Deliverable Report

Deliverable No:	D7.4	**Delivery Month:**	38
Deliverable Title	Protocol for shipping grey mullet eggs		
WP No:	7	**WP Lead beneficiary:**	P4. IOLR
WP Title:	Reproduction and Genetics-grey mullet		
Task No:	7.5	**Task Lead beneficiary:**	P25. DOR
Task Title:	Establish a shipping protocol for grey mullet eggs		
Other beneficiaries:	P4. IOLR		
Status:	Complete	**Expected month:**	36
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Other Scientists participating: H. Rosenfeld (IOLR)

OBJECTIVE: Establish a shipping protocol for grey mullet eggs.

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DELIVERABLE DESCRIPTION (from the DOW)

Protocol for shipping grey mullet eggs (*Mugil cephalus*): Establishment of procedures for handling grey mullet eggs in order to allow their transport from Israel to larval rearing facilities around the Mediterranean Sea or short term transports within a country. The deliverable tested a protocol, based on the transport of Atlantic bluefin tuna (*Thunnus thynnus*) eggs, at two grey mullet egg concentrations and seawater volumes in order to achieve the successful arrival at the destination in terms of percent viable eggs, hatching success and pre-larval survival at the end of the hatching day or 0 days post hatching (dph).

INTRODUCTION

The development of a protocol for the transferring of grey mullet eggs to the various partners in the DIVERSIFY project was based on methodology developed earlier by **P4. IOLR** for shipping Atlantic bluefin tuna (BFT) eggs to different Mediterranean partners (Greece, Spain, Malta, Italy) in the EU 7th framework projects SELFDOTT (212797) and TRANSDOTT (311904) (De la Gándara 2012, Bridges, 2014). In brief, this protocol recommends the stocking of 10 l of filtered (10 µm) seawater with 10-15,000 gastrula-stage BFT eggs l^{-1} in a 20 l cubitainer (stiff, translucent, plastic 6 sided, square bottom containers used in the wine industry), which is placed in a Styrofoam container (**Fig. 1a**). Pure oxygen is added to supersaturate the container seawater as well as flushing the air layer in the upper part of the cubitainer. One to two ice packs, wrapped in cardboard, are placed adjacent to the air-oxygen layer (not against the water layer) and the package closed (**Fig. 1b**). Transport by air from Spain or Malta to Israel, which was the longest transit time among the partners, included two flights, custom documents arranged prior to arrival that facilitated entry at Ben Gurion International airport and taxi transport that brought the shipment from Tel Aviv to the IOLR (4-5 h) in Eilat. Total transit time from egg collection at sea to arrival at the Eilat facility during the SELFDOTT and TRANSDOTT projects ranged between 26-29 h. The eggs did not hatch in transit. During the TRANSDOTT program BFT egg shipments consistently arrived at the Eilat institute in excellent condition where oxygen (>200%), pH (6.5-7.0) and temperature (ca. 22-23 ^{0}C) were within acceptable limits. The eggs were temperature acclimated in the tanks, pH was incrementally increased by dripping a solution of 0.1 N NaOH into the cubitainer to reach a pH of 7.5 to 8.0, the percent of dead sinking and live floating eggs was calculated and the eggs stocked in the experimental system.

Figure 1. (a) 20 l cubitainer and (b) Styrofoam box with ice pack in cardboard used in the past to transport Atlantic bluefin tuna eggs and used in this study with grey mullet eggs.

MATERIALS AND METHODS

In 2015, the long-term egg transport trial simulation was attempted, but the level of egg fertilization was very low and the results are not presented here. This study was successfully repeated in 2016 and is described below. On the other hand, short-term transports of grey mullet eggs within Israel were carried out from partner **P4. IOLR** to **P25. DOR** and one shipment was made from the kibbutz Ma'agan Michael, which is south of Haifa, to the **P4. IOLR.** They are reported here as anecdotal evidence that higher amounts of eggs and egg stocking densities can be carried out provided that transport times are markedly shorter.

