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PROJET INTERREG IV A France-Manche- Angleterre

CRESH

LIVRABLES

2012

TASK 2

In situ observations of natural substrata where spawning females attach their eggs

Observation des supports naturels utilisés pour la ponte sur des sites pilotes

Objective/*Objectifs*: *In situ* observation of natural substratum where spawning females attach their eggs (in both English and French coastal waters). Analysis of environmental variability of eggs and hatchlings habitats (diving observations and experimental fishing).

Observation des supports naturels utilisés pour la ponte sur des sites pilotes (côtes anglaises et françaises). Etude de la variabilité des substrats et des conditions environnementales entourant les œufs et les juvéniles (observations en plongée et pêches expérimentales).

Work progress until 30/07/2012
Etat d'avancée des travaux au 31 juillet 2012

Task 2 database available in the DVD included in claim 4 parcel
La base de donnée de la tâche 2 est disponible sur le DVD inclus dans le colis de la remontée de dépenses n°4

I. 2012 Task Results Summary / Résumé des résultats obtenus en 2012

Subtidal observations of natural egg laying structures and habitats were undertaken within two seagrass beds at the study site of Torbay on the UK coast. These observations were completed as part of a time series which was carried out from 2010 to 2012. Surveys were conducted in May, June and July using a 100 m^2 line belt transect. The results of this research showed that densities of eggs within these two areas was significantly lower than in 2011 and that this may be the result of increased natural disturbance (e.g. storms) that affected the spatial dynamics of the seagrass beds in this area in 2012, thereby reducing the available area and quality of structures available for spawning.

Des observations subtidales des structures naturelles de pontes ainsi que des habitas ont été effectuées sur deux lits d'algues du site d'étude de Torbay, sur la côte britannique.
Ces observations ont été effectuées dans le cadre d'une étude chronologique de 2010 à 2012, et ont été conduites par transect au moyen d'une ceinture de 100m2 en mai, juin et juillet.
Les résultats de cette recherche montrent que les densités d'œufs au sein des deux aires étudiées sont bien moindres qu'en 2011. Cela peut être la conséquence d'une perturbation naturelle en augmentation (comme par exemple due à des tempêtes) qui affecterait les dynamiques spatiales des lits d'algues dans cette aire en 2012, et par conséquent diminuerait l'espace ainsi que les structures disponibles pour la ponte.

II. Actions carried out in 2012/ *Bilan des opérations réalisées en 2012*

- Subtidal observations of cuttlefish spawning within two seagrass beds at the study site of Torbay on the UK coast (May, June and July).
- Analysis of qualitative and quantitative data obtained within the English Channel on both the UK and French coastlines between 2010 and 2012.

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- Observations subtidales de frayères de seiches de deux lits d'algues du site d'étude de Torbay sur la côte britannique (mai, juin, juillet).
- Analyse de données qualitatives et quantitatives obtenues dans la Manche sur les côtes anglaises et françaises entre 2010 et 2012.

III. Scientific outputs / *Détail des travaux scientifiques de l'année*

In many species, migratory patterns like those exhibited by *S. officinalis* have evolved as a result of the need for spawning adults to deposit their eggs in a habitat in which the ecological and environmental conditions are optimal (spatially and/or temporally) for survival and growth of their offspring (e.g. Dodson, 1997, Pierce et al., 2008). At spawning, *S. officinalis* females deposit their eggs on structures attached to the seabed, which means that developing embryos remain at the site of spawning. As such, the location (oviposition site) that mothers 'select' to lay their eggs can dramatically affect offspring performance and fitness by determining the local environment and conditions in which their offspring will develop (Marshall et al., 2008), yet specific details of the structural components and substratum types of these inshore spawning habitats is lacking.

Spawning intensity is thought to vary both spatially and temporally across the English Channel coastline. The patterns of spawning in benthic species like *S. officinalis* can be described directly through natural observations of spawning areas. It is expected that such *in situ* observations will help to better understand the range of habitats and structures used for spawning and the factors and processes that influence variability in spawning patterns.

Study sites:

Subtidal surveys were conducted at study sites along the English Channel coastline to obtain the data for both qualitative and quantitative analysis of the natural spawning structures used by *S. officinalis* within these inshore waters. Surveys were restricted to shallow coastal areas, due to both the safe limit for scientific diving and pre-existing knowledge of the lifecycle of this species, which indicates that spawning adults migrate to the shallow inshore areas of the English Channel to spawn. Five study sites were selected from areas that supported active inshore cuttlefish trap fisheries during the spring and summer, and incorporated sites in both the Eastern and Western English Channel. This was considered important as these areas are known to be distinctive in terms of their oceanographic and hydrodynamic conditions as well as their fisheries and general ecology (e.g. Araujo et al., 2005). The sites selected were Torbay, Poole and Selsey on the UK coast and Langrune-sur-Mer and Agon-Coutainville on the French coast (Figure 1).

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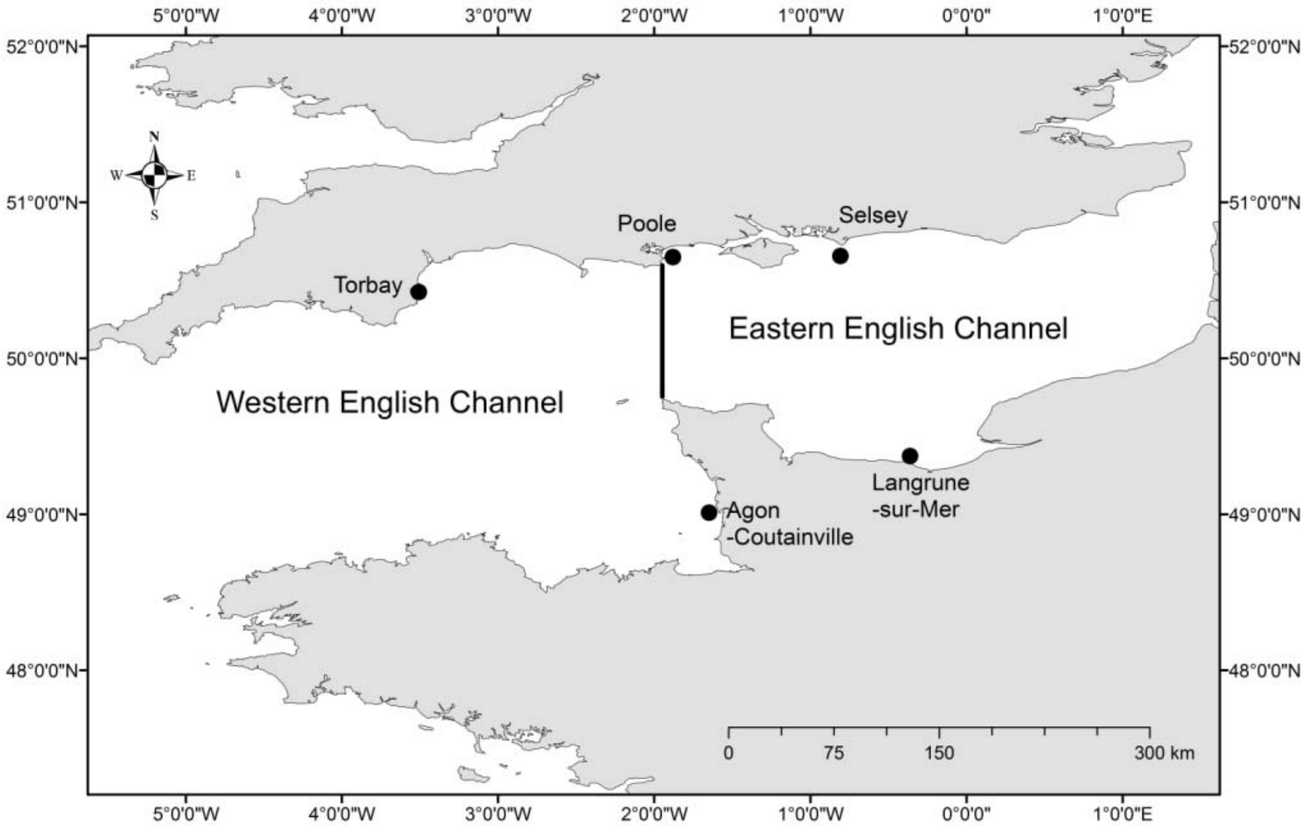

Figure 1: English Channel Study Sites. The location of each study site are indicated on the map and include : Torbay, Poole and Selsey on the UK coast and Agon-Coutainville and Langrune-sur-Mer on the French coast. The vertical black line indicates the split between the Eastern and Western basin of the English Channel as proposed by Pomerol (1977).

Survey methods :

A variety of methods, using SCUBA, were used to obtain data for both the quantitative and qualitative analysis of natural spawning structures. During this research, five study sites along the UK and French coast were surveyed (Torbay, Selsey, Poole Bay, Agon-Coutainville and Langrune-sur-Mer). The variation in hydrological conditions among study sites was high, with tidal systems ranging from megatidal at Agon-Coutainville to microtidal at Poole Bay and additionally large differences in current regimes were also evidenced. Therefore, a single unified survey method was not considered suitable for standardised use across all study sites. Whilst every effort was made to keep the survey method as standardised as possible, methodological changes were required among sites to allow surveys to be undertaken safely. These methods included 50 m^2 circular belt transects (Figure 2a), 100 m^2 line belt transects (Figure 2b), which were used in areas with low currents and timed global positioning system (GPS)-tracked drift transects (Figure 2c), which were suitable for areas with strong currents.

Survey design:

At all sites stratified random sampling was used to obtain the start locations of transects. This was done using ArcGIS (Esri, v.9.3) to construct a grid that overlaid the study site. Each square within the grid was assigned a unique identifying code and then a random subset of squares were selected using a random number generator in Excel (Microsoft Ltd, 2007) to obtain random start locations for transects.

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Qualitative assessment of spawning structures:

For this study all three methods were utilised to obtain data for qualitative analysis. Surveys were conducted between 2010 and 2012 and took place between April and July, to align with the cuttlefish spawning season. On the UK coast all surveys were subtidal, whilst on the French coast, due to the large tidal range both subtidal (SCUBA) and intertidal (walking) surveys were undertaken.

Comparison of spawning strata (structures):

A comparison of spawning between two strata with different structure types, seagrass beds (seagrass stratum) and mixed seaweed habitat (mixed stratum) was undertaken. A preliminary trial within Torbay was conducted in May and July 2010, where the study area was delimited by the natural geographic boundary of the Bay and within the area of the 10 m depth contour. Four seagrass and four mixed stratum sites were surveyed at Torbay in May 2010 and five seagrass and five mixed stratum sites in July 2010. For each survey site, three replicates were undertaken using 50 m^2 circular belt transects. The main study was undertaken in June 2011 at two study sites (Torbay and Poole Bay) to compare spawning patterns between seagrass and mixed stratum. The study site for the Torbay area was altered for the main study in 2011, with the new study area extending from Hollicombe Head around the headland at Hope's Nose and on to Babbacombe Bay. This was done to better reflect the study area at Poole Bay which extended from the Branksome reef around the headland at Handfast point and onto Ballard Pinnacle. For both sites the study area was restricted to within the 10 m depth contour. Transect start points for all surveys (2010 and 2011) were randomly assigned within the two strata. In June 2011, our seagrass and four mixed stratum sites were surveyed at each study site, with eight replicates at each stratum site. All surveys were completed using 100 m^2 line belt transects, although due to adverse diving conditions, it was not always possible to undertake a full set of replicates.

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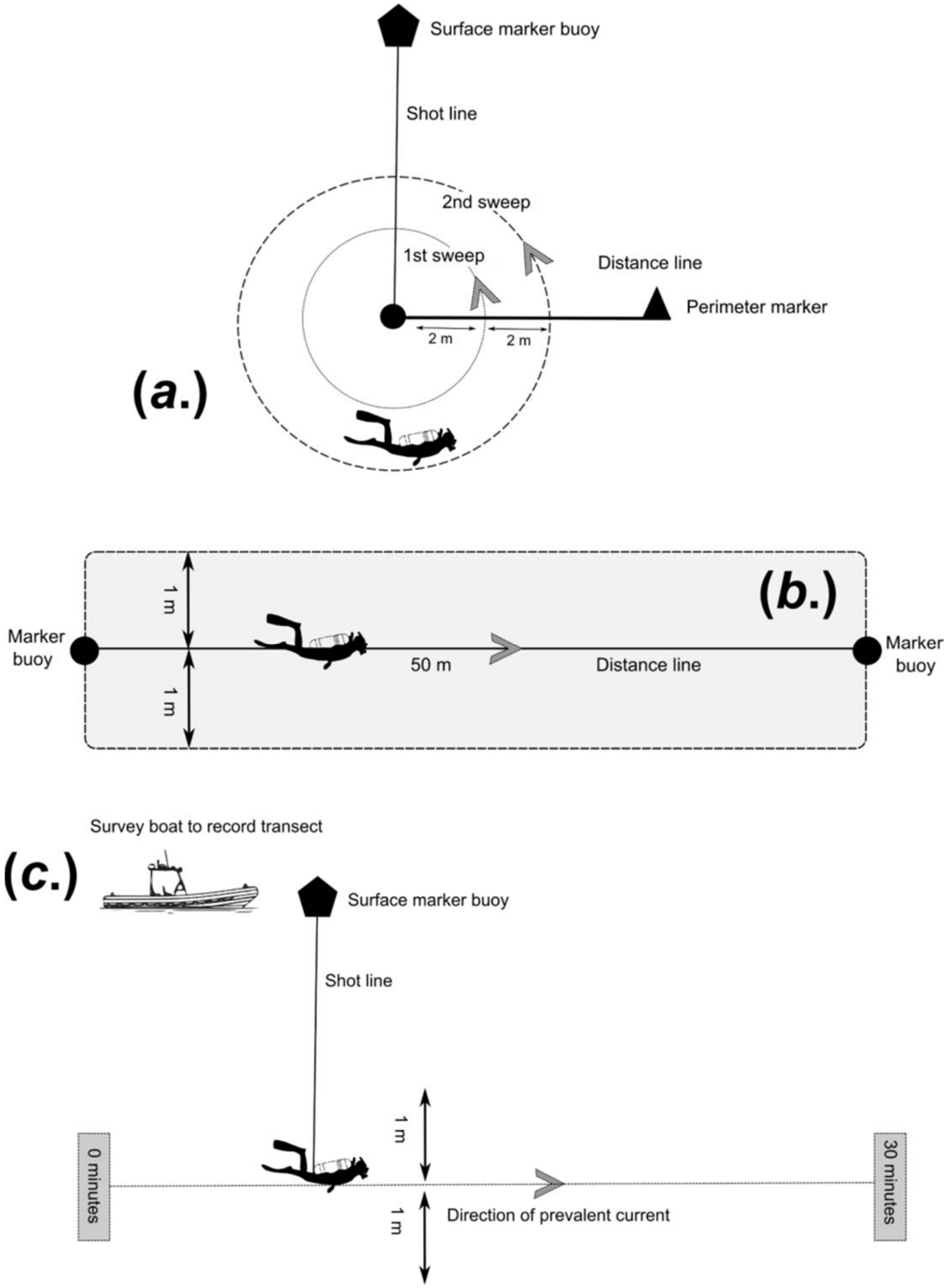

Figure 2: Survey methods : (a.) Circular belt transect, (b.) Line belt transect, (c.) GPS-tracked drift transect.

Temporal analysis of spawning in seagrass beds:

A comparison of spawning patterns and intensity within a season (May, June and July) was undertaken at two seagrass beds (Millstones Bay and Torre Abbey Sands) between 2010 and 2012.

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The extents of the seagrass beds were delimited according to the results of the 2006 Torbay coast and countryside trust (TCCT) seagrass project (TCCT 2006). Transect start points were randomly assigned within each seagrass bed, however, due to the dated nature of the available seagrass maps, a drop-down video camera was also used to verify the presence of seagrass prior to the deployment of transects. If seagrass presence could not be verified at a transect start position, an alternative location was selected. At Millstones Bay and Torre Abbey Sands four replicates were undertaken at each site in 2010 (May and July) using 50 m^2 circular belt transects and eight replicates undertaken at each site in 2011 and 2012 (May, June and July) using 100 m^2 line belt transects, although due to adverse diving conditions, it was not always possible to undertake a full set of replicates.

Results:

Qualitative assessment of spawning structures:

A total of 15 different spawning structures were recorded in this study. The height of these structures varied between 20 and 800 cm and the widths of sections used for egg attachment varied between 0.6 and 15 mm. A summary of the key attributes of these structures and the patterns of spawning observed are presented in Table 1, whilst photographic examples of spawning structures are presented in Figure 4, Figure 5, and Figure 6. The patterns of spawning observed varied between structures, for example *Chorda filum* is composed of cylindrical fronds which are formed from hollow tubes of approximately 0.6 mm in diameter and which grow up to 8 m in height (Bunker et al., 2010). Whilst the diameter of fronds is small, observations of egg laying on this species showed that multiple fronds were grouped together in order to achieve a suitable size for egg attachment (Figure 3) with eggs observed attached to large portions of the total length of this structure. A different type of structural composition and pattern of egg attachment was observed in *Halidrys siliquosa*. This species reaches heights of between 30 and 120 cm and has compressed fronds (< 1 cm wide) with air bladders attached. It grows as a bushy structure with thalli which attach via a strong discoid holdfast (Bunker et al., 2010). The rigid nature of this structure enables it to support multiple egg cluster to be attached to the branches of this plant with coverage of large areas (Figure 5c). In the angiosperm *Z. marina* which grows up to 2 m in height and can form large meadows or beds, with egg attachment observed on both the stem and to groups of leaves (Figure 6a). In addition to plant structures, egg laying was also observed on sessile animals, for example, *Sabella pavonina* which is a polychaete worm that grows up to 30 cm in height and 4 mm in width and can form small forests (Wood, 2007). Eggs were observed attached to the tubes of the worms and their rigid nature supported attachment along the entire length of the tube (Figure 6c).

Table 1: A table indicating the characteristics of natural structures recorded during qualitative subtidal and intertidal surveys (2010-2012).

Species	Structure				Cluster			
	Type	Max depth	Height (cm)	Width (mm)	IT/ST	FR/UK	median size	Total recorded
C. crispus	seaweed (R)	24	22	xx	IT	FR	20	188
C. filum	seaweed (B)	xx	800	0.6	IT	FR	200	1
D. ligulata	seaweed (B)	9	200	2-7	ST	FR	75	3
D. sanguinea	seaweed (R)	30	25	xx	ST	UK	50	1
F. lumbricalis	seaweed (R)	12	30	2	IT/ST	FR	35	6
F. serratus	seaweed (B)	xx	60	20	IT	FR	75	41
Gymnogongus Sp.	seaweed (R)	xx	10	xx	IT	FR	35	6
H. siliquosa	seaweed (B)	xx	120	10	ST	UK	35	30
Nemertesia Sp.	hydroid	xx	25	xx	ST	FR	150	16
Porifera Sp.	sponge	xx	xx	15	ST	FR	50	9
S. chordalis	seaweed (R)	5	20	2	ST/IT	FR	40	3
S. latissima	seaweed (B)	30	150	xx	ST	FR	35	4
S. muticum	seaweed (B)	xx	200	xx	ST/IT	FR	20	116
S. pavonina	fan worm	xx	30	4	ST	UK/FR	75	82
Z. marina	seagrass	9	200	xx	IT/ST	UK/FR	10	1007

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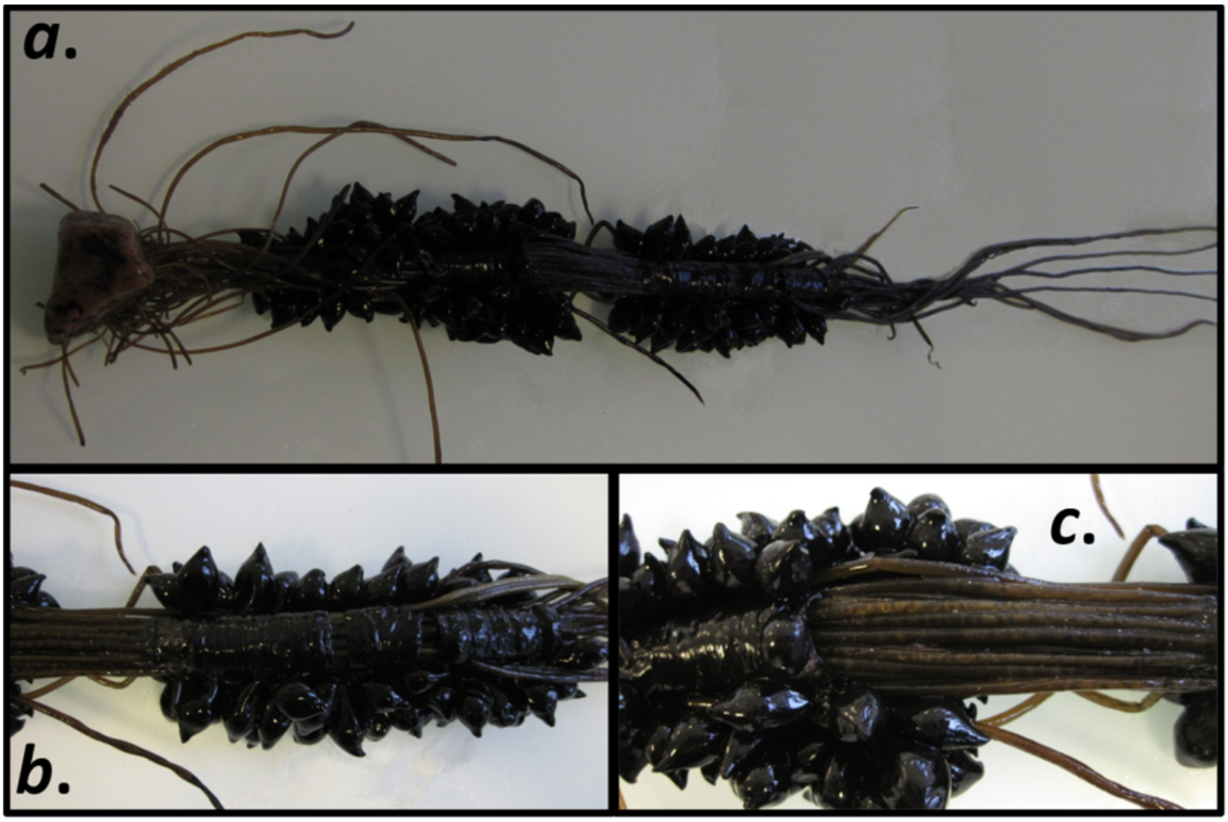

Figure 3: ***C. filum*** **with cuttlefish eggs attached. (a.) egg cluster on** ***C. filum,*** **(b.) and (c.) close up of egg attachemnt, illustrating the use of multiple fronds to obtain a suitable diameter for egg attachment.**

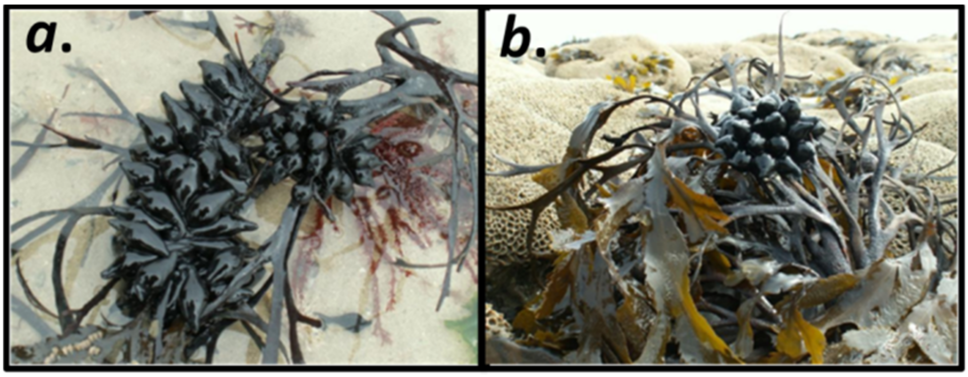

Figure 4: Examples of egg clusters laid on spawning structures intertidally. (a.) ***Chondrus crispus*****, (b.)** ***Fucus serratus*****. Photographs courtesy of University of Caen**

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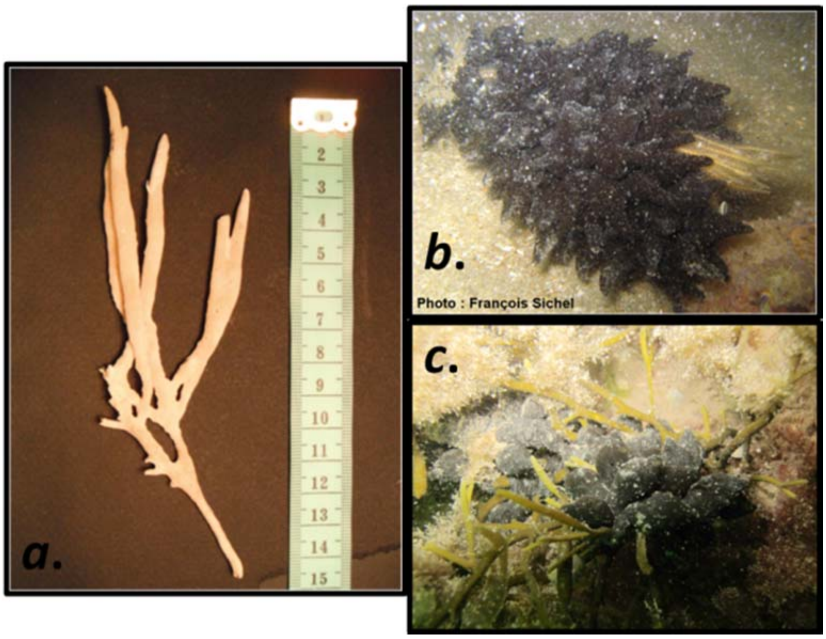

Figure 5: Examples of egg clusters laid on subtidal spawning structures. (a.) a piece from a *Porifera Sp.* on which eggs were found, (b.) *Nemertesia antennina* (photograph by Francois Sichel) and (c.) *H. siliquosa*. Photographs *a* and *b* courtesy of University of Caen.

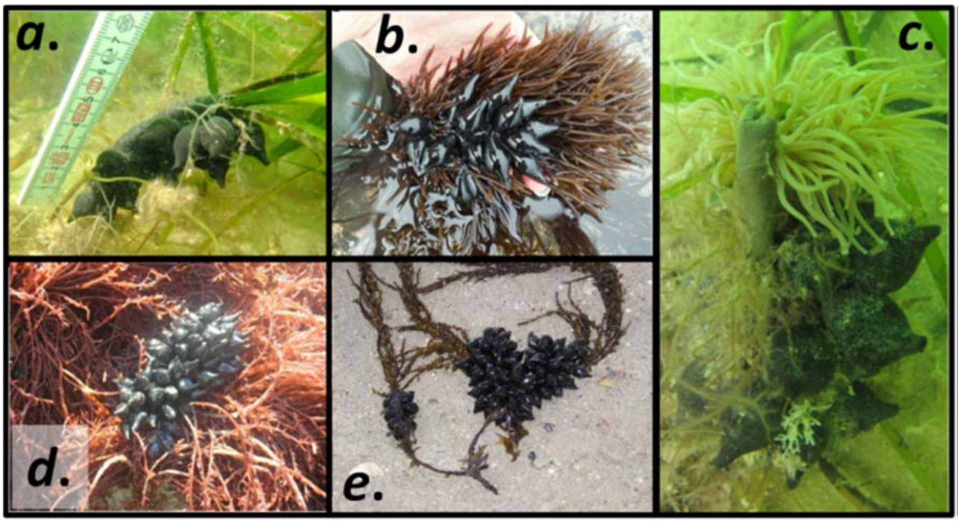

Figure 6: Examples of egg clusters laid on spawning structures both subtidally and intertidally. (a.) *Z. marina*, (b.) *Furcellaria lumbricalis*, (c.) *S. pavonina,* (d.) *Solieria chordalis* and (e.) *Sargassim muticum*. Photographs *b, d* and *e* courtesy of University of Caen.

Structure use among sites :

The diversity and type of spawning structures utilised was found to vary among sites. On the UK coast only three different spawning structures (Table 1) were identified across the three study sites (*Z. marina* 997 egg clusters, *H. Siliquosa* 30 egg clusters and *S. pavonina* three egg clusters) with Z. marina providing the highest numbers of egg clusters recorded for UK sites . On the French coast a total of twelve different spawning structures were identified with the most egg clusters (188) recorded on *C. crispus*. The most diverse range of structures was identified at Agon-Coutainville (Table 1) with ten types of spawning structure identified within the subtidal and intertidal ranges. *Z. marina* plants recorded the highest number of egg clusters when pooled across all sites (UK and France) and across all years with 1007 egg clusters recorded in total.

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Variation in cluster size among structures:

Figure 7 shows the variation in egg cluster size (number of eggs per cluster) with structure type. A few key points regarding cluster size among structures can be highlighted. For example in structures such as *S. pavonina* where the entire length of the tube (up to 30 cm) can be utilised for egg laying, the box plot indicated a relatively high median, range and maximum number of eggs per cluster. A similar pattern is shown for *C. filum* where almost the entire length of the structure (up to 800 cm) can be utilised for egg laying . In contrast for structures such as *S. latissima* where only a small fraction of the total structure (e.g. Stipe) is available for egg attachment, the box plot indicated a relatively low median, range and maximum number of eggs per cluster. In order to test whether the difference in egg cluster size among structures was significant a Kruskal-Wallis test was performed. The results of this test indicated a significant difference among structure type in the numbers of eggs laid per cluster ($H = 431.49$, $DF = 10$, $P < 0.0001$). The following structure types were excluded from the analysis as they contained fewer than five samples per group: *Solieria chordalis*, *Desmarestia ligulata*, *Algae Z*, *C. filum*, *Delesseria sanguinea* and *Saccharina latissima*.

Comparison of spawning strata (structures):

In May 2010 a total of 147 egg clusters were recorded across all sites surveyed within Torbay. Egg clusters were recorded at all four seagrass sites attached to *Z. marina* plants. However, no egg clusters were recorded at any of the four mixed structure sites where seaweeds were present. Seaweeds recorded as present within mixed substrate sites included short faunal turf and mixed red and brown seaweed species. During July 2010, ten sites (five seagrass and five mixed) were surveyed within Torbay with four replicates at each site (excluding Torre Abbey Sands where adverse weather conditions forced the survey to be terminated for safety reasons), with a total of 45 egg clusters recorded across all sites. Egg clusters were recorded at all five seagrass sites attached to *Z. marina* plants. However, no egg clusters were recorded at any of the five mixed structure sites where seaweeds were present. The results of these two studies indicated that a significant difference existed between the spawning patterns of these two strata within Torbay, with eggs only recorded attached to *Z. marina* plants within the seagrass stratum. In June 2011, egg clusters were recorded at all seagrass sites at Torbay. Egg clusters were also recorded attached to a steel rope at Outer Millstones and to the brown seaweed *H. siliquosa* at Babbacombe Bay, there were no egg clusters recorded at the remaining mixed substrate sites. Across the eight sites surveyed at Torbay, a total of 173 egg clusters was recorded. In Poole Bay egg clusters were recorded at two of the four seagrass sites where eggs were attached to both *Z. marina* and *S. pavonina*. Only a single egg cluster was recorded within the four mixed substrate sites attached to a ghost trap within the site at Handfast Point. Structures recorded as present during surveys of the area included *S. muticum*, *H. siliquosa*, Kelp (*Laminaria hyperborea*), short faunal turf and large quantities of red foliose algae. Over the eight sites a total of 100 egg clusters was recorded at Poole Bay. The replicates undertaken at both sites were pooled by strata and a Mann-Whitney U-Test performed to compare the distributions of the two groups. This was done to compare egg clusters and number of eggs observed within transects. For both situations the results indicated a significant difference between the two distributions, with the mean ranks indicating that seagrass beds were found to have significantly more eggs and egg clusters per transect than mixed seaweed habitats (Table 2).

