Advances in Meagre (*Argyrosomus regius*) Research in 2014

During the first year of the project (Dec 2013-Nov 2014), a variety of research activities have been undertaken with meagre, and a summary of the most relevant results is provided below.

1. Reproduction

The genetic variation of a large number of the available captive meagre broodstocks of different research institutes and SMEs around Europe has been carried out by Fundacion Canaria Parque Cientifico Tecnologico de la Universidad de las Palmas de Gran Canaria (FCPCT, Dr. J.M. Afonso) using 2 multiplexes. The examined broodstocks appeared to come from three populations and across all stocks had sufficient genetic variation to form a base population for a breeding program (Fig. 1). However, care will be needed in selecting families to avoid problems and ensure improvement of desirable traits.

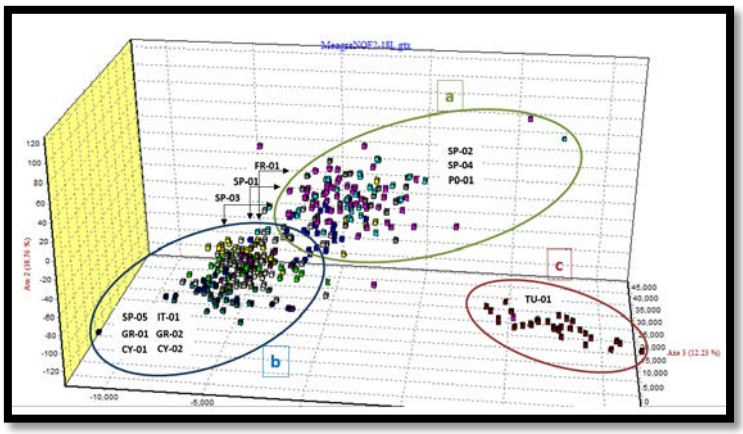


Fig 1.- Graph of Factorial Correspondence Analysis from 18 loci and 376 fish distributed in 13 Mediterranean populations of meagre maintained in captivity for research or aquaculture production.

Paired crossing with six pairs of matured females (21.2 kg) and males (16.1 kg) was carried out in Institute de Recerca I Technologia Agroalimentaries (IRTA, Dr. N. Duncan). Spawning was induced with GnRHa injections (15 µg Kg^{-1} for females and 7.5 g kg^{-1} for males) every 7-10 days. Breeders that did not spawn after 2-3 induced spawning attempts were replaced. A total of 41 different pairs were induced to spawn, of which 10 pairs produced >500,000 eggs, 16 pairs produced >250,000 eggs and 19 pairs produced >100,000 eggs that hatched (Fig. 2). Poor spawning results were not caused by maturity status, repeated spawning or inductions, and different individuals had clear differences in egg production and quality.

