TUNA OCEAN RESTOCKING (TOR) PILOT STUDY - SEA-BASED HATCHING AND RELEASE OF ATLANTIC BLUEFIN TUNA LARVAE THEORY AND PRACTICE

C.R Bridges[1,2]*, D. Nousdili[2], S. Kranz-Finger[2], F. Borutta[2], S. Schulz[2], S. Na'amnieh[2], R. Vassallo-Agius[2,3] M. Psaila[3] and S. Ellul [3]

SUMMARY

In April 2018 a pilot broodstock cage (30 m in diameter and 20 m deep) containing 48 adult Atlantic bluefin tuna (ABFT) was established 6 km off the coast of Malta. When sea water temperatures reached 22°C+, an egg collector net was attached to the downward current side of the cage. On the 21st June approximately 500,000 eggs were collected and on the following days approximately 1.7 million eggs were collected in total. Pilot experiments were carried out monitoring egg fertilisation, hatching rates and YSL survival after incubation both in the sea and in the laboratory. Eggs incubated in-situ sea incubator nets showed hatching rates between 80 and 90%; corresponding to the laboratory value. YSL survived up to 8 days in the laboratory. Molecular analysis of parental contribution of the egg batches indicated that at least 25 mothers had contributed towards egg production. Based on the figures obtained for egg numbers, hatching rates, the numbers of contributing mothers and the theoretical maximum fecundity approximately 75 million DNA-tagged larvae were released back into the sea.

RÉSUMÉ

En avril 2018, une cage pilote de géniteurs (30 m de diamètre et 20 m de profondeur) contenant 48 thons rouges de l'Atlantique (ABFT) adultes a été placée à 6 km au large de Malte. Lorsque la température de l'eau de mer a atteint 22°C, un filet de collecte d'œufs a été fixé sur le côté du courant descendant de la cage. Le 21 juin, environ 500.000 œufs ont été collectés et les jours suivants, environ 1,7 million d'œufs ont été collectés au total. Des expériences pilotes ont été menées pour surveiller la fécondation des œufs, les taux d'éclosion et la survie des larves lécithotrophes après l'incubation en mer et en laboratoire. Les œufs incubés dans des filets d'incubateurs marins in situ ont présenté des taux d'éclosion compris entre 80 et 90%, ce qui correspond à la valeur laboratoire. Les larves lécithotrophes ont survécu jusqu'à 8 jours en laboratoire. L'analyse moléculaire de la contribution parentale des lots d'œufs a fait apparaitre qu'au moins 25 femelles avaient contribué à la production d'œufs. Sur la base des chiffres obtenus concernant le nombre d'œufs, les taux d'éclosion, le nombre de mères contributrices et la fécondité maximale théorique, environ 75 millions de larves marquées par ADN ont été relâchées dans la mer.

RESUMEN

En abril de 2018 se estableció una jaula piloto de reproductores (30 m de diámetro y 20 m de profundidad) que contenía 48 atunes rojos del Atlántico adultos a 6 km de la costa de Malta. Cuando la temperatura del agua del mar alcanzó los 22°C+, una red de recogida de huevos se colocó en la parte de la corriente descendente de la jaula. El 21 de junio se recogieron aproximadamente 500.000 huevos y en los días posteriores aproximadamente se recogieron en total 1,7 millones de huevos. Se realizaron experimentos piloto haciendo un seguimiento de la fertilización de los huevos, las tasas de eclosión y la supervivencia de larvas citotróficas (YSL) tras la incubación tanto en el mar como en el laboratorio. Los huevos incubados in situ, en redes incubadoras en el mar presentaban tasas de eclosión de entre el 80 y el 90 %, correspondiéndose con el valor del laboratorio. YSL sobrevivieron hasta 8 días en el laboratorio. El análisis molecular de la contribución parental de los lotes de huevos indicaba que, al menos, 25 madres

[1] Institute for Metabolic Physiology/ Ecophysiology, Heinrich Heine University, Düsseldorf 40225, Germany (bridges@hhu.de)
[2] TUNATECH GmbH , Merowingerplatz 1A, 40225 Düsseldorf, Germany
[3] MFF Ltd, Hangar, Triq it - Trunciera, Marsaxlokk, MXK 1522, Malta

habían contribuido a la producción de huevos. Basándose en las cifras obtenidas para los números de huevos, las tasas de eclosión, el número de madres que contribuyó y la fecundidad teórica máxima, aproximadamente 75 millones de larvas marcadas con ADN fueron liberadas de nuevo en el mar.