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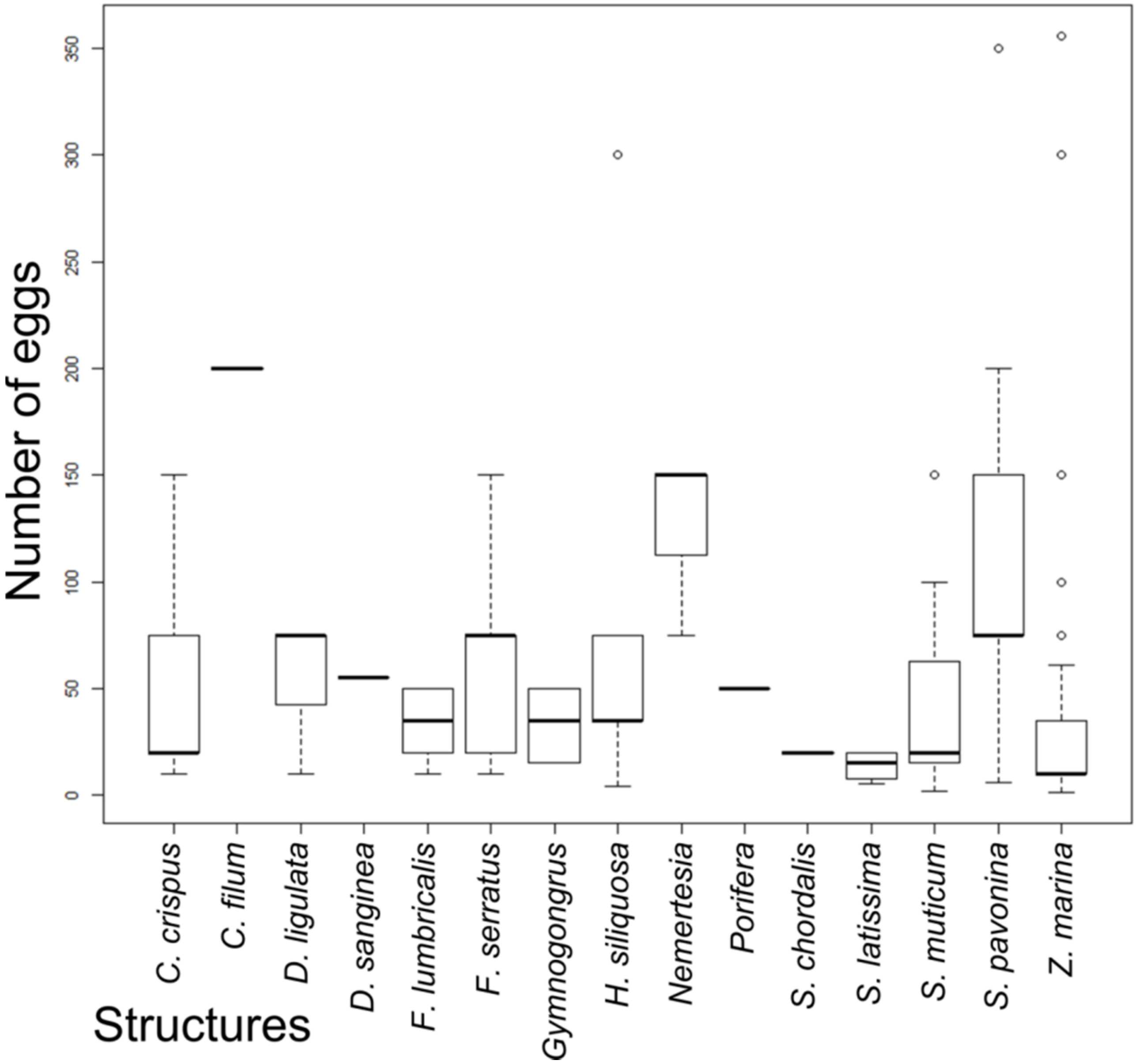

Figure 7: A box plot showing the variation in egg cluster size (numbers of eggs per cluster) among natural spawning structures.

Table 2 : Results of the Mann-Whitney U test for differences between seagrass and mixed strata (Poole and Torbay, June 2011). MR = Mean Rank.

Condition	Test	Z value	MR Seagrass	MR Mixed	Significance
No. egg clusters	Mann-Whitney U	3.18	59.30	44.56	0.0015
No. eggs	Mann-Whitney U	2.78	57.91	45.23	0.0055

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Temporal analysis of spawning within seagrass beds (Torbay 2010 – 2012) :

Egg density :

The egg densities for each month and year are presented in Table 3 for Millstones Bay and Table 4 for Torre Abbey Sands. The data for 2010 are presented for both sites, although it should be noted that a different methodology was used to collect data for this year. At Millstones Bay the highest egg density recorded was in May 2010 (11.3 eggs per m^2) and the lowest egg density recorded was in May 2012 (0.025 eggs per m^2). Egg densities at this site were lower in 2012 (for all months) compared to 2010 or 2011 (Table 3). At Torre Abbey Sands the highest egg density recorded was in July 2011 (3.9 eggs per m^2) and the lowest egg density recorded was in June 2012 (0.008 eggs per m^2). Egg densities at this site were lower in 2012 (for all months) compared to 2010 or 2011 (Table 4).

Table 3: Temporal variation in mean egg density at Millstones Bay seagrass site (2010 -2012). Estimates of total eggs calculated for the total area of Millstones Bay (15.500 m^2). Estimates of potential spawning females calculated using the total number of eggs at a site divided by an average fecundity of 2,000.

Year	Egg clusters			Egg density			Total eggs at site			Potential spawning females		
	May	June	July	May	June	July	May	June	July	May	June	July
2010	117	-	23	11.3	-	1.4	175150	-	21700	88	-	11
2011	8	116	29	0.3	3.7	1.4	4650	57350	21700	2	29	11
2012	20	77	77	0.025	0.1	0.1	388	1550	1550	1	1	1

Table 4: Temporal variation in mean egg density at Torre Abbey Sands seagrass site (2010-2012). Estimates of total eggs calculated for the total area of Torre Abbey Sands (595,000 m^2). Estimates of potential spawning females calculated using the total number of eggs at a site divided by an average fecundity of 2,000.

Year	Egg clusters			Egg density			Total eggs at site			Potential spawning females		
	May	June	July	May	June	July	May	June	July	May	June	July
2010	19	-	12	2.8	-	3.8	1666000	-	2261000	833	-	1131
2011	29	67	74	0.9	2.9	3.9	535500	1725500	2320500	268	863	1160
2012	56	6	44	0.07	0.008	0.055	41650	4760	32725	21	2	16

Discussion :

The range of different spawning structures identified in this study indicated that within the English Channel *S. officinalis* was not confined to a specific spawning structure or habitat. A total of 15 different spawning structures were recorded across the two coasts, whilst it would appear that the use of a structure for spawning is likely to be constrained by a maximum diameter or width, to allow the basal rings of the eggs to be securely attached around the structure. As previously indicated in the literature, which suggests a maximum diameter of 1 cm described as suitable for spawning structures (Boletzky, 1983). However, within this study a minimum diameter or width was indicated to be less important, with cuttlefish adapting structures with smaller diameters by clumping or aggregating multiple leaves or thallus until a suitable diameter or width is achieved. This was observed for both *C. filum* on the French coast and *Z. Marina* on the UK coast and indicated that spawning could occur across a wider range of spawning structures than has previously been suggested. The characteristics of spawning structures was varied, however what drives the criteria and processes behind selection of suitable structures by females remains unknown.

In 2010, studies were undertaken within Torbay (delimited by the extent of the bay) to assess spawning patterns between two different strata, seagrass beds and mixed seaweed habitats. During these surveys (completed in May and July), it was found that within the geographical extent of this Bay, eggs were only recorded attached to *Z. marina* plants within seagrass beds. Despite the presence of a variety of seaweed species (e.g. *C. filum*, *S. muticum*, *S. latissima* and red foliose algae) within the mixed seaweed stratum that are known to be utilised as spawning structures at other study sites, no

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egg clusters were recorded within this substratum. Whether these results indicate a 'preference' for seagrass as a spawning structure/habitat within this study site remains to be determined. Seagrass has often been cited in the literature as providing important nursery areas for a variety of commercial marine species, providing food and relative safety and protection for vulnerable ELS (Jackson et al., 2001). However, demonstrating that a higher density of eggs exist within a habitat does not provide conclusive evidence of the nursery role of that habitat (Beck et al., 2001). A number of key considerations on the species, habitats and variables, for example habitat connectivity, must also be considered (Beck et al., 2001). Further research is now required to gain a full understanding of the relationship between *Z. marina* beds and cuttlefish spawning within this Bay.

In 2011, studies were undertaken within Poole Bay and Torbay, although for this study the geographic extent of the Torbay study site was adjusted to include the area from Torbay to Babbacombe Bay (which extends outside of the geographic extent of the Bay). Within the Torbay study site egg clusters were recorded at all four seagrass sites, but within the mixed substratum only at one site on *H. Siliquosa* (Babbacombe Bay), although a single egg cluster was recorded at Outer Millstones but attached to a section of steel rope. At Poole Bay, eggs were located on natural structures only within the seagrass stratum (although a single egg cluster was recorded at Handfast Point attached to a ghost trap). A significant difference between the numbers of eggs and the numbers of egg clusters recorded within each stratum was demonstrated with seagrass dominating in both categories. As was the case in the 2010 study, a variety of seaweed species (e.g. *H. siliquosa, S. muticum, S. latissima* and red foliose algae) were recorded within the mixed stratum that are known to be utilised as spawning structures at other study sites. A number of theories can be proposed to account for the difference in spawning patterns that was observed within these two strata: (1) a 'preference' for seagrass structures/habitats exists at this sites, as it provides an appropriate ecological environment for the development and survival of ELS (e.g. food and shelter); (2) in contrast to seaweed sites, the hydrodynamic conditions within seagrass areas (e.g. reduced current flows and reduced exposure), may provide better conditions for spawning making it easier for female cuttlefish to attach eggs to structures within sheltered areas. The mechanisms for spawning site and spawning structure/habitat selection need to be investigated in further detail and an investigation into the effect of exposure or current strength on patterns of spawning intensity now made.

The output from Task 2 and Task 3 are included in the CRESH funded PhD thesis of Isobel Bloor submitted on the 14th of October 2012:

BLOOR, I. 2012. The ecology, distribution and spawning behaviour of the commercially important common cuttlefish (*Sepia officinalis*) in the inshore waters of the English Channel. Plymouth University, Plymouth.

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TASK 3
Spatial analysis of potential spawning areas

Analyse des aires potentielles de reproduction

Objective/Objectifs: To examine the environmental conditions (for example depth, temperature, salinity, exposure, structural habitat, sediment type) of known cephalopod spawning grounds. To build a model using relevant environmental data layers to predict areas where the environmental conditions are within the preferred ranges for spawning (potential spawning habitat).

Examiner les conditions liées à l'environnement (telles que la profondeur, la température, la salinité, l'exposition, l'habitat structurel, le type de sédiment) des aires de reproduction connues des céphalopodes, ainsi qu'élaborer un modèle utilisant des couches de données environnementales pertinentes afin de prévoir les aires où les conditions liées à l'environnement sont dans la gamme des valeurs préférées pour la reproduction (aires potentielles de reproduction).

Work progress until 30/09/2012
Etat d'avancement des travaux au 30 septembre 2012.

Task 3 maps are available in the DVD included in claim 4 parcel
Les cartes de la tâche 3 sont disponibles sur le DVD inclus dans le colis de la remontée de dépenses n°4

I. 2012 Task Results Summary / Résumé des résultats obtenus en 2012

A presence-only maximum entropy (MaxEnt) modeling approach was used to predict the distribution of benthic cuttlefish egg clusters (a true measure of spawning) using pre-existing records collated from a variety of sources. The model showed very good performance in terms of predictive power and accuracy and among the explanatory variables used to build the model, distance from coastline, chlorophyll-a concentration (used here as a proxy for turbidity) and depth were shown to be the greatest determining factors for the distribution of Sepia officinalis spawning. As part of the model outputs, maps (logistic and binary) of the predicted spawning distribution of S. officinalis within the English Channel were produced.

Une méthode de modélisation d'entropie maximum basée sur la présence seulement (MaxEnt) a été utilisée dans le but de prédire la distribution des grappes benthiques d'œufs de seiches (une mesure réelle du frai) à l'aide d'archives préexistantes collectées à partir de diverses sources.
Le modèle fut très performant en termes de prédiction et d'exactitude. Parmi les variables d'explication utilisées pour construire le modèle, la distance depuis la côte, la concentration en chlorophylle-a (utilisée ici pour représenter la turbidité), et la profondeur se sont révélées être les meilleurs facteurs déterminants de la distribution des frayères de Sepia officinalis.
Un des résultats du modèle a été la production de carte (logistique et binaire) de la distribution prévue des frayères de S. officinalis dans la Manche.

II. Actions carried out in 2012/ *Bilan des opérations réalisées en 2012*

- Following the trial of several modelling techniques in 2011 a final modelling technique was selected for use in this study.

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- Data for environmental predictor variables were collated and prepared for incorporation into the modelling software and occurrence records of *S. officinalis* egg clusters were extracted from the geo-database.
- A model was produced using a presence-only modelling technique (MaxEnt) and predictive maps of potential spawning locations (logistic and binary) were produced.
- The model was evaluated for its predictive ability and an analysis of the output completed.

★ Après l'essai de plusieurs techniques de modélisation en 2011, une technique a finalement été sélectionnée pour cette étude.
★ Des données ont été collectées pour servir de variables de prédictions environnementales et préparées afin d'être incorporées dans le logiciel de modélisation. Des archives de cas de grappes d'œufs de S. officinalis ont été extraites de la géo-database.
★ Un modèle a été produit à partir d'une technique de modélisation basée sur la présence seulement (MaxEnt), et des cartes de prédiction de lieux potentiels de pontes (logistique et binaire) ont été faites.
★ La capacité prédictive du modèle a été évaluée et une analyse des résultats effectuée.

III. Scientific outputs / *Détail des travaux scientifiques de l'année*

Presence-only model selection:

This current study used presence-only sample points of *S. officinalis* egg clusters (a true measure of spawning) to provide the first species distribution model for spawning of this species within the English Channel. The type of environment that *S. officinalis* use for spawning in the English Channel were investigated by modelling the probability that at least one cuttlefish egg cluster is present at a particular location within the modelling extent, given a particular set of environmental variables. One of the greatest challenges within the marine environment remains the collection of high quality datasets containing both presence and absence records, as collection is often difficult, due to the intrinsic nature of the marine environment which makes sampling costly (and often prohibitive) in terms of both time and expense (Tsoar et al., 2007). As a result there has been an increase in interest in methods that allow the utilisation of pre-existing and readily available presence-only data sets. For this study sources of species, presence records were collated from archival datasets, incidental observation databases, fisheries surveys, bibliographic records and the research undertaken as part of Task 2.

MaxEnt:

Maximum entropy is a general purpose technique for estimating a probability distribution from partial information (Jaynes, 1957) and was first proposed as an approach for probability distributions by Jaynes (1957). The principle of this approach stems from the concept that any estimate of probability distribution that is based on only partial information should be maximally non-committal in order to ensure that no arbitrary assumptions are introduced (Jaynes, 1957). Maximising entropy is a desirable aim in species distribution modelling as to do otherwise would be to impose additional (unfounded) constraints on the predicted species distribution (Phillips et al., 2009). The modern day approach to maximum entropy species distribution modelling was developed within the machine learning community by (Phillips et al., 2004, Phillips et al., 2006) and is delivered through a free-ware software platform known as MaxEnt, which is available for download from: http://www.cs.princeton.edu/~schapire/maxent.

Whilst *S. officinalis* has yet to be modelled using presence-only methods, the EU Interreg IV funded Channel Integrated Approach for Marine Resource Management (CHARM II) project modelled the distribution of *S. officinalis* in the eastern English Channel (for July and October) using GLM and GAM methods and a series of structured fisheries survey data from the ground fish survey (October) and bottom trawl survey (July) that enabled presence-absence modelling methods to be utilised

TASK 3

(Carpentier et al., 2009). These models use data from adult and juvenile life stages and as such do not focus on the spawning locations and conditions of this species.

Species occurrence data:

In Task 1 a geo-database was created and populated with a total of 1966 sample points of presence records of all stages of *S. officinalis*. These records were obtained from five main sources which included:

- Fisheries surveys data (e.g. Centre for Environment, Fisheries and Aquaculture Science [CEFAS] and French Research Institute for Exploration of the Sea [IFREMER]), where cuttlefish are not the target species, but information has still been collected on their presence and abundance
- Bibliographic records (e.g. current and historical published literature)
- Current research (e.g. Cephalopod Recruitment from English Channel Spawning Habitats [CRESH]. subtidal surveys)
- Historical data archives (e.g Data Archive for Seabed Species and Habitats [DASSH])
- Current sightings scheme (e.g. Cuttle-Watch)

For Task 3, the geo-database was searched to extract a subset of data by life stage (eggs). This subset of data containing only the records of egg clusters (Figure 1) was extracted and saved as a comma-delimited (CSV) file with the life stage and associated latitude and longitude coordinates extracted for each record, ready for incorporation into the MaxEnt software. Records of eggs, rather than records of spawning adults, were used as a basis for the model as eggs represent a true measure of spawning. Presence data included a total of 217 records of *S. officinalis* eggs within the English Channel area from 1995-2012, and between March and September. The data was further subdivided by MaxEnt into two randomly allocated data sets, a larger training data set (163 records) and a smaller test data set (54 records).

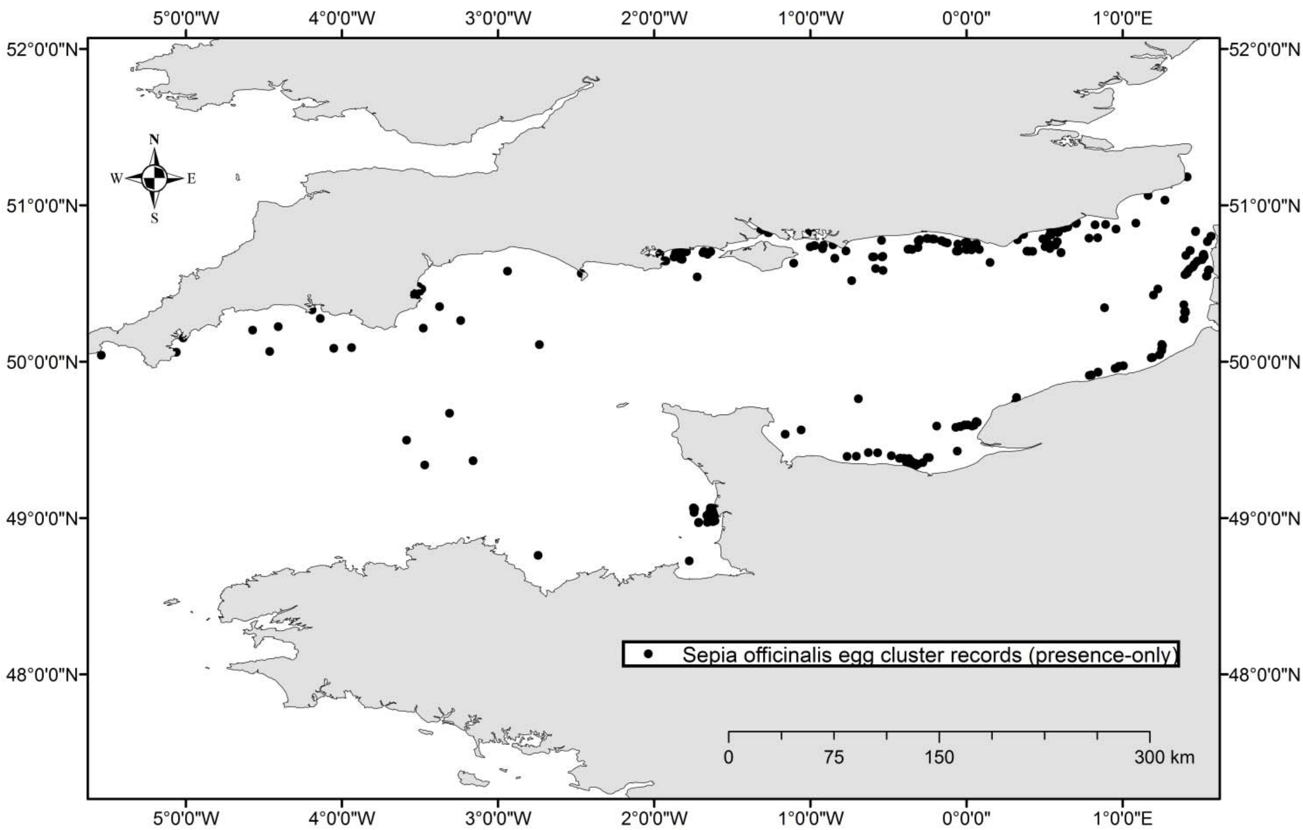

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Figure 1: Sample points for *S. officinalis* egg cluster occurrence within the English Channel (March to September, 1995-2012).

Environmental predictor variables:

A thorough review of the literature concerning spawning in *S. officinalis* was undertaken in order to ascertain which environmental and physical variables were ecologically relevant and as such could be used as predictor variables within the model. The list of variables highlighted included both categorical and continuous forms of data such as: sediment type, depth, distance from coastline, sea surface temperature, sea bottom temperature, sea surface salinity, productivity, thermal fronts, turbidity, current velocity and habitat type.

Of the variables highlighted in the literature a set of eight (Figure 2) were selected to incorporate into the model, based on knowledge of the species life cycle in the English Channel, and/or accessibility of data layers at resolution and quality high enough for useful inclusion:

- Attenuation coefficient K490 (representing turbidity)
- Chlorophyll -*a* (representing turbidity and primary productivity)
- Sea surface temperature
- Depth (bathymetry plus mean sea level)
- Bed shear stress (representing current flow and sediment type)
- Distance from coastline
- Sea surface salinity
- Sediment type

Collinearity among predictor variables:

As it is not recommended to use highly correlated variables within the MaxEnt modelling process, co-linearity among predictor variables was assessed and only variables with a Pearson correlation between -0.7 and 0.7 were included in the model construction phase (for this reason the variables for attenuation coefficient K490 and Sediment type were both excluded).

Preparation of environmental layers:

A brief description of the sourcing and pre-processing of these data layers has been summarised in Table 1**.** For variables with a temporal element (SST, Chl-*a* and K490) ArcGIS was used to assemble long-term averages (median) over the period 2000-2010 for months when eggs were recorded as being present in the English Channel (March to September). This was done as MaxEnt does not have the ability to include time as a variable in this way. To execute the model, data layers (saved in ASCII format) for all predictor variables were converted to the same resolution (cell size), projection system and clipped to the same pixel extent to ensure data availability for every cell and to provide MaxEnt with information on the modelling extent. Data layers were processed using spatial analyst tools in ArcGIS.

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Table 1: A list of predictor variable datasets obtained for use in the MaxEnt model together with description and source (National environment research council earth observation data acquisition and analysis service (NEODASS): www.neodaas.ac.uk; Channel integrated approach for marine resource management (CHARM) Sextant Portal: www.ifremer.fr/sextant/en/web/charm/geocatalogue; International council for exploration of the seas oceanographic data centre (ICES ODC): www.ocean.ices/dk/data/surfacce/surface.htm)

Parameter	Sensor/Model	Units	Resolution	Years	Source
Sea surface chlorophyll-*a*	MERIS	Mg mm^{-3}	1 km	2002-2010	NEODAAS
Sea surface temperature	AVHRR	°C	1 km	2000-2010	NEODAAS
Attenuation coefficient K490	MERIS	m^{-1}	1 km	2002-2011	NEODAAS
Depth	SHOM and MARS	M	1 km	-	CHARM III Sextant
Substrate type	-	-	0.009	-	Larsonneur et al., 1979
Sea surface salinity	-	-	Point data	1981-2012	ICES ODC
Bed shear stress	POL	$N.m^{-2}$	1 km	-	CHARM III Sextant
Distance from coastline	-	M	0.009	-	ArcGIS (ESRI V.10)

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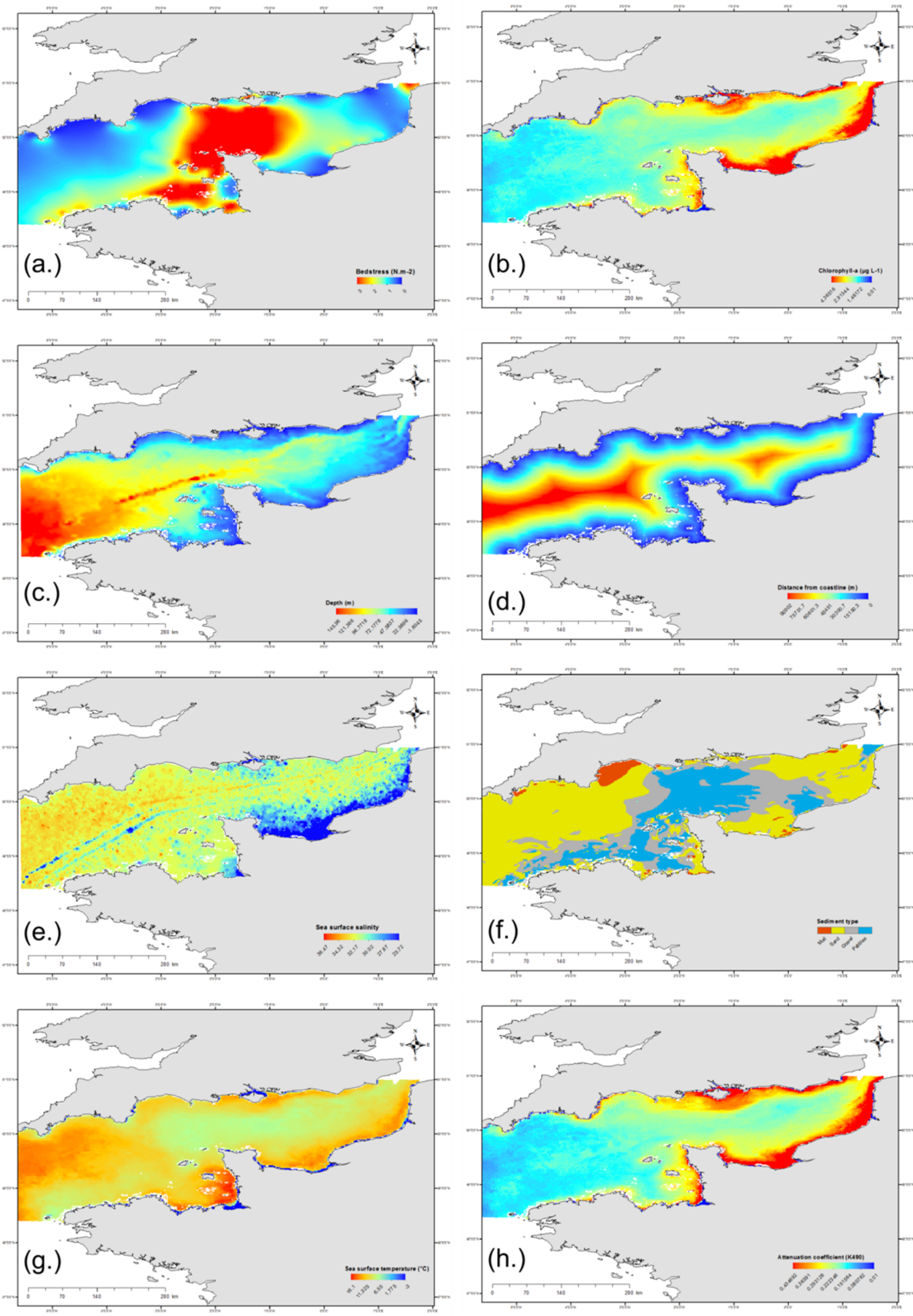

Figure 2: Maps showing the data for each of the predictor variables considered for use in the MaxEnt model, (a.)Bed shear stress, (b.) Chlorophyll-*a*, (c.) Depth, (d.) Distance from coastline, (e.) Sea surface salinity, (f.) Sediment, (g.) Sea surface temperature , (h.) Attentuation coefficient K490.

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Presence maps

To reduce spurious effects the model model generation was replicated 15 times using a random seed option, to allow a different training and test partition to be created for each run, with a subsampling replication method. The presence maps created from the MaxEnt model (Figure 3) show the areas within the English Channel that have suitable conditions predicted for *S. officinalis* spawning. Figure 3a illustrates the average predictions (based on 15 replicated runs of the model) on a logistic scale from 0 to 1, with warmer colours (e.g. red (1)) showing areas with better predicted conditions than cooler colours (e.g. blue (0)).

Figure 3b illustrates the average predictions (based on 15 replicated runs) following adjustment to the ten percentile training presence logistic threshold (0.182) with the output data divided into two categories: areas of suitable habitat (above 0.182) and areas of unsuitable habitat (below 0.182).

To investigate the prediction in more detail the U.K. coast was taken as an example. Here it is evident that a large portion of the inshore area has been predicted as suitable, with a larger proportion in the east of the Channel than the west (Figure 3b), for cuttlefish spawning to occur. The map highlights several areas along this coastline as suitable for spawning, where major inshore cuttlefish trap fisheries are known to occur (labelled as 1 - Torbay, 2 - Poole, 3- Selsey and 4- Hastings on (Figure 3b). However, there are also several areas along this coastline which the map has highlighted as unsuitable for cuttlefish spawning and where major inshore cuttlefish trap fisheries do not occur (labelled as 5 - St Austell area, 6- West Lullworth area, Figure (Figure 3b). In addition the entire offshore, deep water area in the centre of the English Channel was also been predicted as unsuitable for cuttlefish spawning.

Model evaluation:

Area under the receiver operating characteristic curve (AUC):

The model was evaluated using the AUC test statistic. The receiver operating characteristic curves (ROC) for test and training data sets are provided in Figure 4. The red line in Figure 4a represents the fit of the model to the original training data, whilst the blue line represents the fit of the model to the testing data and is a good indicator of the models predictive power. The AUC value (averaged over 15 replicates) for the training data was 0.938 (SD ± 0.005) and the AUC value for test data was 0.909 (SD ± 0.017) (Figure 4a) which is higher than by chance (AUC =0.5). Figure 4b illustrates the ROC curve (averaged over 15 replicates) for the test data, plus and minus one standard deviation (SD), in order to indicate the variability. The training and test AUC values indicate excellent predictive ability of the model (Excellent prediction ≥ 0.9 (Phillips et al., 2009, Hosmer and Stanley, 2000, Swets, 1988).

