University of Brighton

Supporting sustainable Sepia stocks

IFCA-UoB Agreement UB-18-2802

Project report

Assessing the survival rate and egg quality under laboratory conditions

Biochemical composition of eggs and energy content assessed under different laboratory conditions (temperature, substrate and water currents); discuss the results in the context of likely hatching and survival rate of eggs deposited on removable receptors.

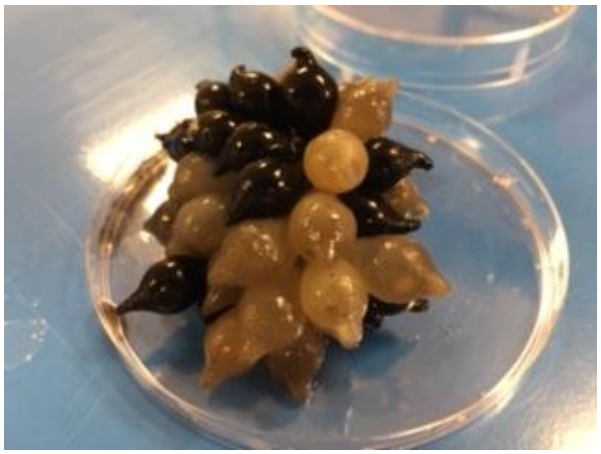

Fig.1. Clusters of black and white eggs (own photo).

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Summary

This yearlong study evaluates the potential of new management strategies directed to support the sustainable fishery of cuttlefish in the English Channel.

The ability of eggs dislocated from cuttle traps to develop and hatch under different laboratory conditions was tested. All laboratory experiments were designed to replicate environmental conditions at the time of egg deposition and development (substrate, water temperature, light regime). The analysis of egg diameter distribution showed high variations between egg size, suggesting either different laying events or a mixture of younger and older females spawning at the same time. This study combining laboratory experiments and first hand data recording has the potential to offer solid information regarding reproduction success and hatching in eggs dislocated from artisanal traps, and to contribute to the recovery of cuttlefish stocks in the framework of a broader management plan.

Life history traits and exploitation

The common cuttlefish *Sepia officinalis* (Linnaeus, 1758) is native to the Mediterranean Sea and the Eastern Atlantic, however nowadays it is encountered in the far north (Baltic and the North Sea) as well as in the waters around South Africa. It is a demersal species, abundant in coastal waters rich in seaweed and seagrasses, where it is intensively exploited with bottom otter trawls, trammel nets, and traps depending on its spatial and temporal distribution patterns. In the English Channel, common cuttlefish is exploited seasonally by different fishing strategies and techniques. In spring, adult cuttlefish concentrate in coastal spawning grounds, where they are targeted by small-scale artisanal fisheries; in autumn, juveniles migrate to deeper offshore waters, where they are caught by bottom trawlers, either as a target species or as bycatch (Matozzo et al, 2015).

Historically, the English Channel population of the common cuttlefish was considered a non-target species for commercial fishers; this position has dramatically changed in recent years (Dunn 1999). The English Channel represents one of the most important fishing grounds for cuttlefish in Europe, with 60% of all catches between 1993 and 2003 occurring in the English Channel, ICES areas VIId and VIIe (Royer *et al,* 2006). In 2017, the UK cuttlefish fishery recorded a ten year high with landings of 7182 tonnes and the Sussex cuttlefish fishery recorded a ten year low with landings of 157 tonnes (Sussex IFCA report).
Similarly, survey data collected in the Adriatic Sea in the past 10 years, have demonstrated a significantly lower abundance and biomass of S. *officinalis*. The situation may be a direct result of the removal of adults and juveniles by fishing activities and/or an indirect effect of the destruction of essential habitats and therefore egg laying substrates, resulting in poor recruitment (UN Environmental Programme, 2014).

European cuttlefish fisheries are not managed and recent scientific reviews suggest that *S.officinalis* is exploited in almost every area of its migration cycle; therefore, there is little scope for a sustainable fishery.

S. *officinalis* has a lifespan of about two years; the spawning season is followed by mass adult mortality. Since egg masses are attached in clusters to seagrass, tubeworms, drowned trees, ropes, and traps, recruitment strongly depends on the availability of spawning substrates. This may be a critical factor, especially in the English channel/Sussex coast, where habitat loss and degradation are particularly severe due to human activities (Royer et al, 2002). The choice of the spawning substrate depends on the female's preferences, which seem to look for tubular surfaces (vegetation, ropes, metal bars). Eggs take up to 50 days to hatch, depending on water temperature. However, egg removal from artisanal fixed gears (cuttle traps) is common in order to ensure gear efficiency (Melli et al, 2014).

Egg biochemistry

Like all cephalopods, cuttlefishes are gonochoristic (separate sex) and reproduce only once in their lifetime. Eggs are usually laid at water temperatures between 13°C and 15°C. The cuttlefish life cycle is 12-24 months, varying according to the environmental conditions (Fig.2). Growth is quite fast, the individuals born in summer from eggs laid in spring, usually spawn in the autumn of the following year, while those born in the autumn clutch, spawn in the spring of their second year of life (Jereb and Roper 2005).