KEYWORDS

Thunnus thynnus, Reproductive cycle, Spawning, Fish eggs, Fecundity, Hatching, Fertilization, Paternal contribution, DNA-Tagging

1. Introduction

Since the early 1970s it has become clear that the overfishing of the Atlantic bluefin tuna (ABFT) in the Mediterranean has led to a large depletion of the biomass present for spawning. The International Commission for the Conservation of Atlantic Tunas ICCAT initiated a recovery plan which was based on strict yearly quotas starting with 12,750 t in 2008 and progressing with limited increases yearly until a predicted quota of 32,000 t in 2020 (ICCAT, 2007). Whether the indicative, increasing biomass observed in the last three years will continue is still unknown. There have been many previous attempts at restocking marine finfish species (for review see Blaxter, 2000; Leber, 2013) the relevance of such exercises has always been called into question since the success or failure has been difficult to prove and this has usually involved the use of cultivated juveniles or post-larvae. When considering fishing stocks many agencies have been involved in classical "mark and recapture" experiments (Miyake, 1990; Cort *et al.*, 2010) or the use of electronic tags (Cermeno *et al.*, 2015; Hanke *et al*., 2019) and recently new molecular techniques such as " Close-Kin Mark and Recapture" (CKMR) have been established for Southern Bluefin tuna (SBFT) (Bravington *et al*., 2016).The aim of the Tuna Ocean Restocking (TOR) pilot-project is to provide millions of DNA tagged larvae for release into the sea, thus thwarting egg predators before they hatch and assisting the spawning of ABFT. At the same time the use of molecular markers will be used, thus overcoming the problems of previous mark and recapture experiments in terms of numbers and assessment of success rates. The present paper outlines a pilot study carried out with a commercial tuna ranching operation in which naturally spawned eggs from a wild broodstock are collected, incubated at sea and then released back into the environment. Details of the theory behind the study are given together with a report on the pitfalls and problems that occurred during the first year of a three-year study period.

2. Material and Methods

Initially 48 ABFT (average weight 90-150 kg) were placed in a sea cage (Ø 30 m, depth 20m) as a broodstock at a low stocking density. Broodstock management and supplementary feeding was according to proven methods during the decade of research carried out in EU DOTT projects. No antibiotics were used for the captive broodstock and no chemical treatments of the larvae took place. Egg collection was carried out after natural spawning and incubation of the eggs until hatching on a sea-based platform. The restocking events consisted of larva release (millions of larvae each year) into natural spawning grounds since each female can produce up to 3 million eggs per spawning event (multiple-spawners). The traceability of the offspring was assured through using molecular biological techniques of DNA parental markers for males and females that have been sampled underwater according to techniques used in the SELFDOTT and TRANSDOTT EU projects (SELFDOTT, 2011; TRANSDOTT, 2014). This pilot program was planned over a 3-year trial period which could be extended and broodstock can be released for scientific purposes at the end of the project. The individual tasks are outlined below:

2.1 Broodstock Cage

In April 2018 a pilot broodstock cage (30 m in diameter and 20 m deep) containing 48 adult Atlantic bluefin tuna (ABFT) was established by Malta Fish Farming Ltd (MFF) under the direction of TUNATECH at a site (**Figure 1a**) approximately 6 km off the south east coast of Malta. These fish had been transferred (**Figure 1b**) under ICCAT rules from fish previously caught between Malta and Tunisia. The fish were fed with a supplemented diet beginning in April to improve the egg quality and production. Close monitoring of the feeding and feeding amounts were carried out with MSC certified baitfish and the modified supplemented diet was used as outlined in the SELDOTT (2011) and TRANSDOTT (2014) EU projects.

2.2 In Situ Temperature Monitoring

This was carried out using the standard "Hobo" data loggers as shown below (**Figure 2**) which can be attached at various positions on the cage itself and then at specific intervals read by a diver-assisted readout system. Cage surface and cage bottom temperatures were measured at 15 minute intervals together with light recordings at the top and bottom of the cage and data downloaded at weekly intervals. Measurements were made throughout the year such that careful monitoring of environmental conditions could be made. A temperature window above 20 - 22 C° has been deemed necessary for spawning in previous EU studies but great variability has been seen in the field (Reglero *et al.*, 2018).

2.3 Egg Collection

Using a modified plankton net (**Figure 3**) which was attached on the „Lee-ward" side of the cage, eggs were continuously collected after release which was usually between midnight and 05:00 in the morning. After collection, eggs were submitted to incubation in offshore incubation systems (**Figure 4**) Initially a solid frame system was established next to the broodstock cage as shown in the diagram below. Egg collection was commenced early in June through the implementation of the net structure around the cage. A more detailed description of the protocol and eggs obtained is given in the results section. This set up was unfortunately damaged through a storm as the structure was not flexible enough to withstand the forces generated by the wave action and swell. A second incubator consisted of a net that was rapidly commissioned and set up *in situ* at the offshore site as indicated below. There were also problems with the egg collector net due to attraction of trigger-fish that came to feed on jellyfish larvae that get caught in the egg collector. The trigger-fish destroyed the net in their attempt to feed on the jellyfish larvae inside the egg collector.

