Deliverable Report

Deliverable No:	D24.9	**Delivery Month:**		52
Deliverable Title	Determination of effective treatments for common monogenean parasites in meagre			
WP No:	24	**WP Lead beneficiary:**		P1. HCMR
WP Title:	Fish health - meagre			
Task No:	24.3	**Task Lead beneficiary:**		P3. IRTA
Task Title:	Anti-parasitic treatments			
Other beneficiaries:	P1 HCMR	P3. IRTA		
Status:	Delivered (delayed)		**Expected month:**	48
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Lead Scientist preparing the Deliverable: A Roque (IRTA)

Other Scientists participating: N Duncan (IRTA), A Estevez (IRTA), E Gisbert (IRTA), P Katharios (HCMR)

Objective: Parasites such as *Sciaenacotyle panceri,* a monogenean found on the gills of meagre (Merella et al. 2009), are also known to cause mortality in farms in the Mediterranean and require development of appropriate treatments. A test was performed to evaluate the tolerance of the fish to each chemical product that was considered to be a potential treatment. At the end of each test, fish were sacrificed humanely and samples of the gills and blood taken. Gills were fixed and analysed by histology in order to evaluate any damage caused at the tissue level either by the potential treatment or by the parasite should they be encountered in the test population during the course of the treatment trials. Plasma was collected from the blood and general stress indicators were measured, including cortisol, glucose and lactate. Potential treatments were repeated.

Description: Description of the work done and results

Initial work involved a literature review on meagre diseases and on strategies to handle them. Most diseases in meagre are of parasitic etiology (Toksen et al. 2007; Duncan et al. 2008; Merela et al. 2009; Quilichini et al. 2009; Ternengo et al. 2010; Soares et al. 2012; Soares et al. accepted). Unfortunately, these reports describe well the outbreaks but not how to control them. Except for the study of Soares et al. (2012), they all

refer to Monogenean parasites, which have a direct cycle, making it extremely difficult to break the life-cycle. A list of medicines tried on monogeneans is offered in Shinn and Bron (2012). However not a single report refers to meagre. There is not much information published on how to treat these parasites but monogeneans are normally treated with praziquantel or levamisole, both of which are used in human medicine also to treat parasite infections (Redman et al. 1996; Robertson and Martin, 1993).

The route of administration was also considered. Since meagre is normally cultivated in sea cages, bath treatment is feasible, but presents obvious technical difficulties as the physiological strain placed on the host during such treatments increases significantly susceptibility to re-infection (Ohno et al. 2009). Moreover, potentially there could be a harmful effect for the surrounding environment. Parasiticides obtained from natural sources, which can be administered with the feed, would be tremendously convenient and safe for use.
Since many pharmaceuticals are not licensed for aquaculture, a review on the use of essential oils was made and the present work focused on the use of essential oils with vermicide properties. Essential oils have been successfully used to treat diseases in fish (Hirazawa 2000; Pessoa et al. 2002; Macedo et al. 2010, El Gahlil 2012; Maggiore et al. 2012; Militz et al. 2014).

ORIGIN OF FISH, MAINTENANCE OF FISH

Fish used throughout the study were all born in the facilities of P3. IRTA and grown to juveniles. They were not used in other experiments and were used from a body weight of 50 g onwards. Once weaned from live feed, fish were fed pelleted feed from the line EFICO SIGMA 862 (Biomar, Spain) throughout their nursery grow out phases. They were stocked in 1000 L round tanks with a stoking density of 2 kg m^{-3} at 15ºC and with a natural photoperiod. For all the trials fish were transferred to a RAS with a volume of 400 L tanks. All tanks had independent air and water supply and were run with flow through, whilst the experiment was performed.

Trial 1

An initial trial was performed to evaluate the acceptability of the different essential oils (EO) by juvenile meagre. Known quantities (30 g) of the meagre feed pellets were coated with 6 ml of the desired EO and were left to dry after they absorbed the oil. Essential oils tested were: Bergamote, Chamomile, Cinnamon, Clove, Eucalyptus, Lavander, Lemon, Mint, Niaouli, Pine, Rosemary, Thyme, and Tea tree.