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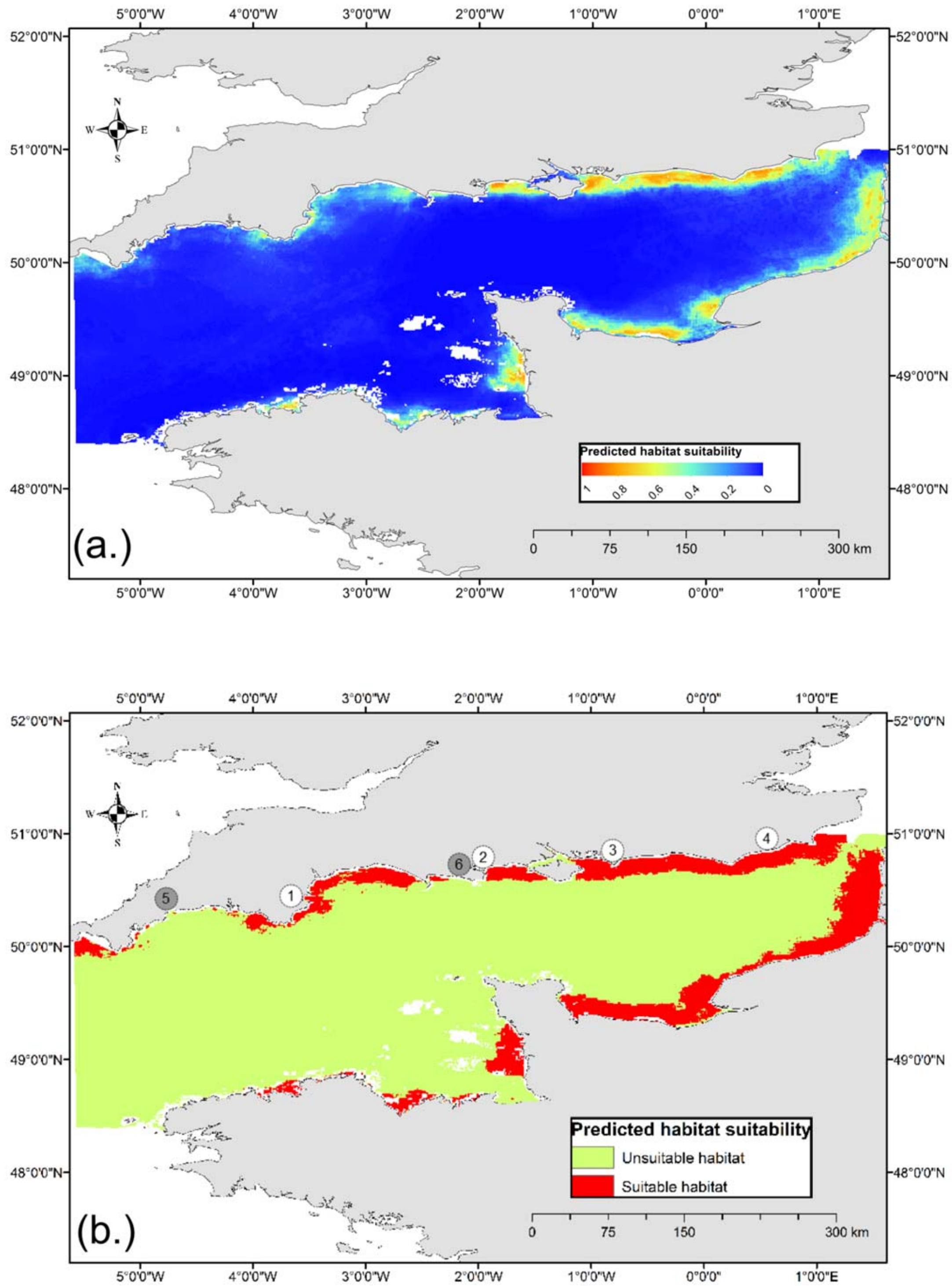

Figure 3: Predicted habitat suitability map for *S. officinalis* spawning distribution within the English Channel. (a.) Logistic output, presence predicted from 0 to 1. The warmer colours (e.g. red and orange) indicate a high probability that the conditions at that location are spawning to occur, whilst the cooler areas (e.g. blue and green) indicate a low predicted probability that the conditions at a location are suitable for spawning, (b.) Binary output, predictions have been classified into two categories 'unsuitable habitat' and 'suitable habitat' using a threshold of 0.182 as specified by the ten percentile training presence logistic threshold. The map highlights several areas along the UK coastline as suitable for spawning (1-Torbay, 2-Poole, 3- Selsey and 4- Hastings) and several areas as unsuitable (5- St Austell area, 6- West Lullworth Area)

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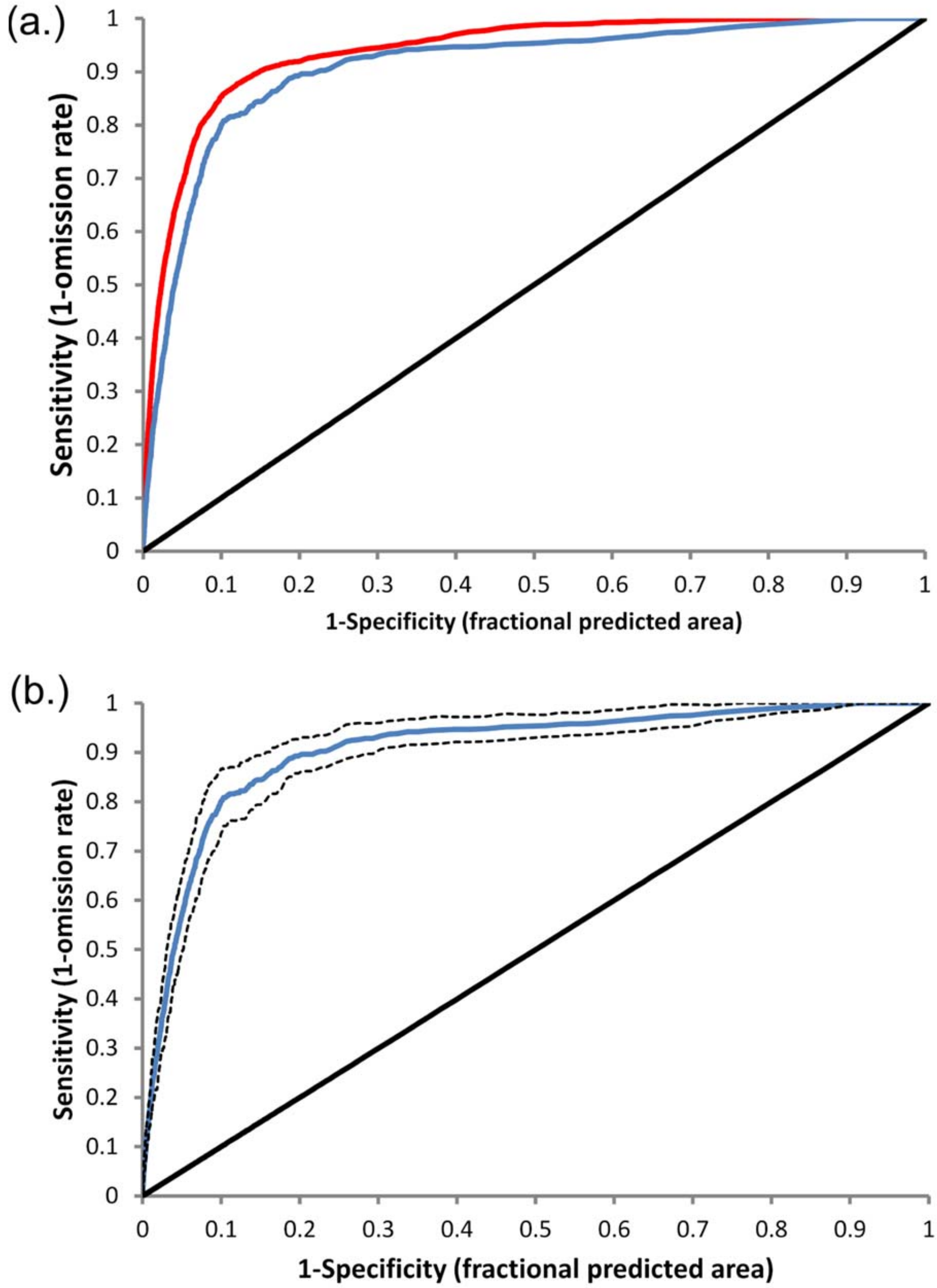

Figure 4: Receiver operating characteristic (ROC) curve averaged over 15 replicate runs for both training and test data, (a.) ROC curve for both training and test data. Training data (red line) has an area under the ROC curve (AUC) of 0.938; Test data (blue line) has an AUC of 0.909; Random prediction (black line) has an AUC of 0.5; (b.) ROC curve for test data, with 1 standard deviation shown (black dotted lines).

Predictor variable importance:

The relative contributions of the predictor variables to the MaxEnt model are indicated in Table 2. The variable with the highest percentage contribution was distance from coastline with 27.5 %, closely followed by depth (23.9 %). Chlorophyll-*a* concentration (22.6 %) and bed shear stress (20 %). However, whilst distance from coastline, depth and bed sheer stress all have accompanying high permutation importance (36.5 %, 23.8 % and 33.6 % respectively), chlorophyll-*a* has the lowest permutation importance of all the variables with a value of only 1.4 %.

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Table 2: Relative contributions of the predictor variables to the MaxEnt model

Variable	Percentage contribution	Permutation importance
Distance from coastline	27.5	36.5
Depth	23.9	23.8
Chlorophyll-*a*	22.6	1.4
Bed shear stress	20	33.6
Sea surface salinity	4	2.5
Sea surface temperature	1.9	2.3

The remaining two variables (sea surface salinity and sea surface temperature) both have a low percentage contribution (4 % and 1.9 % respectively) and a low permutation importance (2.5 % and 2.3 % respectively).

Jackknife:

The results of the jackknife test are displayed within a series of three bar charts (Figure 5). In Figure 5a, the predictor variables with the highest regularised training gain when used in isolation were depth (gain =1.17), chlorophyll-*a* (gain =1.06) and distance from coastline (gain =1.02) (longest black bars in Figure 5a), indicating that in isolation these variables provide the most useful information for predicting the presence of *S. officinalis* spawning, with a good fit to the training data. In addition, the predictor variable that decreases the training gain most when removed from the model was bed shear stress (shortest grey bar in Figure 5a), indicating that this variable may contain the most information that is not present within the other variables.

A comparison of the three bar charts is useful for exploring the model further, for example for this model, depth, chlorophyll-*a* and distance from coastline have the highest gain under both training and test conditions, when used in isolation, suggesting that these predictor variables might be the most transferable between models.

Marginal response curves:

The marginal response curves (Figure 6) give an indication of the range of values that have the highest and lowest predicted probability of suitable conditions. For depth, the highest response in terms of predicted probability of suitable conditions is between 3 and 26 m and declining from this point onwards. For chlorophyll-*a* the highest response in terms of predicted probability of suitable conditions falls between 1.0 and 2.5 $mg.m^{-3}$ whilst the lowest response falls between 2.5 and 5.0 $mg.m^{-3}$. For distance from coastline the highest response is between 2,200 and 12,1000 m from the coastline, after which the response again declines. For bed shear stress the lowest response in terms of predicted probability of suitable conditions falls between 1.5 and 3.0 whilst the highest response falls between 0.5 to 1.5, indicating a preference for areas with weaker bed shear stress. For sea surface salinity the marginal response curve is difficult to interpret, although there is a change in response between approximately 33 to 35. The marginal response curve for sea surface temperature is also difficult to interpret but again there is a change in response pattern between 14 and 18 °C.

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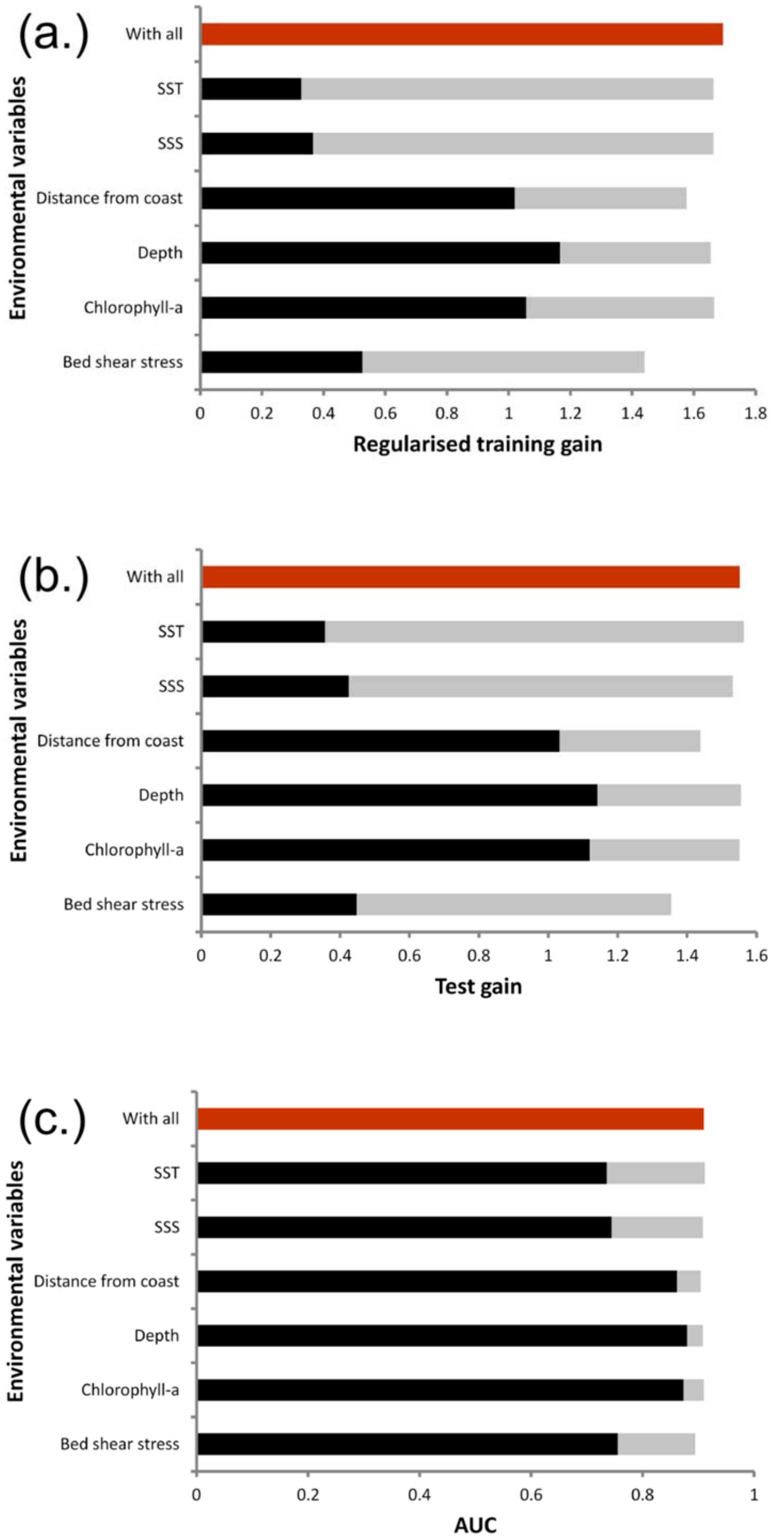

Figure 5: Jacknife of (a.) regularised training gain, (b.) test gain and (c.) AUC, for predicted spawning distribution of *S. officinalis* within the English Channel. The black bars represent model gain using only that variable and the grey bars represent the effect of removing that variable from the model, the red bar indicates the total gain for the model with all variables.

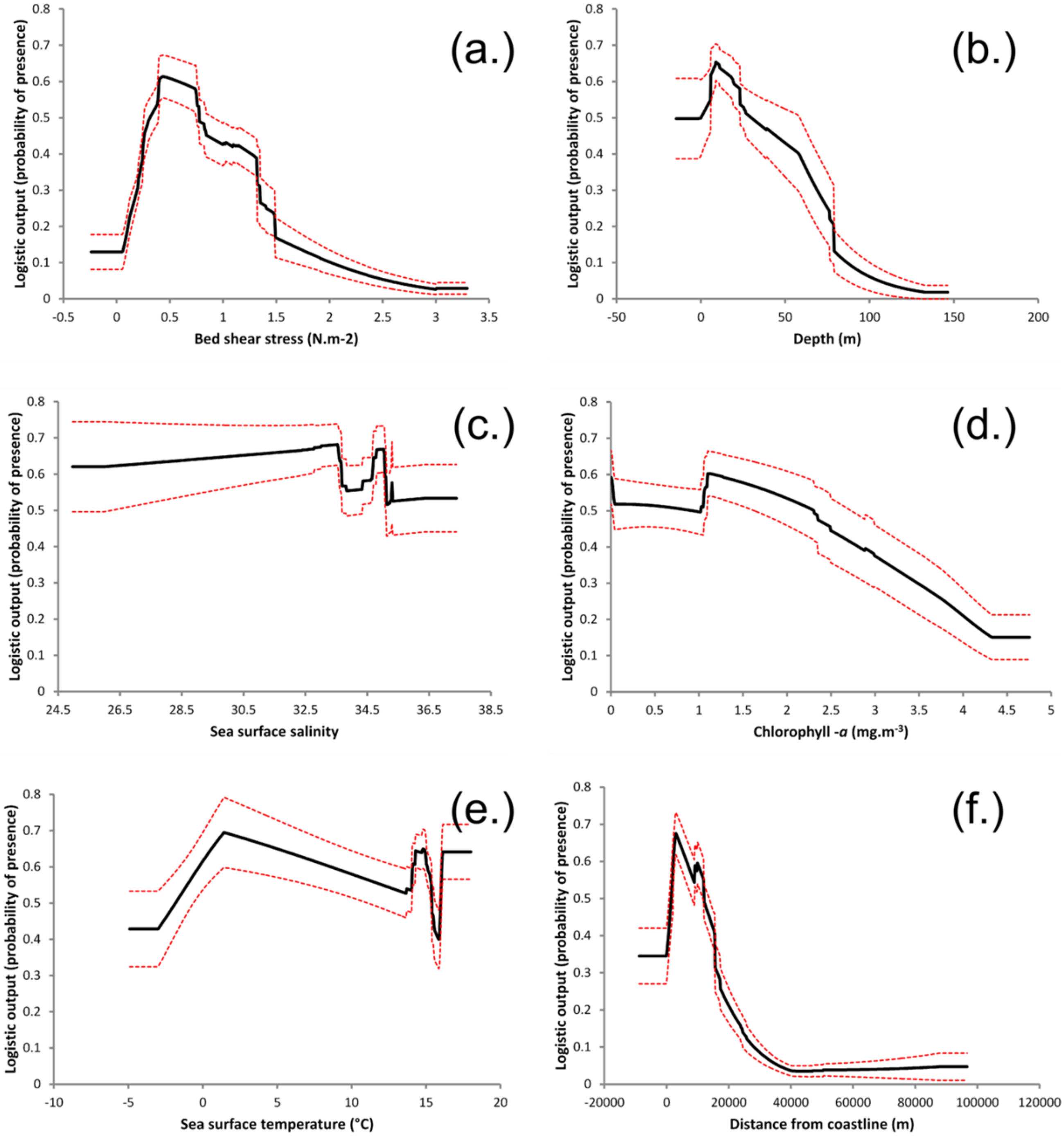

Figure 6: The average marginal response curve (black line) for each predictor variable, with standard deviation (dotted red lines) displayed (a.) Bed shear stress, (b.) Depth, (c.) Salinity, (d.) Chlorophyll-*a*, (d.) Sea surface temperature, (e.) distance from coastline. The curves indicate how the logistic prediction (y-axis) changes as each target environmental variable is varied (x-axis), with all other variables remain constant (at their average sample values).

Discussion:

The aims of this study were to describe the potential spawning distribution of *S. officinalis* within the English Channel and to understand the influence of environmental and physical conditions on this predicted distribution pattern. This study used a novel presence-only species distribution modelling technique known as MaxEnt to enable readily available presence-only data sets, collated from a range of sources including both archival and ad-hoc records) of benthic egg clusters, as a means to represent a true measure of spawning. The results of this study have begun to address this knowledge gap by

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providing the first data available on predictive spawning habitat suitability across the entire area. Such data will enable an evaluation of the important spawning location for *S. officinalis* and could feed into potential future management measures for this species, or in an assessment of the need to protect inshore spawning habitats.

Spawning distribution of S. officinalis within the English Channel

The model predicted areas suitable for *S. officinalis* spawning in coastal areas on both sides of the English Channel, with a predominance of suitable habitat predicted in the eastern part and a smaller fraction, of more discrete sites, predicted in the western part. Areas where important targeted coastal trap fisheries for *S. officinalis* were all identified by the model as suitable areas for spawning, included: Torbay, Exmouth, Poole, Selsey, Eastbourne and Hastings on the UK coast and Agon-Countainville, Langrune-sur-mer on the French coast. In addition, all areas predicted by the model as suitable for spawning were inshore, coastal areas whilst the deeper, offshore area in the middle of the Channel was predicted as unsuitable (Figure 3).

Western and Eastern English Channel:

The MaxEnt model predicted a larger proportion of suitable spawning habitat in the Eastern English Channel relative to the Western English Channel. There are several possible theories as to why this spatial distinction in the model prediction might have occurred. The first is based on sample selection bias, which indicates a slight predominance of samples in the Eastern Channel compared with the Western Channel. Another possible explanation for the higher fractional predicted area of suitable spawning habitat in the eastern part of the Channel compared with the western part may be the innate difference in hydrodynamic and physical conditions which vary distinctly between the two areas, as outlined in (Dauvin, 2012). For example, in the western part of the Channel, the hydrological and oceanographic conditions are mainly dominated by the input of water from the Atlantic; whilst in the eastern part, the large fresh water input from the Seine estuary plays an important role in dictating the conditions, particularly on the French coast (Dauvin, 2012). The sediment in the Western Channel is generally coarser than that found in the Eastern Channel with a decrease in the benthic species from west to east (Pawson, 1995). The Western Channel is known to account for approximately 63 % of the English Channel, covering a total area of 56,452 (Stanford and Pitcher, 2004), however, despite its size, there are still many aspects of its features that remain unknown. For example, whilst several studies have studied the benthic macrofaunal assemblages within the Eastern Channel (e.g. Sansvicente-Anorve et al., 2002), a detailed study regarding the relationships of these assemblages within the Western Channel has yet to be undertaken (Araujo et al., 2005)**.** Further research is therefore required to elucidate the true nature of the differences between these two areas.

Species-habitat relationship:

The internal validation procedure of the MaxEnt model indicates that it performs well in terms of predictive ability (test AUC =0.909), and identifies three variables as being most relevant for predicting the spawning distribution of *S. officinalis*, these are depth, chlorophyll-a concentration and distance from coastline, with bed shear stress providing additional useful information. Spawning occurred between the months of March to September, in shallow (4 to 23 m depth), inshore (2,200 to 12,100 m distance from the coastline) areas, at warmer temperatures (between 14 to 18 °C, SST), within a moderate range of salinity (34.6 to 35.2), in areas of weaker bed stress (0.39 to 0.75 $N.m^{-2}$) and with relatively low Chlorophyll-a concentrations (1.1 to 2.2. mg m^{-3}). These conditions are discussed in more detail below for the four variables shown to be important for predicting the distribution of spawning in *S. officinalis* within the English Channel.

Depth was found to be an important factor in determining the spatial distribution of spawning in *S. officinalis*, with a relatively high percentage contribution (23.9%) together with a relatively high percentage permutation importance (23.8%). In addition, the jackknife test indicated that this variable, when used in isolation, was the most important in terms of both training gain (1.17) and test gain (1.14) when averaged over all 15 replicates. This suggests that of the predictor variables it might be the most important and the most transferable between models, its transferability is likely given the static nature of depth as a long-term factor, when compared with the temporally changing variables such as SST or Chlorophyll-a concentration. The marginal response curve indicated that the values for depth that predicted the best conditions suitable for spawning were between 4 and 23 m, which is congruent with what is known about the species life-history traits, with migration of spawning adults to the shallow coastal waters of the English Channel in Spring (Boletzky, 1983, Boucaud-Camou and Boismery, 1991), as well as with data collated from other populations of *S. officinalis* within the eastern Mediterranean (Valavanis et al., 2002), which suggests that cuttlefish spawning occurs at depths < 50 m. English Channel fisheries data for this species also supports this assertion with deep water, offshore trawling ceasing during the summer period (from March onwards) when both adult and sub-adult cuttlefish have moved inshore (Dunn, 1999).

However, the sample point data for egg clusters that were collected for the model, indicate that a small proportion of egg clusters have been located in the centre of the English Channel (Figure 3). A study by Challier et al. (2005a) that investigated trends in recruitment of *S. officinalis* within the English Channel indicated that although the majority of recruitment occurred in autumn, some recruitment was found to occur throughout the year. One of the explanations that the authors made to account for these findings was that as spawning takes place from spring through summer, when spawning adults occur both offshore (prior to or during their migration) and inshore (following migration), that eggs could potentially be laid in both shallow, warm waters as well as deeper, cold waters (Challier et al., 2005a). As a result of the temperature dependent rate of embryogenesis in this species, eggs spawned in deeper, colder waters would take longer to develop and hatch later than those spawned inshore (Challier et al., 2005a). Whilst it is entirely possible that some spawning does occur in the deeper waters of the Channel as evidenced both by the findings of Challier et al. (2005a) and the location of cuttlefish egg clusters within the presence-only dataset used for model construction, the model constructed in this study indicates that spawning predominately occurs in shallow waters. In addition, the benthic nature of cuttlefish spawning, requires that females have access to a range of benthic supports to attach their eggs to, in order that the eggs receive adequate aeration and water circulation, such structures are likely to be limited in some deeper offshore waters of the Channel where gravel and pebble sediments dominate (e.g. Figure 2f). Although some structures which are suitable for spawning (e.g. Hydroids and *Porifera* spp.) may occur in these deeper offshore waters, such spawning (if apparent) is likely to be limited in contrast to shallow inshore spawning, possibly occurring only under certain environnemental conditions or situations.

Chlorophyll-a, was also found to be an important factor in determining the spatial distribution of spawning in *S. officinalis*, with a high percentage contribution (22.6 %) but a low percentage permutation importance (1.4 %). In addition, the jackknife test indicated that this variable was one of the two most important in terms of training gain (1.06) and test gain (1.12), when used in isolation. The marginal response curve also indicated that the values of chlorophyll-a concentration that predicted the best conditions suitable for spawning was between 1.1 and 2.2 mg m^{-3}. Whilst the sea surface concentration of chlorophyll-a is not likely to directly determine the distribution of spawning in this nekto-benthic species, which attaches its eggs to structures that radiate from the seabed, the role of chlorophyll-a has been shown in other studies to provide a useful indicator of primary productivity (Pierce et al., 2002). Chlorophyll-a concentration is often used as a proxy for primary production (Friedland et al., 2012), which may in turn related to food availability and abundance, and has also been found to be positively correlated with fisheries yield in some ecosystems (e.g. Friedland et al., 2012). These factors are believed to be important in cephalopod distribution and in particular for the distribution of paralarvae and hatchlings (e.g. Vidal et al. 2010). A similar relationship was found between the distribution of *Octopus vulgaris* and chlorophyll-a in the Mediterranean, when modelled with MaxEnt (Hermosilla et al., 2011), although this study was not focused on spawning distribution in particular. A study by Smyth et al. (2010) which investigated the environmental conditions in the Western English Channel state that typical background chlorophyll-*a* concentrations for this area are around 1 mg.m-3 throughout the year. The authors also note that whilst in winter (October to March) coastal areas appear to be characterised by higher chlorophyll-*a* concentrations, that this elevation could actually be caused by an artefact of increased suspended particulates and

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dissolved organic matter which causes an increase in the levels recorded by the satellite (Smyth et al., 2010). This, combined with the co-linearity of the attenuation coefficient (K490) (representative of turbidity) and chlorophyll-a concentration that was recorded in this study and required the removal of one of these variables from the model building process, could indicate that cuttlefish use areas with low chlorophyll-a concentration (which would also represent low concentrations of K490) as these areas have a lower turbidity that areas with higher chlorophyll-a concentrations.

In addition to this, the English Channel is subject to spring blooms where chlorophyll-a values increase in the English Channel within June, July and August (Smyth et al., 2010), the necessity for creation of a long-term average of the chlorophyll-a satellite data which provided a median of 10 years' satellite derived chlorophyll-*a* data over an entire 7 month period (March to September). The averaging process may have the effect of smoothing out point events and lessening their impact. As such, the impact of variations both spatially and temporally in primary production relating to the availability of food within these inshore coastal areas might have been reduced within this model and alternative ways to incorporate these long-term, variable datasets need to be investigated in the future.

Distance from coastline, was also found to be an important factor in determining the spatial distribution of spawning in *S. officinalis*, with a high percentage contribution (27.5%) and the highest percentage permutation importance (36.5%). In addition, the jackknife test indicated that this variable was one of the three most important in terms of both training (1.02) and test (1.03) gain. The marginal response curve also indicated that the values of distance from the coastline that predicted the best conditions suitable for spawning, were between 2,200 and 12,100 m from the nearest coastline. Within the English Channel, *S. officinalis* undertake seasonal migrations, from the deeper offshore waters where they spend the winter months, to the shallow, inshore waters in the spring and summer, when mature adults spawn. The results of the model are congruent with this aspect of their life cycle, predicting the area between 2,200 and 12,100 m from the coastline to be the most suitable for spawning to occur within. A study undertaken by Valavanis et al. (2002) which developed a marine information system for cephalopod fisheries in the eastern Mediterranean found that *L. vulgaris* and *S. officinalis* selected areas to spawn that were closer to the coast when the coastline was rocky and sharp and further away from the coast when the coastline was smooth and sandy. Future analysis using information on the composition of the coastline could be of interest to see if a similar effect is observed in the English Channel.

Finally, bed shear stress, was found to be an important factor, within this model, for determining the spatial distribution of spawning in *S. officinalis*, contributing to the model the most information that was not contained by any other variable producing the lowest training (1.44) and test (1.35) gain value when excluded from the model during the jackknife test. In addition, the percentage contribution to the model (20 %) from this variable was relatively high with a corresponding high value for permutation importance (33.6%). The marginal response curve for this variable indicated that the values of shear bed stress that predicted the best conditions suitable for spawning were between 0.39 to 0.75 N. M^{-2}, indicating a preference for areas with weaker shear bed stress.

As part of the CHARM II project, a distribution model of all life stages of *S. officinalis* was created in the eastern English Channel for the two months of July and October. The results of the July model, which occurs during the spawning season, indicated similar results to those reported in this study, suggesting that the species is tied to areas of weak bed shear stress (Carpentier et al., 2009). Such areas are often found in sheltered bays and are defined by the presence of fine sand and mud as a result of the associated weak currents (Dauvin, 2012). For *S. officinalis* which are benthic spawners, the importance of shear bed stress as a factor may be two-fold, to begin, the task of attaching eggs to a structure, which is performed by the female using her tentacles to 'tie' each individual egg around the structure, by means of a basal ring. This is a complex task that may be best achieved in areas where the currents are low and thus the effect of water movement, on both the structure and the spawner may be reduced. In addition, in areas of weak bed stress, the currents are reduced and the areas are defined by finer sediments which have a higher degree of retention in such conditions, the type of sediment present will additionally affect the type of structures available for spawning and may be important in determining the degree to which a site is utilised for spawning, with cuttlefish spawning grounds thought to occur predominantly in sandy areas (Nixon and Mangold, 1998).