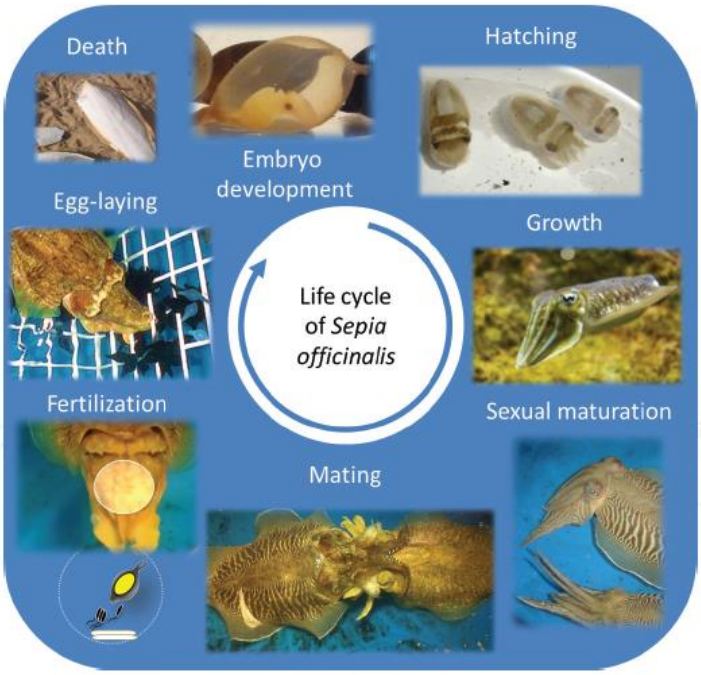


Fig. 2. Main successive steps of the life cycle of *Sepia officinalis* (Zatylyn-Gaudin *et al*, 2018).

Egg masses are spawned in specific mating and spawning coastal areas in the English Channel, where adult cuttlefish aggregate in the spring. Environmental cues (water temperature, currents) are clearly involved in the aggregation process; also, chemical communication plays a determining role: neuropeptides, ovarian regulatory peptides and sex pheromones. Recent transcriptomics studies demonstrate that successive steps of egg laying are mainly regulated by neuropeptides, involved in the integration of environmental cues and ovarian regulatory peptide, associated with egg capsule elaboration (Zatylyn et all, 2018). The main nidamental gland secretes the main polysaccharides and glycoproteins, such as *Sepia Egg Case Proteins*, involved in capsule formation and in embryo protection (Zatylny-Gaudin and Henry, 2018). After egg-laying, embryo protection is ensured for 8-10 weeks by a multilayer capsule (Fig.3).

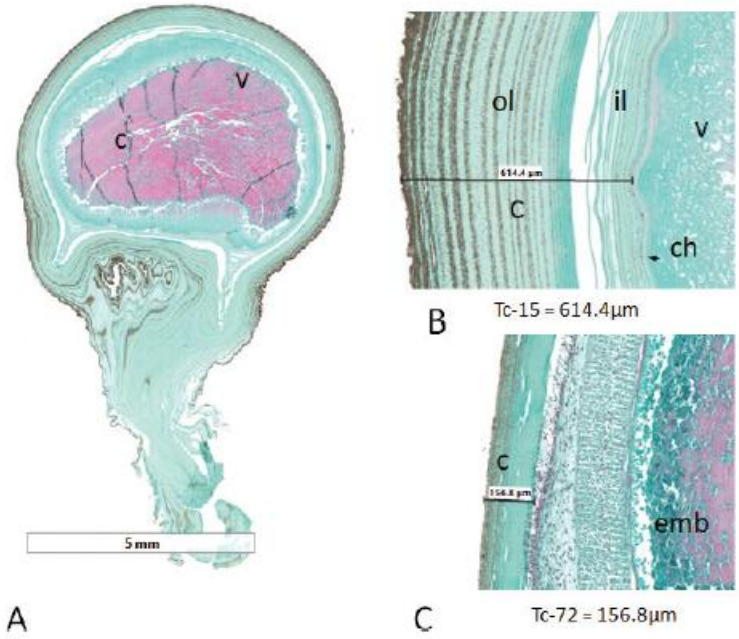


Fig. 3. Longitudinal section of the egg after 15 days incubation (A). Magnification of egg capsule after 15 days (B) and 72 days incubation (C). c = capsule or egg case; ch = chorion; emb = embryo; il = inner layer; ol = outer layer; Tc = capsule thickness; v = vitellus (Zatylyn C. *et al*, 2018).

Cuttlefish eggs are large oocytes, rich in nutrient reserves required for embryo development. The protective egg capsule (or egg case) has an important role in assisting the oocytes to withstand the physical and microbial threats in the marine environment. This egg case is composed of two distinct envelopes: the inner layer in direct contact with the chorion surrounding the oocyte and the outer layer secreted by the two nidamental glands inside the mantle cavity and stained with ink (Boletzky, 1986).

Eggs, and in particular yolk, provide the main constituents for embryonic development, such as proteins, lipids and carbohydrates that support the formation of cell membranes and further operate as energy storage (Matozzo *et al*, 2015).

According to Bouchaud and Galois, 1990, maternal nutritional history is likely to affect the egg development and survival of early life stages, because of the quantity and quality of yolk allocated per egg. Nutrient allocation is influenced by the food availability to mature females; therefore, environmental conditions affect female energy reserves, egg size and the future of the offspring.
To date, only a small number of studies have investigated the biochemical composition of cuttlefish eggs during the embryonic development.

UoB Study

Hatching Rates under laboratory conditions

Hatching rates were investigated under laboratory conditions in summer of 2018, to enable the understanding of the direct effect of environmental factors (substrate, sea currents, and water temperature) on egg development. On 21st of June 2018, clusters of cuttlefish eggs were placed in 9 indoor tanks provided with continuous seawater flow and aerators at the Hastings campus Experiment room, University of Brighton. The egg samples were provided by Sussex IFCA and Hastings fishers, and obtained by manually removing the eggs from the traps and placing them in a bucket with seawater. Less than one-week-old eggs were selected by size and colour, counted and placed in the tanks. Some of the eggs were very gently dislocated from the clusters and placed in the tanks, as free-floating individual eggs.
Eggs were subjected to different conditions (Table 1); hatching success was estimated at the end of the experiment, day 46. Tanks were checked every morning and newly hatched cuttlefish were immediately released in the environment.

