* (pp. 12–16).

Cerezo Valverde, J., & García García, B. (2004). Influence of body weight and temperature on post-prandial oxygen consumption of common octopus (*Octopus vulgaris*). *Aquaculture*, *233*(1), 599–613.

Cuccu, D., Mereu, M., Cau, A., Pesci, P., & Cau, A. (2013). Reproductive development versus estimated age and size in a wild Mediterranean population of *Octopus vulgaris* (Cephalopoda: Octopodidae). *Journal of the Marine Biological Association of the United Kingdom*, *93*(3), 843–849.

Daniel, W. W. (1990). Kruskal-Wallis one-way analysis of variance by ranks. *Applied Nonparametric Statistics,* 226–230.

De Wolf, T., Lenzi, S., & Lenzi, F. (2011). Paralarval rearing of *Octopus vulgaris* (Cuvier) in Tuscany, Italy. *Aquaculture Research*, *42*(9), 1406–1414.

Dilly, P. N., & Nixon, M. (1976). The cells that secrete the beaks in octopods and squids (Mollusca, Cephalopoda). *Cell and Tissue Research*, *167*(2), 229–241.

Fernández-Gago, R., Heß, M., Gensler, H., & Rocha, F. (2017). 3D reconstruction of the digestive system in *Octopus vulgaris* Cuvier, 1797 embryos and paralarvae during the first month of life. *Frontiers in Physiology*, *8*, 1–11.

Feyjoo, P., Riera, R., Felipe, B. C., Skalli, A., & Almansa, E. (2011). Tolerance response to ammonia and nitrite in hatchlings paralarvae of *Octopus vulgaris* and its toxic effects on prey consumption rate and chromatophores activity. *Aquaculture International*, *19*(1), 193–204.

Fiorito, G., Affuso, A., Basil, J., Cole, A., de Girolamo, P., D'Angelo, L., … Andrews, P. L. (2015). Guidelines for the Care and Welfare of Cephalopods in Research –A consensus based on an initiative by CephRes, FELASA and the Boyd Group. *Laboratory Animals*, *49*(2), 1–90.

Forsythe, J. W. (2004). Accounting for the effect of temperature on squid growth in nature: From hypothesis to practice. *Marine and Freshwater Research*, *55*(4), 331–339.

Forsythe, J. W., DeRusha, R. H., & Hanlon, R. T. (1994). Growth, reproduction and life span of Sepia officinalis (Cephalopoda: Mollusca) cultured through seven consecutive generations. *Journal of Zoology*, *233*(2), 175–192.

Forsythe, J. W., & Hanlon, R. T. (1988). Effect of temperature on laboratory growth, reproduction and life span of *Octopus bimaculoides*. *Marine Biology*, *98*(3), 369–379.

Franco-Santos, R. M., Perales-Raya, C., Almansa, E., De Troch, M., & Garrido, D. (2016). Beak microstructure analysis as a tool to identify potential rearing stress for *Octopus vulgaris* paralarvae. *Aquaculture Research*, *47*(9), 3001–3015.

Fuentes, L., Sánchez, F. J., Lago, M. J., Iglesias, J., Pazos, G., & Linares, F. (2011). Growth and survival of *Octopus vulgaris* (Cuvier 1797) paralarvae fed on three Artemia-based diets complemented with frozen fish flakes, crushed zooplankton and marine microalgae. *Scientia Marina*, *75*(4), 771–777.

Fuentes, L., Sánchez, F. J., Otero, J. J., Lago, M. J., & Iglesias, J. (2009). Utilización de zooplancton congelado y artemia en el cultivo larvario de pulpo *Octopus vulgaris*. *XII Congreso Nacional de Acuicultura. Madrid*.

Garrido, D., Martín, V. M., Rodríguez, C., Iglesias, J., Navarro, J. C., Estévez, A., … Almansa, E. (2016). Meta-analysis approach to the effects of live prey on the growth of Octopus vulgaris paralarvae under culture conditions. *Reviews in Aquaculture*, *0*, 1–12.

Garrido, D., Varó, I., Morales, A. E., Hidalgo, M. C., Navarro, J. C., Hontoria, F., … Cardenete, G. (2017). Assessment of stress and nutritional biomarkers in cultured Octopus vulgaris paralarvae: Effects of geographical origin and dietary regime. *Aquaculture*, *468*, 558–568.