The second incubator consisted of a lightweight net with weight suspended at one end and a buoy system suspended at the other end, that could easily be stocked with eggs (**Figure 5**). The aim of the net was to incubate the fertilised eggs so that just prior to hatching, they can be moved further offshore, downstream, away from the tuna culture pens. This was done so that any wild predatory juvenile fish that occupy the vicinity of the tuna cages would not feed on the eggs, thus increasing the chances of survival of these fragile larvae until the onset of feeding.

2.4 Field and Laboratory Egg and YSL incubation

Upon egg collection, the following protocol was carried out as seen in the figure below (**Figure 6**) indicating the batch timeline as well as the handling of the eggs within the laboratory and in the field. Eggs collected were sub-sampled for molecular analysis, and the measurement of fertilisation rates and laboratory incubation helped us assess the fertilisation and hatching rates under controlled conditions. Eggs placed within the *in situ* incubator were then returned to the sea after subsampling and again and incubating them in the laboratory on plates under control conditions. In this way egg collection served primarily to stock the in situ incubators with some samples for controls in the laboratory, followed by a 2nd subsampling to investigate the effects of in situ incubation. Hatched eggs were continuosly monitored throuh the YSL stage in the laboratory for up to 8 days.

3. Results

Temperature

In **Figure 7** below indicates the results of the continuous temperature monitoring in both top and bottom of the cage system. After 21st May the temperature rose rapidly, and the spawning window was reached early in June with 24°C at the surface. From this early peak in June, bad weather prevented the collection of eggs and the number of days for egg collection. It was not until after the 20th June that the first eggs were collected in any large numbers. This corresponded also with the temperature of over 24°C the top of the cage as seen in the figure below.

Egg Collection

The the overall results for all egg collections shown in the table below (**Table 1**). On three consecutive days approximately over 1,600,000 eggs were collected and these samples were analysed for biometric data. Although egg collections were continued and the fish were also induced to spawn using GnRHa analogue implants, no further eggs were found in the collection net. This could be due to various reasons and we assume that the spawning season had started earlier probably due to a temperature trigger in May, above the threshold of 24°C. However, bad weather and further temperature drops down to 18°C may have terminated spawning prematurely.

Alternatively, there could also be the possibilities that the fish never reached their final maturity and due to the drastic fluctuations in water temperatures as a result of the inclement and inconsistent weather. Another scenario could be that many eggs were spawned and released however due to the intense currents and changing weather conditions that included multiple fluctuations as explained above, the spawned eggs may have not been collected. It must be remembered that in the wild, fish may migrate to other areas of warm water when such occurrences take place. Obviously in a cage situation this is not possible.

Field and Laboratory Incubation Experiments

Further laboratory analysis and field incubations were then carried out on the different batches. The results of which are so shown in the figure on the next page (**Figure 8**). Hatching rates in all 4 batches were above 80% and even after sea incubation this level was not inhibited. Indicating that sea incubation is not a deterrent to successful propagation hatching. After this, hatching yolk sac larvae (YSL) were continued to be incubated within the laboratory at constant temperature in 24 well plates. The results of these investigations are summarised in **Figure 9**. In the experiments where both laboratory and field incubations in the sea were compared then on the whole there was no significant difference between the results. In fact yolk sac larvae which emerge on hatching were able survive on average for a period of 3 days and some larvae as long as 7 to 8 days. A comparison in batch 2 which was the largest number of eggs collected show that incubated larvae either directly taken from the subsample from the sea or after 24 hours incubation showed no difference in their survival times indicating the acceptance of the method for *in situ* incubation.

Molecular assessment of parentage

After the collection of subsamples of eggs from each of the spawning batches from I to IV an analysis of mitochondrial DNA extracted from these eggs was carried out to determine the number of spawning females in each batch. Only the Mitochondrial DNA from the mother is transferred to each egg and this is therefore possible to estimate the contribution that each mother makes to the total egg production in a given batch of eggs. The results of this analysis are shown in **Figure 10**. It can be seen for instance that mother 8 (M8) contributed to spawning on 4 days whereas mother 9 and 14 only on single days etc. From the full analysis at least 4 females contributed to the spawning of each batch with a maximum of 8 females contributed to batch 1.