Feed was offered in 5 g portions over a 6-day period. There were 14 tanks (13 given feed with EO + 1 give the control, untreated diet). Each tank contained 3 fish. Over the 6-day period the fish were observed for 5 min immediately after the feed was offered and then at 30 min. After 60 min leftover food was quantified.

After this period, 5 EO were rejected since fish would not consume the feed. Rosemary, pine, cinnamon, bergamote, niaouli, lavender, clove and mint were totally consumed at least 4 days out of the 6 and for the other days, food was partially eaten.

Trial 2

A second experiment was then performed to evaluate the toxicity of EOs in the feed since the literature suggested long term treatments. Four treatments were setup for six weeks (Athanassopoulou et al. 2004; Hirazawa et al. 2000). The best approach in determining the efficacy of any new drug or treatment is to compare the results obtained in trials with the new drug, a proprietary drug with known efficacy and untreated groups. Experimental treatments were fed with either mint or cinnamon essential oils and controls were given an untreated feed or feed containing praziquantel (Hirazawa et al. 2000). Feeds were prepared as described before and fish were offered daily doses of 2 g per tank during a 45 min period, after which

leftover food was collected. Leftover food was dried in an oven at 95ºC overnight and then weighed in order to estimate daily consumption.

Treatments were run in triplicate and each tank had 6 fish. In the end of the trial 4 fish per tank were sampled for immunology and stress (plasma) and histology (liver, intestine and gill). Fish were sacrificed with an overdose of anesthetic, one milliliter of blood was withdrawn from the caudal vein and gills, intestine, kidney and liver were then fixed in 10% formalin. Plasma was collected from blood and frozen at -80ºC until further analysis and fixed organs were processed for histology, staining the slides with hematoxylin and eosin. After fixation, 3 µm histological sections were cut from the paraffin embedded tissues and mounted on slides which were stained in hematoxylin-eosin and observed under a light microscope Optech Biostar B5ICS. Plasma analysis included glucose using the Cromatest kit (Linear Chemicals SL, Catalonia), following the manufacturer's instructions, lysozyme and complement factor.

The protocols used for immune parameters are described briefly below. Alternative complement pathway activity (ACH50) of serum was measured, by modifying the method of Sunyer et al. (1995) by incubating the serum with commercial rabbit red blood cells in the presence of 10 mM EGTA. Absorbance was read at 414 nm and estimations were made based on the highest haemolytic activity and on the total haemolysis of the sample versus the blank. They are expressed in ACH50.

Lysozyme activity in plasma was measured according to Ellis (1990). Briefly a sample of 10 µl of plasma was added to 200 µl of *Micrococcus luteus* solution (0.2 mg/mL) in a 0.1M sodium phosphate buffer (pH 6.8). Absorbance was measured at 540 nm, every 10 min for 50 min. Egg white lysozyme was used as a standard to build a standard curve. Each unit (kilounits/L) is defined as the amount of sample causing a decrease in absorbance of 0.001 per min. All chemicals and reagents used for evaluating different hematological immune parameters were purchased from Sigma-Aldrich (Madrid, Spain).

Biochemical parameters were statistically analysed with an ANOVA using poptools complement for excel (Hood, 2010).

Initial mean weight of the fish was 61g. Six weeks later, mean (±SEM) final weight was 71.8±2.83 g and per group final weights were 67.5±4.84 g (Mint), 71.5±5.89 g (cinnamon), 63.3±5.38 (praziquantel) and 85±4.98 g (untreated). No mortalities were recorded. Average consumption and final weight per treatment are shown in **Figure 1**. As it can be seen, although fish fed with praziquantel was consumed more than experimental treatments, final fish weight was less.

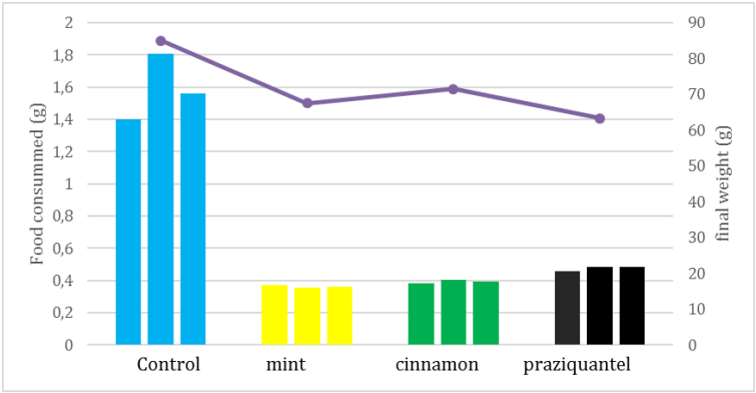

Figure 1- Average consumption of food per tank over a six-week period. Final mean weight per treatment.