Tank 1 (197 eggs) Sand Water at 17 degrees No water currents	**Tank 2 (153 eggs)** Gravel (coral chips) Water at 17 degrees No water currents	**Tank 3 (164 eggs)** Pebbles Water at 17 degrees No water currents
Tank 4 (163 eggs) Sand Water at 17 degrees 2 fans providing robust water movement	**Tank 5 (211 eggs)** Gravel (coral chips) Water at 17 degrees 2 fans providing robust water movement	**Tank 6 (161 eggs)** Pebbles Water at 17 degrees 2 fans providing robust water movement
Tank 7 (171 eggs) Sand Water at 21 degrees No water currents	**Tank 8 (166 eggs)** Gravel (coral chips) Water at 21 degrees No water currents	**Tank 9 (153 eggs)** Pebbles Water at 21 degrees No water currents

Table 1. Experimental design – tank set up to investigate cuttlefish hatching rates in eggs dislocated from the traps, under different environmental factors

A **B**

Fig. 4. Cuttlefish eggs (clustered and free floating) placed in tanks with gravel substrate (A) and pebbles (B) (own photo).

Cuttlefish eggs were housed in 9mm glass tanks (ClearSeal) of 600mm x 300mm x 300mm dimension. Each tank contained an airline and a stone, an outflow pipe and a thermometer; additionally, a heater and a fan were placed in selected tanks (Table 1). There were also valves of saltwater feeds, inputting water into the tanks from a closed system of saltwater equipped with UV filtration. Overhead fluorescent room lights were used on a 10L:14D regime. Temperature, salinity, Ph and dissolved oxygen were measured every other day.

Tank ID/date: 22.06.2018	Free floating/clustered	Width egg (mm)	weight wet embryo mg
3	F	9.04	0.1187
3	F	8.67	0.2519
3	F	8.95	n/a
3	C	9.01	0.2012
3	C	10.64	0.4952
3	C	10.3	n/a
3	C	10.2	0.4527
3	C	10.53	0.5204
6	F	10.34	0.2941
6	F	9.04	0.3041
6	F	9.56	0.3047
6	C	10.21	0.3677
6	C	8.88	0.1758
6	C	9.41	0.3542
6	C	10.2	n/a
6	C	10.11	0.3743

Table 2. Raw data - Characterisation of eggs collected during embryo development, prior to biochemical analysis.

Biochemical analysis of the eggs – materials and methods

Harvest and sample preparation

Eggs were collected once from each tank replicate (Table 2) comprising either clutch grouped eggs (C) or free floating eggs (F). For Clutch eggs, five eggs were harvested, whereas three free eggs were selected each time. After harvest, eggs were kept on ice for transfer to the lab where egg case width, total egg weight and wet weight of the embryos were measured (average egg size range: 15.74mm to 8.8mm, expressed as egg width measured from one to the other side of the egg and perpendicularly to the major axis). After careful extraction of the embryos (Matozzo et al 2015) they were stored at -20 until further use. Only eggs where the complete embryo was successfully removed/measured were recorded and used for the experiments.

For each batch of embryos, samples were homogenised using an Omni Probe homogeniser with Soft tissue probes. Fresh clean probes were used between each batch of egg embryos, with probes sprayed with both 5% Biocleanse and 70% alcohol prior to use. Batch samples were homogenised in 5ml each of pre chilled RO water before being kept on ice. After aliquoting to 1.5ml tubes, samples were centrifuged at 13K g for 5 mins to remove insoluble material. The supernatant was drawn off and stored in 1.5ml tubes in the -20^{o}C till further analysis.

Test assays were run on test embryos taken on an earlier date to assess the expected range of components. All sample assays were run in triplicate, with each result being used to calculate the respective amount in µg/mg wet tissue weight taking into account the sample dilution, and total weight per egg batch.

Total lipid levels were extracted and then quantified according to the method of Mann & Gallagher, 1985. Absorbance was measured at 590 nm and a standard curve of stearic acid was prepared. Total protein content was measured according to the colorimetric method of Lowry et al.1951, the absorbance was measured at 590 nm and a standard curve of bovine serum albumin (BSA) was prepared. Carbohydrate and glycogen levels were determined by the colorimetric method of DuBois et al. 1956. Absorbance was measured at 490 nm and a standard curve of glucose was prepared. For each egg yolk pool, the measurement of the biochemical component was repeated twice and results are expressed as µg/mg egg yolk wet weight.

Carbohydrate content

Carbohydrate levels were quantified by the colorimetric method of DuBois e*t al.* 1956, using the phenol-sulphuric acid reaction. Standards were prepared of concentration of 0, 20, 40, 60, 80 and 100µl/ml using 0.1mg/ml glucose stock solution and distilled water. Samples were diluted and made up to 1ml volume in triplicate in test tubes. 1ml of 5% phenol was added to each tube, then 5ml of 96% sulphuric acid rapidly added

and gently mixed. After standing for 10 minutes, tubes were placed in a water bath at 30^{o}C for 20 minutes. Absorbance was read at 490nm and a standard curve constructed using a linear line.

Protein content

Protein levels were quantified using a Coomassie (Bradford) Protein Assay kit (thermos fisher). Briefly a standard curve was constructed of 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml using a stock solution of 2mg/ml. Samples were diluted so as to take readings from the middle of the standard curve. For each sample/standard point, 20ul of sample was pipetted into 1.2ml of Bradford Dye reagent and mixed well. After standing for 10 minutes, 1ml was transferred to a cuvette for measurement at 595nm. A polynomial curve with 3 orders was used as the standard curve.