2014-Deliveries to P25. DOR fish farm

During 2014, three shipments of mullet eggs were made to **P25. DOR** fish farm from **P4. IOLR** and one shipment was made from the kibbutz Ma'agan Michael to the **P4. IOLR**. A modified BFT protocol was employed where the final volume of the water in the 20 l cubitainer was 15 l and egg density ranged from 55-84,000 gastrula-stage eggs l^{-1}. Pure oxygen was added to supersaturate the container seawater as well as flushing the air layer in the upper part of the cubitainer. One to two ice packs, wrapped in cardboard, was placed adjacent to the air-oxygen layer (not against the water layer) and the package closed. **Table 1** lists shipping conditions as well as percent oxygen and hatching results at final destination for grey mullet eggs in 2014 from the **P4. IOLR** facility to the **P25. DOR** fish farm. Shipment results of eggs sent from kibbutz Ma'agan Michael to the **P4. IOLR** were also included, which has a slightly longer transit time of 11 h. In contrast, the eggs shipped from the Ma'agan Michael hatchery to **P4. IOLR** utilized similar high egg densities (66-110,000 eggs/l), but were sent using thick plastic bags filled with pure oxygen. Domestic shipments necessitated only a single 1 h internal flight and vehicle transport to the destination. In all shipments, eggs arrived in excellent shape and were used for on-going studies.

Table 1. Shipping conditions as well as percent oxygen and hatching results of grey mullet eggs sent from **P4. IOLR** to **P25. DOR** in 2014 using the BFT egg shipping protocol, as well as eggs sent from Ma'agan Michael (approximately the same transit time as shipments sent to DOR, ~10 h), which were sent in plastic bags in Styrofoam boxes with no ice packs.

Spawning date	Box no.	Egg vol. sent (ml)	Egg number	Total Vol. sent (l)	Eggs/l	% O_2 *	Time in transit (h)	% hatching*
	Eggs sent from IOLR to DOR							
16.8.14	1	350	1.26×10^6	15	84000	272	9.0	85
16.8.14	2	250	0.825×10^6	15	55000	280	9.0	85
3.10.14	1	250	1.6×10^6	15	55000	330	10.5	90
3.10.14	2	250	1.6×10^6	15	55000	330	10.5	90
16.10.14	1	350	1.26×10^6	15	84000	265	9.0	85
	Eggs sent from Kibbutz Ma'agan Michael to IOLR							
14.8.14	1	500	1.65×10^6	15	110000	270	11	96
14.8.14	2	300	99000	15	66000	265	11	97

*Measured at arrival destination

2016-long term transport simulation

The eggs used in this study were spawned at 05:00 on November 2, 2016, with a diameter of 796.3 µm±24 (n=34) in 40 ‰ seawater at 25.2°C. The eggs were stocked at gastrula 1(G1) in 12 translucent rectangular

shaped cubitainers in order to test two egg densities (10,000 and 15000 eggs l^{-1}) in two cubitainer seawater volumes (10 and 15 l) in triplicate replicates and is described in **Table 2**. Pure oxygen was added to each container and a data logger was placed in one of the cubitainers. All cubitainers were placed in a Styrofoam boxes and two icepacks were placed on the upper part of the cubitainer where there is air and no water (**Fig. 1**). All boxes were then placed in a non-air conditioned room (25°C) and were gently and periodically shaken (every 90 min during the day). After 26 h the containers were opened and the ammonia, oxygen, temperature and pH were measured. In addition, from each cubitainer, eggs were taken for (1) examination under a microscope and (2) stocking in 5 ml well plates (1-2 eggs $well^{-1}$; n=3), which were placed for 17 h in an incubator at 22.4°C in order to determine percent (%) hatching and survival at the end of 0 dph (**Fig. 2**).