The average value of glucose in the blood of the experimental fish shown in **Figure 2**. Maximum value was measured in a fish of the control with 395.3 mg/dL and the minimum was 20.3 mg/dL in a fish fed food with cinnamon EO. Overall the fish in the control treatment showed significantly higher values of glucose ($p < 0.001$) than in all the other treatments indicating fish were either more stressed or better fed.

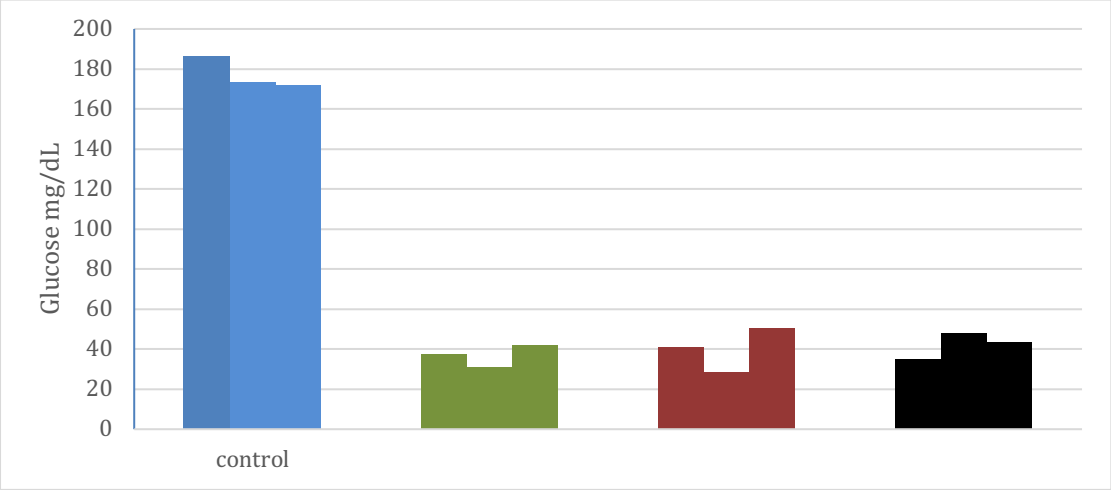

Figure 2 - Levels of blood glucose measured in the different treatments

Figure 3 shows the average lysozyme values measured per tank and treatment. Lysozyme level is an important index of innate immunity of fish and is ubiquitous in its distribution among living organisms. It is well documented that fish lysozyme possesses lytic activity against bacteria. It is also known to be opsonic in nature and activates the complement system and phagocytes. It is present in mucus, lymphoid tissue, plasma and other body fluids of fish (Saurabh and Sahoo, 2008). Lysozyme has also been used as an indicator of acute stress together with cortisol and adrenaline (Demers and Bayne, 1996). For this parameter the minimum value was found in a control fish 13.7 U/mL and the maximum in a fish fed food containing cinnamon EO, 375 U/mL. The range of values is within the values found for different fish species but unfortunately we did not find values published for meagre and there were no significant differences ($p=0.2$) associated with the treatment, possibly due to variable results both in mint and control groups.