Lipid content

Lipid extraction was performed as per Mann & Gallagher, 1985. Briefly, to 300µl of sample in 15ml plastic centrifuge tubes, 100 µl of sterile water and 1.5 ml of 1:2 v/v $CHCL_3$: CH_3OH was added, vortexed and allowed to stand for 10 mins. After centrifuging at 1000g for 10 mins, the supernatant was removed to a new tube. To the remaining pellet 1.5ml of 2:1 v/v $CHCL_3$: CH_3OH was added, vortexed and then left to rest for 10 mins. After centrifuging at 1000g for 10 mins, the supernatant was pooled and 950µl of 0.7% w/v sterile NaCl solution was added, mixed and stood at 4^{o}C for >30 mins. After centrifuging at 500g for 10 min, the bottom layer was removed ($CHCL_3$) to analyse. Subsequent quantification was performed by gravimetric analysis, whereby 1ml of the lipid sample was put in pre weighed aluminium caps overnight and re-weighed.

Results

Significant differences in total egg width and egg yolk weight were recorded among batches. Fresh width of eggs ranged from 8.67 to 10.64 mm, whereas the weight of egg yolk ranged from 0.1187 to 0.4982 g. After 25 days of incubation under laboratory conditions, the egg width range changed to 8.8 to 15.7 mm.

Regarding egg yolk biochemical components, total proteins represented the main fraction, followed by carbohydrates (including glycogen) and lipids (Fig. 5, 6, 7, 8). Statistically significant differences among batches (free floating and clustered eggs) were recorded in egg yolk protein levels (Kruskall Wallis, $P < 0.001$), lipids ($P < 0.01$) and carbohydrates ($P < 0.001$). No significant correlations were found between egg yolk weight and protein/lipid/carbohydrate content.

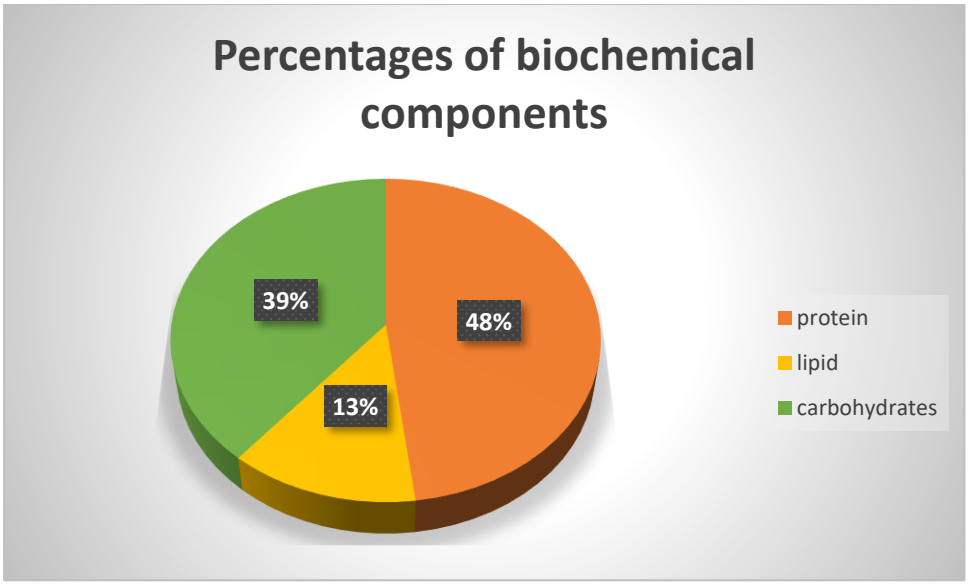


Fig. 5. Percentages of biochemical components in cuttlefish eggs, at the start of the incubation period.

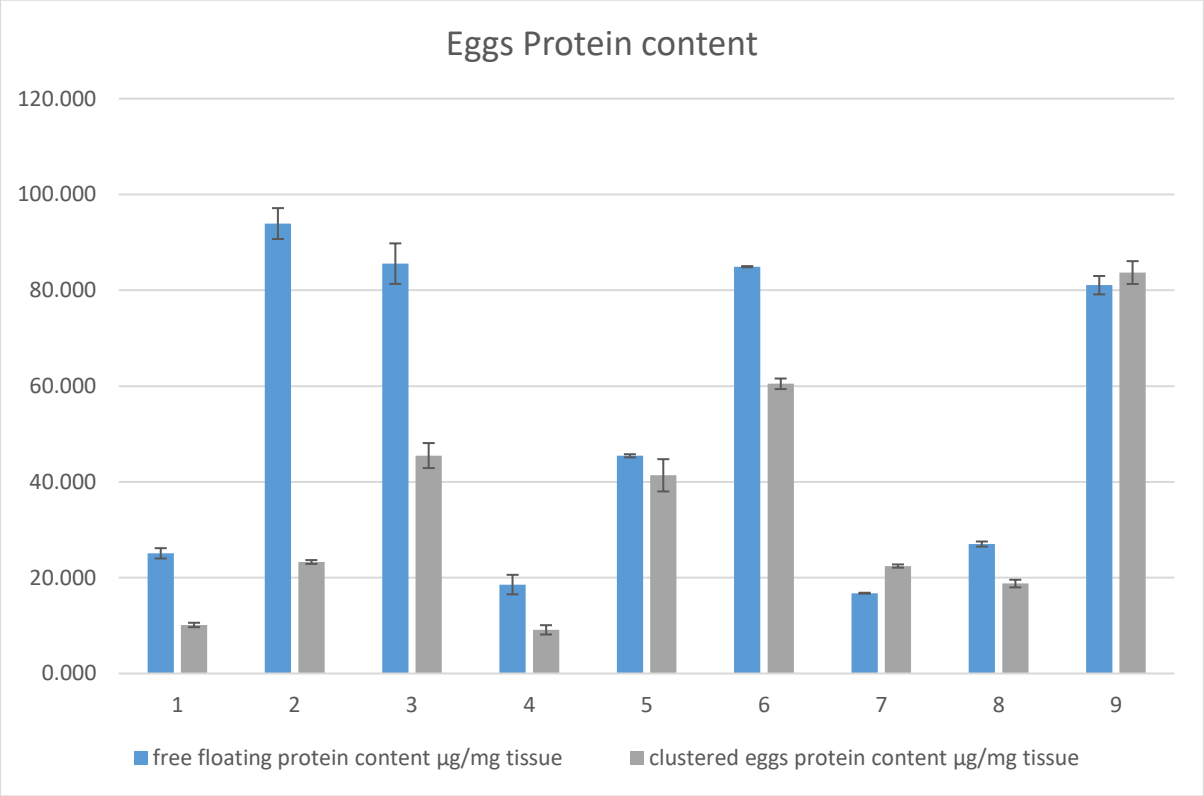


Fig.6. Protein content in eggs measured after 25 days of incubation, in tanks 1-9. Average content displayed, +/- SD.