Giménez, F. A., & García García, B. (2002). Growth and food intake models in *Octopus vulgaris* Cuvier (1797): Influence of body weight, temperature, sex and diet. *Aquaculture International*, *10*(5), 361–377.

Hamasaki, K., & Morioka, T. (2002). Effects of Temperature on Egg Incubation Period, and Paralarval Survival and Growth of Common Octopus, *Octopus vulgaris* reared in the Laboratory. *Suisanzosho*, *50*(4), 407–413.

Hatfield, E. M. C. (2000). Do some like it hot? Temperature as a possible determinant of variability in the growth of the Patagonian squid, *Loligo gahi* (Cephalopoda: Loliginidae). *Fisheries Research*, *47*(1), 27–40.

Hernández-López, J. L., Castro-Hernández, J. J., & Hernández-García, V. (2001). Age determined from the daily deposition of concentric rings on common octopus (*Octopus vulgaris*) beaks. *Fishery Bulletin*, *99*(4), 679–684.

Iglesias, J., & Fuentes, L. (2014). *Octopus vulgaris*. Paralarval Culture. In R. Iglesias, José. Fuentes, Lidia. Villanueve (Ed.), *Cephalopod Culture* (pp. 427–450). Springer.

Iglesias, J., Fuentes, L., Sánchez, J., Otero, J. J., Moxica, C., & Lago, M. J. (2006). First feeding of *Octopus vulgaris* Cuvier, 1797 paralarvae using Artemia: Effect of prey size, prey density and feeding frequency. *Aquaculture*, *261*(2), 817–822.

Iglesias, J., Otero, J. J., Moxica, C., Fuentes, L., & Sánchez, F. J. (2004). The completed life cycle of the octopus (*Octopus vulgaris*, Cuvier) under culture conditions: Paralarval rearing using Artemia and zoeae, and first data on juvenile growth up to 8 months of age. *Aquaculture International*, *12*(4–5), 481–487.

Iglesias, J., Sánche, F. J., Moxica, C., & Otero, J. J. (2000). Culture of octopus (*Octopus vulgaris*, Cuvier): Present knowledge, problems and perspectives. *Cahiers Options Méditerranéennes*, *321*(47), 313–321.

Iglesias, J., Sánchez, F. J., Bersano, J. G. F., Carrasco, J. F., Dhont, J., Fuentes, L., … Villanueva, R. (2007). Rearing of *Octopus vulgaris* paralarvae: Present status, bottlenecks and trends. *Aquaculture*, *266*(1–4), 1–15.

Iglesias, J., Sánchez, F. J., & Otero, J. J. (1997). Primeras experiencias sobre el cultivo integral del pulpo (*Octopus vulgaris* Cuvier) en el Instituto Español de Oceanografía. *VI Congreso Nacional de Acuicultura*, 221–226.

Iglesias, P., Picón, P., Nande, M., Lago, M. J., Otero, J. J., Trujillo, V., & Iglesias, J. (2016). Effect of low salinity on survival and ingested food of the common octopus, *Octopus vulgaris* Cuvier, 1797. *Journal of Applied Aquaculture*, *28*(3), 267–271.

Itami. K, Izawa. Y, Maeda. S, N. K. (1963). Notes on the laboratory culture of the octopus larvae. *Nippon Suisan Gakkaishi*, *29*(6), 514–520.

Jackson, G. D., & Moltschaniwskyj, N. A. (2001). The influence of ration level on growth and statolith increment width of the tropical squid *Sepioteuthis lessoniana* (Cephalopoda: Loliginidae): An experimental approach. *Marine Biology*, *138*(4), 819–825.

Josupeit, H., Wang, W., & Dent, F. (2016). *Globefish European Fish Price Report. Issue 1/2016*.

Kamler, E. (1992). Endogenous feeding period. In E. Kamler (Ed.), *Early Life History of Fish* (pp. 107–175). Springer.

Kim, T. K. (2015). T-test as a parametric statistic. *Korean Journal of Anesthesiology*, *68*(6), 540–546.

Levene, H. (1960). Robust tests for quality of variances. *Contribution to Probability and Statistics*, *1*, 278–292.

Lyon, H. (1991). Tissue Processing: VII. Post Treatment. In H. Lyon (Ed.), *Theory and Strategy in Histochemistry* (pp. 215–219). Berlin: Springer.