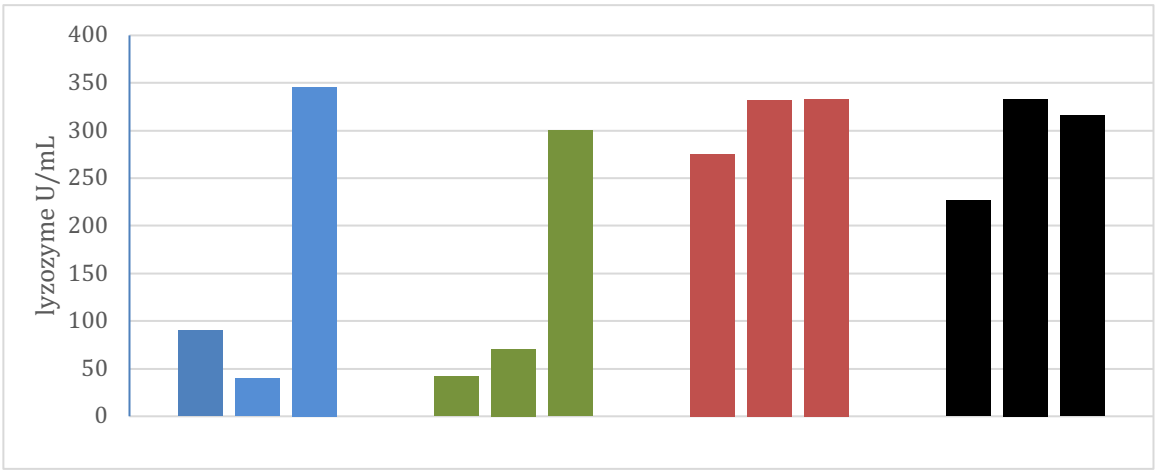

Figure 3 - Levels of lysozyme measured per treatment.

Figure 4 shows the average complement activity in the alternative pathway measured per tank and treatment. In two cases, there was not enough serum to perform this analysis. The complement system is a part of the immune system that enhances (complements) the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism, promotes inflammation, and attacks the pathogen's cell membrane. In addition, activation of the complement system contributes significantly in the orchestration and development of an acquired immune response. The alternative pathway is one of three complement pathways that opsonize and kill pathogens. The pathway is triggered when the C3b protein directly binds a microbe (i.e., viruses, bacteria, fungi, parasites). It can also be triggered by foreign materials and damaged tissues. As it can be seen in Figure 4, fish fed with food enriched with mint EO have significantly lower values of complement activity (p=0.03) than the other groups indicating the immune system was less prepared for a microbial attack. As for lysozyme, the maximum value of activity was found in a fish from the praziquantel treatment (481143.3ACH50) and the minimum, as expected, was found in the mint group (129.35 ACH50).

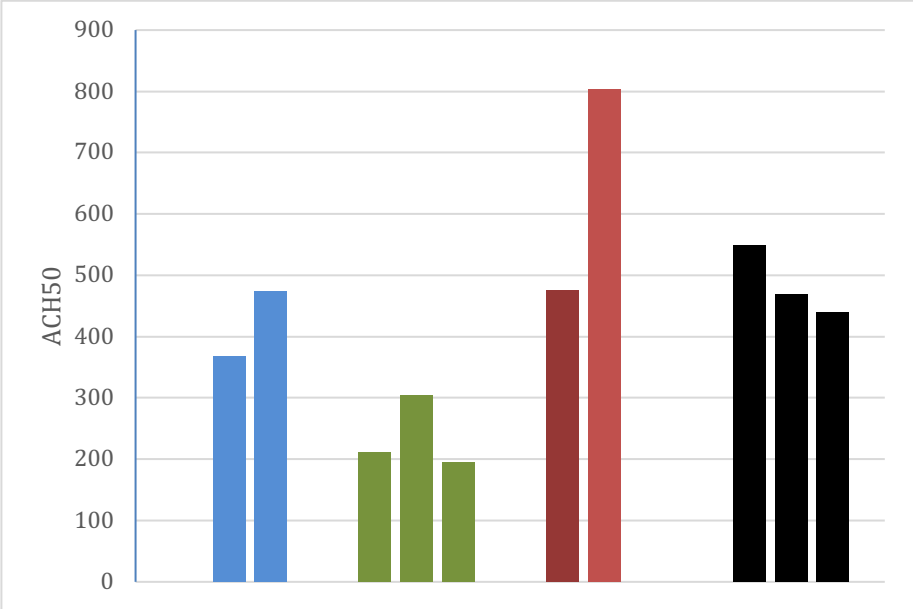

Figure 4 – Complement activity measured per treatment.

According to the histology results, the livers presented some necrosis and some also had congestion, but the intestine did not present any alterations. There were no clear differences related to treatment, though some individuals had the liver in worse state than others.