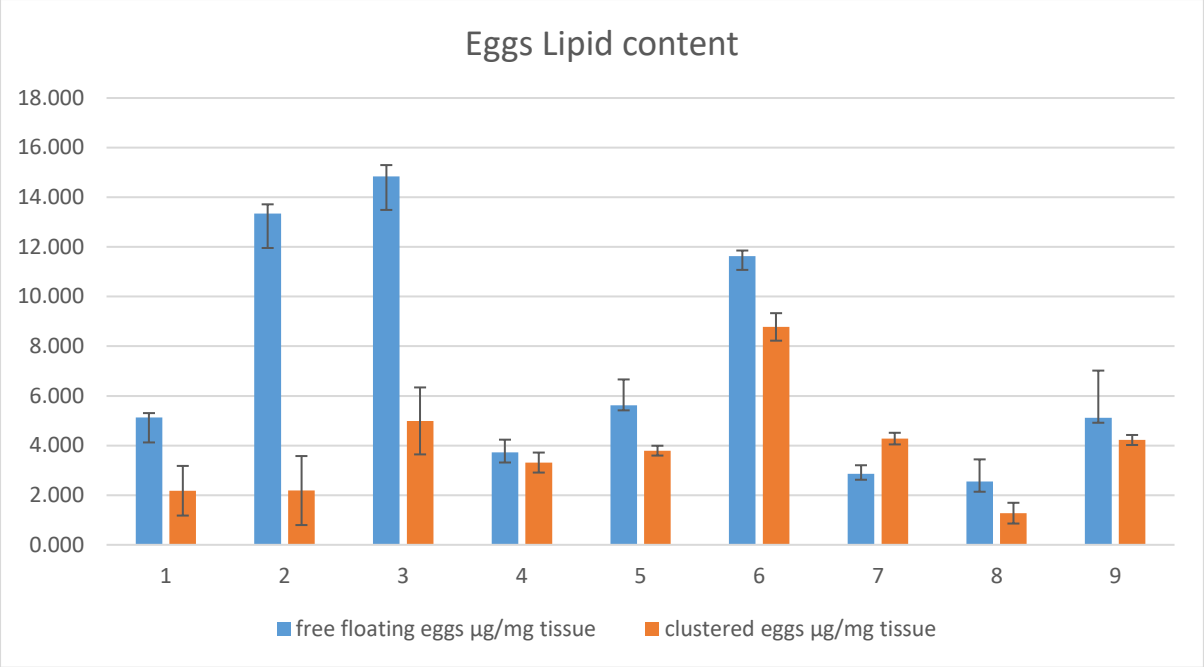


Fig.7. Lipid content in eggs measured after 25 days of incubation, in tanks 1-9. Average content displayed, +/- SD.

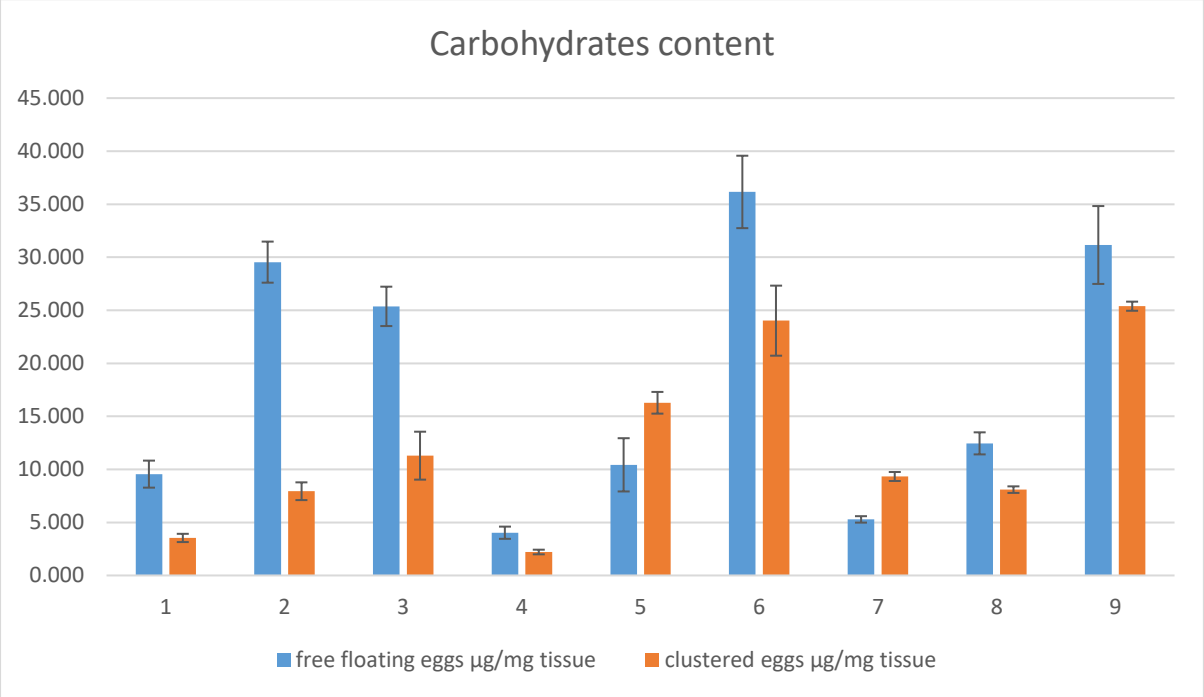


Fig.8. Carbohydrates content in eggs measured after 25 days of incubation, in tanks 1-9. Average content displayed, +/- SD.