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TASK 4

Study on the impact of the different spawning sites of the English Channel on the physiological performance of juvenile cuttlefish, Sepia officinalis L.

Etude de l'impact de différents sites de pontes de la Manche sur les performances physiologiques des juvéniles de seiche, Sepia officinalis L.

Objective/*Objectifs*: Monitoring cuttlefish eggs and s hatched in different areas (experimental fishing). Influence of environment quality on egg hatching and afterwards hatchlings survival, and eco-physiological performances of juveniles.

Suivi des œufs et des juvéniles de seiche issus de différents habitats (pêches expérimentales). Influence de la qualité du milieu sur le développement des œufs, l'éclosion, la survie et les performances éco-physiologiques des juvéniles.

2009-2012 Work progress.
Etat d'avancée des travaux 2009-2012.

I. Task Results Summary / *Résumé des résultats obtenus*

The CRESH project Task 4 aimed at studying cuttlefish eggs quality as well as the physiological performance of cuttlefish juveniles in their early stages in different spawning sites of the English Channel coasts. Four main spawning sites were monitored for three years: two French sites (Agon Coutainville and Baie de Seine), and two English sites (Torbay and Selsey). Seasonal monitoring revealed differences between local cuttlefish populations in the Channel. Indeed, from the egg incubation period to the new recruits migration into the central stock, local environmental factors play an important role in the cuttlefish early life stages. Significant differences in hatching rates were observed between the four sites. The differences were more important between the English and the French sites, where English sites had a higher hatching rate than the French ones. These differences are mainly due to incubation temperatures, cooler in English sites, inducing a better absorption of yolk in embryos during their development. After hatching, juveniles will develop on the coast until autumn where cooling water triggers the migration of these new recruits towards the western part of the Channel.

An experimental approach regarding juveniles' growth was performed under this task, so as to compare and relate juveniles' growth performance to their site. It has been observed that the juveniles' growth rate was similar for all batches (i.e. Baie de Seine, Agon Coutainville, Torbay and Selsey) when these animals were reared under the same biotic and abiotic conditions. However, pre-recruits collected in the natural environment showed that growth differed significantly across sites due to local environmental factors that vary from one site to another.
Our experimental approach also revealed an important point regarding juveniles' survival. Several high mortality cases were recorded during the monitoring year. These episodes were localised in time and space, i.e observed for specific sites and years. Mortality cases were observed in the site of Selsey in 2010 and the site of Torbay in 2011 and may affect the contribution of these sites to the recruited cuttlefish stock.
High mortality rates could explain the interannual fluctuations observed in the recruitment of this short life cycle species (2 years). Episodes of high mortalities in a major spawning site (that highly contributes to the central stock), will affect recruitment over two years, and therefore have an impact on the cuttlefish fisheries.
Eggs, reared juveniles and pre-recruits samples were used to study the immune and digestive systems of the cuttlefish as well as to assess the cuttlefish quality in its first life stages, according to its hatching site. Immune enzymes (Lysozyme-like family, antiproteases and phenoloxidase-prophenoloxidase complex) were

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adapted to juveniles, something that had never been done on small cuttlefish and cephalopods in general. We studied afterwards the immune system of collected pre-recruits. We observed that pre-recruits from the Baie de Seine had a disturbed immune system compared to Agon-Coutainville ones. The immune enzymes high activity recorded (Lysozyme-like, protease and prophenoloxidase-phenoloxidase complex) showed that the site of the Baie de Seine is a stressful environment for juveniles. These results suggest that the strong anthropic pressure of this area affects juvenile cuttlefish immune systems. This information is important because a disturbed immune system weakens animals and leads to early mortality before pre-recruits migration.

Juveniles' quality at hatching in the Bay of Seine showed a lower protein content. This content result from the yolk quality deposited by the mother in the egg (quality that showed no difference between sites), and of incubation conditions that affects yolk absorption. Although this absorption depends in part on abiotic factors, it does also depend on stress factors. Any stress factor induces a decrease from the yolk used for growth at the expense of the part allocated to breathing, which increases during episodes of stress. Results obtained for eggs and hatching quality are consistent with the ones obtained regarding the immune system. The Baie de Seine habitat is more disturbed than other sites'.

The study of the digestive system maturation showed that eggs incubation conditions in their natural environment has a significant effect at different levels. First, on the digestive gland maturation where significant differences between sites were observed in the digestive gland development at hatching (in digestive cells size, as well as their contents -digestive vesicles-). These histological observations were coupled with enzymatic assays, enzymes activities being very dependent from the maturation of the digestive cells. This helped us understanding the observed performance afterwards as the digestive system maturation is key early stages development.

In the end, the task four of the CRESH project allowed us to describe the interactions between environmental conditions and physiological performance of pre-recruits in four main spawning sites of the Channel. This will help to provide answers in understanding the contribution differences between sites.

La tâche 4 du projet CRESH avait pour but l'étude de la qualité des œufs et des performances physiologiques des juvéniles de seiche durant leurs premiers stades de vie au sein de différents sites de ponte des côtes anglo-normandes. Quatre principaux sites de ponte ont été suivis pendant 3 ans : deux sites français (Agon Coutainville et Baie de Seine), et deux sites anglais (Torbay et Selsey). Un suivi pendant plusieurs saisons a permis de révéler des différences entre les populations locales de seiche en Manche. En effet, de la période d'incubation des œufs jusqu'à la migration des nouvelles recrues dans le stock central, les facteurs environnementaux locaux jouent un rôle important sur les premiers stades de vie des seiches. Des différences significatives de taux d'éclosion ont été observées entre les quatre sites. Ces différences étaient très marquées entre les côtes anglaises et françaises, les sites anglais présentant des taux d'éclosion plus élevés que ceux des sites français. Ces différences sont dues aux températures d'incubation plus froides dans les sites anglais induisant une meilleure absorption du vitellus chez les embryons au cours de leur développement. Après éclosion, les juvéniles vont se développer le long de la côte jusqu'à l'automne, où le refroidissement des eaux déclenche la migration de ces nouvelles recrues vers le large.

Nous avons effectué dans cette tâche une étude expérimentale de la croissance des juvéniles issus des œufs des quatre sites afin de comparer leur croissance. Ce suivi sur plusieurs saisons nous a permis d'observer que le taux de croissance des juvéniles était identique quel que soit le site d'origine (Baie de Seine, Agon-Coutainville, Torbay et Selsey) lorsque ces animaux étaient élevés dans les mêmes conditions biotiques et abiotiques. En revanche, la récolte de pré-recrues du milieu naturel a montré que la croissance diffère significativement selon les sites. Ceci est dû aux facteurs environnementaux locaux qui varient d'un site à l'autre.

Cette étude expérimentale a aussi révélé un point important sur la survie des juvéniles. En effet, plusieurs épisodes de forte mortalité ont été enregistrés au cours des années de suivi. Ces épisodes étaient localisés dans le temps et dans l'espace c'est-à-dire que ces mortalités ont été observées dans des sites précis et sur des

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années précises. Ces mortalités ont été observées pour le site de Selsey en 2010, et pour le site de Torbay en 2011, et influenceraient la contribution des sites au stock de seiche recruté.
Les forts taux de mortalité pourraient être mis en relation avec les fluctuations interannuelles observées dans le recrutement de cette espèce ayant un cycle de vie court de deux ans. Un épisode de forte mortalité dans un site important (qui contribue beaucoup au stock central recruté) va affecter le recrutement sur deux ans. Ceci va se répercuter sur les pêcheries de seiches.

Les échantillons d'œufs, de juvéniles d'élevage et de pré-recrues du milieu naturel, ont permis l'étude du système immunitaire et digestif ainsi que la qualité des seiches aux premiers stades de leur vie en fonction de différents sites de ponte. L'étude du système immunitaire (Enzymes de la famille des lysozymes, des antiprotéases et du complexe phenoloxidase-Prophenoloxidase) a été adapté sur les juvéniles (travail qui n'avait jamais été fait chez la seiche et très peu chez les céphalopodes en général). Nous avons ensuite étudié le système immunitaire des pré-recrues récoltées. Ceci nous a permis de constater que les pré-recrues de Baie de Seine avaient un système immunitaire plus perturbé que celles d'Agon-Coutainville. Les fortes activités des enzymes immunitaires montrent que le site de Baie de Seine est un milieu stressant pour les juvéniles. La forte pression anthropique dans cette zone affecte le système immunitaire des juvéniles de seiche. Cette information est importante car le système immunitaire perturbé fragilise ces animaux et induit des mortalités précoces avant la migration des pré-recrues.

L'étude de la qualité des juvéniles à l'éclosion en Baie de Seine a montré que ces individus étaient les plus pauvres en contenu protéique. Ce contenu résulte de la qualité du vitellus déposé par la mère dans l'œuf (qualité qui n'a pas montré de différence entre les sites), et des conditions d'incubation qui vont agir sur l'absorption du vitellus. Cette absorption est certes en partie dépendante des facteurs abiotiques du milieu, mais elle est aussi dépendante des facteurs de stress. Tout facteur de stress induit une diminution de la part du vitellus utilisée pour la croissance au dépend de la part allouée à la respiration. Cette dernière augmente lors des épisodes de stress. Les résultats de la qualité des œufs et des juvéniles à l'éclosion sont en accord avec ce qui a été observé sur le système immunitaire. L'habitat de Baie de Seine est plus perturbé que les autres sites.
L'étude de la maturation du système digestif a montré que les conditions d'incubation des œufs dans le milieu naturel avaient un effet important à différents niveaux. Premièrement au niveau de la maturation de la glande digestive, où l'on a observé des différences significatives entre les sites dans le développement de la glande digestive à l'éclosion (taille des cellules digestives et leur contenu, les vésicules digestives).
Ces observations histologiques furent couplées avec des dosages enzymatiques, les activités enzymatiques étant très dépendantes de la maturation des cellules digestives. Ceci nous aida à comprendre les performances de croissance observées par la suite car la maturation du système digestif est une étape clé au cours de ces premiers stades de développement.

Au final, la tâche 4 du projet CRESH nous a permis de décrire les interactions entre conditions du milieu et les performances physiologiques des pré-recrues dans 4 principaux sites de ponte en Manche. Ceci va permettre de donner des éléments de réponse pour la compréhension des différences de contributions entre ces sites.

II. Actions carried out during the project/ *Bilan des opérations réalisées pendant le projet*

2009 Survey (June-September 2009)

During the first sampling survey, we developed the sampling collection and rearing of juveniles' protocol. Rearing began in July and ended in September. The number of samples collected is as follows:

The number of juveniles : 450

The number of eggs collected: 3704 from Baie de Seine, 1034 from Agon-Coutainville and 925 from Torbay.

Laboratory analysis (October 2009 - March 2010)

During this period, protocols were developments of for enzyme assays (Cathepsines, trypsines, acid and alcalin phosphatases). Then, the assays were carried out on all samples collected during summer.

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2010 survey (April 2010 - September 2010)

During the second sampling campaign, we continued monitoring juveniles rearing by adding a second English site (Selsey's). Rearing began in July and ended in September. Moreover, in September, juvenile cuttlefish were collected on French coastal sites and at Torbay, England. The number of samples collected is as follows:

The number of juveniles : 600

The number of eggs collected : 800 from Baie de Seine, 1100 from Agon Coutainville, 1500 from Torbay and 2000 from Selsey.

The number of pre-recruits studied : 30 from Agon Coutainville, 30 from Baie de Seine and 10 from Torbay).

Laboratory analysis (October 2010 - March 2011)

During this period, the development of immune assay protocols was performed (Lysozyme-like, phenoloxidase-prophenoloxidase complex,
antiprotease). Then, immune and enzyme assays were made. In addition to these biochemical assays, histological sections were conducted on reared juvenile in order to describe the maturation of the digestive system by studying digestive gland slices.

Year 2011 (April 2011 - September 2011)

During this third sampling campaign, we continued rearing juveniles at four sites. Rearing began in July and ended in September. Moreover, in November, juvenile cuttlefish were collected on French coastal sites and at Torbay, England. The number of samples collected is as follows:

The number of juveniles : 600

The number of eggs collected : 2340 from Baie de Seine, 931 from Agon Coutainville, 1000 from Torbay, and 1160 from Selsey)

The number of pre-recruits studied :30 from Agon Coutainville, 30 from Baie de Seine 30 and 10 from Torbay.

Laboratory analysis (October 2011 - October 2012)

During this period, immune and enzyme assays were performed on samples of 2011. In addition to these biochemical assays, new histological sections were conducted on cultivated juveniles to clarify certain aspects of the digestive system description. Based on these analyses, results processing and manuscript writing began. Today, every result has been processed, and two publications are almost finished. Other publications are on their way.

Campagne 2009 (Juin – Septembre 2009)

Au cours de cette première campagne d'échantillonnage, nous avons mis en place le protocole de collecte des échantillons et d'élevage de juvéniles. Les élevages commencent en juillet et se terminent en septembre. Le nombre d'échantillons collecté est le suivant :

Le nombre de juvéniles élevés : 450

Le nombre d'œufs collectés : 3704 dans la Baie de Seine, 1034 à Agon-Coutainville et 925 à Torbay.

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Analyses au laboratoire (Octobre 2009 – Mars 2010)

Pendant cette période, la mise en place des protocoles de dosage enzymatique (Cathepsines, trypsines, phosphatases acides et alcalines) ont été effectués. Ensuite, les dosages ont été faits sur l'ensemble des échantillons collectés en été.

Campagne 2010 (avril 2010 – septembre 2010)

Au cours de cette deuxième campagne d'échantillonnage, nous avons continué les suivis d'élevage de juvéniles en rajoutant un deuxième site Anglais (le site de Selsey). Les élevages commencent en juillet et se terminent en septembre. De plus, en septembre, des juvéniles de seiche ont été récoltés dans les sites côtiers français et le site de Torbay en Angleterre. Le nombre d'échantillons collecté est le suivant :

Le nombre de juvéniles élevés : 600

Le nombre d'œufs collectés : 800 en Baie de Seine, 1100 à Agon Coutainville, 1500 à Torbay et 2000 à Selsey.

Le nombre de pré-recrues étudiées : 30 d'Agon Coutainville,30 de Baie de Seine et 10 de Torbay.

Analyses au laboratoire (octobre 2010 – mars 2011)

Pendant cette période, la mise en place des protocoles de dosages immunitaires a été effectuée (Lysozyme-like, complexe phenoloxidase-prophenoloxidase, antiprotease). Ensuite, les dosages immunitaires et enzymatiques ont été faits. Parallèlement à ces dosages biochimiques, des coupes histologiques ont été menées sur les juvéniles d'élevage de façon à décrire la maturation du système digestif par l'étude des coupes de glande digestive.

Campagne 2011(Avril 2011 – Septembre 2011)

Au cours de cette troisième campagne d'échantillonnage, nous avons continué les suivis d'élevage de juvéniles sur les quatre sites. Les élevages commencent en juillet et se terminent en septembre. De plus, en novembre, des juvéniles de seiche ont été récoltés dans les sites côtiers français et le site de Torbay en Angleterre. Le nombre d'échantillons collecté est le suivant :

Le nombre de juvéniles élevés : 600

Le nombre d'œufs collectés : 2340 de Baie de Seine, 931 d'Agon Coutainville, 1000 de Torbay et 1160 de Selsey.

Le nombre de pré-recrues étudiées : 30 d'Agon Coutainville, 30 de Baie de Seine et 10 de Torbay.

Analyses au laboratoire (octobre 2011 – octobre 2012)

Pendant cette période, les dosages enzymatiques et immunitaires ont été effectués sur les échantillons de 2011. A côté de ces dosages biochimiques, de nouvelles coupes histologiques ont été menées sur les juvéniles d'élevage de façon à préciser certains points de la description de la maturation du système digestif. Après ces analyses, le traitement des résultats et le manuscrit ont débuté. Aujourd'hui tous les résultats sont traités et deux publications sont quasiment finies. Les autres publications sont en cours de préparation.

III. Scientific outputs/ *Détail des travaux scientifiques*

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La forme des articles présentés ci-dessous n'est pas définitive. Ces articles, qui sont en cours de préparation seront soumis à des journaux internationaux.

TASK 4

"This paper is in preparation to be submitted in a journal – All figures are separated from the text and are at the end of this paper"

CHARACTARISTICS OF CUTTLEFISH, SEPIA OFFICINALIS L., EARLY LIFE HISTORY IN RELATION WITH SPAWNING LOCATIONS

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Abstract

Spatial and temporal variability in the cuttlefish spawning sites of the English Channel were investigated in this study. A delay in the spawners arrival and egg laying period between coastal habitats was observed. Spawning began in the south western French coasts in Mid-April (the west coast of Cotentin Peninsula) and then moved toward the eastern French coast at the end of April, early May (Eastern coast of Cotentin Peninsula) and to the English ones in May. Furthermore, spawning strategy varied also between sites. The western French coast was the only spawning site that had high egg concentrations in the intertidal zone. This special ecosystem seemed to be very suitable for spawning to occur in the local cuttlefish population. After being laid, eggs underwent local environmental variables that influenced the embryonic development time such as temperature, salinity and turbidity that differed between spawning areas. Significant differences were observed afterwards in hatchlings weight and length due to these local variables. Juveniles from the south western French coasts were the smallest at hatching after undergoing warmer coastal waters in the incubation period. Moreover, hatching rate and juvenile survival rate showed interannual variability between sites. The English spawning sites had higher hatching rates but showed also episodes of high juvenile mortalities. In the French coast, the Bay of Seine site (East Cotentin Peninsula) had the lowest hatching rate over the monitoring years. These variations were probably related to eggs quality (maternal impact) but also to local factors such as water oxygenation and water pollution. Once hatching occurred, hatchlings were reared under controlled experimental conditions. Monitoring hatchlings growth over several seasons has allowed us to see that there were no significant differences in hatchlings growth rate due to spawning sites. Exponential models fitted by applying linear models to log transformed weight-at-age data revealed parallel growth between cuttlefish batches when reared in the same biotic and abiotic conditions. Differences observed at hatching are thus maintained in experimental rearing and the local environmental variables seemed to be the only responsible of growth variations observed in wild cuttlefish pre-recruits of the English Channel populations. Monitoring four major spawning sites of the English Channel coasts over several seasons has allowed us to better understand the spring migration pathways of this species and then its local spawning strategy, the embryonic development with eggs and hatchlings characteristics that differed between sites and years. To our knowledge, this is the first time where spawning sites impact on cuttlefish eggs development and hatchlings growth is undertaken. This study is important for assessing the importance of these spawning grounds and constitutes a first approach in understanding the contribution of these sites to the cuttlefish recruited stock.

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a. Introduction

The geographical distribution of the common cuttlefish, *Sepia officinalis* L., 1758 covers the Mediterranean Sea and the waters of the Eastern Atlantic from southern Norway and northern England to the northwestern coast of Africa. It is also found in Madeira and in the Canary Islands (Guerra, 2006). Throughout its distribution range, this species have marked seasonal migrations. In the English Channel, the populations of *Sepia officinalis* make large migrations offshore in winter and inshore in spring for reproduction (Boucaud-Camou and Boismery, 1991). The littoral zones of the English Channel are thus important spawning locations for *Sepia*. Once mating occurred, cuttlefish lay their eggs in coastal waters between April and June and die afterwards (Boucaud-Camou and Boismery, 1991). Eggs are incubated in the coastal spawning sites and the main hatching peak is observed between July and the middle of August (Challier et al., 2002). Hatchlings grow in the coastal habitats during the summer and early autumn before migrating towards the central zone of the Channel for wintering. These early stages of cuttlefish life cycle are called pre-recruited stages. Indeed, once the juveniles have entered the cuttlefish central stock, they are considered as new recruits (Challier, 2005).

The main cuttlefish exploiting countries in the English Channel are France, England, Wales, Scotland, Belgium, Netherlands and the Channel Islands (ICES, 2012). This exploitation undergoes large inter-annual fluctuation in landings that fluctuates between 8500 t and 18000 t in the ICES VIId,e (English Channel) divisions (Wang et al., 2003;, ICES, 2012). Fluctuations in abundance of exploited stocks may be due to a great variety of factors. However, in short-lived animals such as cephalopods, where abundance depends on strength of recruitment, it is well known that abundance is highly influenced by the environmental conditions, which affect recruitment (Sobrino et al., 2002). Cephalopods seem to respond to environmental variation both “actively” (e.g. migrating to areas with more favored environmental conditions for feeding or spawning) and “passively” (growth and survival vary according to conditions experienced). Environmental effects on early life stages can affect life history characteristics (growth and maturation) as well as distribution and abundance (Pierce et al., 2008). Given the cuttlefish short life span of around two years and the inter-annual fluctuations in landings, it is expected that marine environment conditions have an important impact on cuttlefish recruitment and distribution (Wang et al., 2003). Moreover, the early life stages of any cephalopod species are closely related with the recruitment process, and knowledge of recruitment to a fishery is particularly important in short-lived species where there is a complete turnover of biomass every 1-2 years. (Guerra, 2010). The environmental conditions probably affect the early life stages of *Sepia* in its pre-recruited stages. Variations during these stages will directly affect the recruited stock over the next two years.

From April until September, many cuttlefish enters coastal areas to lay their eggs. Several million eggs are deposited on natural and artificial supports. This egg mass, which represents the largest part of the cuttlefish stock renewal leaving the coast in autumn, is subject to environmental variables that influence their development and survival (Bouchaud and Daguzan, 1989). The relationship between the cuttlefish abundance and the early life history of juveniles in the coastal spawning sites still not well documented. Spawning grounds are the basis of the cuttlefish stock renewal and studying these habitats could give important elements for understanding their contribution to the central stock recruited.

The aim of this work is thus to investigate some aspects of these early life stages of juvenile cuttlefish. The impact of four major spawning sites on eggs development and hatchlings characteristics were studied in the English Channel. Are there any differences in eggs development, hatching rates, hatchlings survival rate and characteristics between these sites? Do we have inter-annual variations in these early life stages of *Sepia officinalis* in the Channel? Is there a difference in juvenile growth due to the incubation conditions in the coastal habitats and their associated ecosystems? If there are differences, which factors might explain them?

b. Material and methods

Study spawning sites

This study was interested in four sites among the main spawning grounds of cuttlefish in the English Channel (Boucaud-Camou et Boismery, 1991 ; Dunn, 1999 ; Challier, 2005). Two spawning sites were located on the French coast (Agon Coutainville (AC; 49°02‘35”N, 1°34’32’’W) and Bay of Seine (BS; 49°18‘53‘’N,

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0°21'0''W)) and two others were located on the English coast (Torbay (TB; 50°27'08''N, 3°33'25''W) and Selsey (S; 50°44'06''N, 0°47'23''W)) (**Figure 1**). **T**he French sites were on the eastern (BS) and western (AC) coasts of Cotentin peninsula. The west coast is exposed to general currents of the North Atlantic drift, which are propagated eastward and deflected northward along the coast. The maximum spring tidal range is as much as 12 m, and among the highest in the world. Thus, ecosystems of the west coast are megatidal (maximal bottom current speed is 10 knots and intertidal zone covers a very large surface) (Lefebvre et al., 2009, Dauvin et al., 2012). Furthermore, this site offers wide sandy expanses with scattered rocks and seaweeds. The Bay of Seine (BS), located east of the Cotentin Peninsula, is a very large bay that is completely open to the Channel but which nevertheless remains more protected from the prevailing winds and marine currents than ecosystems on the west coast. The maximum tidal range is 8 m, describing a macrotidal environment (maximal bottom current speed is 5 knots and intertidal zone is smaller than the west coast) (Lefebvre et al., 2009, Dauvin et al., 2012). This site offers sandy areas and rocky plateaus with high seaweed concentrations. Seaweeds are important as natural support for egg clusters and sandy bottoms are areas for hatchlings to hide. Indeed, the young animals, with a sand covering behavior that begins very early, stay on the bottom and swim up only to capture prey (Boletzky, 1983).
About the English coastal sites, we have chosen to study the Torbay site, which is a smaller and more protected bay then the BS one, and the Selsey site which is a site widely open to the English Channel. Torbay is at the west end of the Lyme Bay and is protected from the western currents. It is a habitat that is very rich with seagrass meadows (Hirst and Atrill, 2008). As for Selsey, …

Eggs collection and handling

Eggs from *Sepia officinalis* L. were collected during July from2008 till 2011. Two protocols were used: the intertidal protocol and the subtidal one. The intertidal protocol consited in making linear transect that were perpendicular to the coast at low tide. Five to eight persons were aligned over a 1 kilometre distance and moved towards the sea. Each cluster found was geo-localised with a GPS than transferred in a box half filled with seawater and algae to keep it fresh and stabilized. The subtidal protocol also consisted in linear transects with three to five scuba-divers. Eggs were geo-localised and conditioned as for the intertidal protocol.
Afterwards, eggs were separated in groups (~ 100) and placed in sieves (36 x 28.6 x 9 cm) distributed in large tanks containing circulating seawater, with temperature between 18-19°C. Hatchings were recorded daily for the final hatching rate determination. The hatching rate was then calculated as follows:
Hatching percentage = (number of hatchlings / total number of collected eggs) x 100

Experimental design

Once eggs were installed in the rearing structures, they were acclimatized for 3 days, to avoid premature hatchlings use in the rearing due to transport stress. Afterwards, hatchlings were collected daily to determine their hatching day. Throughout the hatching period, hatching peaks were observed due to the important amount of collected eggs. Juveniles used in the rearing were collected during these peaks in order to have 150 cuttlefish per site, which hatched in the same day, to be used for the growth survey. The experimental growth survey ran afterwards for 35 days between July and September from 2008 till 2011. Cuttlefish of each site were placed into 6 rectangular tanks (36 x 28.6 cm; 25 hatchling /tank). All batches were under the same controlled and standardised conditions and were fed *ad-libitum* with frozen shrimps, *Crangon crangon*.

Rearing system and parameters

Rearing was conducted in a semi-enclosed system that limited the presence of debris that causes turbidity of the water. This system allowed 80% of seawater to be renewed in the tanks per day to avoid problems with evaporation, loss of water due to tank cleaning, changes in salinity and pH, and accumulation of nitrate (NO_3^-), nitrite (NO_2^-), ammonium(NH_4^+), and ammonia (NH_3) concentrations (Koueta and Boucaud-Camou 2003). The base of the rearing system consisted of a 1000-l storage tank containing a heating system. The natural seawater used to renew the circuit ran through UV lamps before filling the tank which also contained oyster shells that acted as biological filters. Water was driven by an automatic pump into a protein skimmer, which removed dissolved organic material and returned the seawater to the superior tray containing the filters.
Physical and chemical parameters were maintained by constant renewal of fresh seawater. The photoperiod was 12:12. Weekly measurements made with colorimetric kits (Tetra Test Kit) always revealed concentrations

of NO_3^- < 10 mg/l, NO_2^- < 0.1 mg/l, NH_4^+ < 0.5 mg/l and NH_3 < 0.02 mg/l. The pH of seawater was 8 and the oxygen was optimised by adding bubblers to each tank. As for the temperature, it was maintained stable by the heating system.

Food preparation

The food preparation started before the rearing period and lasted for more than one month (between June and July). When rearing started, cuttlefish were fed every afternoon with wild caught frozen brown shrimps (*Crangon crangon*). The shrimps were divided into three classes based on their length (**Table 1**). It is important to rank prey according to their size to have shrimp smaller or at the same size as cuttlefish. After the size class, the prey mean weight was used for the preparation of standard prey bags with 2g (Mini), 3g (Small) or 4 g (Average) of shrimps. The bags were then frozen at -80°C and used afterwards to feed each cuttlefish tank (25 cuttlefish / tank). The same food quantity was placed in each tank and the amount of ingested food was estimated by weighing every day the remaining shrimps. The food pre-preparation allowed us to properly regulate the food quantity and to have no bias between cuttlefish batches due to food availability.

Growth analysis

Weight and length measurements were conducted once a week on 24 juvenile cuttlefish per site (4 per tank). A sliding calliper was used to measure dorsal mantle length (mm) and a Sartorius balance was used to weigh all animals with a precision of 0.1 mg (Koueta and Boucaud-Camou., 1999). Furthermore, the quantity of food eaten by each cuttlefish batch was calculated. These measures allowed us to determine the mean length (mm) and weight (g) of cuttlefish at each sampling date and to follow several growth parameters such as:

Weight increase (g): final weight – initial weight

Ingested rate (IR - % body weight/day): (IF/average W(t)) x 100, where IF is the ingested food (g) and average W(t) is the average wet weight (g) of the cuttlefish during the time period ($g.d^{-1}$).

Instantaneous growth weight (IGR - % body weight/day): (ln W2 – ln W1) x 100/t, where W2 and W1 are, respectively, final and initial weight (g) of each animal and t (35 days) the duration of the experiment in days.

Conversion rate (CR - %): (weight increase (g) / Ingested Food (g)) x 100

Data modeling and statistical analysis

Juvenile cuttlefish have an exponential growth in their early life history due to a high growth rate when reared in optimal conditions as described in the rearing parameters. Exponential models were thus fitted by applying linear models to log transformed weight-at-age data. Natural logarithmic transformations were formed for comparison of growth.

For statistical analysis, preliminary tests of normality and homoscedasticity allowed the use of parametric methods. In juvenile's weight, growth parameters and survival rate, statistical comparisons were made afterwards with analysis of variance (ANOVA) using R program for statistical computing. When differences were present, a Tukey's test was used to determine differences between batches. In the hatching rate data, a GLM binomial test was used for differences analysis (GLM function with R program).

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c. Results

Hatching and juveniles survival rates

The mean hatching rate (HR) over four years monitoring revealed significant differences between the four studied sites (**Table 2-A**). The lowest mean HR found was for French sites (BS (62.2%) and AC (65.9%)) and the highest in English sites (TB (74.4%) and S (91%)) (**See figure 1** for station location). Only one exception was observed for TB in 2010 were HR was lower than the French ones (**Table 2-B**). Furthermore, differences in the eggs location, in the hatching rates and in the survival rates were observed between the sites in each year and between the years (**Table 2-B**). For the eggs location, AC had high egg concentrations in the intertidal zone, as for the other sites, the highest egg concentrations were in the subtidal zone between 5 and 10 meter depth. The comparison between eggs from subtidal or intertidal zone in the French sites showed that the eggs location in BS had no impact on the hatching rates that were already the lowest. On the other hand, in AC site, the subtidal zone seemed to be more favorable for a better hatching rate (93% in 2011) compared to the ones found in intertidal zone (between 60 and 73 %). The high hatching rate of AC site in 2011 is also comparable to those found in the English sites. However, beside the eggs location impact, an interannual variability was also observed in the hatching rate when eggs were collected in the same zone. Indeed, in AC intertidal zone, HR varied from 60% to 73% between 2008 and 2010 and in TB, HR in subtidal zone varied from 66% to 93% between 2009 and 2011.

As for the mean survival rate after 35 days of rearing, no significant differences were observed between the four studied sites (**Table 2-A**). The mean survival rate for all batches was around 90% excepting three points (TB in 2010 (98 ± 1.5%), S in 2010 (84 ± 5.4%) and TB in 2011 (76 ± 6%)) (**Table 2-B**).