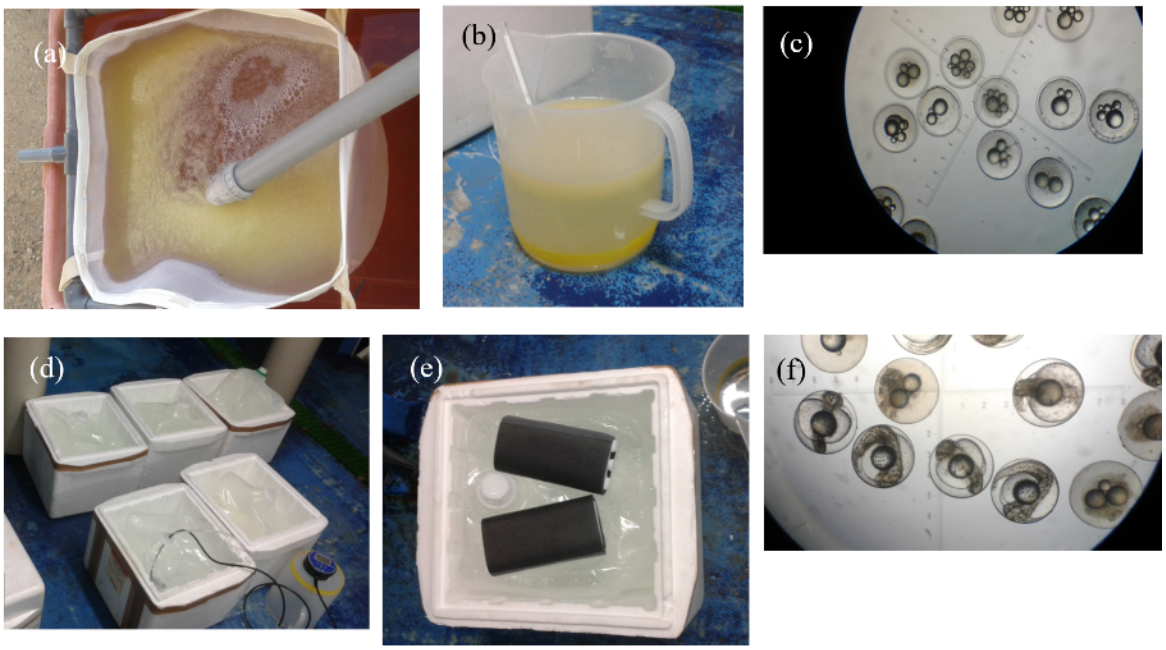

Figure 1. Grey mullet eggs (a) in a collector beside spawning tank, (b) separated into viable and non-viable eggs, (c) stocking at G1 stage in (d) cubitainers with (e) ice packs and (f) after 26 h of simulated transport.

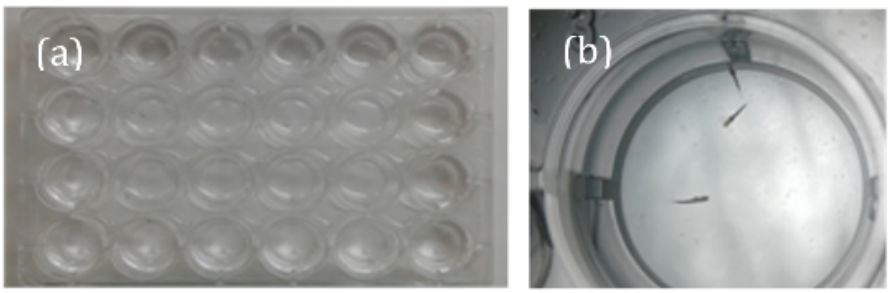

Figure 2. (a) 24 well plate where 1-2 grey mullet eggs were stocked in each 3 ml well and (b) hatched pre-larvae in the well.

Table 2. The egg simulated transport treatments testing two densities and two water volumes.