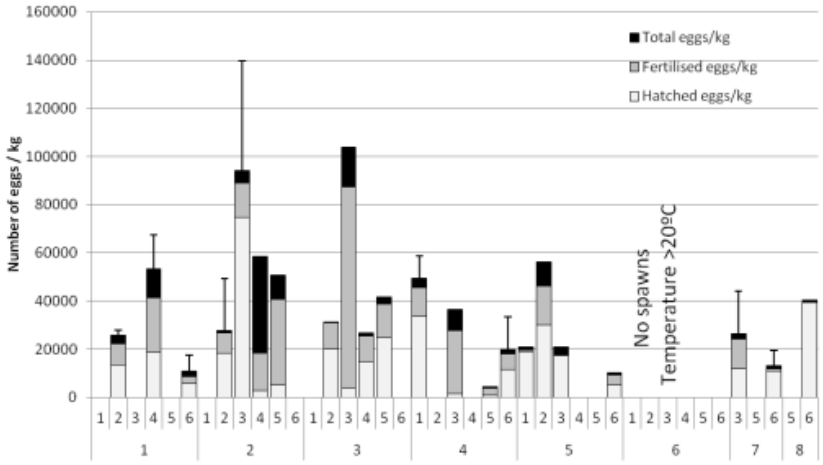


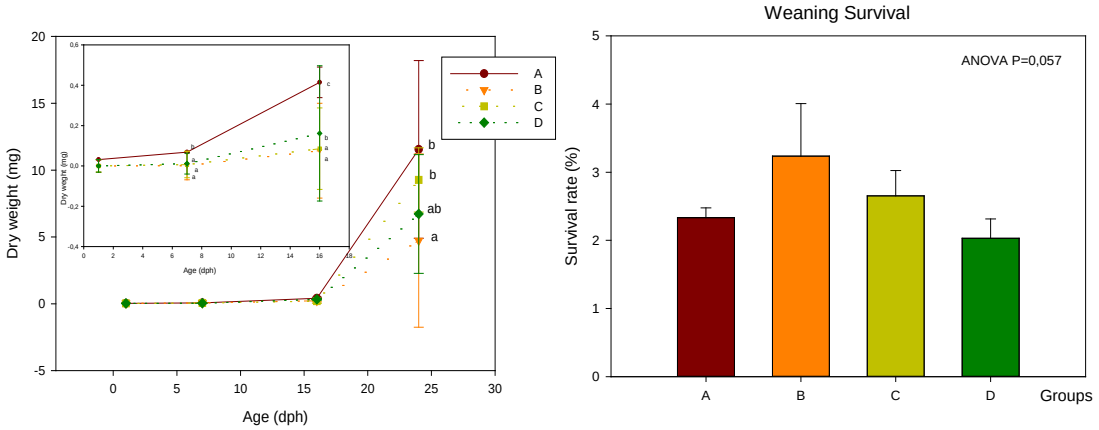
Fig 2.- Mean (± SD) daily number of meagre eggs per kg produced in response to multiple GnRHa injections. Total number of eggs was multiplied by percentage fertilization and hatch to determine number of fertilized eggs and eggs that would hatch

An additional experiment was also carried out at the Hellenic Center for Marine Research (HCMR, Dr. C.C. Mylonas) with four pairs of breeders to determine how many successful spawns can be produced in response to consecutive weekly injections of GnRHa. Up 17 consecutive spawns were obtained with high quality eggs that had >80% hatching success and larval survival to 5 days post hatch. These two trials demonstrated that paired spawning of high quality eggs is possible, and the method could be used in breeding selection programs.

Photograph 1.- Taking an ovarian biopsy to determine stage of maturity

2. Larval culture

A weaning assay was carried out in IRTA (Dr. A. Estevez) to advance the time for weaning in meagre. Larvae were weaned either at age 12, 15 and 20 (the usual age) days post hatch (dph) using half the amounts of enriched *Artemia* metanauplii and a commercial weaning diet (Gemma Micro, Skretting). Growth (Fig. 3), survival rate (Fig 4), fatty acid composition as well as digestive system development (histology and enzymes) were analysed. A high incidence of cannibalism was detected from day 12 dph onwards, resulting in very low survival (2-3.3%). The experiments will be repeated in 2015 and several new approaches will be taken, including increasing the photoperiod to give more chances of the fish to eat the weaning diet or increase the initial stocking density.



Figs 3 and 4.- Growth (dry weight in mg) and survival (%) of larvae of the different groups after weaning

3. Nutrition

A trial was conducted by FCPCT (Dr. L. Robaina) to investigate the requirements of meagre larvae for n-3 HUFA and its nutritional interrelation with vitamin E (vit E) and vitamin C (vit C). After feeding the larvae with different combination levels of n-3 HUFA (0.5% and 3.5%) and vit E and vit C (150 vit E+180 vit C, 300 vit E+180 vit C and 300 vit E+360 vit C) from day 14 to 28 dph, results showed a clear improvement in growth, particularly body weight, when dietary HUFA levels were raised from 0.5 to 3.5%, whereas the effects of vit E or vit C and the interaction between both nutrients and the n-3 HUFA levels were not significant. Regarding biochemical composition, larval contents of n-3 HUFA reflected clearly dietary levels, being significantly higher in larvae fed fish oil, and elevation of dietary HUFA and vit E+vit C tended to increase larval lipid contents. Study of larval foregut histological characteristics showed that larvae fed 0.5% HUFA presented very pigmented enterocytes with centered nucleoli and very little lipid vacuoles while larvae fed higher levels of dietary HUFA, such as in the 3.5/150/180 combination, showed larger and more developed enterocytes containing lipid vacuoles around the nucleous, reflecting the higher lipid absorption activity. These results suggest that there is a high requirement of this species for HUFA to promote growth and vit E and vit C to prevent fatty acid oxidation during larval stages. Thus, weaning diets for larval meager must be supplemented with increased n-3HUFA, vit E and vit C in order to be improved. Selected diets were used to conduct studies on resistance to handling stress, stress bio-markers such as gene expression of HSPs (FCPCT), specific fish behaviour, evaluation of metabolic cost after sub-lethal stress, video analysis of activity, escape responses and sensory acuity (Danmarks Tekniske Universitet, Dr. Ivar Lund) and digestive enzyme (protease, amylase and lipase) and gut ATPase activities (University of La Laguna, Dr. Covadonga Rodriguez).