It was estimated that, based on the figures obtained from the egg collection numbers, viability and the numbers of contributing mothers and their theoretical maximum egg production value, that 75 million DNA tagged larvae were released back into the sea (see **Table 2**). However, in 2019 we hope to increase the numbers of actually incubated eggs and larvae prior to release, so they can have a better chance of survival when released a further distance away from the broodstock cage, where wild predatory fingerlings and other small fish are not abundant.

4. Discussion

The present pilot study can be seen as the first step in determining the viabilty of incubating naturally spawned tuna eggs *in situ* and then releasing them into the sea. It is well known from previous work in EU Projects (De Metrio *et al.*, 2010; SELFDOTT, 2011 ; TRANSDOTT, 2014) that it is possible to obtain large amounts of eggs from captive wild broodstock. There is also evidence from towing cages Gordoa *et al.*, (2009); Gordoa, and Carreras (2014) that considerable amounts of fertilized eggs can be released and in a recent study Medina *et al*., (2016) suggested that farmed fish which spawn can have a significant impact on the ecosystem and affect ABFT recruitment. The present study has used a wild broodstock that was caught within Tunisian waters not far from Malta. Therefore, the release of the larvae away from the cages are certainly within the geographical area between Malta and Tunisia which is a candidate for spawning ABFT. We estimate that we are only collecting approximately 5% of the eggs which are released as shown in **Table 2.** Fertilization rates are high in all batches and sea incubation did not hamper fertilization and hatching rates. *In situ* net incubation was not as successful as expected, averaging between 30 and 40%. However, this was due to the improvised nature of the net bags used which could have caused abrasion on the fertilised eggs. In 2019, a new model with a supporting frame was successfully tested. Maternal contributions to spawning were easily determined by mitochondrial DNA analysis as in the study of Gordoa *et al.*, (2015) a more detailed investigation using microsatellites was also provided by TRANSDOTT (2014). In the present study a total of 25 spawning females were identified and it was clear that as multi-spawners, some contributed on more than one day to the eggs collected (**Figure 10**). Gordoa *et al.*, (2015) also reported that specific mothers could contribute to up to 8 specific spawning events. Therefore, as can be seen in **Table 2** the number of released tagged eggs is a conservative estimate based on only one spawning event per mother. The actual figure may be considerably larger. At the present time this is only a pilot study to look at the feasibility of

both the theory as well as the difficulties involved in practice. However, through international sponsorship we should in 3 years have a solid data bank for both broodstock and have distributed well over 300 million tagged larvae into the sea. Early egg survival rates in the sea are not known but certainly extremely high mortality rates from mobile predators can be expected against the non- mobile eggs themselves. The YSL are more motile and independent and therefore protecting eggs before and during the hatching phase should increase the chances of survival. Gordoa *et al.*, (2013) have shown that *Pelagia noctiluca* ephyrae are quite capable of feeding on Atlantic Bluefin Tuna Eggs and reports from Japan indicate that small predatory fish species gorge themselves on spawned Pacific bluefin tuna eggs within and around the broodstock cages.

Since Malta is at the center of the tuna ranching industry within the Mediterranean, it is proposed that this will serve as the main sampling point for DNA material from the fisheries within the future. During harvesting, fin clipping from observers could provide material for comparison using SNP´s panels or NGS systems to compare broodstock databanks with farmed material. This novel technique harnesses the ranching industry as a sampling tool for the whole of the Mediterranean. Cost analysis can be defrayed through levies on individual fish which would be only very small fraction of the income per fish. Broodstock maintenance in other centers around the Mediterranean with a limited number of fish together with production cage management, egg sampling and monitoring from the ranching sector could certainly help to support classical and digital tagging systems with molecular techniques such as CKMR and at the same time to give us feedback on the viability of such a restocking/tagging enterprise and provide additional data for stock assessments.

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Table 1. Results of egg collection in 2018 from 4 batches of eggs collected from 21-26 June. Details of egg biometrics and fertilization rates are given.

Year: 2018		Species: *Thunnus thynnus*			
Batch Number		1	2	3	4
Date/Month		21. Jun	22. Jun	23. Jun	26. Jun
Egg Production	Units				
Buoyant eggs	(ml)	400	950	50	100
Buoyant eggs	(no. of eggs)	449.600	1.067.800	56.200	112.400
Fertilized eggs *1	(ml)	400	950	48	10098
Fertilized eggs *1	(no. of eggs)	449.600	1.067.800	53.952	110.152
Egg Quality					
Fertilization rate					
Buoyant eggs	(%)	100	100	96	98
Abnormality rate *2	(%)	14	20	8	2
Abnormal fertilised eggs *2	(no. of eggs)	62.944	213.560	4.316	2.203
Mean Egg diameter (x50 magnification) *3	(mm)	1,106	1,046	1,081	1,089
Mean Oil-glob. diameter (x50 magnification) *3	(mm)	0,292	0,287	0,294	0,292

*1 Morphologically normal and fertilized eggs at the morula stage.