Magro, M. (2015). *Effect of temperature on embryonic development, larval viability and biomarkers in the first stages of life of Octopus vulgaris (Cuvier , 1797)*. Universidade do Algarve.

Mancuso, M. (2014). *Octopus vulgari*s new species for the diversification in the Mediterranean aquaculture. *International Journal of Marine Science and Ocean Technology (IJMO)*, *1*(1), 1–2.

Mangold, K. Boletzky, S. von. (1973). New data on reproductive biology and growth of *Octopus vulgaris*. *Marine Biology*, *19*(1), 7–12.

Mangold, K. (1983). *Octopus vulgaris*. In P. R. Boyle (Ed.), *Cephalopod Life Cycles* (Vol. 1, pp. 335–364). London: Academic Press.

McDonald, J. H. (2014). *Handbook of Biological Statistics*. (J. H. McDonald, Ed.). Baltimore: Sparky House Publishing.

Morillo-Velarde, P. S., Cerezo Valverde, J., Hernández, M. D., Aguado-Giménez, F., & García García, B. (2012). Growth and digestibility of formulated diets based on dry and freeze-dried ingredients in the common octopus (*Octopus vulgaris*). *Aquaculture*, *368–369*, 139–144.

Moxica, C., Fuentes, L., Hernández, J., Iglesias-Estévez, J., Lago-Rouco, M. J., Otero-Pinzas, J. J., & Sánchez-Conde, F. J. (2011). Efecto de Nannochloropsis sp. en la supervivencia y crecimiento de paralarvas de pulpo *Octopus vulgaris*. *IX Foro Dos Recursos Mariños E Da Acuicultura Das Rías Gallegas*, *9*, 255–261.

Moxica, C., Linares, F., Otero, J. J., Iglesias, J., & Sánchez, F. J. (2002). Cultivo intensivo de paralarvas de pulpo, *Octopus vulgaris* Cuvier, 1797, en tanques de 9 m^3. *Boletín Instituto Español De Oceanografía*, *18*(May 2014), 31–36.

Naef, A. (1928). *Die Cephalopoden. Embryologie*. *Fauna und Flora Golf von Neapel* (Vol. 35). Berlin: Friedländer & Son.

Nande, M., Iglesias, J., Domingues, P., & Pérez, M. (2016). Effect of temperature on energetic demands during the last stages of embryonic development and early life of *Octopus vulgaris* (Cuvier, 1797) paralarvae. *Aquaculture Research*, 1–11.

Navarro, J. C., Monroig, Ó., & Sykes, A. V. (2014). Nutrition as a key factor for cephalopod aquaculture. In J. Iglesias, L. Fuentes, & R. Villanueva (Eds.), *Cephalopod Culture* (pp. 77–95). Springer.

Navarro, J. C., & Villanueva, R. (2000). Lipid and fatty acid composition of early stages of cephalopods: An approach to their lipid requirements. *Aquaculture*, *183*(1–2), 161–177.

Nemenyi, P. B. (1963). *Distribution-Free Multiple Comparisons, Princeton University NJ, 1963*. Princeton University.

Nixon, M. (1971). Some parameters of growth in *Octopus vulgaris*. *Journal of Zoology (London)*, *163*(3), 277–284.

Norman, M.D., Finn, J.K. & Hochberg, F. G. (2016). Family Ocotopodidae. In J. K. Jereb, P. Roper, C.F.E. Norman, M.D., Finn (Ed.), *Cephalopods of the world. An annotated and illustrated catalogue of cephalopod species known to date. Volume 3. Octopods and Vampire Squids. No. 4. Vol. 3. Rome* (pp. 36–215). FAO.

Okumura, S., Kurihara, A., Iwamoto, A., & Takeuchi, T. (2005). Improved survival and growth in *Octopus vulgaris* paralarvae by feeding large type Artemia and Pacific sandeel, *Ammodytes personatus*: Improved survival and growth of common octopus paralarvae. *Aquaculture*, *244*(1–4), 147–157.

Olcott, G. W. (1960). Preparation and use of a Gelatin Munting Medium For Repeated Oil Immersion Of Minerals. *The American Mineralogist*, *45*, 1099–1101.