Trial 3

Because previous results indicated that fish were not consuming the food properly and that the digestive system showed alterations, the experiment was repeated by sampling fish every week in order to understand whether the batch of fish had these signs from the beginning or whether they developed them with time. To fulfill this objective three tanks were set up with 12 fish with a mean weight of 54 g. Each tank was fed 2% of its biomass daily. Treatments were feed with EO of mint, feed with EO of cinnamon and control feed. Every week three fish from each tank were sampled for histology to understand when the perceived alterations started. The experiment lasted 4 weeks.

Observation of the histology slides established that from the beginning a moderate inflammation of the liver with haemocytic infiltration and some slight alterations in the gill structure, such as some lamellar fusion, were apparent. Two weeks later this had not changed for any of the treatments, but by the end of four weeks the fish that were fed with cinnamon had reduced the hepatic alterations, whereas the others had not. The same occurred in the gill. Throughout the experiment the digestive tract appeared unaltered.

Trial 4

In order to evaluate the therapeutant's effects, infected fish were needed. Usually, *Scianocotyle panceri* is found to affect fish in the Mediterranean sea cages when water temperatures are higher (over 15ºC, Micaela Bras, pers. Comm.). It affects all sizes of meagre in cages, but as a source of parasites larger fish are better, since not only they can have more parasites but they die less from it. Meagre is harvested in Spain around 3 to 4 kg weight. However, transport of live fish stresses them very much and the transport of heavily parasitized fish is even of bigger concern, since it puts their survival at risk. Therefore, two alternative infection models were devised.

1) Place live adult parasites directly on the gills of naive anesthetized juvenile fish
2) Place eggs in the tank water and wait to see whether infection of naive juvenile fish would happen.

To perform this task, a trip was made to a fish farm in the Valencian coast 5h away from P3. IRTA and several gills of freshly dead fish were collected, after they were confirmed to be infected with *Scianocotyle*. Gills were then brought to P3. IRTA in damp conditions by placing them in sterile Petri dishes filled with paper wet with sterile seawater, then they were sealed with parafilm and placed inside cool boxes.

Upon arrival, gills containing the parasite eggs were placed inside a 400 L tank containing 15 fish of around 100 g in body weight. Two lots of 20 parasites were also collected to place directly on the gills of two fish. These two fish were placed in a tank by themselves. A fortnight later the two juveniles exposed directly to adult parasites and another two fish from the tank containing parasite eggs, were sacrificed and gills inspected for parasites. The 2 juveniles exposed directly to the parasite were infected, however the ones from the tank containing the eggs were not. Unfortunately, no published information was found on the length of the lifecycle of this species of Monogenean. Another week later, two more fish were sacrificed, which again did not have any parasites. A week later (4 weeks), two fish were sacrificed and one of them did have some parasites on the gills, therefore the leftover 9 fish were all sacrificed to estimate the prevalence, which was 2 out of 11 (around 20%). From this result, it was decided that it was worth using this challenge model for future studies.

Trial 5

For the final experiment, it was decided to work only with cinnamon but not with mint, since fish did not consume it very well and cinnamon appears to be an immunostimulant, which mint does not.

A final experiment was set up to test the efficiency of cinnamon as a parasiticide for meagre infested with *S. panceri*. In order to perform this experiment, infected fish were needed, and so more eggs from infested meagre were collected from a farm in the Mediterranean coast. For 5 weeks, 180 juveniles were exposed in 400 L tanks with 20 fish that had infected gills with eggs attached. Throughout this process some fish were lost. In the end of the 5 weeks, fish were confirmed to have parasites and the experiment started. To do this, 10 fish from each tank were anaesthetized and gills observed under a strong light with the help of a magnifying glass. For the treatment experiment three diets were prepared as described before, untreated diet, diet with EO of cinnamon and a new diet prepared with an aqueous extract of Echinaecea. Echinacea was selected since recently many studies showed its immunostimulant properties in fish (Bulfon et al, 2017; Guz

et al 2014; Oskoi et al 2012) and it was decided to investigate whether an immunostimulant would benefit the fish with parasites.