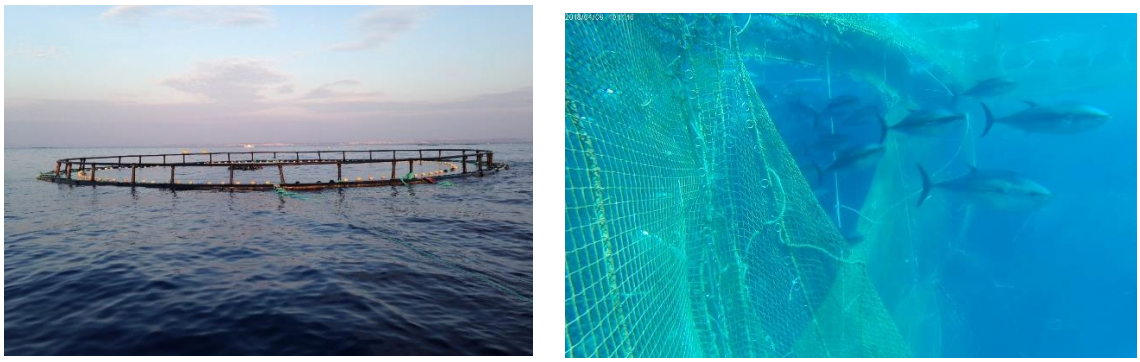
*2 Egg with more than 1 oil-globule.

*3 n = 20

Table 2. Viability of eggs after *In situ* incubation and the calculated release number of tagged eggs. Identification of Mt DNA was used to determine the numbers of contributing mothers.

Viability of Sea Incubated Eggs *Year: 2018*	*Species: Thunnus thynnus*				
Batch Number	1	2	3	4	Cumulative
Date/Month	21. Jun	22. Jun	23. Jun	26. Jun	
Viable (%)	32,3	39,3	/	/	
Dead (%)	67,7	59,7	/	/	
Expt.DNA Tagged larval release	145.221	419.645	/	/	
Theoretical Calculations					
Nr. Collected eggs	449.600	1.067.800	53.952	110.152	1.681.504
Ident. mtDNA Nr. Females	8	7	6	4	25
Calculated Overall					
Tagged Larval Release *	24.000.000	21.000.000	18.000.000	12.000.000	75.000.000
Egg Collection Efficiency %	1,87	5,08	0,30	0,92	8,18

* Estimated 1 female = 3 million eggs per spawn



a) Malta offshore (6km) Broodstock cage **b) Fish Transfer from Towing Cage to Broodstock**

Figure 1. a. Shows the offshore broodstock cage at the MFF facility in Malta. The cage dimensions were 30m Diameter and 20m deep. B. Shows the ICCAT monitored transfer of the broodstock of approximately 80 kg to the broodstock cage.

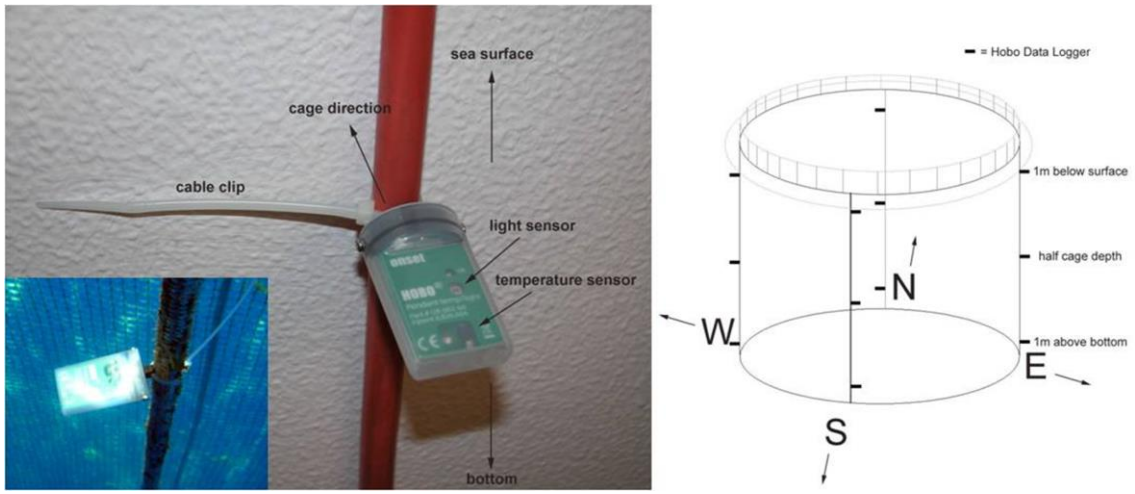


Figure 2. The cage temperature monitoring system conssisting of a light and temperature sensor attached to the cage walls – They can be distributed to all 4 points of the compass and record at intervals of 15 minutes for up to periods of 1 year. In this study only top and bottom temperatures were recorded.