The essential fatty acid requirements will be examined in grow out diets (Skretting Aquaculture Research Center, Dr. Ramon Fontnillas) for meagre by feeding six levels of docosahexaenoic, eicospentaenoic and araquidonic acids (FCPCT). During the last three months of this reporting period, information on the nutritional requirements of meagre and related species have been collected and a basal diet formulation has been defined.

Table 1.- Culture performance and morphometric parameters of larval meagre (initial total length 4.07±0.26 mm and dry weight 0.058±0.01 mg) fed early weaning diets containing several n-3 HUFA, vitamin E and vitamin C levels from 14 dph to 28 dph.

	Diet					
	0.5/150/180	**0.5/300/180**	**0.5/300/360**	**3.5/150/180**	**3.5/300/180**	**3.5/300/360**
Total length (24dah)	4.754±0.44^{b}	4.999±0.39^{a}	4.906±0.40^{ab}	4.955±0.45^{a}	4.964±0.48^{a}	5.055±0.38^{a}
Total length (28dah)	5.155±0.46^{ab}	5.198±0.43^{ab}	5.139±0.51^{ab}	5.290±0.44^{a}	4.969±0.31^{b}	5.340±0.59^{a}
Dry weight (24dah)	0.192±0.04^{c}	0.208±0.02^{bc}	0.202±0.03^{bc}	0.207±0.02^{bc}	0.223±0.02^{ab}	0.238±0.03^{a}
Dry weight (28dah)	0.233±0.02	0.214±0.04	0.207±0.03	0.267±0.05	0.234±0.05	0.244±0.04
Survival (%)	12.09±4.96	8.04±5.20	15.12±4.14	14.16±8.29	16.68±3.45	15.16±7.67

*Values (mean ± standard deviation) with the same letters are not significantly different; ANOVA, $P_{Length} < 0.01$; $P_{Weight} < 0.05$.

4. Ongrowing

Size variability in juvenile pre-grow out makes regular grading essential to avoid cannibalism, and grades of smaller fish may be related to poor performance when transferred to sea cages. Experiments were carried out by IRTA using meagre juveniles of a mixture of 5 known families, to simulate the commercial hatchery situation and in order to study differences in growth rate. Juveniles were stocked into tanks at the same initial density and fed the same commercial diet. After 4 months the distribution of all the size grades across the different tanks / grades was compared and 70% of the population was observed to be in the size range of 15-30 g (Fig 5).

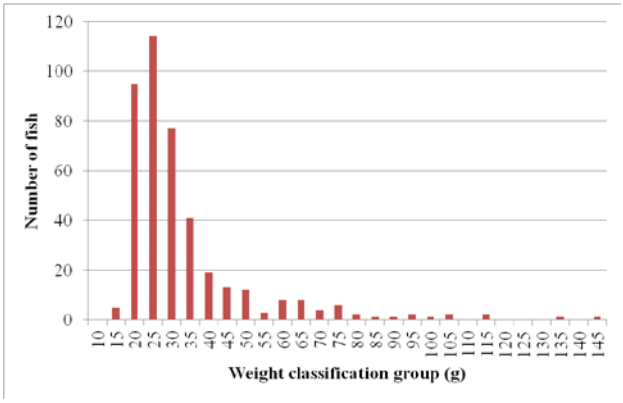


Fig 5.- Frequency distribution of fish in each 5-g size classification. The weight shown is the upper value of the classification, for example classification 15 g contains fish from 10.1 to 15 g.

The population was skewed to larger fish with 30% of the population in the range of 30-145 g and this wide dispersion of sizes made management difficult. The normally distributed 70% of the population was graded into three grades of 73 large fish (25-30 g), 89 medium fish (20-25 g) and 86 small fish (15-20 g) and growth was monitored.

A random sample of 50 fish from each group was weighed and measured (length) every 3 weeks. The large fish have grown from 27.2±1.5 g to 113±21.0 g, medium fish have grown from 22.7±12.2 g to 94.2±19.8 g and small fish have grown from 17.9±1.8 g to 71.6±31.31 g (Fig. 6). On all sample dates there have been significant differences ($P<0.05$) between the grades and the fish in each group

have grown significantly (P<0.05). The different size grades appear to have very similar growth potential. The trial finished on 11th December 2014 and the fish will be characterised genetically for parentage assignment (HCMR, Dr. C. Tsigenopoulos) to establish if differences in growth were a consequence of genetic origin.