Parra, G., Villanueva, R., & Yúfera, M. (2000). Respiration rates in late eggs and early hatchlings of the common octopus, *Octopus vulgaris*. *Journal of the Marine Biological Association of the UK*, *80*(80), 557–558.

Pechenik, J. A. (1999). On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. *Marine Ecology Progress Series*, *177*, 269–297.

Perales-Raya, C., Almansa, E., Bartolomé, A., Felipe, B. C., Iglesias, J., Sánchez, F. J., … Rodríguez, C. (2014b). Age Validation in *Octopus vulgaris* Beaks Across the Full Ontogenetic Range: Beaks as Recorders of Life Events in Octopuses. *Journal of Shellfish Research*, *33*(2), 481–493.

Perales-Raya, C., Bartolomé, A., García-Santamaría, M. T., Pascual-Alayón, P., & Almansa, E. (2010). Age estimation obtained from analysis of octopus (*Octopus vulgaris* Cuvier, 1797) beaks: Improvements and comparisons. *Fisheries Research*, *106*(2), 171–176.

Perales-Raya, C., Jurado-Ruzafa, A., Bartolomé, A., Duque, V., Carrasco, M. N., & Fraile-Nuez, E. (2014a). Age of spent *Octopus vulgaris* and stress mark analysis using beaks of wild individuals. *Hydrobiologia*, *725*(1), 105–114.

Prato, E., Portacci, G., & Biandolino, F. (2010). Effect of diet on growth performance, feed efficiency and nutritional composition of *Octopus vulgaris*. *Aquaculture*, *309*(1–4), 203–211.

Quintana, D., Márquez, L., Arévalo, J. R., Lorenzo, A., & Almansa, E. (2015). Relationships between spawn quality and biochemical composition of eggs and hatchlings of *Octopus vulgaris* under different parental diets. *Aquaculture*, *446*, 206–216.

Rama-Villar, A., Faya-Angueira, V., Moxica, C., & Rey-Méndez, M. (1997). Engorde de pulpo (*Octopus vulgaris*) en batea. In *VI Congreso Nacional de Acuicultura* (pp. 1–6).

Raya, C. P., & Hernández-González, C. L. (1998). Growth lines within the beak microstructure of the octopus *Octopus vulgaris* Cuvier, 1797. *South African Journal of Marine Science*, *20*(1), 135–142.

Reis, D. B., Acosta, N. G., Almansa, E., Tocher, D. R., Andrade, J. P., Sykes, A. V, & Rodríguez, C. (2016). Composition and metabolism of phospholipids in *Octopus vulgaris* and *Sepia officinalis* hatchlings. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *200*(Supplement C), 62–68.

Roper, C. F. E., Sweeney, M. J., & Nauen, C. E. (1984). *FAO species catalogue: vol. 3 Cephalopods of the world; an annotated and illustrated catalogue of species of interest to fisheries*. (C. E. Roper, Clyde. F. E., Sweeney, M. J. Nauen, Ed.) (3rd ed.). Food and Agriculture Organization of the United Nations.

Roura, A., Gonzalez, A. F., Redd, K., & Guerra, N. (2012). Molecular prey identification in wild *Octopus vulgaris* paralarvae. *Marine Biology*, *159*(6), 1335–1345. https://doi.org/10.1007/s00227-012-1914-9

Sánchez, F. J., Fuentes, L., Otero, J. J., Lago, M. J., Linares, F., Pazos, G., & Iglesias, J. (2013). Effect of tank volume on the growth and survival of reared *Octopus vulgaris* paralarvae. *Aquaculture Research*, *44*(4), 667–670.

Seixas, P., Rey-Méndez, M., Valente, L. M. P., & Otero, A. (2008). Producing juvenile Artemia as prey for *Octopus vulgaris* paralarvae with different microalgal species of controlled biochemical composition. *Aquaculture*, *283*(1–4), 83–91.

Seixas, P., Rey-Méndez, M., Valente, L. M. P., & Otero, A. (2010). High DHA content in Artemia is ineffective to improve *Octopus vulgaris* paralarvae rearing. *Aquaculture*, *300*(1–4), 156–162.

Shapiro, S. S., & Wilk, M. B. (1965). An Analysis of Variance Test for Normality (Complete Samples). *Biometrika*, *52*(3/4), 591.