Figure 3. Show an aerial view of a drone above the broodstock cage. The MSC fish feeding box can be see in the centre and also the position of the pelagic egg collector positioned in the main-stream down current.

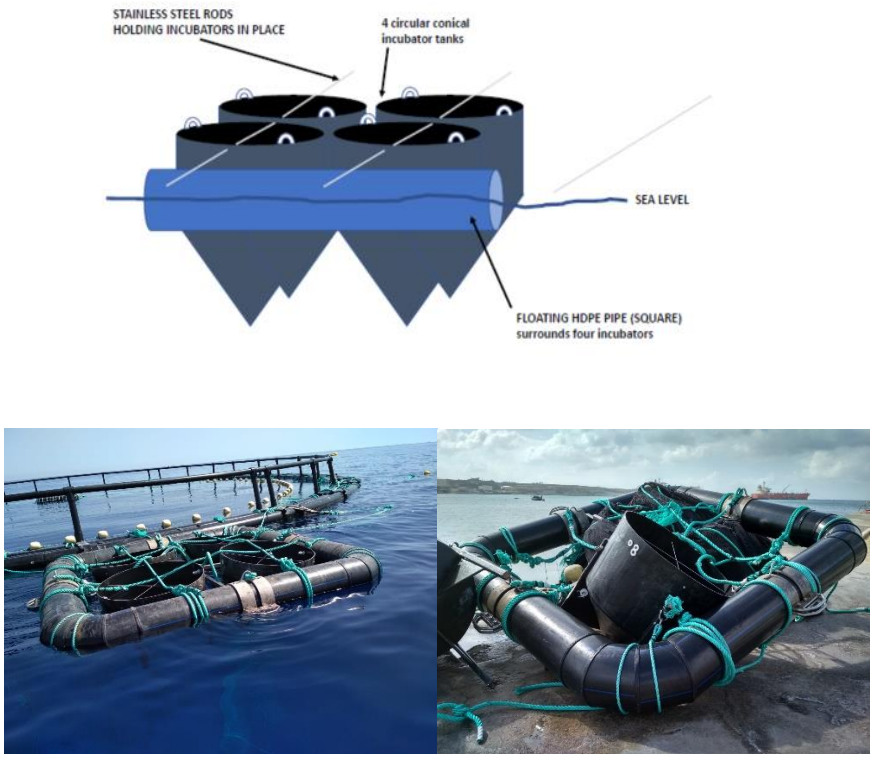


Figure 4. In the top panel a schematic of the *in-situ* prototype egg incubator made up og 4 Artemia rearing tanks anchored together in a floating frame made of HDPE tubing. The lower left panel shows the incubator placed at sea alongside the broodstock cage. The right panel shows the damaged incubator after a Force 6 storm. A too rigid structure was not applicale to this type of mounting and an alternative protocol was used.

Figure 5. Alternative floating net *in situ* egg incubator. Eggs are deposited in the weighted net and hung vertically in the water. They were incubated for just over 24hrs and then reseampled for laboratory control and released. A plastic supporting frame has since been added recently to prevent net rubbing and a Hobo data logger was used to record temperature.