Experimental growth survey

Significant differences ($p < 0.05$) were observed between spawning sites in juvenile's weight at hatching (Day 1) and these differences were maintained till day 35 after hatching in experimental conditions (**Table 3**). Indeed, the comparison between the two French sites showed marked differences between BS and AC over four monitoring years with BS juveniles being significantly bigger than those of AC. Only one exception was noticed in 2010 where AC juveniles weight after 35 days were not significantly smaller ($p > 0.05$) than BS ones. As for the English sites, no significant differences ($p > 0.05$) were observed between TB and S. At the end of the rearing, the English juvenile weights were closer to those observed for BS. A linear growth model was considered appropriate to describe the juvenile's growth in these experimental rearing conditions (**Figure 2**). This model highlighted the parallel growth observed between the cuttlefish batches all along the experiment duration. Indeed, AC juveniles were always smaller than the other batches and with the linear model, the AC juvenile linear growth was thus represented below the other ones.

During the experimental rearing, the ingested rate (IR - % cuttlefish body weight/day), the conversion rate (CR - % cuttlefish body weight/day) and the instantaneous growth rate (IGR - % cuttlefish body weight/day) did not show significant differences between cuttlefish groups when compared in each year (**Table 3**). The water temperature, which was the only abiotic parameter that varied between years, was also stable in each year with no differences between cuttlefish batches. However, significant differences ($p < 0.05$) were noticed between the years (from 2008 till 2011) in the growth parameters where a decrease in the IR, CR and the IGR was observed. The same pattern was also noted for the juvenile's weight after 35 days of rearing and in the water temperature. In 2008, water temperature was 20.55 ± 0.12°C with juvenile weight at day 35 around 3.5 g, a mean IR of 38%, a mean IGR of 7.5% and a mean CR of 23%. In 2011, the juveniles mean weight (Ca. 1.3 g) was almost three times smaller than in 2008 when reared in water temperature around 19.2°C with a mean IR of 20%, a mean IGR of 5% and a mean CR of 15%.

Hatchlings and environmental parameters: The French sites

We have already observed that the spawning sites had an impact on juvenile cuttlefish hatching weight. Here we tried to make a relationship between the hatchlings weight and the environmental parameters. For the two French sites, we have a four year data that showed significant differences between BS hatching weight and the AC ones (Figure 3). In 2008, the egg clusters were collected in the intertidal zone for the two sites and hatchlings had significantly ($p < 0.05$) different weights. This difference was also observed in 2009 and 2010 with different collection zones (Subtidal-Intertidal) and in 2011 when eggs were collected in the subtidal zone

for the two sites. Different observations, in the site characteristics and environmental parameters, enabled us to get some answers for these differences.
Site general characteristics in Agon Coutainville were shown to be different from those in Bay of Seine (Table 5). Indeed, the west coast describes a megatidal ecosystem with very large intertidal zone due to a high tidal range up to 15 m and a different bathymetry. In these conditions, the egg clusters were found in high concentrations in the intertidal zone in opposite to what is observed in the Bay of Seine. This is the first major difference between the two sites that affects the egg incubation conditions. Furthermore, the environmental parameters showed also differences.
We actually looked into the coastal water temperatures and salinities, from 2008 till 2011, between April and July which correspond to the eggs incubation period in the spawning sites before collecting them (Table 4). The mean water temperature (°C) and salinity (‰) were higher in Agon Coutainville coastal waters compared to Bay of Seine. This means that the eggs are incubated in warmer coastal seawater in the west coast with approximately 1°C more and higher salinities with more than 1.5‰.more during the their incubation period.

IV. Discussion

Due to their shallow depths and terrestrial inputs, coastal ecosystems are characterised by high spatial and temporal variability of environmental factors such as freshwater runoff, levels of irradiance, temperature, water movement and other factors. These parameters affect the coastal spawning grounds and all organisms living in the inshore waters such as cuttlefish in their early life stages. Understanding the relationship between a species and its surrounding abiotic environment is relevant for the fundamental ecology of a species but also useful in terms of conservation ecology, e.g. through the creation of marine protected areas, for managing marine resources and for evaluating the consequences of climate changes on populations (Loots et al., 2010).

Coastal spawning sites and egg clusters characteristics

After four years monitoring, we were able to observe, describe and discuss four important spawning sites of the cuttlefish, *Sepia officinalis* L., in the English Channel. Spatial and temporal differences were observed between the sites. Indeed, the egg clusters were differently concentrated depending on site, with Agon Coutainville (AC-West Cotentin) having the highest concentrations in the intertidal zone as for the three other sites, the highest concentrations were in the subtidal zone. This intertidal habitat in the West Cotentin coast seemed to be an important spawning area for cuttlefish with hatching rates that were similar to what was found in the Bay of Seine (BS-East Cotentin) subtidal zone. Successful embryonic development in this particular ecosystem can be due to several reasons. First of all, the clusters were generally found at the algae base that covered the eggs at low tide and probably protected them. Secondly, the thickness of the egg envelope with its several layers protects the embryonic development at least till the stage 26 of Lemaire (1970) from which there is a decrease in the thickness till stage 30 (= hatching stage) (Paulij et al., 1991, Lacoue-Labarthe et al., 2010). Concerning the spawning behavior, the difference was marked between AC and the other 3 sites. But this spawning strategy in AC could be due to hydrodynamic stress and coastal habitat typology that is different from the other sites. Hydrodynamic stress caused by currents can damage eggs directly and may also dislodge attached eggs and remove them to a less favorable location which may result in severe rates of mortality (Probst et al., 2009). This should influence the selection of the spawning area. Cotentin West coast is exposed to strong Atlantic currents and is less protected than the eastern part of the English Channel (Lefebvre et al., 2009). The West Cotentin coast sustained bottom current speed (10 knots) twice higher than the eastern Bay of Seine part (5 knots) and maximal tidal range that is almost twice higher in the west coast (15 m) compared to the Bay of Seine (8m). (Dauvin et al., 2012). The eggs distribution in the West Cotentin coast (AC) could be thus the result of the influence of hydrodynamic stress coupled with the coastal habitat typology.

Temporal differences were observed in the field with the beginning of the spawning period and in the laboratory with hatchings. The comparison between the West (AC) and the East (BS) Cotentin in the eggs laying period revealed one to two weeks delay between the two coasts. Eggs were started to be layed around mid-April in AC and at the end of April/ early May in BS. This is in agreement with Boucaud-Camou and Boismery (1991) where these authors observed an earlier arrival of spawners on the west coast. In lab experiments, 10 to 20 days delay were also observed in hatchings between the French and the English coast.

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This suggests the idea of a probable shift between the two coasts that could be due to different egg laying period and to different environmental conditions. The English Channel constitutes a transition area between the warm-temperate Atlantic oceanic system and the boreal North Sea and Baltic Sea continental systems of the northern Europe (Dauvin et al., 2012). Average bottom temperatures of the English Channel present a decreasing gradient between the western part towards its eastern basin (Boucaud-Camou and Boismery, 1991). Furthermore, during summer, Atlantic water enters the English Channel from its south side along the northern Brittany coast toward the western Cotentin coast (Wang et al., 2003). The amplitude of the movements of the Channel cuttlefish stock is clearly linked to the temperature without being the only factor involved (probably currents and photoperiod too) and *Sepia officinalis* seems to be only active above 10°C (Boucaud-Camou and Boismery, 1991; Boletzky, 1999; Wang et al., 2003). If we compare the coastal water temperatures between the French and the English coasts (Table 4 and 7) between April and July, we can see clearly a shift between the two coasts. Indeed, the English waters are colder than the French ones at the same periods. In April, when spawners arrive to West Cotentin coast (11.5±1.1°C), the Bay of Seine mean water temperature is (10.4±0.1°C) and the English waters mean temperature is around 9±1°C. These differences could explain the delay between the two French sites and the shift between the English and the French coast. Adult cuttlefish arrives in the West-Cotentin around mid April with the warm Atlantic currents that reaches this coast first. One to two weeks later, cuttlefish gets to the Eastern-Cotentin coast at the end of the same month followed by the English coast in May. In addition, this scheme would be in agreement with our field observations. Cuttlefish arrived in inshore waters when water temperature was between 11 and 12°C.

Another shift was observed between the French and the English Coastal spawning habitats that concerned the eggs hatching rate. When considering the total amount of collected eggs between 2008 and 2011 in each site, the English ones had significantly higher hatching rates than the French ones. This observation might be related with the shift observed in the egg laying period. The delay in the migration to the English coast with different pathway to get there may influence the food availability and quality. Fluctuations in environmental conditions, particularly food availability, experienced during the female's reproductive season, can have significant flow on effects to the subsequent population structure (Kerrigan, 1997). In *Sepia officinalis,* egg yolk lipids quantity varies with egg size, suggesting that differences in cuttlefish egg quality may exist. Furthermore, the lipid requirements for correct cephalopod development are not understood; however, phospholipids and long-chained polyunsaturated fatty acids, particularly EPA and DHA, are likely to be essential constituents (Boucaud and Galois, 1990; Navarro and Villanueava, 2000, 2003). Thus, eggs quality due to maternal transfer could be a major factor in understanding the hatching success. But other factors could also be involved such as the spawning strategy and the local environmental variables. In AC (West Cotentin), we found a similar hatching rate with those of the English sites when eggs were collected in the AC subtidal zone. This French site presented two separated spawning habitats that seemed to affect differently the hatching rate. This final remark should be confirmed by increasing the number of eggs collected in the subtidal zone and compare it with eggs collected in intertidal zone. But the fact that the intertidal zone in AC had the highest egg concentrations, the mean hatching rate of these two habitats will still be lower than the English ones. In the Bay of Seine (BS) case, the low hatching rate could not be the result of a different spawning strategy as the eggs were spawned in subtidal zone like the English sites. But the BS site is also a particular ecosystem. The BS site is impacted by high river inputs and has thus the lowest salinity rates in the Channel which impacts the hatching rate (Blanc, 1998; Dauvin, 2007, 2012). The decrease in salinity is positively correlated with hatching decrease in *Sepia officinalis* (Palmegiano and D'Apote, 1983). Episodes of heavy rainfall and river discharge can increase this salinity impact too. Furthermore, the Seine estuary is highly contaminated by metals, with high levels of Cd, Hg and Ag, in addition to PAHs and PCBs making it one of the most contaminated in Europe. More recently, significant levels of new contaminants, including antibiotics and estrogens, have been reported (Dauvin et al., 2007 and 2012). Heavy metal affects mechanism of development and also whole developmental and hatching process in many cephalopods such as *Loligo vulgaris* (Halil and Ugur, 2007) and *Sepia officinalis* (Le Bihan, 2004; Lacoue-Labarthe 2008, 2009a, 2009b, 2010). The combined effect of all these parameters could be implicated in the low hatching rate observed in this spawning site. Another environmental factor is also important in the embryonic development and the hatching success. Eggs oxygenation is the key factor leading to hatching (Gutowska and Melzner, 2009) and embryos develop normally when oxygen concentrations are close to saturation (Boletzky et al., 2006). Selsey site being completely open to the Channel may have a better rate of water oxygenation than the other sites leading to higher hatching rates.

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An inter-annual variability was also observed in the hatching rate in all the studied sites. Since the incubation conditions in the rearing structures were identical over the four year monitoring, it is unlikely that these variations could be due to experimental bias. These variations seem more likely to be related with environmental and eggs quality variations. As described above, the reduction in lipid quantity and quality seems to be responsible for elevated mortality rates due to lake of fuel for embryogenesis (Steer et al, 2004, Leporati et al., 2007).Thus inter-annual variations may be the result of egg quality evolution between years due to changes in the composition of the reserves transferred by females. But fluctuations in environmental parameters such as the salinity, oxygen and temperature could also vary between years affecting thus the hatching rates

Impact of the environmental factors on eggs incubation and hatchlings weight

Looking into correlations between hatchling weight and environmental parameters is a way to understand eggs incubation conditions in the spawning grounds. An impact of spawning sites on hatchlings weight was observed with some marked differences mainly between the western and eastern coast of Cotentin peninsula. We have chosen thus to look more closely into those sites parameters and try to find correlations between site characteristics and hatchlings weight. According to recent literature, temperature, salinity and oxygen are the major factors that influence the traits of cephalopods early life stages (Guerra et al., 2010). As described above, oxygen seems to impact the embryonic development and thus the hatching rates. As for temperature and salinity, differences were noted between Agon Coutainville (AC- West Cotentin) and Bay of Seine (BS – East Cotentin). Indeed, AC had warmer water temperature during the incubation period than in BS and higher salinity rate. The mean water parameters between April and July are 15.6°C and 34.4‰ in AC vs 14.6°C and 32.6‰ in BS. This is due to the fact that hydrologic and oceanographic features are mainly dominated in the Western basin of the English Channel by the influence of Atlantic waters (Dauvin et al., 2012) and in the Bay of Seine by the fresh water inputs of the Seine estuary. Watersheds impacting the east coast are four times larger than that of watersheds impacting the west Cotentin coast (Lebfevre et al., 2009).But we also observed different spawning strategies. In AC, the high egg concentrations in the intertidal zone could have an additional impact on incubating temperature with warmer waters at low tide.

The embryonic development of many cephalopods has been shown to be highly temperature dependent, with eggs generally developing faster in warmer waters (Semmens et al., 2007). An inverse relationship is observed between temperature and embryonic development duration in many cephalopods such as *Sepia atlantica* (Rodrigues et al., 2011), *Octopus mimus* (Uriarte et al., 2012), *Illew coindetii* (Villanueava et al., 2011) and *Sepia officinalis* (Lemaire, 1970; Bouchaud and Daguzan, 1989; Dickel, 1997; Blanc, 1998). This development duration is proportional to hatchlings length and weight for cuttlefish and *Illew coindetii* (Bouchaud and Daguzan, 1989; Villanueava et al., 2011) but inversely proportional in *Octopus mimus* (Uriarte et al., 2012). Our results are consistent with authors' descriptions as we observed that in colder water, in BS site, hatchlings were significantly bigger than in AC site. The Larger cuttlefish hatchlings are produced at low temperature probably due to better yolk absorption (Bouchaud and Daguzan, 1989). But temperature was not the only parameter that varied. Seawater salinity has also an impact on hatching rate, survival and incubation time in many cephalopods. *Loligo vulgaris* embryonic development was possible at salinity range between 34‰ and 37‰ but was stopped below 31‰ (Halil, 2004). In *Octopus vulgaris*, abundance seemed to be closely related to salinity and mass mortalities were observed with episodes of decreased salinity (Sobrino et al., 2002; Guerra et al., 2010). Blanc (1998) studied the impact of four different salinities (20, 25, 30, 35‰) on *Sepia officinalis* eggs incubation and hatchling characteristics. This author observed that at the lowest salinities (20 and 25‰), no hatching occurred and for the two other salinities, significant relationships were found. Salinity of 30‰ induced a longer incubation period for eggs compared to 35‰ and bigger hatchlings weight and mantle length. The lower salinity rates in BS could also have an impact thus on embryonic duration period and afterwards on hatchlings weight. The combined effects of temperature and salinity in BS on hatchlings weight seems to be consistent with the literature. Other factors such as rainfall, river discharge, water turbidity, solar flux and photoperiodicity and currents may also be important in influencing the eggs incubation duration as they will impact water temperature and salinity. Sobrino et al. (2002) found that *Octopus vulgaris* abundance was highly correlated with the rainfall and river discharge previous to fishing season. Cuttlefish, *Sepia officinalis*, is a migrating species (Boucaud and Boismery, 1991) therefore it is less concerned by coastal environmental conditions. But in its early life history, when eggs are incubating in the coastal habitats, episodes of heavy rainfall and river discharge can locally affect their development by affecting salinities and currents. Furthermore, Paulij et al. (1991) studied the influence of photoperiodicity on hatching of *Sepia officinalis*. These authors observed that in the absence of an external Light-Dark rhythm, the time to hatching increased. In the Bay of Seine, turbidity is high where the mean annual particulate river discharge has been evaluated at 650 000 t of suspended matter (Dauvin et al., 2007) in opposite with west coast were clarity of water is high (Dauvin et al., 2012). The high turbidity in BS could thus have an additional impact on eggs by increasing the incubation duration.

Hatchlings growth and survival

Spawning sites had an impact on hatchlings weight due to differences in the incubation conditions. Hatchlings from BS were generally the biggest at hatching followed by the English ones (Torbay and Selsey) and the AC being the smallest. This weight distribution was found after 35 days of rearing highlighting the importance of the cuttlefish initial size. The initial size and weight of hatchlings plays a major role in their growth performance (Leporati et al., 2007). Differences observed at hatching are thus maintained when juveniles were reared in the same biotic and abiotic conditions. Furthermore, cephalopods growth is exponential during the first part of their life cycle (Domingues et al., 2006) thus applying a linear model to log transformed weight-at-age data seemed appropriate. The use of a linear model in our study was interesting to highlight the parallel growth observed between hatchlings from different spawning sites. Abiotic and biotic factors were standardized in the laboratory in order to avoid any bias between sites. Thus, the only difference between the cuttlefish batches was their origin site. This approach allowed us to see that there was no difference in cuttlefish growth performance due to the spawning sites and their isolation by distance. Whether we were on the French or English spawning sites or in the eastern or western part of the English Channel, hatchlings growth performance were identical. Challier et al. (2005) showed that pre-recruits growth rates differed significantly in inshore waters between the Bay of Seine and Cotentin west coast. These authors suggested the possibility of a difference in growth of pre-recruits between these areas that may be caused by genetic variation or by environmental differences (such as the variability in the rate flow of the Seine) that could impact the growth rate. Our results show that the only difference in growth is thus due to local environmental variations and not to genetic divergence.

During the rearing surveys, different growth parameters were calculated in order to estimate the growth rates and to check the food impact on juveniles. No significant differences were observed in growth parameters between the sites. Ingested rate was similar to all batches indicating that food source had no particular impact on one group compared to another. No differences were observed in instantaneous growth rate (IGR) and the conversion rate (CR) too. IGR and CR values were consistent with what was found by Koueta and Boucaud-Camou (1999), Domingues et al. (2002, 2006) and Sykes et al. (2003). We noted however a decrease in the IGR and the CR between 2008 and 2011 that was clearly related to water temperature decreasing. Cephalopods are poikilothermic organisms and react instantly to increased temperatures by raising all metabolic processes, including feeding and growth (Domingues et al. 2001). But the decrease of IGR and CR were similar to all batches and thus the linear growth was appropriate all along the years monitoring.

Early life stages of cephalopods are known to be a critical phase and are highly important for the renewal of the stock. Ecosystem variability affects biological parameters, such as mortality rate, life span, fecundity, maturity and growth and thereby affects models of population dynamics (Challier et al., 2006). In our study, we had juvenile mortalities but the survival rates were pretty high, around 90%. Similar rates were found by Koueta and Boucaud-Camou (2001) and Forsythe et al. (2002) between 90% and 96.7% survival after 35-40 days of rearing. These high survival rates are probably due to the optimal experimental conditions and standardized parameters. As for the site impact on hatchlings survival, it varied between sites and years. What is interesting to see is the inter-annual variability observed and the lower survival rates in some sites in specific years. These variations could be the result of the egg quality varying from one year to another. Cuttlefish egg quality may differ in its size, reserves content, composition and even the number of eggs per clutch (Boucaud and Galois, 1990; Navarro and Villanueava, 2000, 2003) from one year to another and from one site to another. Eggs of lower quality might produce hatchlings that are more easily subject to infections and diseases. In 2011, Torbay site was the only spawning site with high mortality rates and the same pattern was observed for Selsey in 2010. The eggs quality could vary from one year to another inducing variation in the juvenile's survival rate and thus the potential site contribution to the cuttlefish recruited stock.

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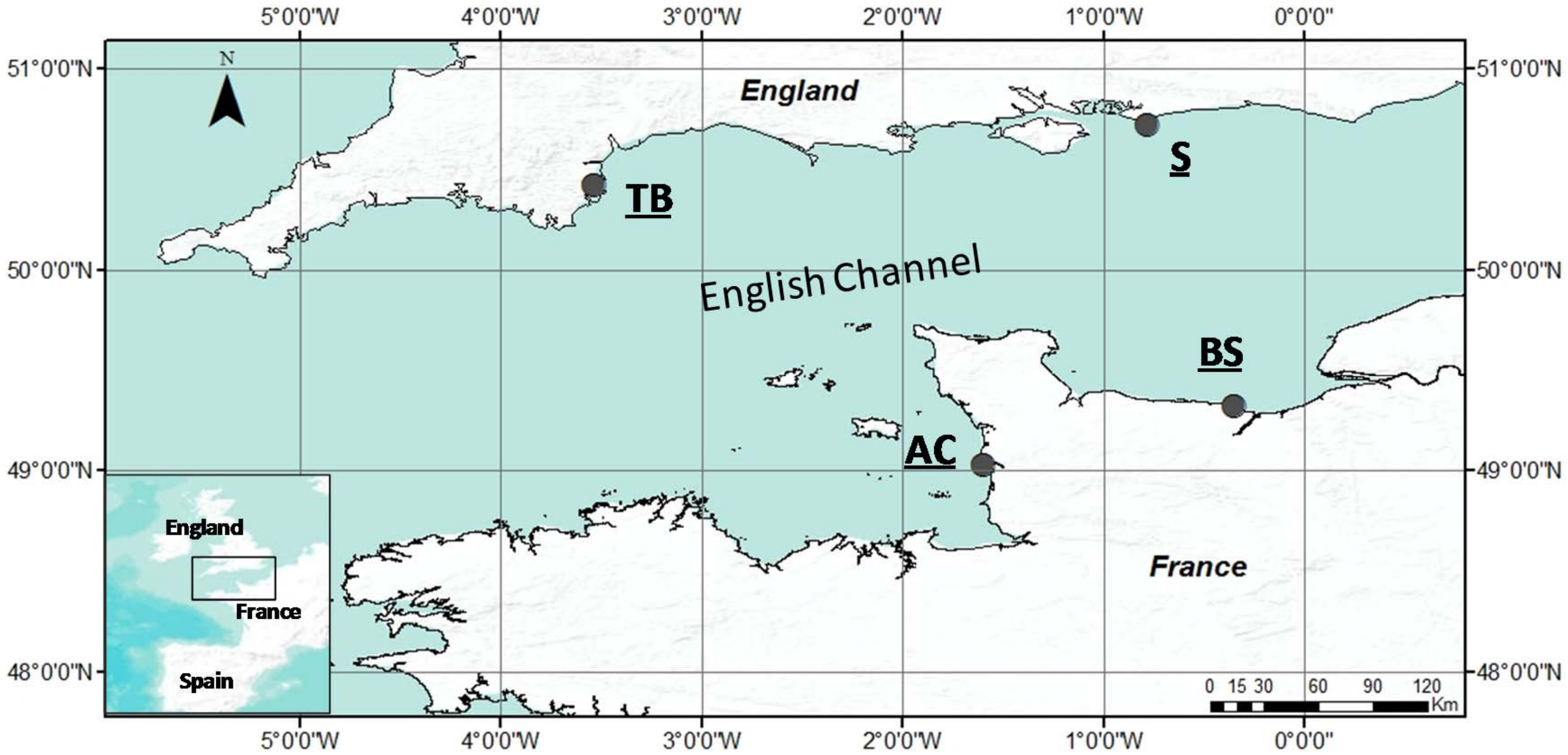

Figure 1: Study spawning sites distribution of *Sepia officinalis* in the English Channel. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK).

TASK 4

Table 1: Size classes, based on brown shrimps (*Crangon crangon*) length and their subsequent mean weight, used to feed *Sepia officinalis* hatchlings.

Size	Length range (mm)	Mean weight (mg)	rearing period (days)
Mini	12 - 16	25.7 ± 5.6	1 - 7
Small	17 - 23	64.5 ± 7.4	8 - 21
Average	24 - 30	112 ± 30.2	22 - 35

Table 2 : Hatching and hatchlings survival (35 days of rearing) rates obtained for cuttlefish eggs collected from different spawning sites over several seasons. A- Mean values of 4 year monitoring. B- Détails for each year and each site. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK). The location of eggs is specified by Inter (intertidal) and Sub (subtidal) and n is the sample size.

A	Site	Total eggs	Total hatchlings	Hatching rate / site	Total reared juveniles	Juveniles survival rate / site
	BS	8510	5289	$62,2^{a}$	600	91 ± 1^{a}
	AC	7967	5248	$65,9^{b}$	600	92 ± 1^{a}
	TB	3437	2558	$74,4^{c}$	450	88 ± 11^{a}
	S	3180	2895	91^{d}	300	87 ± 4^{a}

B		Eggs characteristics			Juveniles characteristics	
Year	**Site**	**Eggs location**	**n**	**Hatching rate (%)**	**n**	**Survival rate (%)**
2008	BS	Inter	1666	57^{a}	150	$92,7 \pm 2,1^{a/b}$
	AC	Inter	4902	$60,1^{b}$	150	$92,7 \pm 2,1^{a/b}$
2009	BS	Sub	3704	$58^{a/b}$	150	$90,4 \pm 3,2^{a/b}$
	AC	Inter	1034	$60,5^{a/b}$	150	$91,2 \pm 3,9^{a/b}$
	TB	Sub	925	$66,4^{c/e}$	150	$91,2 \pm 3,9^{a/b}$
2010	BS	Sub	800	$72,6^{d}$	150	$91,2 \pm 1,5^{a/b}$
	AC	Inter	1100	$73,3^{d}$	150	$90,4 \pm 4,1^{a/b}$
	TB	Sub	1502	$66,8^{c/e}$	150	$98 \pm 1,5^{a}$
	S	Sub	2020	$94,4^{f}$	150	$84 \pm 5,4^{b}$
2011	BS	Sub	2340	$68,8^{e}$	150	$91 \pm 3^{a/b}$
	AC	Sub	931	93^{f}	150	$92,8 \pm 3,4^{a/b}$
	TB	Sub	1010	$93,2^{f}$	150	76 ± 6^{c}
	S	Sub	1160	$85,2^{g}$	150	$90,4 \pm 4,7^{a/b}$

a,b: Numbers not bearing the same subscript letter are significantly different

Table 3: Comparison of mean weight (± s.e.), mean ingested rate (IR %) (± s.e.), mean instantaneous growth rate (IGR %) (± s.e.), and mean conversion rate (CR %) (± s.e.) for all monitored sites over 35 days of rearing and for 4 seasons. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK), and n is the number of individuals used for the mean weight (g) at day 1 and day 35 (rearing end).

Year	Site	Mean weight (g)				Mean IR (%)	Mean IGR (%)	Mean CR (%)	Mean T (°C)
		n	Day 1	n	Day 35	Period 1-35 days	Period 1-35 days	Period 1-35 days	Period 1-35 days
2008	BS	24	$0,26 \pm 0,01^{a}$	30	$3,92 \pm 0,17^{a}$	$38,8 \pm 6,4^{a}$	$7,5 \pm 3,4^{a/b}$	$24,6 \pm 9,5^{a}$	$20,55 \pm 0,12^{a}$
	AC	24	$0,22 \pm 0,006^{b}$	28	$3,29 \pm 0,16^{b}$	$37,7 \pm 7,8^{a}$	$7,5 \pm 1,5^{a}$	$21,1 \pm 10,6^{a}$	$20,55 \pm 0,12^{a}$
2009	BS	24	$0,22 \pm 0,005^{b}$	24	$2,62 \pm 0,09^{c}$	$15,4 \pm 1,6^{b}$	$7 \pm 1,2^{a/b}$	$20,2 \pm 5,5^{a}$	$19,9 \pm 0,3^{b}$
	AC	24	$0,19 \pm 0,003^{c}$	25	$1,52 \pm 0,09^{d}$	$14,8 \pm 2^{b/d}$	$6 \pm 1,7^{a/b}$	$22,6 \pm 6,7^{a}$	$19,8 \pm 0,5^{b}$
	TB	24	$0,20 \pm 0,008^{b/c}$	24	$2,29 \pm 0,12^{c/d}$	$15,1 \pm 2,4^{b}$	$7 \pm 1,5^{a/b}$	$16,9 \pm 6,4^{a}$	$19,9 \pm 0,2^{b}$
2010	BS	24	$0,25 \pm 0,006^{a}$	26	$1,99 \pm 0,11^{e}$	$22,4 \pm 1,9^{c}$	$6 \pm 2,4^{a/b}$	$15 \pm 6,3^{a}$	$19,4 \pm 0,2^{b/c}$
	AC	24	$0,19 \pm 0,004^{c}$	22	$1,75 \pm 0,08^{d/e}$	$22,9 \pm 4,2^{c}$	$6 \pm 2,8^{a/b}$	$17,1 \pm 6,8^{a}$	$19,4 \pm 0,08^{c}$
	TB	24	$0,21 \pm 0,009^{b/c}$	29	$2,02 \pm 0,11^{e}$	$19,9 \pm 3,3^{b/c}$	$6,5 \pm 2^{a/b}$	$19,1 \pm 8,7^{a}$	$19,4 \pm 0,09^{c}$
	S	24	$0,20 \pm 0,004^{b/c}$	30	$1,93 \pm 0,15^{e}$	$20,5 \pm 2,8^{b/c}$	$6,3 \pm 1,8^{a/b}$	$17,9 \pm 8,5^{a}$	$19,4 \pm 0,09^{c}$
2011	BS	24	$0,23 \pm 0,004^{b/d}$	19	$1,43 \pm 0,07^{d}$	$20,4 \pm 3,9^{b/c}$	$5,4 \pm 1,7^{a/b}$	$16 \pm 5,9^{a}$	$19,1 \pm 0,3^{c}$
	AC	24	$0,20 \pm 0,005^{b/c}$	25	$1,15 \pm 0,05^{f}$	$20,5 \pm 4,6^{b/c}$	$4,8 \pm 2,2^{a/b}$	$12,7 \pm 5^{a}$	$19,1 \pm 0,3^{c}$
	TB	24	$0,25 \pm 0,005^{a/d}$	8	$1,39 \pm 0,13^{d}$	$20 \pm 2,5^{b/c}$	$4,9 \pm 1,3^{a/b}$	$14,8 \pm 4,6^{a}$	$19,3 \pm 0,4^{c}$
	S	24	$0,24 \pm 0,005^{d}$	20	$1,41 \pm 0,06^{d}$	$20,8 \pm 3,6^{b/c}$	$4,8 \pm 1,1^{b}$	$16,7 \pm 6,3^{a}$	$19,4 \pm 0,2^{c}$

a,b,c,d,e,f: Numbers not bearing the same subscript letter are significantly different ($p < 0,05$)

TASK 4

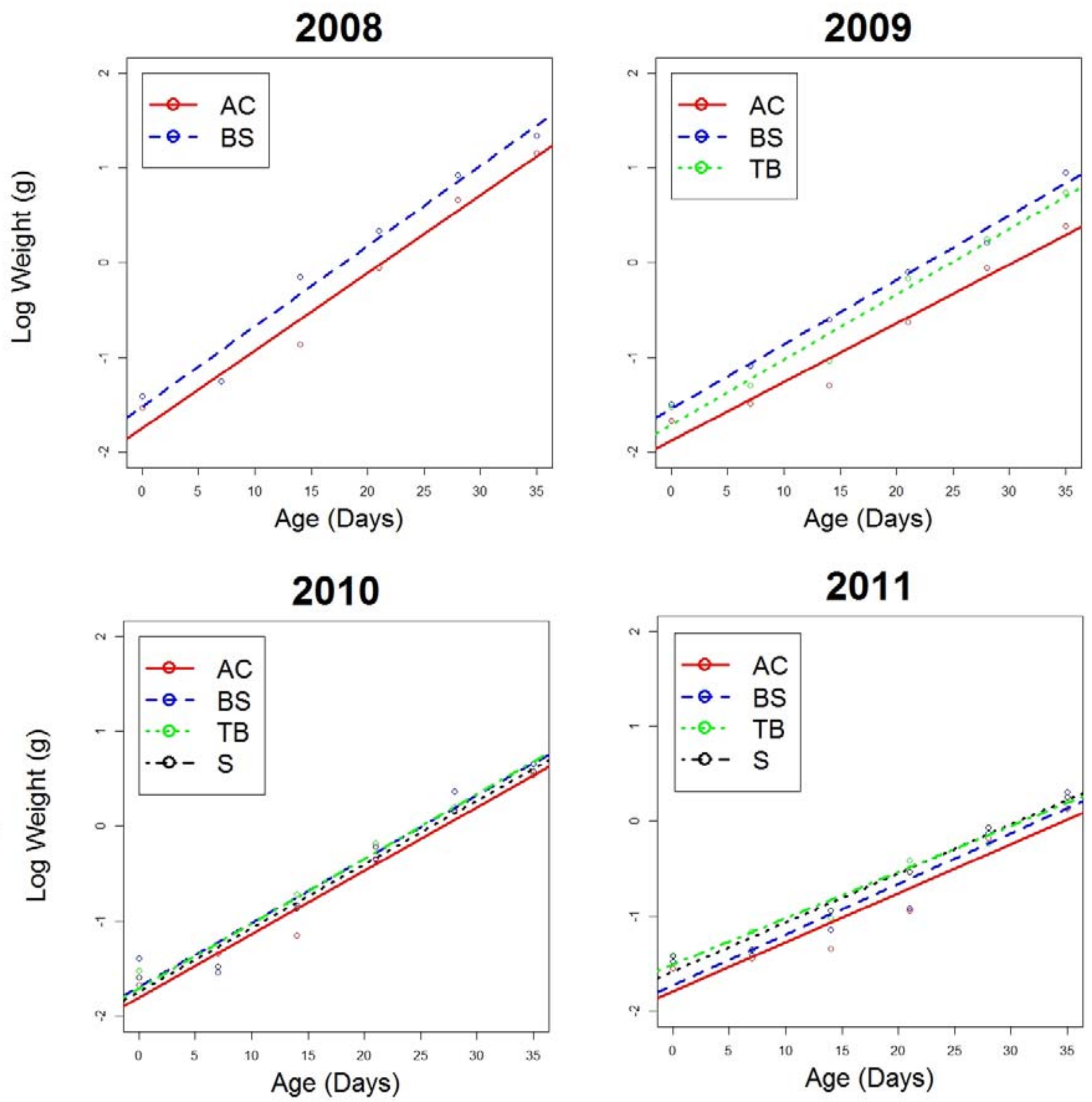

Figure 2 : Linear models fitted to *Sepia officinalis* early juvenile experimental growth from different spawning sites of the English Channel over four years. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK).