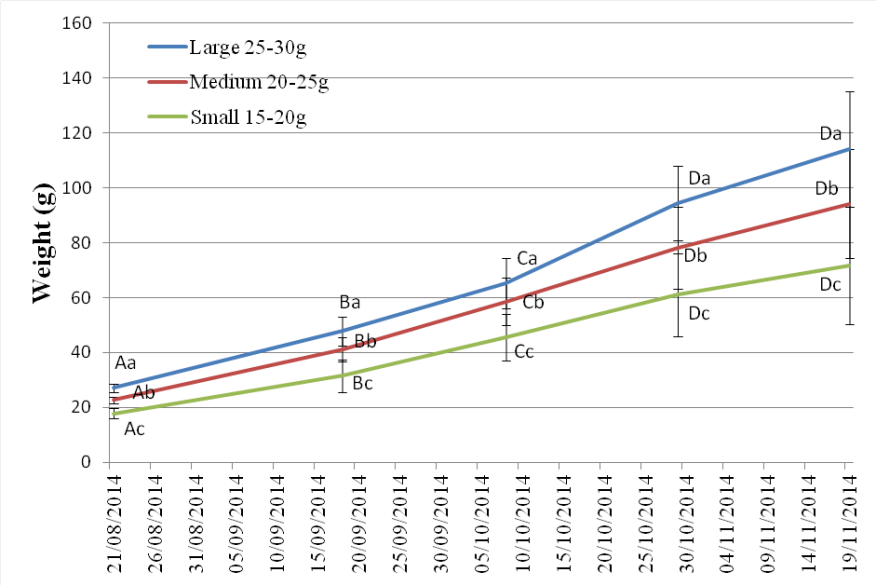


Fig 6.-Growth, mean ± SD wet weight (g) of the juveniles classified to three grades large (initially 25-30 g), medium (initially 20-25 g) and small (initially 15-20 g). These fish represented 70% of the population from five spawns on two different dates. Capital letters represent significant differences (P<0.05) between sample dates for the same size grade. Lower case letters represent significant differences (P<0.05) between size grades on the same sample date.

Photograph 2.- Sampling growth of meagre juveniles

The effect of cage depth on meagre ongrowing was studied by HCMR. The trial started in May 2014 using cages of 180 (6x6x5) and 290 (6x6x8) m^3 at the HCMR Souda Bay pilot farm (Dr. N. Papandroulakis) in duplicates indicated as Shallow and Deep. Fish origin was the hatchery of HCMR. Eggs were from a single spawning and larval rearing was performed at the Mesocosm hatchery. Juveniles of 2 g were transferred at the cage facility and they were reared under similar conditions until the beginning g of the trial. Then, 4 groups were created, two of ~5,150 for the 180-m^3 cages and two of ~8,240 for the 290-m^3 ones. The wet weight at the beginning of the trial was 200±20 g. As the duration of the trial was planned to be 8 months, its termination is expected by the beginning of 2015. During this period, growth performance is estimated with monthly samples (Fig. 7)

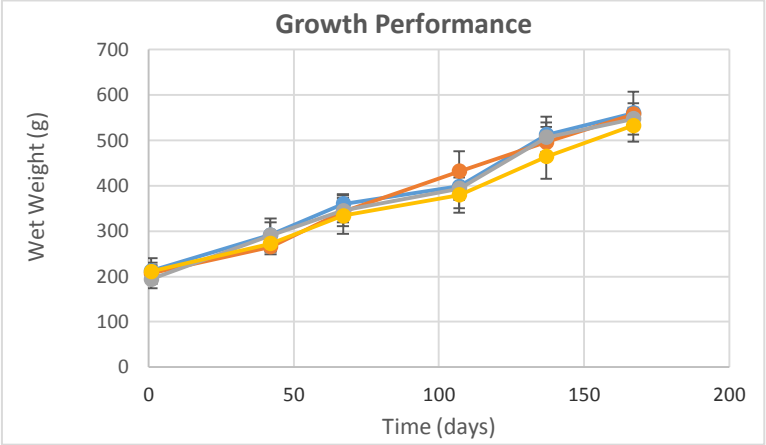


Fig 7.- Mean (±SD) growth performance, mean weight, of meagre a the Souda Bay pilot farm.