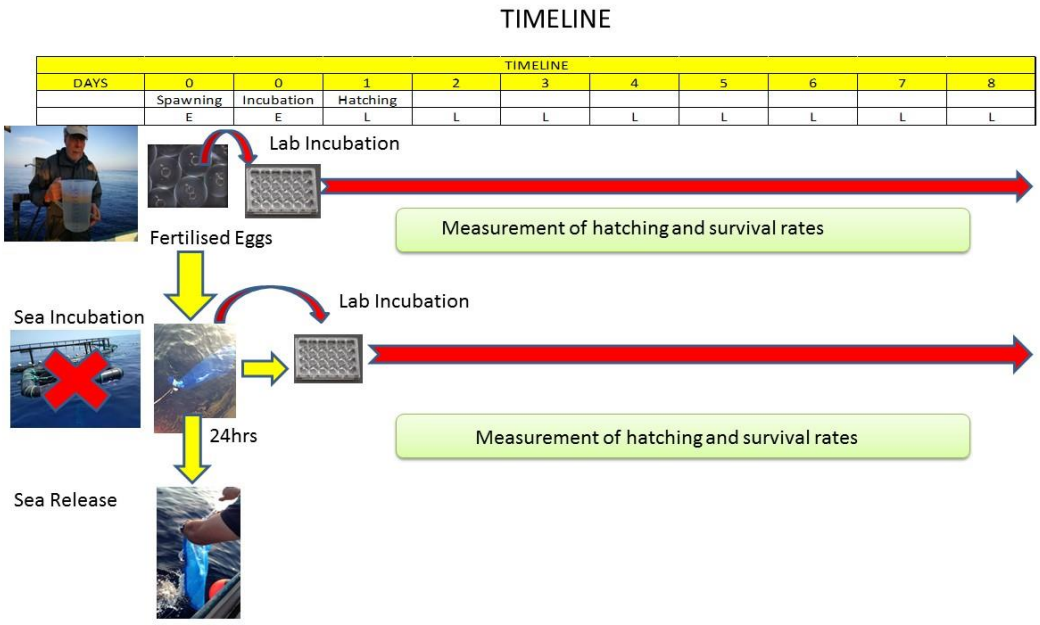


Figure 6. Laboratory protocol schematic showing on the left the collection od the eggs at sea then subsampling for the laboratory incubation on 25 well plates at constant temperature in oxygenated water. The rest of the eggs are incubated in the net incubators for 24hrs and then again subsampled and incubated in the lboratory. Hatching rates and times are recorded and the progress of the yolk-sack larvae was continued to be observed for up to 8 days. No feeding of the larvae was carried out during these incubations.

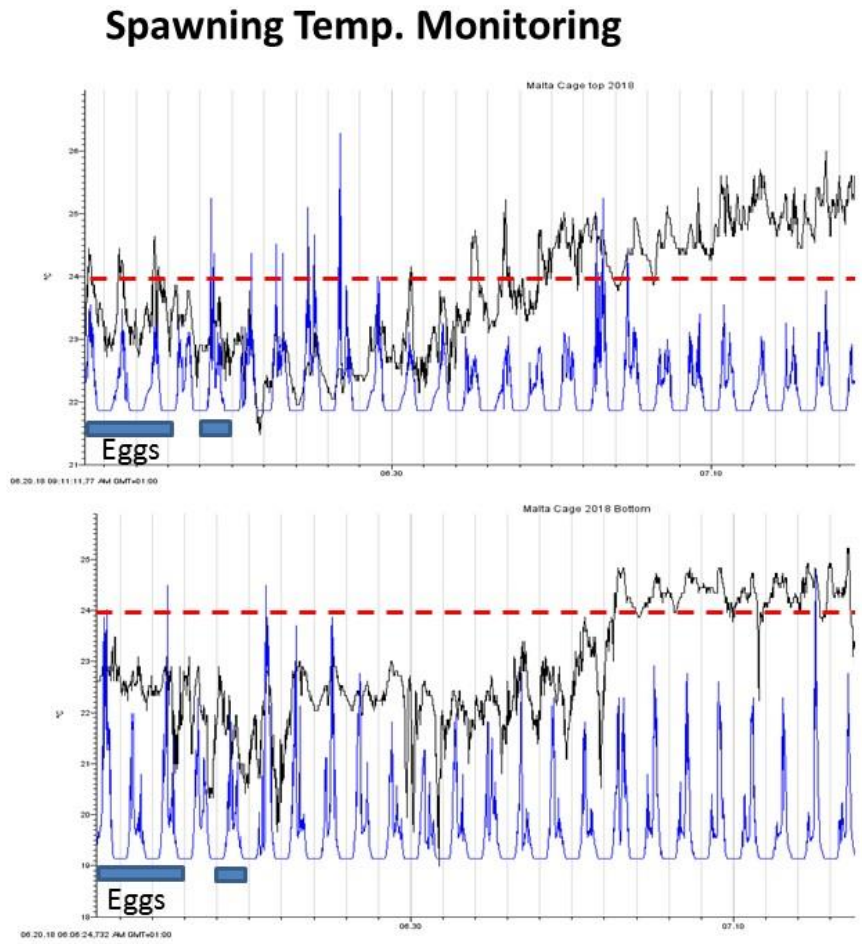


Figure 7. Broodstock cage temperature (black) and light (blue) monitoring data from "Hobo" data loggers set at the surface (top panel) and bottom of the cage (bottom panel). Measurements were made at 15 minute intervals and the 24° C temperature level is indicated by the red dotted line. Eggs were collected at the 20th June and the daily increases in light due to sunrise and sunset can be clearly seen.