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Table 4: Coastal water temperature (°C) and salinity (‰) (mean and SD) for each of the studied French site between April and July from 2008 till 2011. Agon Coutainville and Bay of Seine are respectively the western and eastern studied sites of the Cotentin peninsula of the French English Channel coast.

Site	Year	Temperature (°C) April	Temperature (°C) May	Temperature (°C) June	Temperature (°C) July	Salinity (‰) April	Salinity (‰) May	Salinity (‰) June	Salinity (‰) July
Agon Coutainville (West coast)	2008	10,7 ± 1,29	14,9 ± 0,9	17,5 ± 1	18,9 ± 0,9	33,7 ± 0,01	33,9 ± 0,2	33,9 ± 0,2	34,6 ± 0,06
	2009	11,6 ± 0,9	14,2 ± 1	17,5 ± 1,2	19,3 ± 0,7	34,9 ± 0,5	34,6 ± 0,2	34,6 ± 0,8	34,8 ± 0,06
	2010	10,7 ± 1,4	13,4 ± 1,6	16,9 ± 1,2	20 ± 0,5				
	2011	13 ± 1,1	15,3 ± 0,5	16,7 ± 0,8	18,2 ± 0,7				
Mean/month		**11,5 ± 1,1**	**14,5 ± 0,8**	**17,2 ± 0,4**	**19,1 ± 0,8**	**34,3 ± 0,8**	**34,3 ± 0,5**	**34,3 ± 0,5**	**34,7 ± 0,1**
Bay of Seine (East coast)	2008	10,3 ± 2,2	13,3 ± 2	15,6 ± 0,9	17,5 ± 0,7	32 ± 1	32,4 ± 0,4	32,5 ± 0,1	33,1 ± 0,7
	2009	10,5 ± 1,5	13,3 ± 1,9	16,5 ± 2,3	18,2 ± 0,1	32 ± 0,2	32,5 ± 0,3	32,4 ± 0,3	33 ± 0,4
	2010	10,4 ± 1,1	12,6 ± 1,9	17,3 ± 0,8	19,2 ± 1,5	32,4 ± 0,6	33 ± 0,5	33,1 ± 0,3	33 ± 0,09
	2011	10,6 ± 1,2	13,6 ± 1,3	16,2 ± 0,4	18,5 ± 1,1	32,6 ± 0,3	32,6 ± 0,9	32,5 ± 0,7	32,8 ± 0,06
Mean/month		**10,4 ± 0,1**	**13,2 ± 0,4**	**16,4 ± 0,7**	**18,3 ± 0,7**	**32,2 ± 0,3**	**32,6 ± 0,3**	**32,6 ± 0,3**	**33 ± 0,1**

Environmental data parameters collected from SMEL and SOMLIT

Table 5 : Monthly sea temperature (mean and SD) of five coastal English stations from the English Channel between April and July.

Site Time series	Shoreham 1966 - 1999	Fawley PS 1984 - 2004	Bournemouth 1970 - 2004	Weymouth 1966 - 2004	Plymouth 1957 - 1993
April	9,4 ± 1,2	9,3 ± 1,1	9,1 ± 1,4	8,8 ± 0,7	9,3 ± 1
May	12,9 ± 1,2	12,9 ± 1,5	11,9 ± 1,2	11 ± 0,9	11,2 ± 1
June	15,7 ± 1,3	16,5 ± 1,3	14,9 ± 1,3	13,3 ± 1	13,7 ± 1
July	18 ± 1,5	19 ± 1	17,5 ± 1,4	15,8 ± 0,7	15,3 ± 0,8

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TASK 4

"This paper is in preparation to be submitted in a journal – All figures are seperated from the text and are at the end of this paper"

1- Antiprotease, lysozyme and phenoloxidase activity in the cuttlefish *Sepia officinalis* L.

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Introduction

Most marine organisms inhabit environments that are relatively rich in bacteria and other microorganisms. In this environment, seawater may function as a medium for both transport and growth of microbes, as distinct from air, which has been thought only to function as a transport medium. Marine organisms share thus an ecosystem with microorganisms responsible for their disease and the principal function of their immune system is to mediate interactions of the host with the microbiotic world (Hansen and Olafsen, 1999, McFall-Ngai et al., 2010).This finding helps to understand the need for marine species to have a powerful immune system. In invertebrates, innate cellular and humoral mechanisms are the major lines defence limiting microbial invasions and mediating the clearance or killing of the invading microbes from blood and tissues. Lysozyme-like family, protease inhibitors and phenoloxidase system are constituents of this humoral line defence (Hikima et al., 2003; Armstrong, 2006). Lysozyme, an antimicrobial protein, is an enzyme that cleaves the 1,4-β-glycosidic linkage between N-acetylglucosamine and N-acetylmuramic acid of Gram-positive bacterial cell walls (Fiolka et al., 2012). Most organisms have the genetic capacity to produce multiple lysozymes of different types, and it is presumed that these may have complementary or even different functions (Callewaert and Michiels, 2010). As for the phenoloxidases (POs), a family of copper proteins, which catalysed the first step of melanin production i.e. the oxidation or hydroxylization of phenols to produce quinones. Melanisation is an important immune mechanism among many invertebrate taxa, implied in non-self recognition, toxic compounds production against pathogens, wound healing process, and stimulating cellular defences factor synthesis (Luna-González et al., 2003; Siddiqui et al., 2006; Hellio et al., 2007; Lacoue-Labarthe et al., 2009; Cerenius et al., 2008; 2010; Luna-Acosta et al., 2010, 2011a, 2011b). Specifically involved in invertebrate non-self recognition and already described in arthropods (Cerenius et Söderhall, 2004, Masuda et al., 2012), a zymogenic form of POs called prophenoloxidase (proPO) began to be studied in molluscs. This system consists in the activation of the inactive proenzyme (proPO) to PO by proteolytic cleavage via an endogenous activator or exogenous agents (Hellio et al., 2007) such as lipopolysaccharides or peptidoglycans from bacteria (Cerenius et al., 2008). Finally, antiproteases or protease inhibitors aid in the defence of various organisms by regulating and inhibiting the activities of potentially destructive proteases, they regulate protease activities involved in the coagulation of haemolymph, the activation of proPO system and in the synthesis of cytokines and antimicrobial peptides (Xue et al., 2009). In marine invertebrates, they have been mainly studied in haemolymph and haemocytes of bivalve molluscs (*Crassostrea gigas* (Luna-González et al., 2003 ; Hellio et al., 2007 ; Thomas-Guyon et al., 2009)*, C. virginica* (Jordan et al., 2005)*, Mytillus edulis* (Coles & Pipe, 1994), *M. galloprovincialis* (Carballal et al., 1997), *Nodipecten subnodosus* (Luna-Gonzalez et al., 2003)*, Perna viridis* (Asokan et al. 1997), *Ruditapes decussatus* (Lopez et al., 1997), *Saccostrea glomerata* (Peters and Raftos, 2003).

In cephalopod class, which presents the particularity (in mollusc phylum) to have a closed circulatory system, defence against pathogens involves both humoral and cellular responses. The cellular response is mediated by haemocytes in the haemolymph and tissues, and humoral activity is found in haemocyte, haemolymph and tissues of cephalopods. (Malham et al., 1998; Malham et al., 2002, Saurabh and Sahoo, 2008; Cong et al., 2009). In *Sepia officinalis*, antiproteases were found in the cuttlefish plasma (Armstrong, 2006) and ProPO-PO system was studied during embryonic development (Lacoue-Labarthe et al., 2009). Furthermore, POs studies focused on tyrosinase activity found in the ink gland which is implied in melanogenesis (Palumbo et al., 1997; Naraoka et al., 2003; Fiore et al., 2004; Derby, 2007). Even if scientific interest is growing toward understanding immune enzyme functioning, antiproteases, lysozyme-like family and ProPO-PO system remain poorly studied in cephalopods. The purpose of this work is thus to describe the distribution of these enzymes in cuttlefish body tissues in order to localise their maximum activities and to better understand their role in *S. officinalis* immune system.

Material and methods

Animals and tissue samples

Cuttlefish (*Sepia officinalis*, n = 10, DML = 21.3 ± 3.5 cm, Weight = 1.03 ± 0.38 Kg) were obtained from the marine station traps in front of the marine station at Luc-sur-Mer. The animals were brought into an aquarium in the marine station and were maintained at 15°C for 48h before experimentation.

Animals are anesthetized in a 2 % solution of ethanol in seawater. Once anesthetized, animals are dissected and organs (Figure 1) are sampled and placed afterwards in ice. At the end of the dissection, organs are transferred and kept at -80°C until analyse.

Humoral immune assays

Enzymes extraction

Tissue samples were weighed and grounded in liquid nitrogen. Once we obtained a fine powder, sample is homogenised in a known amount (0.1 g to 10 ml) of Tris buffer pH 8 (10 mM Tris-HCl and 150 mM NaCl) for lysozyme-like and antiprotease-like assays or Tris buffer pH 7 (0.1 M Tris-HCl, 0.45 M NaCl, 10 mM $CaCl_2$ and 26 mM $MgCl_2$) for phenoloxidase assay. The mixture was stored at 4°C for 1 hour, and then centrifuged for 10 minutes at 4°C at 12000 rpm. The supernatant contains the Tris soluble protein used for the enzymes assays. The protein content was assayed according to the Bradford method (1976) using BSA as standard.

Enzymes assay

Antiprotease assay

Antiprotease activity was measured by transferring 10 µl of sample in triplicate in sterile 96-well flat bottom plates (BD, USA). and 5 µl of trypsin (100 µg / ml of 0.05 M Tris buffered -TBS pH 8) with mixing at room temperature for 5 minutes (Thompson et al., 1995). In parallel, intrinsic trypsin activity is measured by replacing 5 µl of trypsin by TBS. 100 µl of Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA, Sigma) substrate solution (10.4 mg/ 20 ml de Tris 0.01 M, pH 7.4) was added to all wells and incubation lasted for 15 minutes at room temperature. A positive control is used without sample. Absorbance is read at 405 nm and antiprotease activity is expressed as percentage of trypsin sample inhibition compared to the positive control. This activity is divided afterwards by the protein sample concentration (µg protein in 10 µl of sample) and the final activity is expressed as antiprotease activity / µg of protein antiprotease activity / µg of protein.

Phenoloxidase assay

Phenoloxidase-like (PO-like) activity was measured spectrophotometrically by recording the formation of o-quinones, as described by Luna-Acosta et al. (2010). PO assays were conducted in 96-well flat bottom plates (BD, USA). 3,4-Dihydroxy-DL-phenylalanine (DL-DOPA, $C_9H_{11}NO_4$, Sigma) was used as substrate, at final concentration of 10 mM (Luna-Acosta et al., 2011c). DL-DOPA was prepared just before being used in Tris buffer (0.1 M Tris-HCl, 0.45 M NaCl, 26 mM $MgCl_2$ and 10 mM $CaCl_2$) adjusted to pH 7. At 25°C, 10 µl of sample was incubated with 80 µl of DL-DOPA and 50 µl of Tris buffer. Several control wells were systematically use : 'buffer control' containing only buffer, 'sample control' containing only sample and buffer, and 'non-enzymatic control' containing only substrate and buffer, always in a final volume reaction of 140 µl. Immediately after DL-DOPA addition, PO-like activity was monitored during 5h by using Mithras LB 940 luminometer (Berthold, Thoiry, France) and by following the increase of absorbance between 3 and 5h at 490 nm.

For enzymatic oxidation, the results were systematically corrected for non-enzymatic autoxidation of the substrate. One enzyme unit (1 U) corresponded to an increase of 0.001 in the absorbance per min, per mg of protein at 25°C.

Phenoloxidase inhibition/activation assays

Tropolone and trypsin TPCK was used as PO inhibitor and activator at final concentrations of 10 mM and 1 $g.l^{-1}$, respectively (Lacoue-Labarthe et al., 2009). PO inhibition/activation assays were performed as described, by Luna-Acosta et al., 2010a, by preincubating 10 µl of PO inhibitor/activator (prepared in Tris buffer) with 10 µl of sample for 20 minutes, at 25°C. Then, PO assay was carried out with DL-DOPA at final concentration of 10 mM. Each organ was tested in triplicate wells and average rates were calculated. Enzymatic oxidation (in the presence of inhibitor or activator) was systematically corrected for non-enzymatic autoxidation of the substrate (in the presence of PO inhibitor or PO activator).

Lysozyme assay

Lysozyme activity was quantified according to Malham et al. (1998). Fifty µl of hen egg white lysozyme (Sigma) (85 µg/ml of Tris buffer pH 8) standard was serially diluted in triplicate in sterile 96-well flat bottom plates (BD, USA). Fifty µl of each sample was added in triplicate to the 96 well plates as well as fifty µl of buffer, as blanks. One hundred and fifty µl of the substrate, freeze dried, *Micrococcus lysodeikticus* (Sigma) (0.075 g / 100 ml of phosphate/citrate buffer pH 5.8 ($Na_2HPO_4.2H_2O$, 4.45 g / 250 ml distilled H_2O; citric acid, 2.1 g / 100 ml distilled H_2O; NaCl, 0.09 g / 100 ml buffer)), was added to each well. The reductions in turbidity in the wells were read on a multiscan spectrophotometer for 5 minutes at 30 seconds intervals at 450 nm using negative kinetics. Lysozyme concentrations were calculated from the standard curve (µg equivalent of lysozyme from hen egg white / ml). This concentration was divided afterwards by the protein concentration in the sample. Final lysozyme activity is thus expressed as lysozyme activity / mg of protein.

Analysis

Organs assays were repeated 10 times to have a final result of 10 assays/tissue. Results are given as mean ± standard error (SE) for adult organs. Values were tested for normality (Shapiro test) and homogeneity of variances (Bartlett test). Normal data (PO and Pro-PO) were compared with an ANOVA followed by a Tukey's test when significant differences ($p < 0.05$) were found. For non normal values (Lysozyme-like and Antiproteases), values were transformed (1/(value+1)) for normality and homogeneity of variances and these transformed values were used afterwards for ANOVA and Tukey's analysis. R software was used for statistics and graphics.

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Results

Antiprotease Assay

Antiprotease activity was detected in all tissue samples of *Sepia officinalis* (Figure 2). The digestive system tissues presented though significantly higher activities ($p < 0.05$) than other organs. The digestive gland had the highest protease inhibitor activity followed by the digestive gland appendage, the stomach and the cecum. The posterior salivary gland had also antiprotease activity that was significantly lower ($p > 0.05$) than what was found in the digestive gland and its appendage but significantly higher than in the optic lobes. As for the circulatory system organs (gills, branchial heart and its appendage, central heart and white body) along with the mantle and the skin, antiprotease activity was significantly lower ($p < 0.05$) than activities found in the digestive system.

Lysozyme Assay

All tissue samples of cuttlefish *Sepia officinalis* L. indicated variable amounts of lysozyme activity (µg equivalent of hen egg white / mg of protein) (Figure 3). The highest activity was detected in the white body, the haemocytes synthesis organ, which was significantly ($p < 0.05$) higher than lysozyme activity in the gills, digestive gland and its appendage. Organs of the circulatory system (branchial heart, branchial heart appendage and central heart) together with the optic lobes and the posterior salivary gland presented all lysozyme activities. As for the digestive system, the stomach had a mean lysozyme activity of 1.39 ± 0.64 µg equivalent of lysozyme from hen egg white / mg of protein, while significantly ($p < 0.05$) lower activities were found in the cecum, digestive gland and in the digestive gland appendage compared to the branchial heart appendage, the central heart or the white body.

Phenoloxidase Assay

At the exception of the mantle, PO-like activity was detected in all sampled cuttlefish tissues. Tropolone inhibit totally (or almost totally) PO-like activity in all tissues (data not shown). When an inhibited PO-like activity was measured, this value was systematically subtracted from proPO and PO-like activity. Highest value of PO-like activity was measured in the skin, following by digestive gland and branchial heart. A pool of organs presents similar activities of about 5 $U.min^{-1}.prot\ mg^{-1}$ (post salivary gland, stomach, digestive gland appendage, white body, central heart, branchial heart appendage and gills). The lowest activities were detected in optic lobe and cecum (Figure 4). Trypsin TPCK seems stimulate PO-like activity in most of the organs except in the skin and the digestive gland. The increasing of initial PO-like activity is significant in seven tissues i.e. in digestive gland appendage, optic lobe, white body, central heart, branchial heart, branchial heart appendage and gills. Highest activated PO-like activity was measured in gills. Then, digestive gland appendage, central heart, branchial heart and branchial heart appendage present similar activated PO-like activity. Finally, the lowest activated PO-like activity was measured in and white body and optic lobe. After comparison between initial PO-like activities and activated PO-like activities, it appears that highest PO-like activity increases were localized in central heart and gills, and lowest in branchial heart. All these data are described in Table 1.

Discussion

In general, antiprotease, lysozyme and phenoloxidase activities are measured in serum or haemolymph and also on cultured blood cells such as haemocytes (Thompson et al., 1995 ; Malham et al., 1998 ; Gollas-Galvan et al., 1999 ; Siddiqui et al., 2006). Between these authors, Malham et al. (1998) worked on determining whether lysozyme and antiprotease activities existed also in various tissues of *Eledone cirrhosa* and the effect of *in vivo* infection of live bacteria on these activities. The purpose of this study was also to show the presence of these activities in different organs in *Sepia officinalis* in order to better understand their distribution and their potential role.

Antiprotease

Protease inhibitor activity was mainly found in the digestive system organs (digestive gland, stomach, cecum and digestive gland appendage) with the highest activities being found in the digestive gland and its appendage. The posterior salivary gland had significantly ($p<0.05$) lower activity but still clearly present. This detected activity could well be involved in the regulation of the powerful proteases present in the saliva (Malham et al., 1998). As for the digestive organs, several authors have described the presence of protease inhibitors in the hepatopancreas and the visceral tissues of squid (Sofina et al., 1988; Kishimura et al., 2001). The protease inhibitors are used as the major form of control once a protease has been activated and in cephalopods, proteolitic enzymes plays a major role in the digestive system. (Jellouli et al., 2009; Balti et al., 2012). In this study, the ability of cuttlefish antiproteases to inhibit trypsin activity was made as described by several authors (Thompson et al. (1995), Malham et al. (1998), Kishimura et al. (2001)). In *Sepia*, there is a strong proteolytic activity in the digestive system among which are the serine proteases. Furthermore, these activities are highly concentrated in the digestive gland and its appendage (Boucaud-Camou, 1974) and this high activity implies the presence of protease regulator (protease inhibitors). The presence of trypsin inhibitor activity in the digestive organs of *Sepia* seems thus quite normal. Results obtained here are consistent with the descriptions of this author. Indeed, the highest antiprotease activity was found in the digestive gland ($p <0.05$) followed by the digestive gland appendage.

In parallel, antiprotease plays an important role in immunity. It aids in the defence of various organisms by regulating and inhibiting the activities of potentially destructive proteases (Malham et al., 1998). This study showed the presence of the trypsin inhibitor activities in the gills, the branchial heart and its appendage and in the central heart. These organs play an important role in the circulatory system. The gills are responsible of the haemolymph oxygenation before its circulation in the other organs and the hearts ensure distribution of haemolymph in the entire body. Beuerlein et al. (1998, 2002) showed the importance of the branchial hearts in the circulatory system of *Sepia officinalis*. These authors proposed that this organ played an important role in the defence and detoxification of haemolymph and prevented thus the contamination of the whole organism. Activity found in the branchial hearts with their appendages and in the central heart may reflect a regulation, in those organs, of the potentially destructive proteases and thus preventing the entire body contamination. Antiprotease activity was also found in the mantle which is the first barrier with the surrounding environment. This activity could be explained thus by the necessity of having immune enzymes against pathogens when injuries occur.

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Phenoloxidase

To our knowledge, this study details for the first time POs and proPO distribution in the major part of cephalopod organs. Highest PO-like activity found in the skin of *S. officinalis* highlighted two roles that phenoloxidases probably play specifically in this tissue i.e. as first defence barrier against exogen invaders and also in the wound healing process (Asokan et al., 1997 ; Söderhäll & Cerenius 1998, Cerenius et al. 2008). However, it is surprising that, contrarly to litterature data, activity measured in the skin is only PO-like activity and not activated PO-like activity. Moreover, our assays allow to think that POs synthesis is local i.e. in the skin, because of the lack of PO-like and activated PO-like activities in the mantle which is the only body part in contact with the skin. This underlines a constant and direct secretion of POs in this tissue able to resist continually to foreign invaders and quickly react to wounds. Second highest PO-like activity has been found in the digestive gland. Recent studies (Luna-Acosta et al., 2011a ; 2011b) described a laccase PO-type activity in the bivalve mollusc *C. gigas* digestive gland without explanations about its potential local role. The important detoxification role of cephalopod digestive gland and the potential various reactions induce by compounds produced from melanisation cascade (Cerenius et al., 2010) underline the potential detoxification role of PO enzymes in *S. officinalis* in this tissue, but further study tissue-specific will have to be undertake to validate this hypothesis. Like in the skin, we did not measure activated PO-like activity in digestive gland. However, a high activated PO-like activity has been registered in digestive gland appendage (DGA), highlighted a potential reservoir function for digestive gland. Indeed, digestive gland hold and synthesis high levels of powerfull proteases (Boucaud-Camou, 1974), which can potentially activate proPO and cause unwanted deleterious effects on host by toxic melanisation intermediates (Cerenius et al., 2008). This reservoir function could explain this high proPO content in digestive gland appendage which have no already described other role that food absorption. Other tissues presented quite constant PO-like activity (i.e. PSG, Stc, DGA, WB, CH, BH, BHA, and Gill), with lowest activities measured in cecum and optic lobe. PO-like activities measured in immune implied organs (i.e. WB, CH, BH, BHA, and Gill) highlighted significance of phenoloxidases in immunity of *S. officinalis*. It is more surprising to find PO-like activity in digestive implied organs (i.e. PSG, Stc and DGA). However, Bailey & Worboys (1960) describe in lamellibranch bivalve molluscs, a phenoloxidase locate in the cristallin style, and Blaschko and Hawkins (1952) reported strong amine oxidase enzimatic activity in posterior salivary gland and digestive gland of both cephalopods *Octopus vulgaris* and *Sepia officinalis*, but without clear role. Even if cephalopods do not possess cristallin style or equivalent in their digestive gland, phenoloxidases could be conserved and play role in marine molluscs digestive system directly or indirectly with compounds produced from melanisation cascade (Cerenius et al., 2010).

proPO

Concerning activated PO-like activities, significantly increase have been measured in immune implied organs (at the exception of DGA and OL), underlined the already described non-self recognition role of the proPO system (Söderhäll and Cerenius, 1998). The highest activated PO-like activity was in CH and Gill. This elevated content of the proPO zymogenic form is probably linked to the position of these organs e.g. at the center of the arterial system and as first circulatory organe next gill for CH, and as main entrance way for foreign organisms and toxic compounds for Gill. These localizations highlighted proPO involvement in non-self recognition mainly described in arthropods (Söderhall and Cerenius, 1998; Luna-González et al., 2003; Cerenius and Söderhall, 2004). Activated PO-like activity value found in WB can be explained by its well describe role in circulant-cell (haemocyte) synthesis (Claes, 1996). Concerning the activated PO-like activity found in the OL, to our knowledge, no immune role of OL have been already highlited, but haemocyte infiltration are often described (personal observation) in the outerlayer of this organ. These data underline the potential income of proPO and PO in this organ to protect it from infection in view of its essential role in the nervous system and more particularly in the locomotor control (Chichery and Chanelet, 1976). Similar coupling activities than those described above with DG and DGA is observed in BH and BHA. The BH, implied in detoxification process (Beuerlein et al., 2002), presents a high level of PO-like activity (the third measured in the studied organs) and the lowest proPO induce activity (significantly increased). Whereas BHA, which have an haemolymph filter role (Malham et al., 1998), presents a medium level of PO-like activity and a level of proPO induce activity similar to DGA, OL and WB. This highlights the potential reservoir function of the BHA to BH, ready to be activated and release in circulatory system in bacterial infection case for example. To our knowledge, this is the first time that organ location of phenoloxidases could play a role in proPO-activation regulation process. These data highlight the tissue–specific heterogeneity of PO activities as already describe in *C. gigas* by Luna-Acosta *et al.* (2011), and the need to increase coupled proPO-PO studies in invertebrates. In the present study, no phenoloxidase family differentiation has been done (i.e. tyrosinases, laccases and catecholases) because of the lack of knowledge on function and repartition of phenoloxidases in cephalopods. Further studies, could differentiate this three types of POs distribution in cuttlefish organs in order to better understand melanisation process and role in cephalopods and more widely in molluscs.

Lysozyme

Cephalopods possess a closed circulatory system with a single type of haemocyte predominante in their haemolymph. These circulating blood cells are formed in the white bodies, a pair of multilobed organs in the optic sinus, where different development stages within this cell line are found (Claes, 1996). Lysozyme-like activity presented an important variability in the various organs but was mainly found in the white body and the adjacent optic lobes. In *Sepia officinalis*, these organs are richly vascularised and especially the optic glands bonded to the optic lobes where lysosomal inclusions were described by Mangold and Frösch (1977). The important vascularisation of these organs may partly explain the presence of lysozyme activity in the optic lobes which could be due to the presence of circulating haemocytes in the capillary system, but this activity may also be due to the presence of lysosomal inclusions that may contain lysozyme (idem PO). Claes M.F. (1996) described the cyto-morphological aspects of the white body cells. This author observed the presence of lysosome-like vesicles in the four categories of the white body cells with the highest density of lysosomal inclusions in the haemocyte ones. The ultrastructural characteristics of the white body described by this author indicate that these cells are endocytotically active. Our results on lysozyme activity in the white body and in the optic lobes are consistent with the descriptions of these authors. These activities are thus found in the organs containing haemocytes or lysosomal inclusions. Assays were subsequently conducted on the organs of the circulatory system. Lysozyme-like activities were found in the central heart, the branchial hearts and their appendages. These activities reflected the lysozyme activity due to the presence of circulating haemocytes in the haemolymph. Furthermore, as for the antiproteases, the presence of lysozyme-like activity in the branchial heart and its appendage along with the central heart reflected the importance of these organs in purifying haemolymph before its distribution in the entire body. Afterwards, lysozyme-like activity found in the skin and mantle could be due to the vascularisation present in the mantle and beneath the skin (Mangold et al., 1989). As for the protease inhibitors, the lysozyme activity in the mantle and in the skin may reflect its action in neutralizing pathogens after injuries or bacterial infection.

In the digestive system, activities were detected mainly in the stomach and in the posterior salivary gland and significantly ($p < 0.05$) lower in the cecum, digestive gland and the digestive gland appendage (Figure 3). The presence of the lysozyme activity in the anterior parts of the digestive system in *Sepia* is nevertheless interesting. Activity in the cuttlefish posterior salivary gland could probably indicate the presence of lysozyme in the oral cavity as a first barrier for food microbes. As for lysozyme-like activity in the stomach, this is a first time description in cephalopods. In some animals, lysozyme has been recruited as a digestive enzyme, enabling them to use bacteria as a food source (Callewaert and Michiels, 2010). This evolution of lysozyme role is described as well in vertebrates as in invertebrates. In ruminants, for example, one of the factors contributing to the success of their development is the acquisition of digestive lysozymes that contribute to the digestion of bacteria in the stomach (Dobson et al., 1984). As discussed for the ruminants, the same mechanism of gene duplication possibly allowed *Drosophila* to recruit lysozyme for a digestive function (Callewaert and Michiels, 2010). Furthermore, the digestive function of lysozyme has been described in bivalve molluscs. To benefit from large amounts of bacteria ingested as a food source, bivalve molluscs need a digestive system capable of breaking down prokaryotic cells. Lysozyme activities detected in the digestive systems of many bivalve molluscs have triggered speculation about a possible digestive role for these lysozymes (Nilsen et al., 1999; Bachali et al., 2002; Takeshita et al., 2004; Matsumoto et al., 2006; Itoh and Takahashi 2007; Xue et al. 2010). Many invertebrates from different phyla have thus lysozymes that show adaptations for a digestive profile. But, to our knowledge, this adaptation has never been described in mollusc cephalopods. Results obtained here show the presence of lysozyme activity mainly in the posterior salivary glands and in the stomach of *Sepia officinalis* L. without being able to confirm their specificity. These preliminary results may suggest the presence of digestive lysozymes in this species of cephalopod. In addition, the food accumulation in early digestive stages in Sepioidae takes place in the stomach that can expand considerably and thus accumulate food (Mangold and Bidder, 1989). It is conceivable that this accumulation in the stomach, as described in ruminants, could be prone to severe degradation of the food bacteria by digestive lysozyme which will be absorbed afterwards. Bacteria may play an important role for marine animals by furnishing cell substances or micronutrients such as essential fatty acids, vitamins, minerals or even enzymes (Hansen and Olafsen, 1999). To confirm the adaptation of these enzymes, as described in other species, this will require the study of the lysozyme expression in the stomach along with the characterization of lysozymes present in the cuttlefish organs. Lysozymes contributing to antibacterial defence are generally expressed in tissues and buidy fluids exposed to the environment or involved in bacterial clearance, while high expression levels of lysozyme in the stomach or gut rather points to a digestive function (Callewaert and Michiels, 2010).