Every second month, blood samples have been taken for haematological (hematocrite, hemoglobin), biochemical (osmotic pressure, glucose, lactic acid, free fatty acids), immunological (lysozyme, myeloperoxidase serum) and hormonal (cortisol) evaluation. The samples are currently being analyzed.

The vertical distribution in cages is monitored using an echo integrator. Although a technical problem has not allowed the monitor during the first month of the trial, an upgraded system (CageEye 1.3, Lindem Data Acquisition AS, Norway) was installed in June 2014 and the trial is implemented as planned without further alterations. The analysis of the data is not completed yet, but an interesting observation has been already made. The vertical distribution of meagre shown for a period of 3 days (Fig. 8), demonstrates clearly that the fish are located mostly at the lower half of the cage for a period of ~12 hours, while the rest of the period are distributed almost homogeneous in the whole available volume of the cage. This observation is independent of the cage depth and it is correlated with the light and dark period of the day. To our knowledge this is the first time that such behavior has been observed.

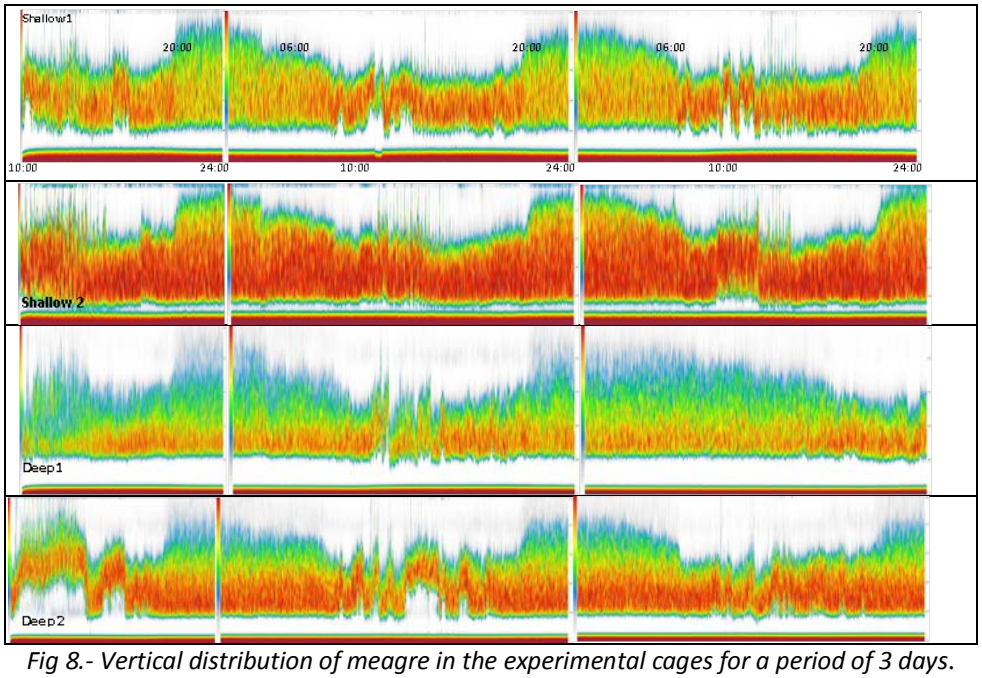


Fig 8.- Vertical distribution of meagre in the experimental cages for a period of 3 days.

5. Health

Meagre were sampled for collecting data on specific growth rate and to collect chronological samples for the immune ontogeny study. Duplicate sets of samples were collected at each time point (Fig. 9); one set was fixed in formalin for histological analysis, and a second set was collected in RNAlater for extraction of RNA to be used in gene expression analysis. As fish became more developed and organ tissues were easily recognized individual tissue samples were collected in formalin and RNAlater. Tissues collected were spleen, head kidney, gills, and intestine. Samples for immune gene expression analysis are being stored at -80°C. Our original plan was to collect animals that were of a medium size, as well as animals from the larger end of the growth spectrum to see how differential growth may lead to premature immune maturation. We eliminated this idea due to a reduction in the overall size of the population. The original population was diminished greatly due to cannibalism during the growout.