Spisni, E., Tugnoli, M., Ponticelli, A., Mordenti, T., & Tomasi, V. (1998). Hepatic steatosis in artificially fed marine teleosts. *Journal of Fish Diseases*, *21*(3), 177–184.

Tukey, J. W. (1949). Comparing Individual Means in the Analysis of Variance. *Biometrics*, *5*(2), 99–114.

Uriarte, I., Espinoza, V., Herrera, M., Zúñiga, O., Olivares, A., Carbonell, P., … Rosas, C. (2012). Effect of temperature on embryonic development of *Octopus mimus* under controlled conditions. *Journal of Experimental Marine Biology and Ecology*, *416–417*, 168–175.

Uriarte, I., Iglesias, J., Domingues, P., Rosas, C., Viana, M. T., Navarro, J. C., … Zuñiga, O. (2011). Current Status and Bottle Neck of Octopod Aquaculture: The Case of American Species. *Journal of the World Aquaculture Society*, *42*(6), 735–752.

Vaz-Pires, P., Seixas, P., & Barbosa, A. (2004). Aquaculture potential of the common octopus (*Octopus vulgaris* Cuvier, 1797): A review. *Aquaculture*, *238*(1–4), 221–238.

Viciano, E., Iglesias, J., Lago, M. J., Sánchez, F. J., Otero, J. J., & Navarro, J. C. (2011). Fatty acid composition of polar and neutral lipid fractions of *Octopus vulgaris* Cuvier, 1797 paralarvae reared with enriched on-grown Artemia. *Aquaculture Research*, *42*(5), 704–709.

Vidal, E. A. G., Dimarco, F. P., Wormuth, J. H., & Lee, P. G. (2002). Influence of Temperature and Food Availability on Survival, Growth and Yolk Utilization in Hatchling Squid. *Bulletin of Marine Science*, *71*(2), 915–931.

Vidal, É. A. G., DiMarco, P., & Lee, P. (2006). Effects of starvation and recovery on the survival, growth and RNA/DNA ratio in loliginid squid paralarvae. *Aquaculture*, *260*(1–4), 94–105.

Vidal, E. A. G., Villanueva, R., Andrade, J. P., Gleadall, I. G., Iglesias, J., Koueta, N., … Wood, J. (2014). Cephalopod culture: Current status of main biological models and research priorities. In E. A. G. Vidal (Ed.), *Advances in Marine Biology* (Vol. 67, pp. 1–198). Academic Press.

Villanueva, R. (1995). Experimental rearing and growth of planktonic Octopus vulgaris from hatching to settlement. *Canadian Journal of Fisheries and Aquatic Sciences*, *52*(117), 2639–2650.

Villanueva, R., Arkhipkin, A., Jereb, P., Lefkaditou, E., Lipinski, M. R., Perales-Raya, C., … Rocha, F. (2003). Embryonic life of the loliginid squid *Loligo vulgaris*: Comparison between statoliths of Atlantic and Mediterranean populations. *Marine Ecology Progress Series*, *253*, 197–208.

Villanueva, R., & Bustamante, P. (2006). Composition in essential and non-essential elements of early stages of cephalopods and dietary effects on the elemental profiles of *Octopus vulgaris* paralarvae. *Aquaculture*, *261*(1), 225–240.

Villanueva, R., Escudero, J. M., Deulofeu, R., Bozzano, A., & Casoliva, C. (2009). Vitamin A and E content in early stages of cephalopods and their dietary effects in *Octopus vulgaris* paralarvae. *Aquaculture*, *286*(3–4), 277–282.

Villanueva, R., & Norman, M. D. (2008). Biology of the planktonic stages of benthic octopuses. *Oceanography and Marine Biology: An Annual Review*, *46*, 105–202.

Wilcoxon, F. (1945). Individual Comparisons by Ranking Methods. *Biometrics Bulletin*, *1*(6), 80.

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., … Wang, J. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature*, *490*(7418), 49–54.

8. Annexes

Annex I. Main diagnostic features unique to *Octopus vulgaris sensu stricto* (from Norman et al., 2016a).