Conclusion and perspectives

In conclusion, antiprotease, lysozyme and phenoloxidase were found in the cuttlefish body tissues with different distributions. Assay of protease inhibitors showed that this activity was mainly detected in the digestive organs and primarily in the digestive gland where strong synthesis of digestive trypsin is found. Regarding the lysozyme assay, this activity was mainly found in the circulatory system organs playing an important role in the immune system. The lysozyme activity found in these organs is probably due to the presence of haemocytes and lysosomal inclusions in these cells. In parallel, lysozyme activity found in the stomach and the posterior salivary gland led us to suggest a possible adaptation of this enzyme into a digestive lysozyme as described in several phyla. This adaptation has never been described in cephalopods and its confirmation will require a characterization of the lysozyme and its expression in cuttlefish organs.

Finally, the assays we made led us to better know the distribution of these enzymes in the body tissues of *Sepia officinalis*. This study was a basic approach of the cuttlefish immune system and will allow us afterwards to work on the adaptation of these enzymes assay on juvenile. Indeed, studies on the juvenile cuttlefish early life stages immunity are still scarce.

Acknowledgments

This work was supported by European funding as part of the CRESH INTERREG IV-A project and by the regional fundings of Basse-Normandie.

TASK 4

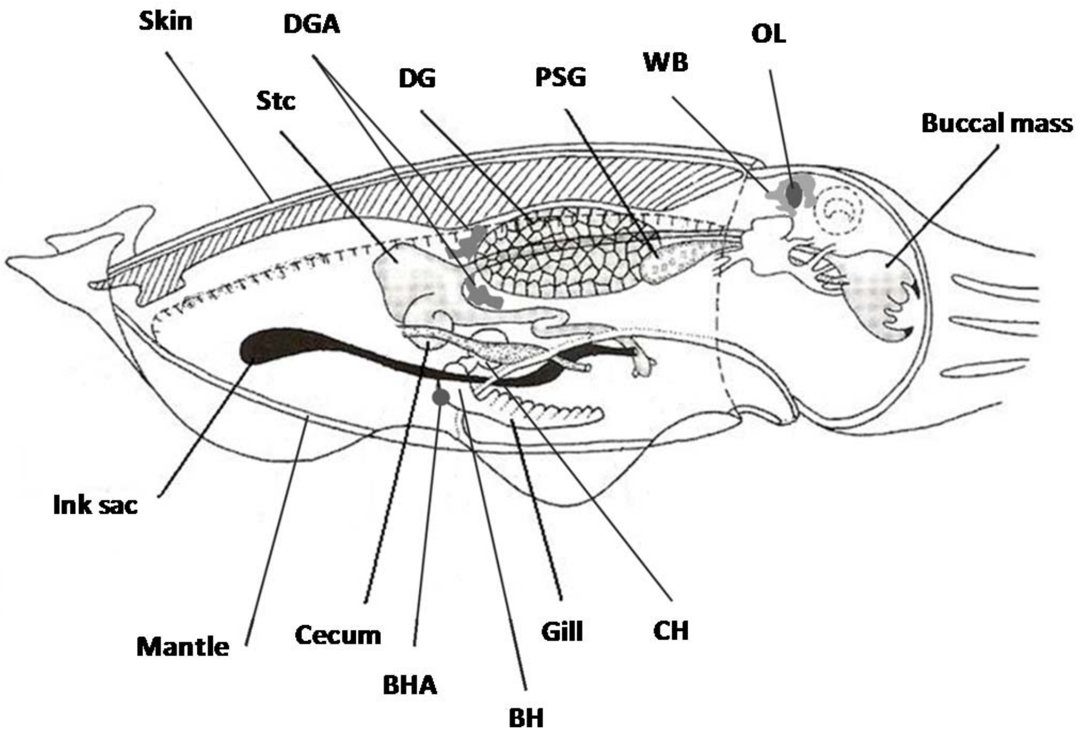

Figure 3: Internal anatomy of cuttlefish, *Sepia officinalis* L., with studied organs. BH: branchial heart, BHA: branchial heart appendage, CH: central heart, DG: digestive gland, DGA: digestive gland appendage, OL: optic lobe, PSG: posterior salivary gland, Stc: stomach, WB: white body.

TASK 4

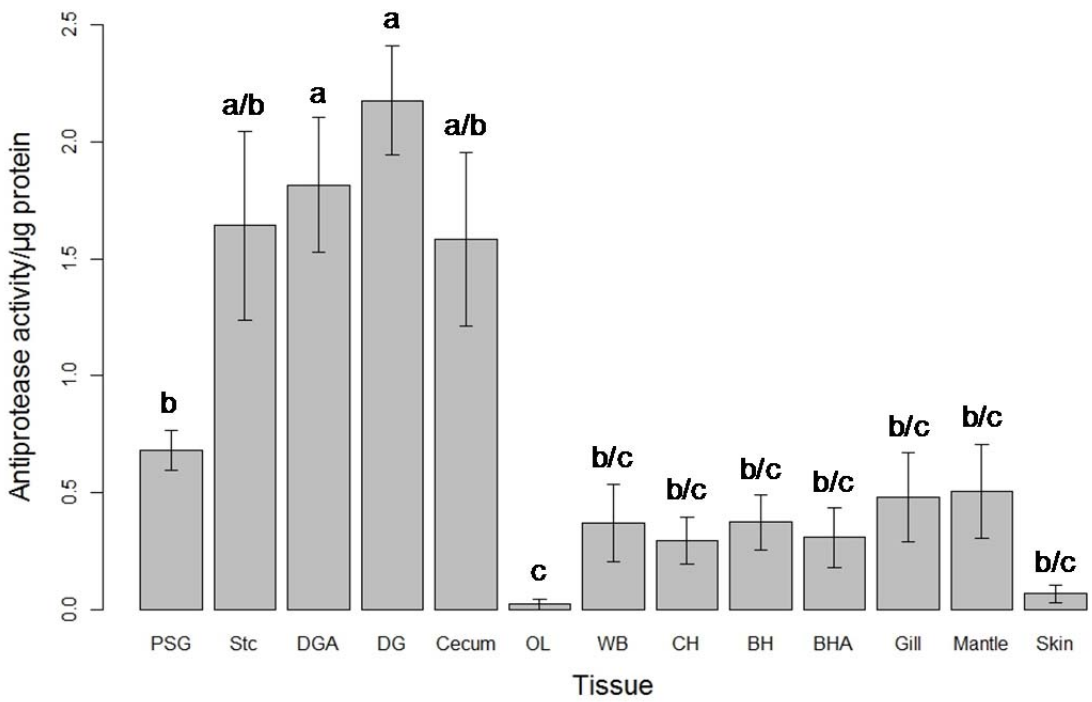

Figure 4: Antiprotease activity (% trypsin inhibition / µg of protein) in various tissues of cuttlefish *Sepia officinalis* L. (n = 10). BH: branchial heart, BHA: branchial heart appendage, CH: central heart, DG: digestive gland, DGA: digestive gland appendage, OL: optic lobes, PSG: posterior salivary gland, Stc: stomach, WB: white body. The bars represent the means (± SE) of 10 animals. Graphs not bearing the same subscript letter are significantly different (p<0.05).

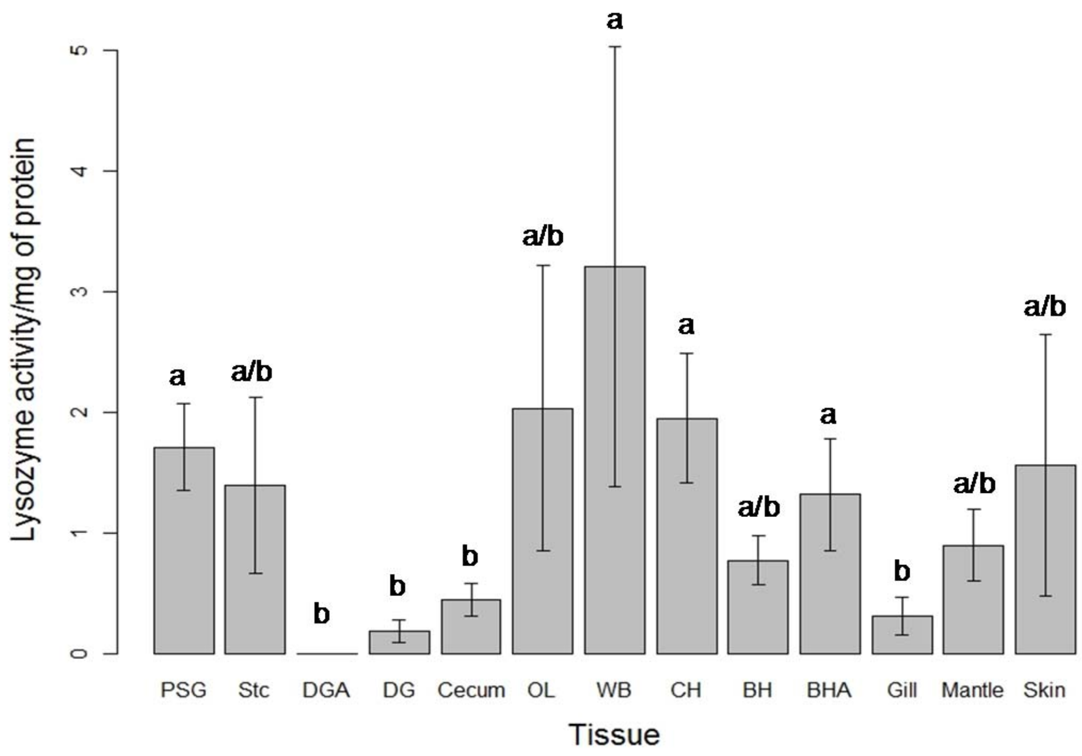

Figure 5: Lysozyme activity in various tissues of cuttlefish *Sepia officinalis* L. (n = 10). BH: branchial heart, BHA: branchial heart appendage, CH: central heart, DG: digestive gland, DGA: digestive gland appendage, OL: optic lobes, PSG: posterior salivary gland, Stc: stomach, WB: white body. The bars represent the means (±SE) of 10 animals. Graphs not bearing the same subscript letter are significantly different ($p<0.05$).

TASK 4

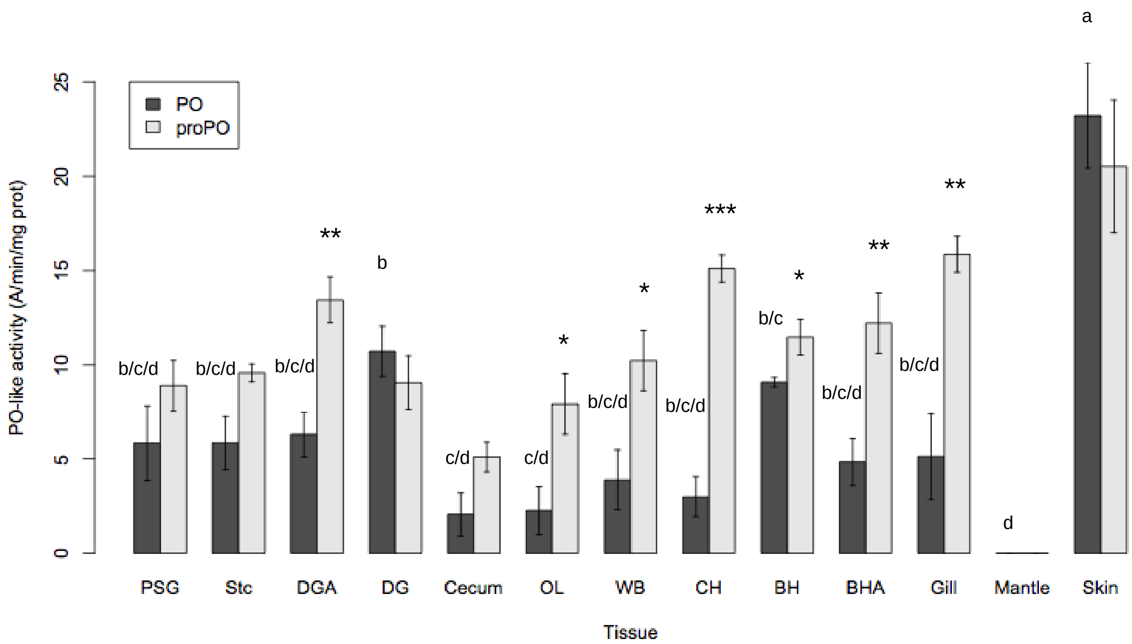

Figure 4: Phenoloxidase and Prophenoloxidase activities in various tissues of cuttlefish *Sepia officinalis* L. (n = 10). BH: branchial heart, BHA: branchial heart appendage, CH: central heart, DG: digestive gland, DGA: digestive gland appendage, OL: optic lobe, PSG: posterior salivary gland, Stc: stomach, WB: white body. The bars represent the means (±SE) of 10 animals. Graphs not bearing the same subscript letter are significantly different (p<0.05). *,**=statistical difference between PO-like and proPO-like activity for p<0.05 and p<0.01, respectively.

Table 1: Phenoloxidase-like, activated phenoloxidase-like and proPO induce activities (mean ± standard error, U.min^{-1}.mg prot^{-1}, n=10) in tissues of *Sepia officinalis* which showed significantly differences (i.e. DGA: digestive gland appendage, OL: optic lobe, WB: white body, CH: central heart, BH: branchial heart, BHA: branchial heart appendage, Gill). *,**,***=statistical difference between PO-like and activated PO-like activity for p<0.05, p<0.01 and p<0.001 respectively. Letters indicate groups significantly different amongst themselves (p<0.05).

Tissue	PO-like activity		Activated PO-like activity			proPO induce activity	
DGA	6,11 ± 1,19	ab	13,44 ± 0,77	**	ab	7,33 ± 1,34	ab
OL	2,23 ± 1,26	b	7,92 ± 1,62	*	c	5,70 ± 1,19	ab
WB	3,84 ± 1,56	ab	10,24 ± 1,59	*	bc	6,39 ± 0,78	ab
CH	2,88 ± 1,01	ab	14,61 ± 0,77	***	ab	11,74 ± 0,69	a
BH	9,04 ± 0,24	a	11,47 ± 0,74	*	abc	2,43 ± 0,75	b
BHA	4,79 ± 1,24	ab	12,21 ± 1,01	**	abc	7,43 ± 1,85	ab
Gill	4,95 ± 2,11	ab	15,87 ± 0,95	**	a	10,91 ± 1,88	a

TASK 4

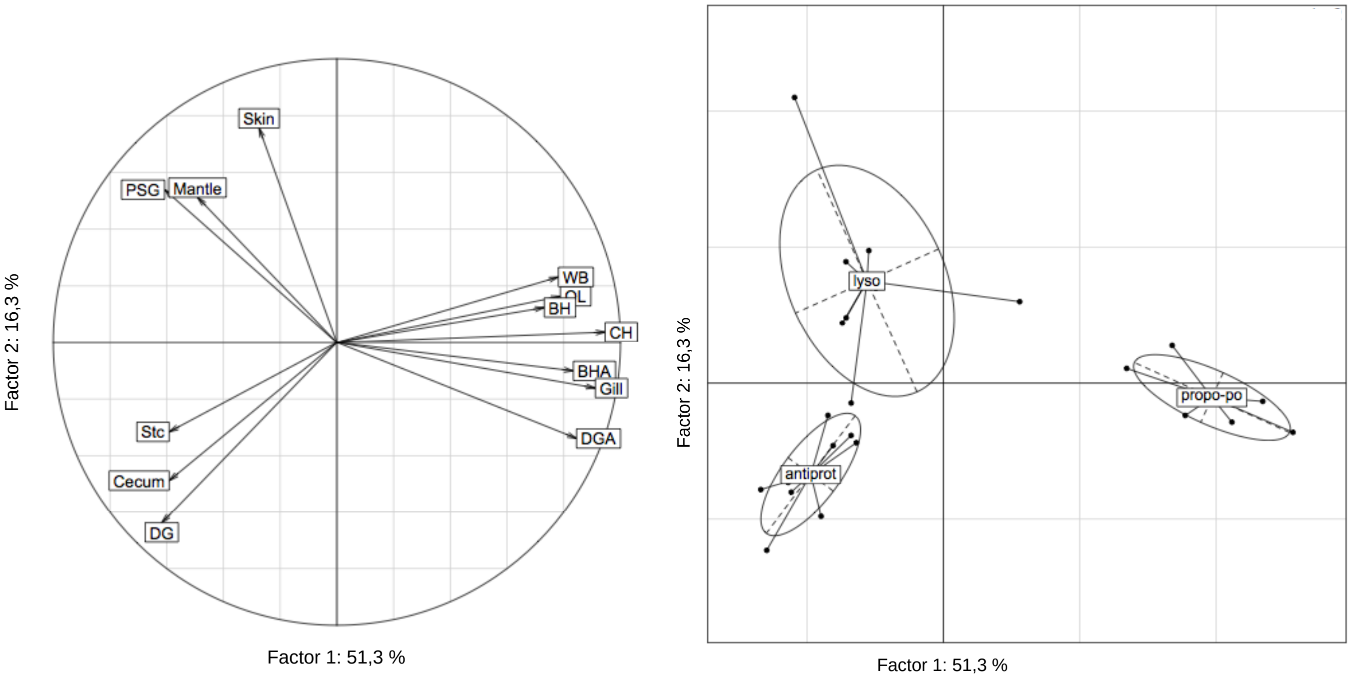

Figure 5: Principal component analysis of antiprotease, lysozyme-like, proPO induce activities in branchial heart (BH), branchial heart appendage (BHA), central heart (CH), digestive gland (DG), digestive gland appendage (DGA), optic lobe (OL), posterior salivary gland (PSG), stomach (Stc) and white body (WB).

TASK 4

"Here are the results of the quality approach. This part is not written yet as a paper form to be submitted but will be written soon"

Cuttlefish pre-recruits quality according to their origin spawning site

Year	Site	n	Protein content (% wet weight)	Total lipid (% wet weight)	Total carbohydrate (% wet weight)
2009	AC	30	7,3 ± 0,3[a/b]	1,9 ± 0,5[a]	2,9 ± 1,1[a/b]
	BS	30	8,4 ± 0,7[a/b]	1,6 ± 0,3[a]	1,2 ± 0,6[a]
	TB	30	9,0 ± 0,4[a]	1,5 ± 0,3[a]	3,7 ± 0,9[b/d]
2010	AC	30	9,2 ± 0,9[a/b]	1,4 ± 0,6[a/b]	3,3 ± 1,4[b/c/d]
	BS	30	13,2 ± 1,2[a/b]	1,3 ± 0,2[a/b]	3,7 ± 1,5[b/c/d]
	TB	30	10,0 ± 1,2[a]	1,5 ± 0,4[a/b]	4,8 ± 1,4[d]
	S	30	10,5 ± 2,2[a]	1,2 ± 0,5[a/b]	3,8 ± 1,3[b/d]
2011	AC	10	15,0 ± 6,9[a]	0,8 ± 0,3[b]	2,6 ± 0,9[a/b/c/d]
	BS	30	10,7 ± 2,0[b]	1,4 ± 0,4[a/b]	2,2 ± 0,2[c]
	TB	30	10 ± 3,6[a/b]	1,7 ± 0,4[a]	2,5 ± 0,8[a/b/c/d]
	S	30	9,7 ± 1,7[a/b]	1,9 ± 0,2[a]	2,6 ± 0,7[b/c]

Figure 6: Eggs composition (Proteins, lipids and carbohydrates) from different spawning sites of the English Channel. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK).

Year	Site	SFA	UFA	MUFA	PUFA	n3	n6	n3/n6
2009	AC	38,2	61,8	8,3	53,6	48,9	4,6	10,7
	BS	38,6	61,4	8,6	52,8	48,4	4,2	11,5
	TB	36,6	63,4	8,9	54,5	50,6	3,6	14,1
2010	AC	40,2	59,8	8,1	51,8	46,4	5,2	8,9
	BS	41,6	58,4	7,0	51,4	46,9	4,3	11,0
	TB	38,3	61,7	8,3	53,5	49,5	3,7	13,5
	S	39,9	60,1	7,8	52,3	47,3	5,0	9,4
2011	AC	38,4	61,6	8,1	53,5	48,6	4,8	10,0
	BS	39,1	60,9	8,3	52,6	48,0	4,3	11,1
	TB	36,8	63,2	8,8	54,3	50,6	3,5	14,5
	S	35,1	64,9	9,1	55,9	52,0	3,9	13,3

Figure 7: Fatty acid composition in eggs from different spawning sites of the English Channel. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK).

TASK 4

The study of the eggs quality (figure 1 and 2) showed no significant differences in vitellus content with protein, lipids or carbohydrates. But the study of fatty acids revealed that the English eggs had more poly unsaturated fatty acids (PUFA) which are essential fatty acids for a better development of embryos. The English eggs are very rich in omega 3 PUFA which is an indicator of better quality eggs.

Year	Site	n	Protein content	Total lipid	Total carbohydrate
2010	AC	8	9,9 ± 2,6 a/c	7,3 ± 2,5 a/b	3,0 ± 0,5 a
	BS	8	7,0 ± 1,3 c	9,2 ± 1,7 a	3,1 ± 0,6 a
	TB	8	11,9 ± 2,3 a/b	7,1 ± 2,3 a/b	3,1 ± 0,7 a
	S	8	13,6 ± 1,9 b	5,3 ± 1,8 b	3,8 ± 0,8 a
2011	AC	8	13,5 ± 3,5 a/b	7,6 ± 1,9 a/b	2,6 ± 0,6 a/b
	BS	8	10,0 ± 2 a/c	6,2 ± 0,6 b	1,7 ± 0,4 b
	TB	8	15,5 ± 1,4 b/d	5,5 ± 1 b	3,1 ± 0,5 a
	S	8	16,0 ± 1,8 b/d	9,9 ± 1,4 a	2,8 ± 0,5 a

Figure 8: Hatchlings (at hatching day) composition (Proteins, lipids and carbohydrates) from different spawning sites of the English Channel. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK).

The eggs quality impacts the hatchlings quality. Indeed, the English juveniles at hatching had a better quality then the French ones. The English hatchlings had higher contents of protein (figure 3) which is essential for the growth energy. The cephalopod metabolism is based on protein and amino acids (Sykes, 2008).

Year	Site	n	Protein content (% wet weight)	Total lipid (% wet weight)	Total carbohydrate (% wet weight)
2010	AC	6	18,6 ± 4,4 a/b	0,5 ± 0,1 a	1,2 ± 0,7 a
	BS	6	21,7 ± 5,4 a/b	1,4 ± 1 a/b	1,5 ± 0,7 a
	TB	6	23,9 ± 4,5 a	1 ± 0,6 a/b	1,8 ± 0,7 a
2011	AC	6	23,6 ± 4,4 a	1,2 ± 0,3 b	1,3 ± 0,2 a
	BS	6	14,9 ± 5,2 b	1 ± 0,9 a/b	1,3 ± 0,3 a
	TB	6	18 ± 4,6 a/b	1,8 ± 1,3 b	1,6 ± 0,5 a

Figure 9: Pre-recruits composition (Proteins, lipids and carbohydrates) from different spawning sites of the English Channel. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK).

As for the pre-recruits collected in the natural environment, only the comparison between the two French sites was possible because these samples were collected in the same period. The Torbay pre-recruits were collected after the French ones and were thus older (1 month older). The comparison between the 2 French pre-recruits showed that the Agon Coutainville ones had a better protein content in their mantle thus being of a better quality than the Bay of Seine ones (figure 4).

Year	Site	Stage	n	Protein content (% wet weight)	Total lipid (% wet weight)	Total carbohydrate (% wet weight)
2010	AC	7	5	9,2 ± 1,4[a/b]	10,6 ± 1,1[a/b]	3,2 ± 0,8[a]
	BS		5	8,2 ± 1,2[a]	9,7 ± 2[a/b]	3,2 ± 0,7[a]
	TB		5	6,5 ± 1,3[a]	7,4 ± 1,5[a/b]	3,1 ± 0,6[a]
	S		5	6 ± 1,8[a]	7,9 ± 2,5[a/b]	3,4 ± 1,6[a/b]
	AC	14	5	12,4 ± 1,7[b]	10,4 ± 1,4[a/b]	5,5 ± 0,5[b/c]
	BS		5	13,5 ± 1,6[b/c]	9 ± 3,4[a/b]	6,4 ± 1,1[c]
	TB		5	13,9 ± 2,1[b/c]	8,2 ± 2,8[a/b]	4,6 ± 0,3[b]
	S		5	15,7 ± 0,9[b/c]	10,6 ± 2,5[a/b]	4,8 ± 0,4[b]
	AC	21	5	14 ± 1,6[b/c]	6,1 ± 2,3[b]	8,1 ± 1,9[d]
	BS		5	18,8 ± 2,7[c]	11 ± 3,5[a/b]	8,9 ± 2,8[d]
	TB		5	11,5 ± 2,1[a/b]	11,7 ± 1[a]	9,2 ± 1,6[d]
	S		5	-	-	-
	AC	28	5	11,2 ± 1,2[b]	5,6 ± 1,6[b]	7,9 ± 1,3[d]
	BS		5	9,8 ± 1,1[a/b]	6,8 ± 2[a/b]	7,7 ± 1,9[c/d]
	TB		5	-	-	-
	S		5	10,3 ± 1,1[a/b]	5,3 ± 1,4[b]	6,6 ± 1[c]
	AC	35	5	10,1 ± 1,3[a/b]	8,7 ± 1,5[a/b]	7,2 ± 0,9[c/d]
	BS		5	8,5 ± 1,4[a]	6 ± 1,7[b]	8,2 ± 1,4[d]
	TB		5	8,9 ± 1,3[a/b]	6,4 ± 1,8[b]	8,9 ± 1,8[d]
	S		5	9,3 ± 1,7[a/b]	9,7 ± 2,8[a/b]	7,9 ± 1,6[d]

Figure 10: 2010 reared juvenile's composition (Proteins, lipids and carbohydrates) from different spawning sites of the English Channel. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK).

Year	Site	Stage	n	Protein content (% wet weight)	Total lipid (% wet weight)	Total carbohydrate (% wet weight)
2011	AC	7	5	11,8 ± 0,8[a/b]	8,2 ± 1[a]	3,2 ± 0,9[a/b]
	BS		5	11,7 ± 0,9[a/b]	6,7 ± 1,4[a/b]	2,4 ± 0,5[a]
	TB		5	13,2 ± 1,1[a]	6,6 ± 1,2[a/b]	2,7 ± 0,5[a]
	S		5	12,1 ± 0,7[a]	8,8 ± 3,2[a]	3,3 ± 0,6[a/b]
	AC	14	5	14,8 ± 2,3[a/c]	7,1 ± 1,6[a/b]	2,6 ± 1[a/b]
	BS		5	9 ± 1,3[b]	8,2 ± 1,6[a]	2,3 ± 0,9[a]
	TB		5	13,3 ± 1[a]	7,4 ± 1[a]	2,2 ± 0,5[a]
	S		5	11 ± 1,3[a/b]	7,7 ± 1,6[a]	2,4 ± 0,5[a]
	AC	21	5	12,4 ± 1[a]	8 ± 1,5[a]	2,3 ± 0,6[a]
	BS		5	12,9 ± 0,6[a]	7,6 ± 1,7[a]	2 ± 0,7[a]
	TB		5	13,5 ± 0,8[a]	6 ± 2,2[a/b]	2,3 ± 0,6[a]
	S		5	11,8 ± 2,2[a/b]	7,9 ± 1,6[a]	2,8 ± 0,5[a/b]
	AC	28	5	14,2 ± 1[a/c]	4,9 ± 1,9[a/b]	3,9 ± 0,7[b]
	BS		5	15,1 ± 0,6[c]	2,5 ± 1,1[b]	4,4 ± 1,2[b]
	TB		5	17,2 ± 3,1[a/c]	2,9 ± 1,4[b]	3,3 ± 0,6[a/b]
	S		5	12,8 ± 1,5[a]	5,8 ± 1,6[a/b]	3,1 ± 1[a/b]
	AC	35	5	13,7 ± 1,1[a/c]	3,5 ± 4,8[a/b]	3,6 ± 0,5[a/b]
	BS		5	12,3 ± 3,1[a/b/c]	3 ± 1,3[b]	3,4 ± 0,7[a/b]
	TB		5	12,9 ± 1,5[a]	5,1 ± 2,2[a/b]	3,6 ± 0,7[a/b]
	S		5	14 ± 1,4[a/c]	7,1 ± 1,7[a]	2,6 ± 0,5[a]

Figure 11: 2011 reared juvenile's composition (Proteins, lipids and carbohydrates) from different spawning sites of the English Channel. The monitored spawning sites are: BS (Bay of Seine-FR), AC (Agon Coutainville-FR), TB (Torbay-UK) and S (Selsey-UK).

The study of rearing juveniles content (Figure 5 and 6) over two years did not reveal any clear scheme. The fact that all juveniles were reared in the same conditions with the same food (*Crangon crangon*) could explain this lake of difference. Indeed, the fact that the food was the same for all batches has finally erased differences between batches. The differences were well observed at hatching (figure 3) and disappeared afterwards with the experimental rearing.