Three replicate tanks were used per treatment with 17 fish per tank. Fish were fed 2x a day with 7.5 g of the corresponding diet, at 8 am and 3 pm for three weeks. Two days before starting the experiment, an initial sampling was performed using 9 fish which were weighed, blood sampled and samples were taken for histology from liver, intestine, stomach, gills and anterior kidney. From the fresh blood sample, a haematocrit was performed and then plasma was separated and frozen for further analysis. Prevalence was estimated from these 9 fish and another 45 fish were anaesthetized to get a better idea of the initial prevalence. Glucose, cortisol, complement activity and haematocrit analysis were performed. Cortisol was measured using the Demeditec kit (Demeditec Diagnostics GmbH, Germany) following the manufacturer's instructions. On days 8 and 15, nine fish (one from each tank) were sacrificed, prevalence was estimated, and blood and organs sampled as above. On the last day of the experiment, day 22, all fish were sacrificed to estimate the prevalence per treatment and nine fish were blood and organ sampled.

Initial weight of the fish was 254 ± 25.95 g, but when after the first week fish were weighed per treatment it was found that the cinnamon group had an average of 185 ± 30.36 g whereas the control had an average of 280 ± 20.26 g and Echinacea had 290 ± 49.42 g. A week later averages of the control was 249 g, Echinacea 297 g and cinnamon 245 g, and on the final sampling day weight was as follows: 232 ± 26.36 g – control, 211 ± 17.81 g- echinacea and 245 ± 63.37 g cinnamon. Only sacrificed fish were sampled on each sampling day, but it seems that over time weight was either maintained or even lost from individual fish, and the only treatment where fish were growing was cinnamon. This is probably due to the parasitism, which was clearly more intense and prevalent in the control and the Echinacea treatments than in the cinnamon treatment. In histology multifocal granulomas were observed at the beginning of the experiment, and in one fish as well as the kidney it had granulomas in the liver. After the treatment only the fish fed with cinnamon had reduced number of granulomas.

Initial mean prevalence in the tanks was around 80 ± 10%, it is difficult to state a value since the fish were not sacrificed and the gills taken out. At the end of the experiment, fish fed with Echinacea were all infested with the parasite and all except one had more than 5 individuals per gill (n=15 fish) and half of them had more than 10 (n=8 fish). In the Control treatment all but two fish were infested also with more than 5 individuals per gill and 2 with more than 10. In the case of Cinnamon, six fish were not infested and the ones that had parasites had less than 5 individuals per gill (see **Table 1**). The experiment only lasted three weeks instead of four to six, which was advisable because even three weeks is not followed by farmers sometimes and an attempt to optimize was made.

Complement activity is shown in **Figure 5**. Complement activity showed no differences among treatments (p=0.6) or along the period of study (p=0.4), with Echinacea showing both the maximum and the minimum values detected.

Table 1. Results summary.

Treatment	Prevalence (%)	Intensity (1-4 ind/ gill)	Intensity (>5 ind/ gill)	Intensity (>8 ind/gill)
Control	100	2	15	2
Echinacea	100	1	15	10
Cinnamon	60	7	0	0

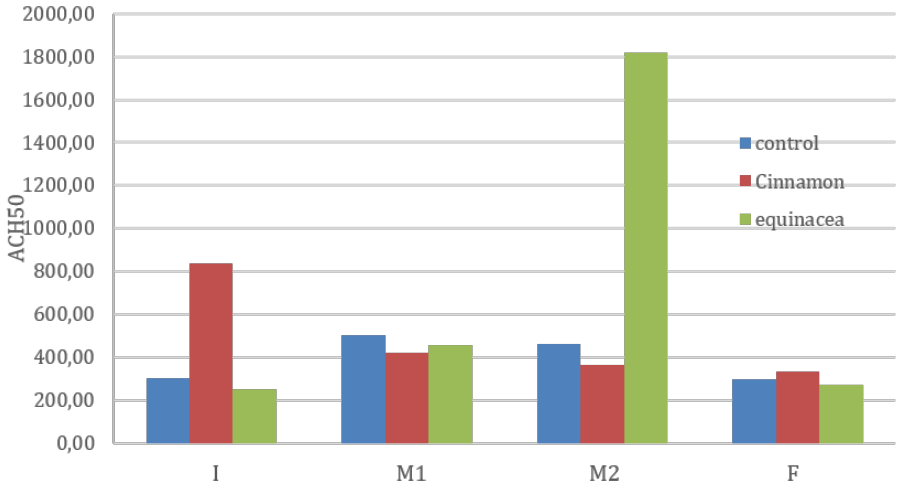

Figure 5 - Complement activity measured weekly. I= Day 1; M1= Day 8, M2 = Day 15 and F= Day 22.