Treatments	Eggs (x10,000)/l	Water Volume*	Total eggs
1	10	10	100,000
2	15	15	225,000
3	10	15	150,000
4	15	10	150,000

*40 ‰ filtered, ambient sea water.

RESULTS AND DISCUSSION

The egg stocking and water parameters before closing of the cubitainers of the different treatments and the opening of them at the end of 26 h of transport simulation or 34 h after spawning are shown in **Tables 3 and 4**, respectively. The results largely support the application of the bluefin tuna egg transport protocol for the long-term shipment of grey mullet fertilized eggs. The **Fig. 3** graphically demonstrates that the ice packs maintained the cubitainer temperature, without any major fluctuations, at ca. 22.5°C in a 25°C room after 26 h, which is a minor decrease from the stocking temperature of 23.4°C. The requirement for packaging that insulates and reduces temperature fluctuation during transport is critical. This is because egg shipments will be exposed to a range of temperatures in the plane's cargo bay and airport holding facilities during and between flights as well as when transported by land to the end user. Temperature increase during egg incubation can be a source of stress on the eggs and affect mortality (Wagner et al. 2009; Thépot and Jerry, 2015), hatching patterns (Laurel and Blood, 2011, Thépot and Jerry, 2015) and larval development after hatching (Jobling, 1997; Laurel and Blood, 2011. The egg treatments in addition to demonstrating relatively consistent temperature during simulation also demonstrated, at the end of the trial, high saturated oxygen levels (>295%) and a moderate reduction of approximately 1pH unit (7.03± 0.1) from ambient sea water (8.0) (**Table 4**). Nevertheless, in treatment 2, which tested the effect of the high egg density and seawater volume (15,000 egg l^{-1} in 15 l) and consequently the greatest number of eggs (225,000 eggs $cubitainer^{-1}$), showed significantly (P=0.0001) higher total ammonia nitrogen (TAN) compared to the other treatments. The TAN is a measurement of the end product of protein catabolism and is comprised primarily of both ionized (NH_4+) and non-ionized ammonia (NH_3) (**Fig. 4**; Wajsbrot et al.1993). The ionized form is more permeable to biological membranes and is many times more toxic than the ionized form. Although there are reports that non-ionized NH_3 is more detrimental to first feeding larvae than yolk-sac larvae and eggs (Rice and Stokes, 1973), the results of the present study suggest a mullet egg sensitivity to this metabolite. The excess unionized ammonia (NH_3) of treatment 2, although not directly measured, was responsible for the marked (P=0.0001) reduction of viable eggs (**Fig. 5**), hatching success (**Fig. 6**), and larval survival to the end of 0 dph (**Fig. 7**). Elevated concentrations of ammonia affected hatching success as well as larval deformity and survival in Nile tilapia (*Oreochromis niloticus*; El-greisy et al 2016) and red drum (*Sciaenops ocellatus*; Holt and Arnold, 1983). It is conceivable that the ammonia was produced from water borne bacteria but a high proportion of viable eggs, good hatchability and survival was also shown in the treatment with 10,000 eggs l^{-1} in a cubitainer volume of 15 l, consequently, it was concluded that the excess ammonia was produced by the eggs, particularly during later development close to hatching (Rønnestad et al., 1994).

Taken altogether, the results suggest that the bluefin tuna protocol for egg transport can be successfully applied to the shipment of live grey mullet eggs (gastrula 1), provided that cubitainers stocked with a maximum of 15,000 eggs l^{-1} should not exceed a total sea water volume of 10 l (total of 150,000 eggs). This procedure will likely ensure, after a 26 h transit time, a high proportion of viable eggs with a good hatching rate and survival to the end of 0 dph.

Table 3. Stocking and water parameters before closing of cubitainers at time 0.