Various studies have highlighted the flexible reproductive strategies in *Sepia officinalis* and the variability of egg sizes (Bloor et al, 2013). In particular, yolk synthesis is

strongly influenced by female metabolism, therefore diet may be an important factor affecting not only cephalopod egg size but also yolk size (Matozzo et al, 2015). In our study, egg sizes and yolk weight differed significantly among tanks and between free floating and clustered eggs. Free floating eggs constantly displayed higher levels of protein, lipids and carbohydrates, after 15 and 25 days of incubation, suggesting a slower embryo development, compared to clustered eggs. Similarly, eggs in tanks 3, 6, and 9 (pebble substrate) showed higher levels of protein, carbohydrates and lipids compared to other tanks, again indicating that the embryo development is lagging behind. Yolk energy components are essential for embryo growth and development. However, very little information is available in the literature regarding the variation in the biochemical composition of egg yolk during embryogenesis. To the best of our knowledge, the present study is one of the very few dealing with the complete biochemical composition of egg yolk in *S. officinalis* from the English Channel.

Embryos rely primarily on endogenous nutrients for development. The results of our study show that proteins are the main energy component (48%), in agreement with Matozzo et all 2015 study and the assumption that proteins constitute the main components of marine invertebrate eggs. Other studies recorded a marked decrease in total protein content during embryonic development in *Sepia esculenta*, (Lei et al, 2013), suggesting that lower levels of protein in developing eggs are a marker of successful embryogenesis.

The relatively high percentage of carbohydrates (39%) recorded in the present study suggested that they are also important energy resources for *S. officinalis* embryos. The results are very similar to those reported by Matozzo et al, 2015, but in sharp contrast with a previous study in *S. esculenta* (Lei et al, 2013). Further studies should investigate differences in carbohydrate content in eggs at different stages of embryo development, to better understand the functional role of carbohydrates.

Total lipids were the lowest biochemical component in egg yolk of *S. officinalis,* suggesting that lipids represent a marginal energy substrate. Sykes et al. 2009 argued that lipids are not used as energetic substrate but as structural components. Interestingly, Lei et al, 2013 highlighted that in *S. esculenta* the contents and use of lipids during embryo development may vary according to both biotic (different species with different metabolism) and abiotic (mainly temperature) factors. Our study recorded lower levels of lipids in almost all clustered eggs compared to their free-floating counterparts (Fig. 7).

Hatching rates

High fishing pressure is generally exerted on spawning adults, with traps exploiting female attraction to deposition substrates and male attraction towards females, capturing almost exclusively mature breeding individuals. Consequently, females

often lay eggs on the trap surface and eggs are actively removed from the fishing gear to prevent a reduction in fishing capacity. In Morbihan Bay, France, it has been estimated that this practice causes the destruction of 18–40 millions of eggs per season (Melli et al, 2014). Egg destruction, the reduction of coastal seagrass and seaweed habitats are likely to contribute to the declines in the cuttlefish fishery. The present study, hosted by the Hastings campus UoB, aimed to investigate the hatching success of the eggs that have been actively removed from the cuttlefish traps and thrown into the sea. Hatching rates were estimated after 46 days of incubation under laboratory conditions. Different substrates (sand, gravel, pebbles), water movement and two different incubation temperatures (17^0C and 21^0C) have been tested during the study.

The different experimental conditions used in this study had a great influence in the hatching rate, as demonstrated by the results below (Fig. 9, 10). Moreover, examining the hatching rates obtained in the laboratory, the manipulation and translocation of eggs from fishing boats to the hatching site (in this case, the laboratory), we can conclude that clustered eggs have more chances to successfully hatch than free floating ones. None of the free-floating eggs has hatched after 46 days of incubation, irrespective of the conditions in the experimental tanks. This result is in agreement with the anecdotal observation frequently reported by divers, that no free floating eggs are observed in the seawater, even at the height of the cuttlefish spawning season. There are several hypothesis accounting for the lack of free-floating eggs, including predation, washing up on the shore and destruction following scraping against the substrate.

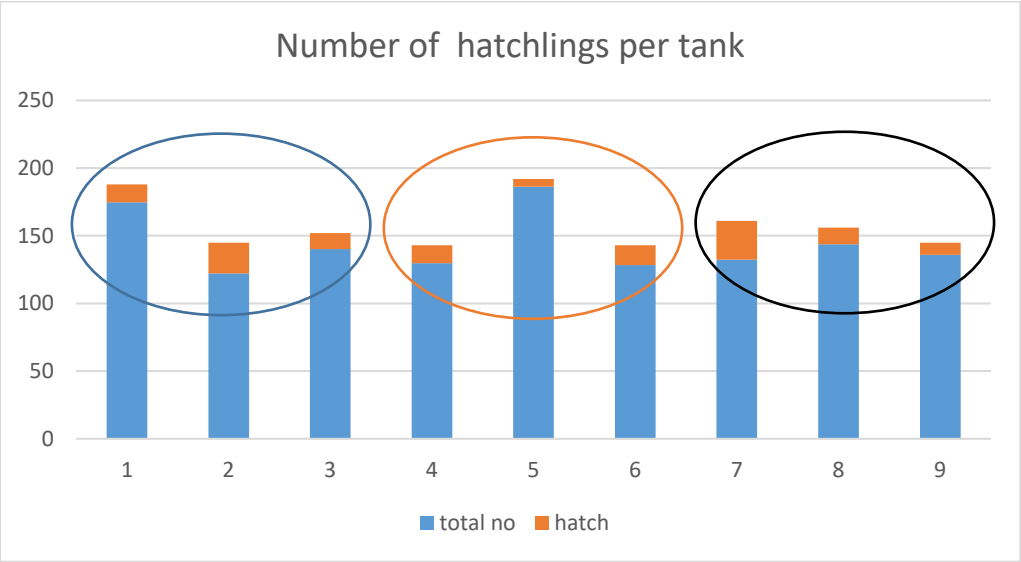


Fig. 9. Number of hatched eggs per tank, grouped as control (1-3), water currents (4-6) and heating (7-9) treatments.