Diagnostic Features	Notes
Colour and body pattern	• Colour in life variable from yellow-brown, to red-brown, dark brown or grey. • Transverse pair of white spots present on the dorsal mantle, slightly anterior to the midpoint of the mantle. • Skin with distinct patch and groove system that forms a dark trellis or reticulate pattern. • A Fixed diamond pattern of four large erectile primary papillae in mid-region of the dorsal mantle.
Sculpture	• Skin texture of regular patch and groove with small circular patches.
Webs	• Deepest on lateral arms, webs between dorsal arms shallowest. • Interbrachial web pouches absent.
Arms and Suckers	• Arms muscular, medium length, 3 to 5 times mantle length. Lateral arms longest (typically 2>3>4>1 or 3>2>4>1). • Arm autotomy at distinct plane absent. • Two rows of suckers on each arm. • Enlarged suckers present in mature males, typically on arms 2 to 3, sometimes on arm 4. • In larger *O. vulgaris*, ~220 to 320 suckers are on each normal arm. Both sexes have 2 to 3 enlarged suckers on the lateral arms at the location of the 15th to 19th proximal suckers (larger in males).
Ink Sac	• Present.
Gills	• 9 to 11 lamellae per outer demibranch.

Annex II. (a) The average temperature and (b) tank parameters for the thermal shock experiment.

Tank Parameters		**Date**			
		09/09/2015		**16/09/2015**	
		CONTROL	TS	CONTROL	TS
Oxygen	*mg/L*	6.5	6.9	6.7	6.7
	Saturation (%)	85	89	89	90
Nitrate (mg/L)		0	0	0	0
Nitrite (mg/L)		0	0	0	0
Renewal rate		63 mL/sg	1.3rev/day	63 mL/sg	1.3rev/days

	Date			
	09/09/2015 – 13/09/2015		**14/09/2015 - 18/09/2015**	
	CONTROL	TS	CONTROL	TS
Average Temperature (°C)	17	17.2	17.9	18.9

Annex III. Protocol for paralarvae cultivation in laboratory conditions for the constant temperature experiment cultivation (created by Paula Barreiro Baceta, Universidade de Vigo, 2015).

Cleaning

1. Before cultivation, clean all materials (mesh, tanks, pipes, etc.) with distilled water and allow to air dry completely.
2. Next, fill the tanks and the other material with distilled water and bleach. Leave for 48 hours.
3. Empty the tanks and rinse with distilled water.

Cultivation Setup

1. Place the meshes in the centre of the tank and add the phytoplankton supply facility (cover drum and place pipes into the tanks).
2. Fill tanks with seawater.
3. Install aeration tube in the centre of the tank.
4. Place (if necessary) heaters in the centre of the tank near the aeration.
5. Set the required photoperiod.
6. Regulate the inflow of water and temperature (at least four days prior to the start of the cultivation).

Table

Tank*	Water warmth at start of cultivation	Heater
14°C	Cold	No
16°C	Hot	Yes
18°C	Cold	Yes
21°C	Cold	Yes

*Two 150L tanks for each temperature.

Annex IV. (a) The tank parameters and (b) average temperature of tanks during the constant temperature experiment.

(a)

Tank Parameters		Date																							
		05/11/2015 (Day 0)								09/11/2015 (Day 4)								16/11/2015 (Day 11)							
		14°C		16°C		18°C		21°C		14°C		16°C		18°C		21°C		14°C		16°C		18°C		21°C	
		T4	T7	T1	T8	T2	T5	T3	T6	T4	T7	T1	T8	T2	T5	T3	T6	T4	T7	T1	T8	T2	T5	T3	T6
Oxygen	mg/L	7.2	6.7	7.1	7.2	6.8	6.8	7.3	6.7	7.2	6.7	7.1	7.3	6.8	6.7	6.8	6.7	6.1	6.2	5.9	6	6.3	6.1	61	6.3
	Saturation (%)	92	86	91	92	89	89	96	86	92	86	91	96	89	86	89	86	78	78	76	76	80	79	83	83
Nitrate (mg/L)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nitrite (mg/L)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pH		7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7

(b)

	Date															
	09/11/2015 - 16/11/2015								17/11/2015 - 19/11/2015							
Tank Temperature (°C)	14°C		16°C		18°C		21°C		14°C		16°C		18°C		21°C	
Tank	T4	T7	T1	T8	T2	T5	T3	T6	T4	T7	T1	T8	T2	T5	T3	T6
Average Temperature (°C)	14.1	14.1	16.5	16.5	18.1	18.2	21.4	20.5	14.3	14.0	16.0	16.3	16.0	18.3	16.0	20.0

Annex V. (a) The tank parameters and (b) average temperature of tanks during the increasing temperature experiment. *EXP* – increasing temperature tank.