Treatment	Cubitainer no.	Temperature (°)	pH	Oxygen (%)	Total eggs	No. liters
1	1	23.8	8	301	100,000	10
	2	23.8	8	289		
	3	23.8	8	298		
2	4	23.8	8	300	225,000	15
	5	23.8	8	295		
	6	23.8	8	302		
3	7	23.8	8	301	150,000	15
	8	23.8	8	295		
	9	23.8	8	300		
4	10	23.8	8	335	150,000	10
	11	23.8	8	342		
	12	23.8	8	316		

Table 4. Water parameters at the end of 26 h in the cubitainers from each of the treatments and 34 h after spawning.

Treatment (eggs/l in final volume)	Cubitainer no.	Temperature (°C)	pH	Oxygen
10,000 eggs/l in 10 l	1	22.3	7.03	235
	2	22.5	6.95	195
	3	22.2	7.01	199
15,000 eggs/l in 15 l	4	22	7.0	200
	5	21.9	6.99	220
	6	22.5	7.01	290
10,000 eggs/l in 15 l	7	22.3	7.03	181
	8	22.2	6.8	200
	9	22.3	7.17	149
15,000 eggs/l in 10 l	10	22.5	7.05	195
	11	22.1	7.2	196
	12	22.2	7.15	203

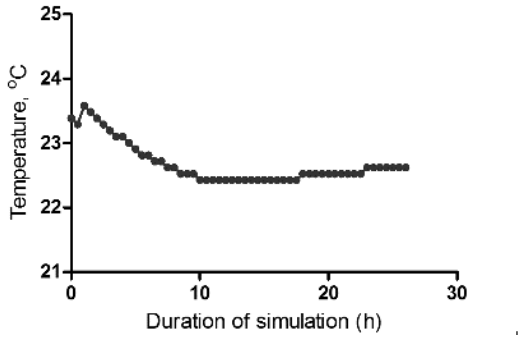

Figure 3. The effect of simulation time (h) on the seawater temperature (°C) in the cubitainer that contained the data logger.

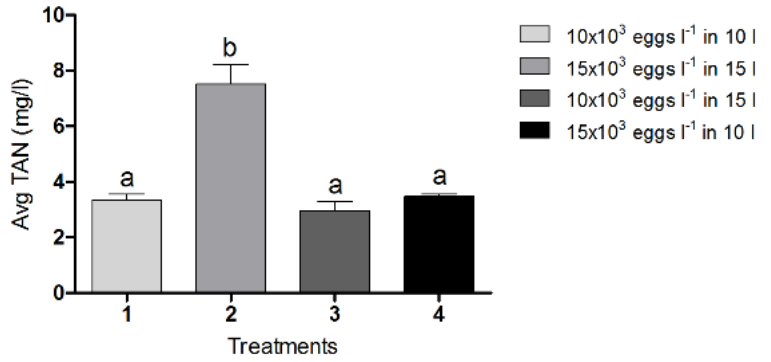

Figure 4. Average (Avg.) total ammonia levels (TAN) (mg/l) in egg sea water after 26 h of simulation in the 4 stocking and volume treatments. TAN values with different letters were significantly (P<0.05) different.

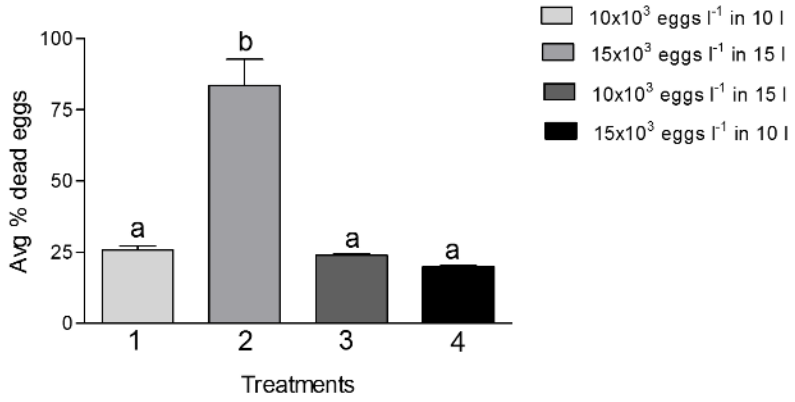

Figure 5. Average (Avg) percent (%) dead eggs after 26 h of simulation in the 4 stocking and volume treatments. Dead egg percentages with different letters were significantly (P<0.05) different.