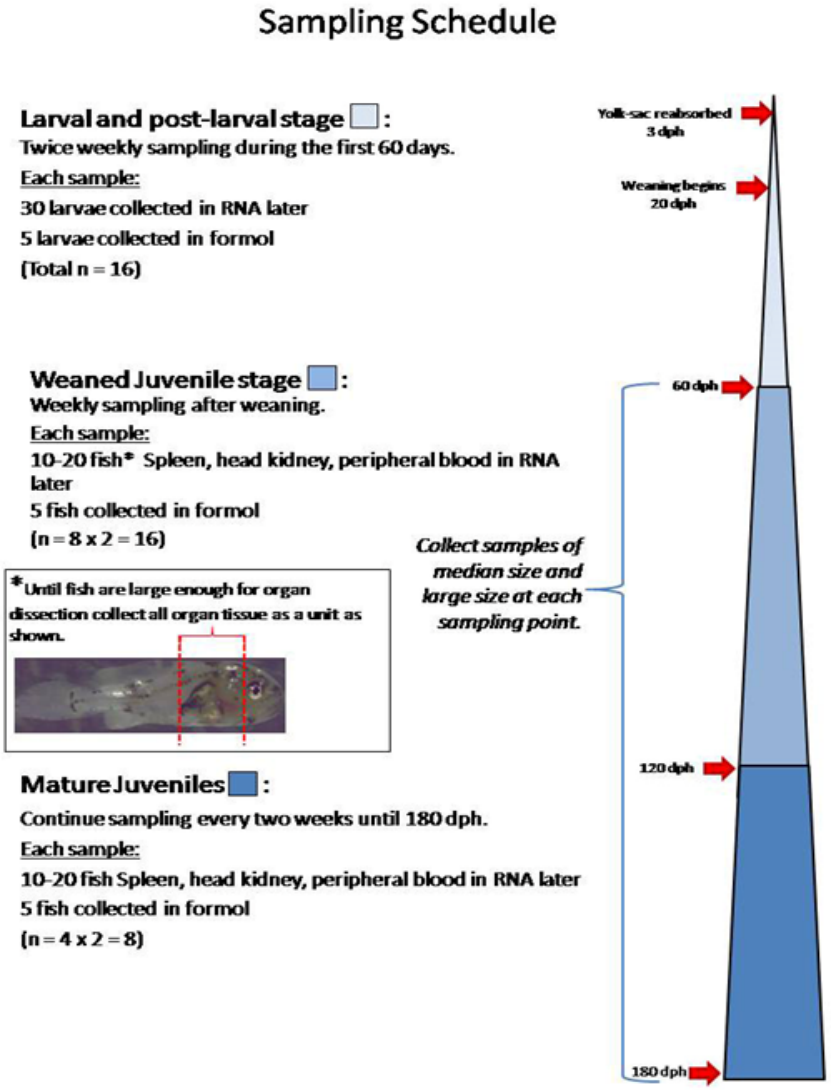


Fig 9.- diagram showing the larval sampling programme for the study of the ontogeny of the immune system

A search of the online database GenBank was performed to identify and collect existing sequences for genes of interest from extant marine teleost species for the study of the immune system. The sequences collected were used for the preparation alignments for designing degenerate/consensus primers for amplification from cDNA of meagre tissues. Samples for the preparation of RNA and subsequent synthesis of cDNA for preparation of these gene expression asssays has already been done during the growout period of fish being used in the earlier experiment. All of this process for isolation of gene sequences and development of the specific gene expression assays will be initiated in the next quarter of 2015.

Table 2.- Genes targeted for characterization of the immune system of meagre. The unknown gene sequences should provide amplicon sizes approximating those shown, if there exists a high degree of conservation between species. These estimates are based upon data from existing sequences found in GenBank.

	Target Gene	Degenerate/ Consensus Primers	Amplicon size
Endogeneous Controls	EF1 (Elongation Factor)	X	230
	GAPDH (Glyceraldehyde Phosphate Dehydrogenase)	X	239
	18S	X	-
Innate Immunity	Piscidin1 ("Defensin")	X	110
	Piscidin2 ("Defensin")	-	-
	Piscidin3 ("Defensin")	-	-
	Lysozyme	X	220
	Metallothionein	X	80
	MX protein	X	570
	NOD2 (Toll Like Receptor - TLR)	X	1390
Adaptive Response	RAG1 (Recombination Activating Gene)		
	IgM		
	IgT		
	TcR (T-cell Receptor)		
	C3 (complement)	X	1202
	TNFa (Tumor Necrosis Factor)	X	250
	IFN alpha (interferon)		
	IFN gamma		
	IL-1beta (Interleukin)		
	IL-2		
	IL-4		
	IL-10		
	IL-17		
	IL-22		
Inflammatory Response	COX2 (cyclooxygenase 2)	X	1500
	MyD88 (myeloid differentiating factor)	X	130