(a)

Tank Parameters		Date																							
		09/09/2015								16/09/2015								24/09/2015							
		CONTROL				EXP				CONTROL				EXP				CONTROL				EXP			
		T1	T4	T6	T8	T2	T3	T5	T7	T1	T4	T6	T8	T2	T3	T5	T7	T1	T4	T6	T8	T2	T3	T5	T7
Oxygen	mg/L	6.8	6.1	6.5	6.2	6.4	6.1	6.2	6.4	6.5	6.1	6.5	6.5	6.3	6.7	6.6	6.6	7.1	7.2	6.7	7.2	6.8	7.3	6.8	7.1
	Saturation (%)	83	78	80	79	83	78	79	81	86	83	85	86	85	90	89	89	91	92	86	92	89	96	89	92
Nitrate (mg/L)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nitrite (mg/L)		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Renewal rate (L/h / rev/day)		16.2 L/h				2.8 rev/day				16.0 L/h				2.6 rev/day											

(b)

Experimental Tanks	Date																							
	14/09/2015 - 16/09/2015								17/09/2015 - 24/09/2015								25/09/2015 - 02/10/2015							
	CONTROL				EXP				CONTROL				EXP				CONTROL				EXP			
	T1	T4	T6	T8	T2	T3	T5	T7	T1	T4	T6	T8	T2	T3	T5	T7	T1	T4	T6	T8	T2	T3	T5	T7
Average Temperature (°C)	16.4	16.4	16.5	16.2	19.7	19.2	18.9	19.8	16.6	16.5	16.5	16.5	20.1	19.9	19.4	19.7	16.4	16.2	16.1	16.4	19.7	19.5	20.1	19.7

Annex VI. Glycerol gelatine mounting protocol for *O. vulgaris* paralarvae.

Preparing the glycerol gelatine

1. At 34-37°C (using a water bath) add 100ml of distilled water, 100g of glycerine, 17g of pure gelatine and 1g/mL of phenol and mix.
2. Remove from the heat once the mixture is pasty and rubbery in appearance.
3. Allow gelatine to dry for one hour.
4. Store in glass/plastic container away from direct sunlight at room temperature.

Extraction

1. Carefully extract the beak from the individual paralarva using a thin precision needle.
2. Clean the beak in distilled water.
3. Heat a small piece (50mm) of gelatine onto a clean slide and heat with a hot air gun till is viscous.
4. Mount the beak in the gelatine and place a glass slide carefully on the top of the slide (leave to cool for 3 minutes).

Analysis

1. Observe increments and stress marks under the microscope with transmitted light with Nomarski-DIC.
2. Count increment on both the left and right inner side of the UJ of the RSS or LWS and identify the first increment.
3. Take pictures of all beak specimens.
4. Count marks independently two times on each side (with the same reader).
5. Analyse age precision using the coefficient of variation (Campana, 2001).
6. Measure the increment width using photo analysis software (e.g. Digimizer, Image J, etc.)

Annex VII. Dry weight protocol for *O. vulgaris* paralarvae (created by Paula Barreiro Baceta, Universidade de Vigo, 2015)

Previous day: Preparation of aluminium cuvettes

1. Turn the oven on to 110ºC
2. Number the aluminium trays
3. Place cuvettes in the oven for 24 hours at 110°C
4. Place cuvettes in a desiccator for 30min.
5. Weigh cuvettes on a 5-digit scale.
6. Return cuvettes to the oven for 30 min
7. Weigh cuvettes twice more.

Sampling day: Preparation of paralarvae weight

1. Turn the oven to 110ºC.
2. Take 3 paralarvae and place them in Petri dishes filled with seawater and anaesthesia (10ml of water with one drop of 70% alcohol).
3. Rinse each paralarva with 100ml of distilled water for 10 seconds using a wide-mouth pipette.
4. Transfer the paralarvae to aluminium cuvettes with as little water as possible.
5. Place cuvettes in the oven for 24 hours at 110°C
6. Place cuvettes in a desiccator for 30min.
7. Weigh cuvettes on a 5-digit scale.
8. Return cuvettes to the oven for 30 min
9. Weigh cuvettes twice more.