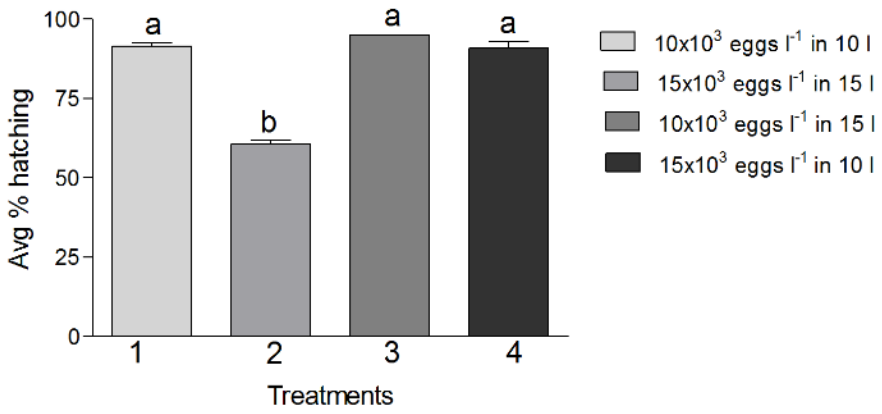

Figure 6. Average (Avg) percent (%) hatching after 26 h of simulation in the 4 stocking and volume treatments. Hatching percentages with different letters were significantly (P<0.05) different.

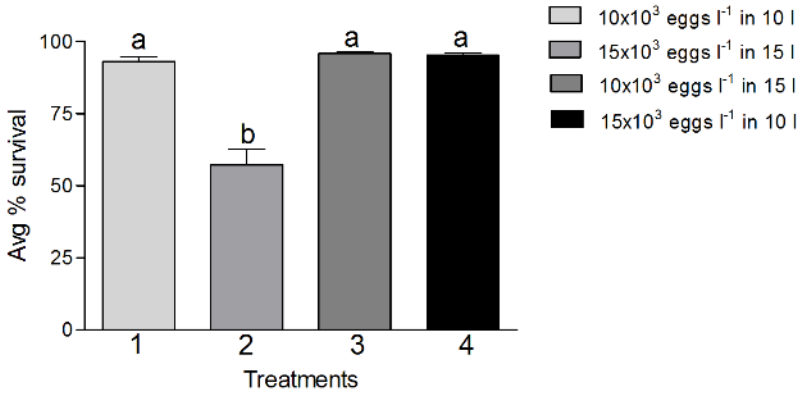

Figure 7. Average (Avg) percent (%) survival to the end of 0 dph in the 4 stocking and volume treatments. Hatching percentages with different letters were significantly ($P<0.05$) different.

CONCLUSIONS

1. Short term shipping (≤1 h) of gastrula stage, grey mullet eggs with very high egg density (55-84,000 eggs l^{-1}), can be carried out using cubitainers or strong plastic bags together with the addition of pure oxygen. One or no freezer packs may be sufficient as long as the shipment does not encounter temperature extremes.

2. Long term shipping (26 h) employing the Atlantic bluefin tuna protocol for gastrula stage egg transport can be successfully applied to the shipment of live grey mullet eggs (gastrula 1), provided that cubitainers stocked with a maximum of 15,000 eggs l^{-1} should not exceed a total seawater volume of 10 l (total of 150,000 eggs).

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DEVIATIONS:

Due to poor egg quality and fertilization rates in 2015, the egg shipment protocol was not thoroughly tested during the first reporting period and a partly-completed deliverable was first submitted on 30 November 2015 [Ares(2015)5460003]. The study was successfully repeated and completed in December 2016 and is reported here.

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