Figure 8. Hatching rate of the different Batches from 1-4 are shown in the diagram with laboratory rates in yellow and sea and laboratory incubation rates in blue. Sea incubation involves maintenance of the eggs within net incubators placed next to the broodstock cage for 24 hours and then placed in 25 well plates in the laboratory for hatching determination.

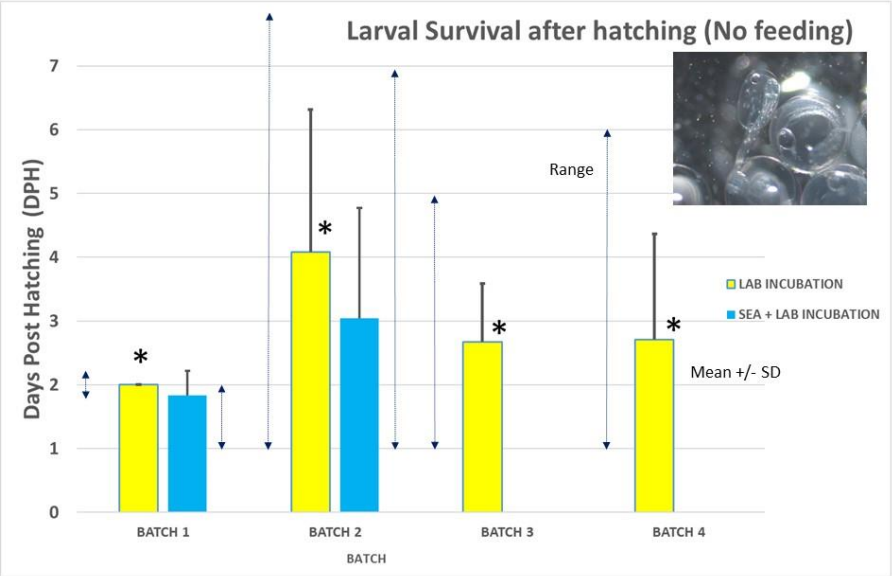


Figure 9. Depicts the survival rates of the hatched larvae (YSL) for both laboratory and *in situ* incubated eggs after retrieval. The overall survival rate was approximately 3 days with a range from 1 to 8 days. Only batch 2 was significantly different in survivability compared to the other batches and there was no difference between survival rates of laboratory incubated and *in situ* incubated eggs and larvae.

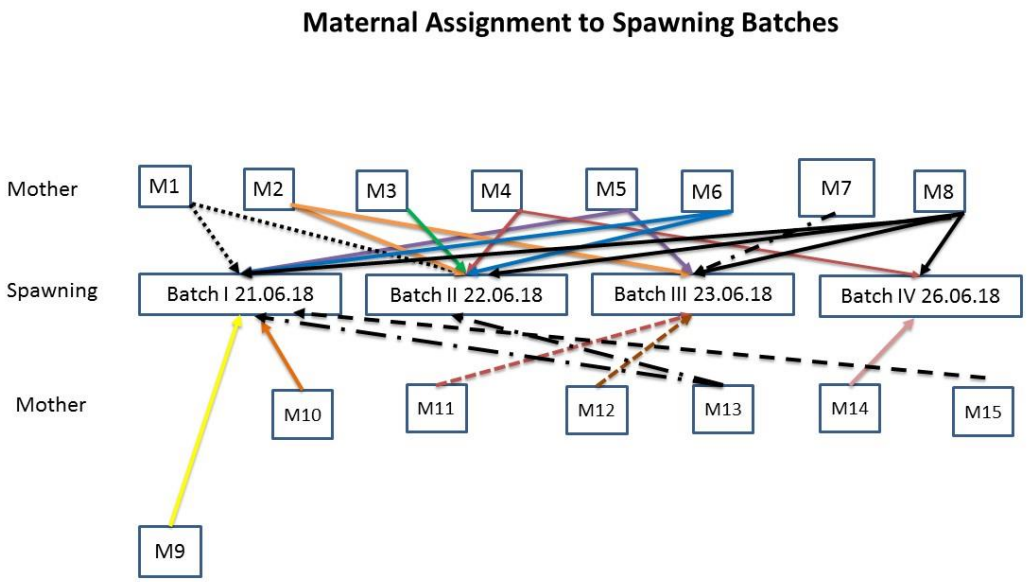


Figure 10. Gives an example of the maternal assignment of specific mothers (M1-M15) to specific spawning events (Batch I-IV). e.g. for example mother M 8 spawned on four days and Mother´s M2, M4, M5 and M13 on two days.