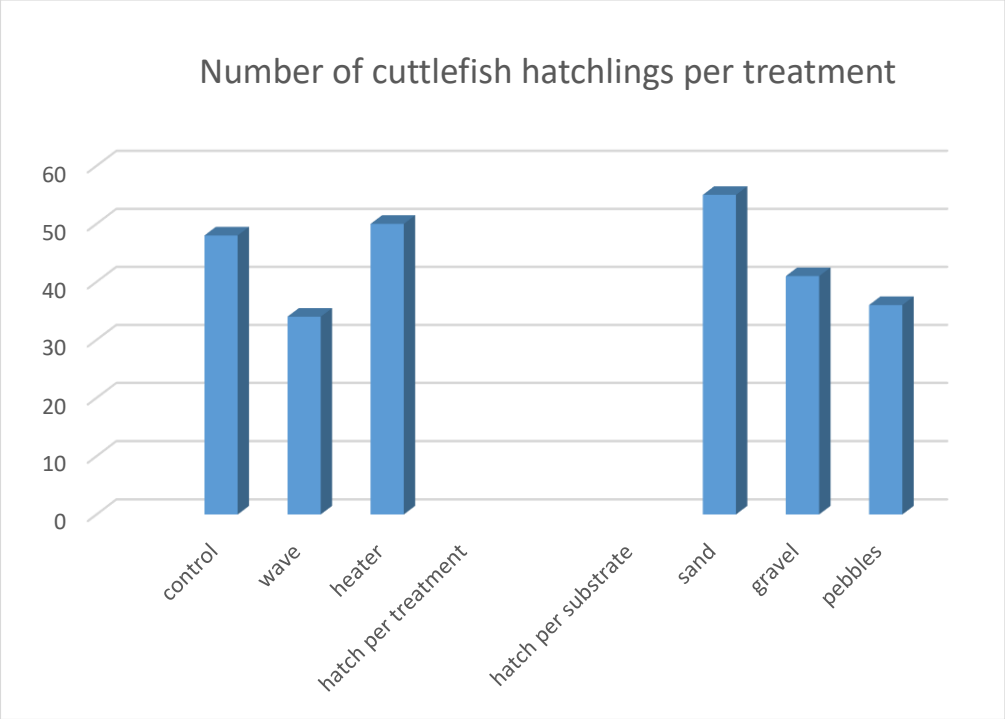


Fig.10. Number of cuttlefish hatchlings per treatment and substrate, counted after 46 days of incubation, under laboratory conditions

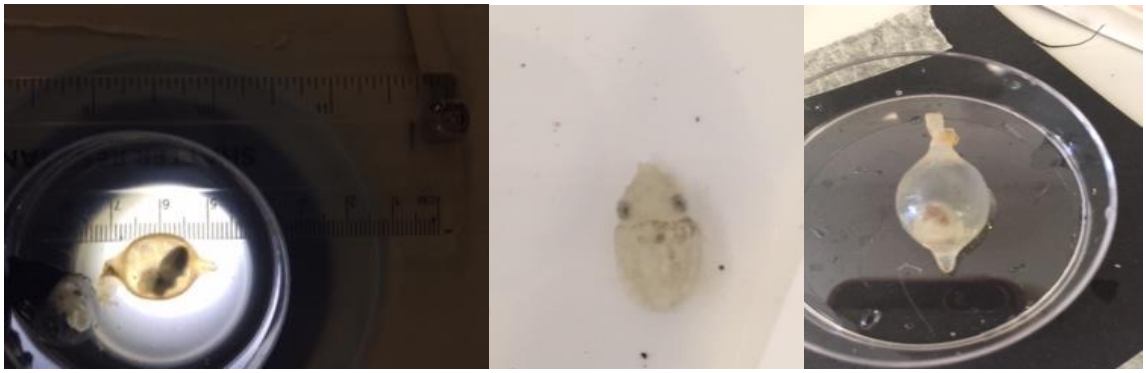
Overall, our study recorded an average of 11% hatchings out of the total number of eggs initially included in the experiment. Highest hatching rates (10.7%) were obtained in the 21^0C water (tanks 7,8,9); the lowest rate was recorded in the tanks equipped with water fans (6.6% in tanks 4,5,6). Regarding the preferred substrate for the optimal development of the embryos, our study revealed that the sand supported the highest hatching rate of 11.5%, as opposed to the pebble substrate, where only 36 hatchlings were recorded out of 454 eggs (7.8% hatching rate).

Our results are in accordance with previous studies (Domingues et al, 2006), indicating that higher temperatures are likely to accelerate the embryonic development in cuttlefish eggs; however, eggs actively detached from the traps and thrown into the sea have more chances to develop on sandy substrate and calm waters, rather than gravel or pebbles and strong currents. Both observations highlight the importance of environmental factors on cuttlefish reproduction success.

Embryology

Sepia officinalis eggs have been proved to be an attractive material for embryological studies due to the possibility of live observations under straightforward conditions (using simple light microscopes and dissecting microscopes). Several observations have been made during our study, here is a summary of findings.