TASK 4

"Here are the results of the digestive system maturation. This part is not written yet as a paper form to be submitted but will be written soon"

Juvenile cuttlefish digestive system maturation: an histological and enzymatic approach

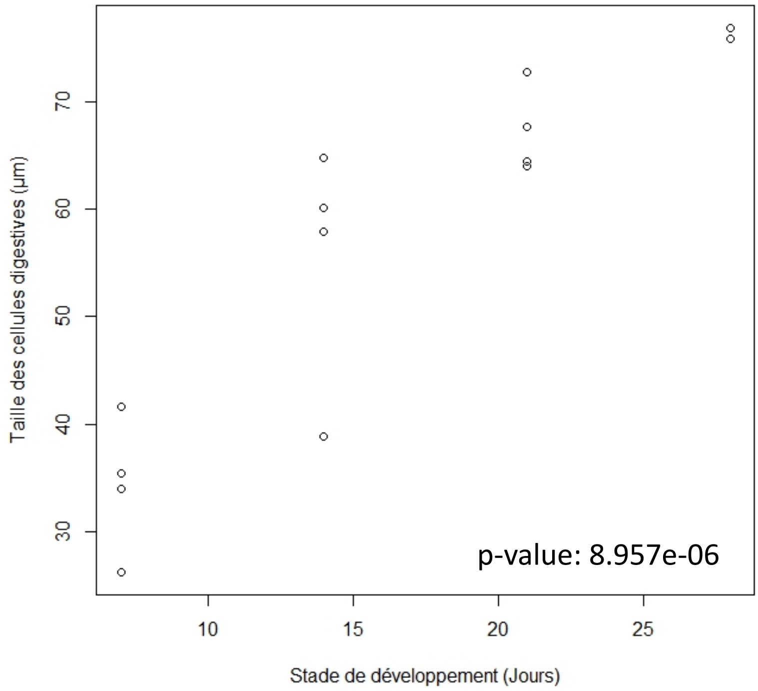

Figure 12: Relation between digestive cells length and juveniles development stages

We observed an increase in the digestive cells length between the hatching time till 30 days after hatching (Figure 7). These cells are immature at hatching with a mean length of 20µm and mature as the length increase with the formation of digestive vesicles in the digestive cells (Figure 8). These digestive vesicles contain digestive enzymes that will be released in the tubules of the digestive gland and will act in the stomach during digestion.

TASK 4

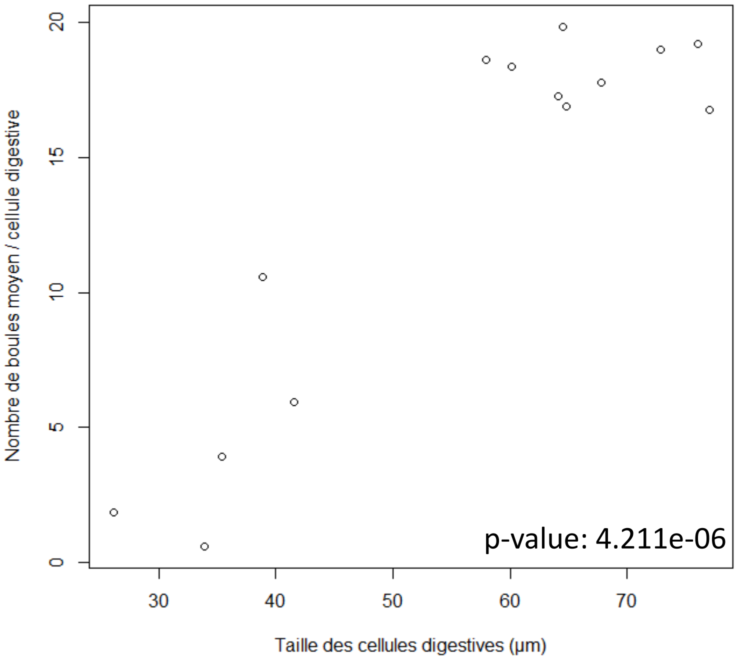

Figure 13: Relation between the mean number of digestive vesicles/ digestive cell and the digestive cell length

Studied enzymes were related afterwards with the histological observations. The acid digestive enzymes had no linear relation either with the cells length and vesicles number (figure 9 and 12). The alcalin digestive enzymes) had a linear relation with the cells characteristics. These relationships show that the acid digestive enzymes that act intracellularly are not related to the vesicles number but would be more related to vacuoles that are formed in the cells and digestion is thus made in the cell. As for the alkalin enzymes, the relation between vesicles and enzymatic activities show that these vesicles contain the alkalin enzymes that are released in the digestive tubules and will act extracellularly in the stomach, cecum and digestive gland appendages.

TASK 4

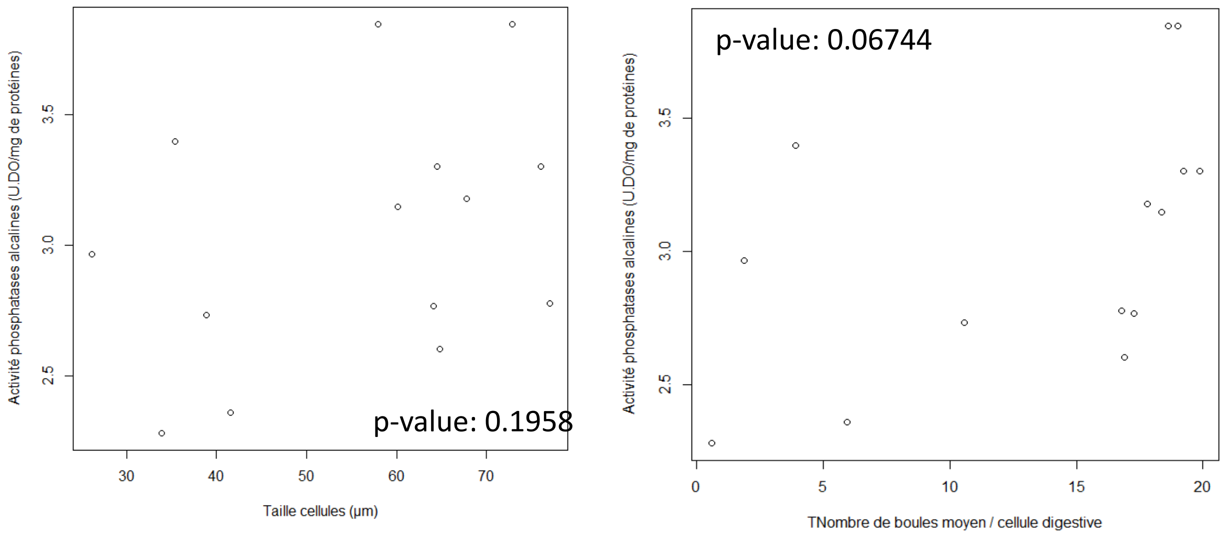

Figure 14: Relationship between Alcalin phosphatase activity and digestive cells characteristics

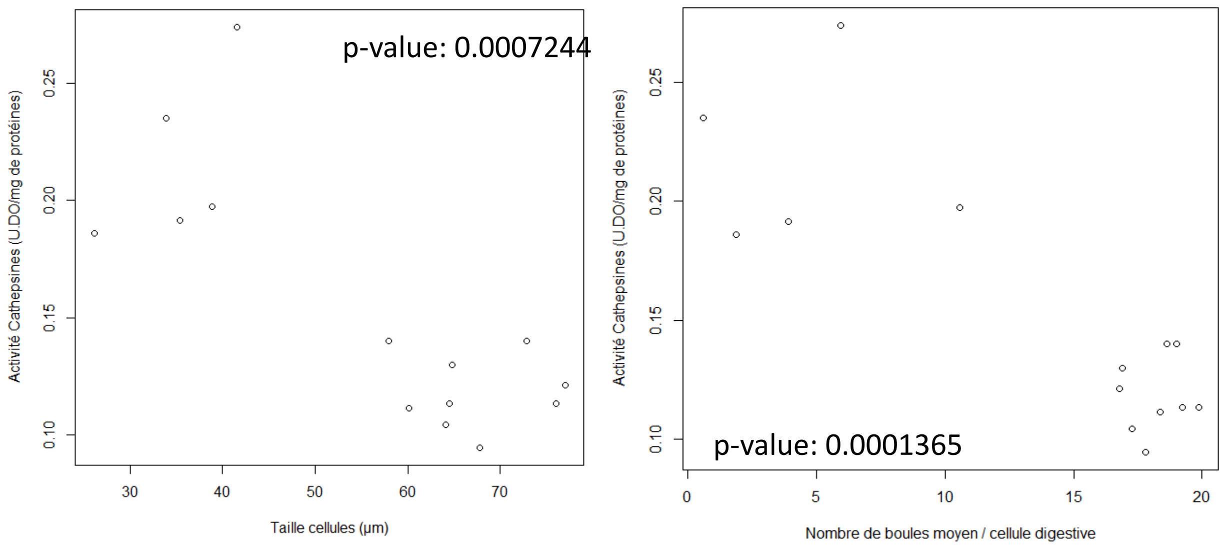

Figure 15: Relationship between cathepsines activity and digestive cells characteristics

TASK 4

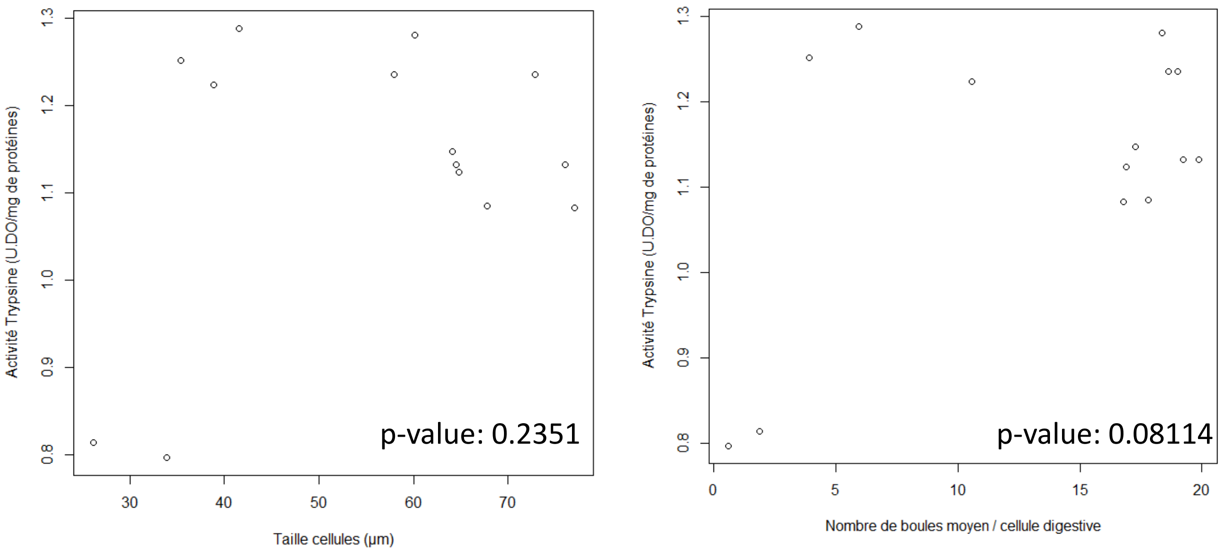

Figure 16: Relationships between Trypsin activity and digestive cells characteristics

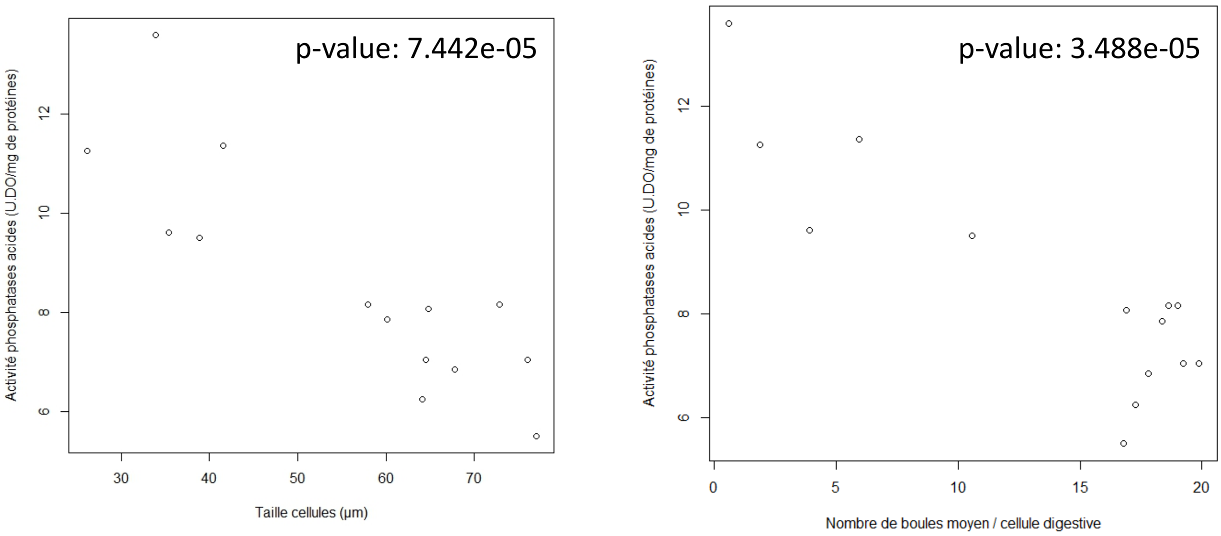

Figure 17: Relationship between acid phosphatases activity and digestive cells characteristics

TASK 5

Relations trophiques aux stades œufs et juvéniles

Trophic relationships in egg-juvenile stages

Objective/*Objectifs*: Interactions between Cephalopod eggs and juveniles and other biotic components of the ecosystem (trophic relationships in egg-juvenile stages).

Interactions entre œufs et juvéniles de Céphalopodes et autres populations de l'écosystème (relations trophiques concernant ces stades).

2009-2012 Work progress

I. Task Results Summary / *Résumé des résultats obtenus*

Trophic ecology information regarding a certain species is essential to understand its role within the trophic network.
The first objective of this work was to identify juvenile cuttlefish food resources, and to study the potential diet differences (prey types and their proportions) between the main spawning grounds of the English Channel. The second objective was to check if the use of stable isotopes as trophic tracers could distinguish juveniles according to their spawning site, and also discern recruits origins within the central stock.

Juvenile cuttlefish trophic ecology has been studied thanks to carbon and nitrogen stable isotopes. Results show that juveniles from west Cotentin (Agon Coutainville, France) and Torbay (England) have a similar isotopic signature, whereas juveniles from the Baie de Seine (Ouistreham) have a different signature, with a carbon and nitrogen enrichment (ca. 2‰).
The mentioned results have been observed during both studied years (2010 and 2011). The main juvenile preys identified are prawns, crabs and little fishes (Gobi). The preys were collected within the two French spawning locations (west Cotentin and Baie de Seine). Results have demonstrated that for both sites cuttlefish diet is mainly composed of a mixed of the aforementioned preys. However, the isotopic ratio difference noticed between both sites indicates a different proportion in each prey within the juveniles diet.

La connaissance de l'écologie trophique d'une espèce est essentielle pour comprendre son rôle au sein du réseau trophique. Ainsi, le premier objectif de ce travail était d'identifier les ressources alimentaires des juvéniles de seiches et d'étudier les différences potentielles de régime alimentaire (type de proies, proportion de chacune) entre les principaux sites de ponte en Manche. Le second objectif était de déterminer si l'utilisation des isotopes stables comme traceur trophique permettait de discriminer les juvéniles en fonction de leur site de ponte, mais également de discriminer l'origine des recrues se retrouvant dans le stock central.

L'écologie trophique des juvéniles de seiches a été étudiée en utilisant les isotopes stables du carbone et de l'azote. Les résultats montrent que les juvéniles provenant des sites de la zone ouest du Cotentin (Agon Coutainville, France) et de Torbay (Angleterre) présentent une signature isotopique similaire. Par contre, les juvéniles provenant de la Baie de Seine (Ouistreham) présentent une signature différente avec un enrichissement en azote et en carbone (ca. 2‰).
Ces résultats ont été observés pour les deux années étudiées (2010 et 2011). Les principales proies des

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juvéniles identifiées sont les crevettes, les crabes et les petits poissons (Gobi). Ces proies ont été récoltées dans les deux zones de ponte françaises (côte ouest Cotentin et Baie de Seine).
Les résultats montrent que pour ces deux sites le régime alimentaire des juvéniles de Seiche est principalement composé d'un mélange de ces différentes proies. Par contre, la différence de signature isotopique entre les deux sites, indique des différences dans la proportion de chacune des proies dans le régime alimentaire des juvéniles.

II. Actions carried out during the project/ *Bilan des opérations réalisées pendant le projet*

Sampling

Samples used for the cuttlefish trophic ecology study came from 3 different sampling types :

- From the open sea from fisheries or scientific boats
  This sampling allowed the study of pre-recruits. Areas sampled were both French sites (Baie de Seine and Agon Coutainville; 1 sampling per site in September 2010 and in November 2011), and one English site (Torbay; 2 samplings – the first one in 2010 and the second one in 2011).

- From the intertidal and the subtidal area
  This sampling allowed the study of eggs (2 sampling per year in Agon and Baie de Seine in 2010 and 2011), and of pre-recruits preys (6 sampling for Baie de Seine and 2 for Agon during summer 2010).

- Port-en-Bessin and Cherbourg fish markets
  This sampling allowed the study of recruits coming from the central stock: 2 samples were made in 2011, and 2 in 2012.

Sample analysis

Samples used for isotopic measurement were processed at the laboratory of the University of Caen Basse-Normandie and analysed with a mass spectrometer. We performed 80 analyses on preys (crabs, prawns and fishes), 100 analyses on pre-recruits and 300 analyses on recruits. For each analysis 2 measurement of stable isotopes were performed (nitrogen and carbon).

Echantillonnage

Les échantillons analysés pour l'écologie trophique proviennent de trois types d'échantillonnages :

- En mer à bord de bateaux de pêches ou scientifiques
  Ce type d'échantillonnage a concerné principalement les pré-recrues.
  Les zones échantillonnées sont les deux sites français (Baie de Seine et Agon Coutainville, 1 sortie par site en septembre 2010 et en novembre 2011), et un site anglais (Torbay, 2 sorties : une en 2010, et une en 2011).

- En zone intertidale et subtidale

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Ce type d'échantillonnage a concerné les œufs de seiches, 2 sorties par an à Agon et en Baie de Seine en 2010 et 2011, et les proies des pré-recrues : 6 sorties en Baie de Seine et 2 à Agon durant l'été 2010.

– A la criée de Port-en-Bessin et Cherbourg.
  Ce type d'échantillonnage a concerné les recrues pêchées dans le stock central. 2 criées en 2011 et 2 criées en 2012.

Traitement des échantillons

Les échantillons utilisés pour les mesures isotopiques ont été préparés au laboratoire de l'Université de Caen et analysés au spectromètre de masse. Nous avons réalisé 80 analyses sur les proies (crabes, crevettes, poissons), 100 analyses sur les pré-recrues et 300 analyses sur les recrues. Pour chaque analyse, deux mesures d'isotopes stables ont été réalisées (azote et carbone).

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III. Scientific outputs/ *Détail des travaux scientifiques*

1. Cuttlefish trophic step fractionation

Consumers experience a trophic step enrichment (also called isotopic fractionation) compared to their prey that needs to be evaluated accurately. This fractionation is assessed under controlled experimental conditions for different organs that incorporate the stable isotopes with different turnover rates.

Two different experimentations were carried out in order to estimate the trophic step fractionation. The first one is based on experiments performed in the task 4 (more details of this work are indicated in the task 4 final report). Data were collected during the rearing of juveniles performed in 2009 and 2010. The second one aimed to assess the trophic step fractionation at the time of hatching. Thus, spawning eggs from one site (Baie de Seine) were incubated in the same rearing structures than those used in the task 4 in order to maintain stable abiotic conditions (water temperature, salinity, nutrient concentrations). The incubation was performed until the hatching time. During the incubation, some eggs were regularly removed. Then, the isotopic ratios (carbon and nitrogen) of embryos and vitelli were assessed for each sample. Results showed that these experiments have successfully been performed. However, the exact estimation of trophic step fractionation of juveniles and eggs requires more process on data and is currently in progress.

2. Cuttlefish trophic ecology

The study of cuttlefish trophic ecology allowed firstly to identify the main trophic resources of pre-recruits in the main spawning grounds of the English Channel, and secondly to contribute to the identification of recruits origins within the central stock. The main results about these two concerns are detailed below.

Pre-recruits trophic resources

The main objective of this part was to identify the main trophic resources of cuttlefish pre-recruits in the main spawning grounds of the English Channel, and to assess the potential differences of cuttlefish diet between these areas.

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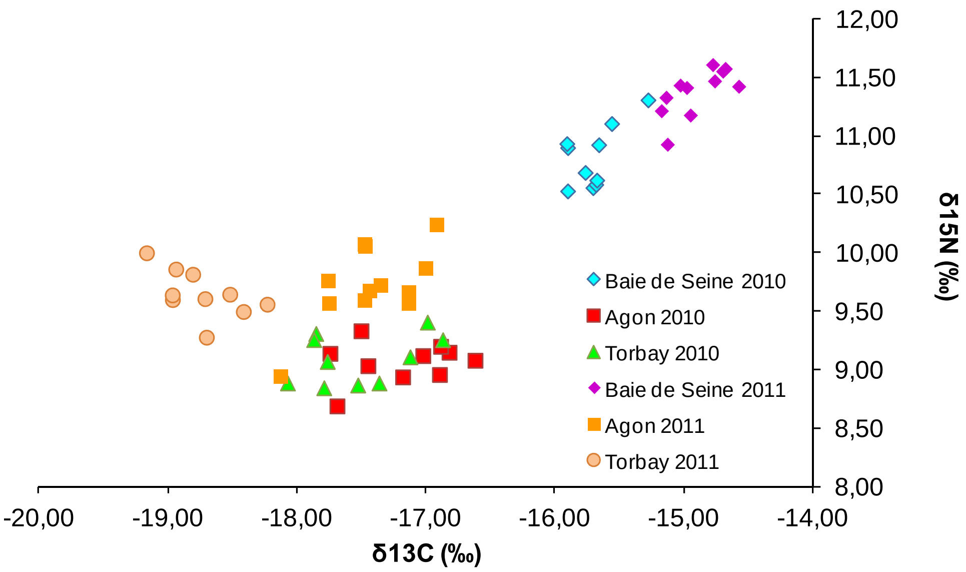
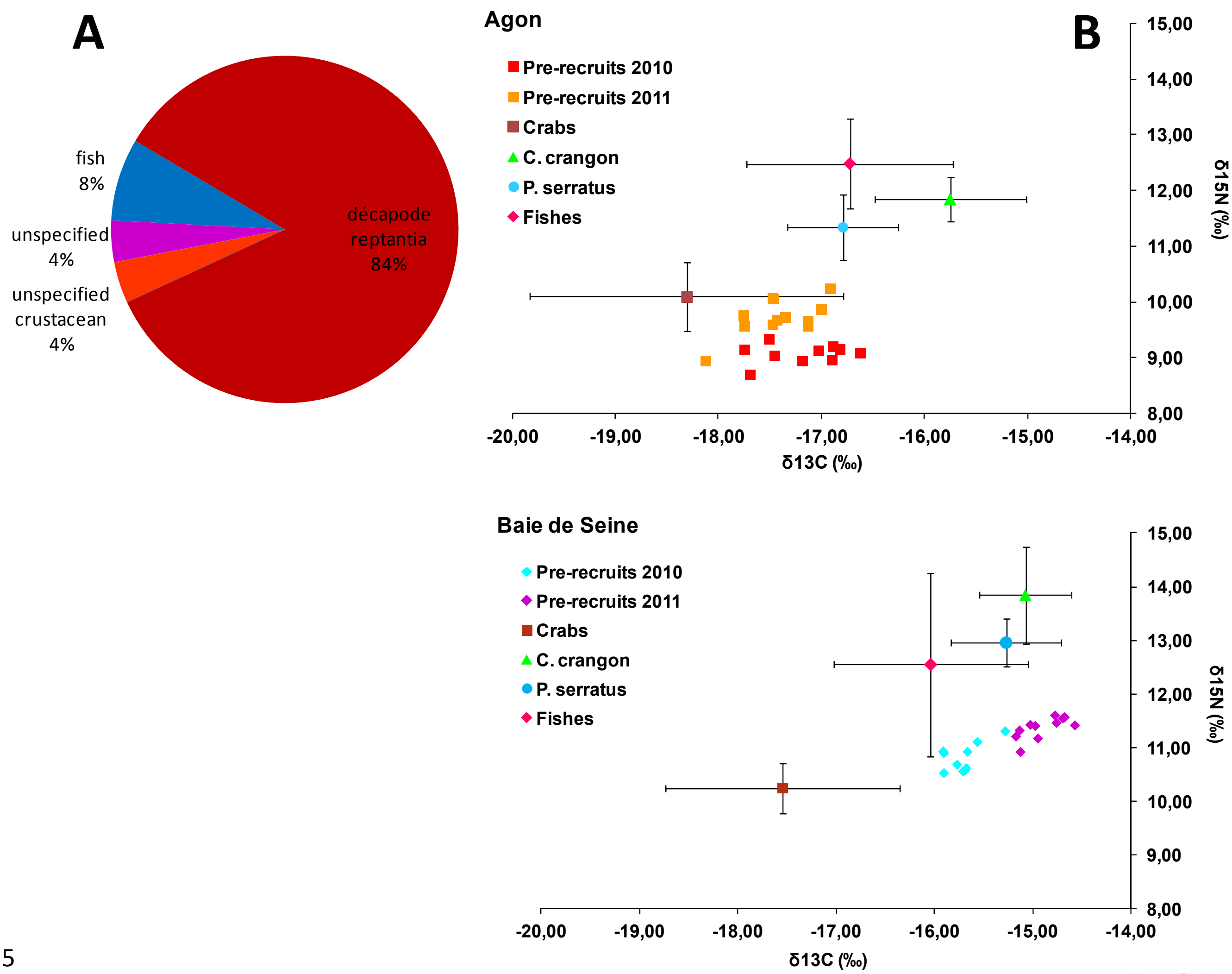


Figure 1: Isotopic values of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) of pre-recruits for the two French study sites (Baie de Seine and Agon) and an English site (Torbay) in 2010 and 2011.

Results highlighted a difference between isotopic values of pre-recruits from Baie de Seine and from Agon and Torbay. For the two years studied (i.e. 2010 and 2011) carbon and nitrogen isotopic values of pre-recruits coming from Baie de Seine was always higher than those coming from Agon and Torbay (figure 1).



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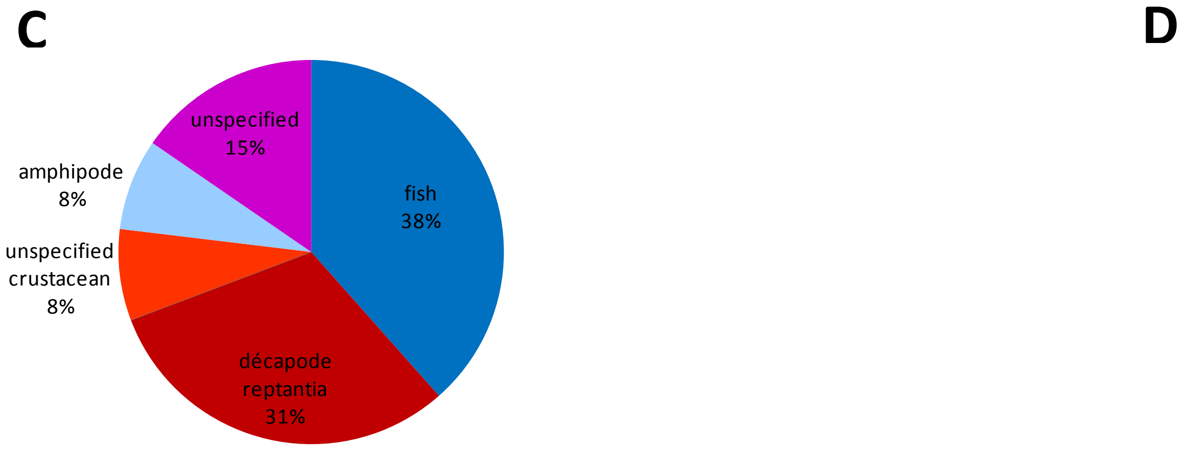

Figure 2: Stomach contents of pre-recruits (A,C) and isotopic values of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) of pre-recruits and of potential food sources (B,D) for Agon (A,B) and Baie de Seine (C,D) spawning grounds.

In order to interpret results of pre-recruits, isotopic values of cuttlefish were compared with those of their potential preys. The studied preys were crabs, prawns and fishes which are the main common prey for cuttlefish. The comparison was only performed for French sites because no prey data were available for Torbay. Isotopic results of cuttlefish were supplemented with the study of the stomach contents of pre-recruits (figure 2).

As a whole, results showed that for the two French study sites, pre-recruits relied on the same food sources: crabs, prawns and fishes. However, the contribution of each source to the cuttlefish diet is very different between these two sites.

For Baie de Seine, results showed that pre-recruits relied on a mix between the aforementioned preys (figure 2 A,B), whereas, for Agon, cuttlefish diet was mainly made up of crabs (figure 2 C,D). For both sites, isotopic results and stomach contents are consistent.

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Origin of recruits within the central stock

The main objective of this part was to identify the recruits' origins within the central stock using isotopic values as a trophic tracer.
Results previously obtained showed that the use of isotopic values distinguishes only pre-recruits coming from Baie de Seine. As for the other study sites (Agon and Torbay), there is no difference in pre-recruits isotopic ratios. Thus, isotopic composition cannot be used to identify pre-recruits coming from these spawning grounds within the central stock.
Young recruits of cuttlefish were sampled in the central stock in December 2010 and in January 2012. For these two years, results of isotopic ratios showed that around 25% of recruits had a similar isotopic composition than those of the pre-recruits which have grown up in the Baie de Seine (figure 3). These results are in accordance with results obtained on trace metals studied in the task 7.

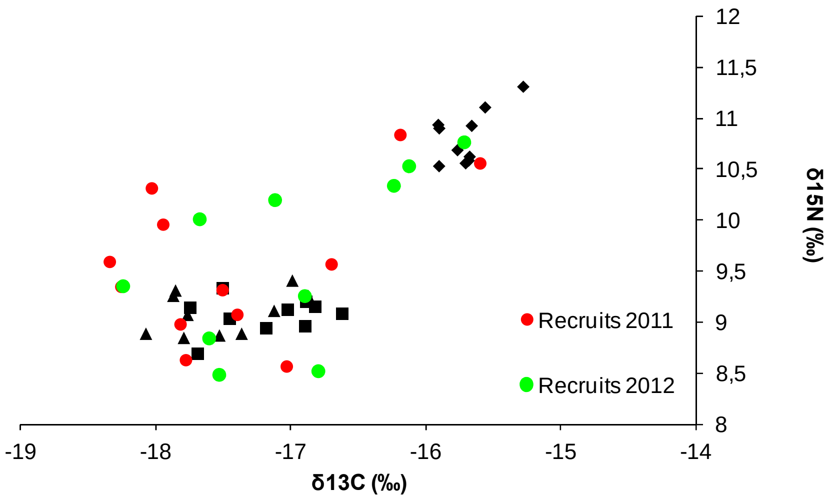

Figure 3: Isotopic values of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) of recruits coming from the central stock for 2011 and 2012. In order to compare the isotopic ratios of recruits and pre-recruits, isotopic values of pre-recruits are also indicated in black.

Conclusion

The work performed in this task allowed a better knowledge of the trophic ecology of the cuttlefish pre-recruits in the main spawning grounds of the English Channel.

- The study of pre-recruits diet highlighted that, whatever the study site the same trophic resources are used by the cuttlefish. The main difference between spawning grounds is the proportion of each source in the pre-recruits diet. More particularly, results showed that pre-recruits which had grown up in the Baie de Seine were more enriched in heavy isotopes and have a more varied diet.
- The study of young recruits within the central stock highlighted that during the first year of life, the isotopes ratios of mantle can be used to identify recruits coming from the Baie de Seine. However, when recruits are in the central stock - whatever their origin - they use the same trophic resources during winter. Thus, during the second year of life, all individuals have the same isotopic ratios and it is no more possible to use the isotopic composition of mantle to identify recruits origins.