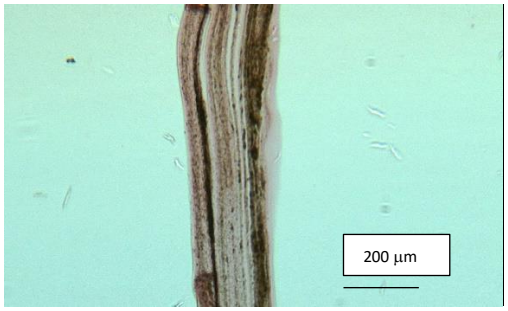
Although premature hatching can occur under stress conditions, thus influencing the size of hatchlings, usually the hatchlings of this species have a mantle length ranging from 6 to 9 mm (Domingues et al, 2006). Our results showed that most of the hatchlings measured between 6.5 and 10mm (Fig.11 a), depending primarily on the egg size. *S. officinalis* newly hatched are strikingly similar to the adults; however, different body colour has been noted in hatchling from white eggs compared to the black eggs (Fig 11 b, c).

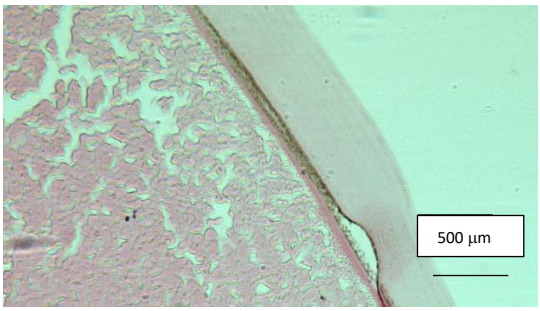


A B C

Fig. 11. Living embryos of cuttlefish photographed through a dissecting microscope (A and C). Embryo developing inside a white egg (C) resulting in a light coloured hatchling (B). (own photo).

Various studies on cuttlefish recorded that occasionally, cuttlefish females produce unstained, translucent, whitish egg cases. In some studies, those eggs have been discarded under the assumption that they are unfertilized. However, other authors have highlighted the great potential for embryonic studies on white eggs, because of the transparent outer envelope and the better observation of the embryo (Boletzky et al, 2006). A very recent paper (O'Brien et al, 2018) has highlighted the effects of stressors applied to reproducing females and developing embryos, with fewer eggs being laid by stressed mothers followed by lower hatching rates. In addition, stressed mothers laid mostly white eggs, lacking the dark pigment characteristic for this species. We have included approximately 10% white eggs in our hatching experiment and the final counts revealed that there were no statistically significant differences between the hatching rates in white eggs compared to the black eggs.





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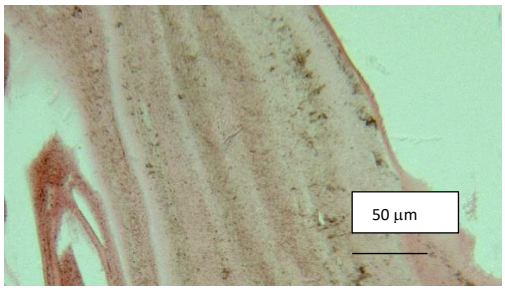

C

Fig. 12. Histological section through a black egg case (A) and a white case (B and C), after 25 days of incubation in laboratory. Tissues stained with Haematoxylin and eosin. Notably less pigment present in the white case. (own photos).

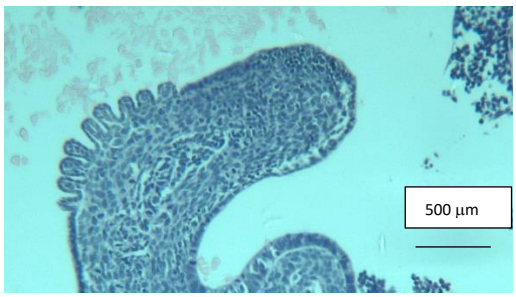

Fig.13. Peripheral structures of a disk shaped embryo developed in a white egg (after 25 days of incubation) – the arm buds are visible in this histological section. Tissues stained with Haematoxylin-eosin. (own photo).

Conclusions

Overall, our results, obtained through integrating experimental data with the knowledge of fishermen and relevant scientific literature, highlight the significant impact of environmental conditions on cuttlefish embryo development and the success of hatching.

Cuttlefish are exploited by different types of fisheries and are likely impacted by the habitat degradation. Therefore, the management of this species is not easy.

However, our study revealed that it is possible for the dislocated eggs to develop and hatch, as long as the environmental conditions are not too harsh (temperature and substrate). Free floating eggs are less likely to develop, as demonstrated by the biochemical characteristics and the lower hatching rates under laboratory conditions.

White eggs, presumably laid by stressed mothers, have less pigment deposited in the egg capsule. Although the light colour case may not offer the necessary camouflage for the developing embryo (and therefore put the eggs at higher risk of predation), the hatching rates proved to be similar to those of black eggs, under laboratory conditions. However, lighter coloured hatchlings have been obtained from white eggs, which again can constitute a disadvantage in terms of camouflage and ink squirting abilities, further suggesting a potentially reduced survival rate.

There are two further ongoing studies within the UoB laboratories at the moment, investigating:

a) The antibacterial properties of the cuttlefish egg cases and their role in protecting the developing embryo in eggs dislocated from the traps.
b) The potential of environmentally friendly materials (algal derived biomaterial) to act as receptors for cuttlefish eggs, in aquaculture and poor habitats.

We wish to thank Sussex IFCA officers Kat Nelson and Daniel Davies for their cooperation in this project, Hastings fishers for assisting in egg collection and colleagues at Hastings Campus for their support in maintaining the right conditions in the experimental room, during a very hot summer. Many thanks to Claude Annels for promoting this study at the UoB seminar in April 2019.

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