The haematocrit estimates the volume of red blood cells in the circulating blood, and indirectly it gives an estimation of the capacity of the individual to transport oxygen to the cells. Hematocrit levels are often elevated during stress to increase the oxygen supply to the major organs in response to the higher metabolic demand (Cnaani et al 2004). In the present study the levels of haematocrit did not vary with time (p=0.06) and were not different among treatments (p=0.6). Values ranged from 28.7 and 44.7% (**Figure 6**), which were assumed to be within the normal range since no information on this species was available, but similar values have been measured in seabass (Yavuzcan et al 2010). Although it was expected these values would rise with the intensity of parasitism or that they would decrease with treatment, no significant differences were detected over time, as p= 0.06 this may be a result of a type II statistical error, not enough individuals sampled to detect the magnitude of difference that could be achieved by the experimental treatments.

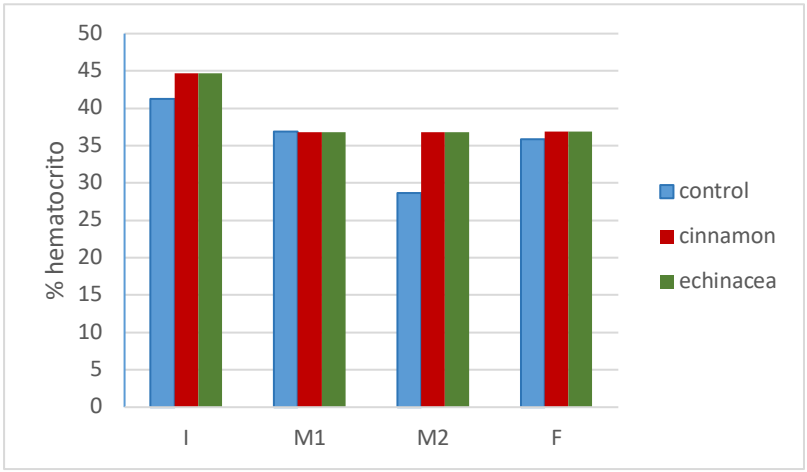

Figure 6- Haematocrit values measured weekly. I= Day 1; M1= Day 8, M2 = Day 15 and F= Day 22.

Levels of cortisol are shown in **Figure 7**. As for the immunology parameters no significant differences were detected among treatments (p=0.4) nor over the period of study (p=0.3).

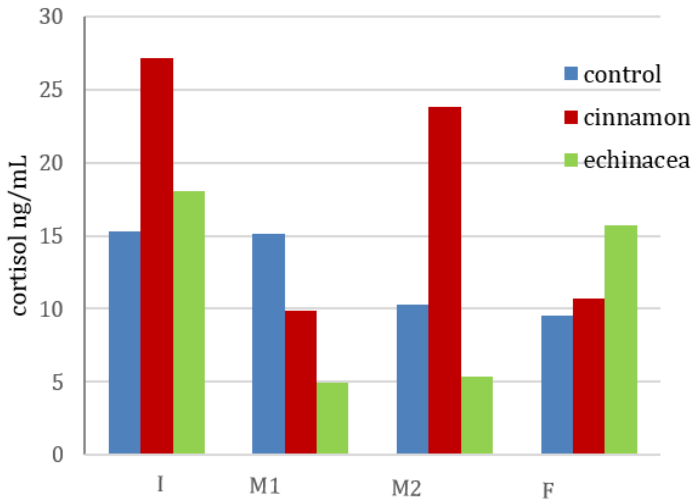

Figure 7- Levels of cortisol measured weekly. I= Day 1; M1= Day 8, M2 = Day 15 and F= Day 22.

Levels of glucose are shown in **Figure 8**. As for the immunology parameters no significant differences were detected among treatments (p=0.3) nor along the period of study (p=0.18).

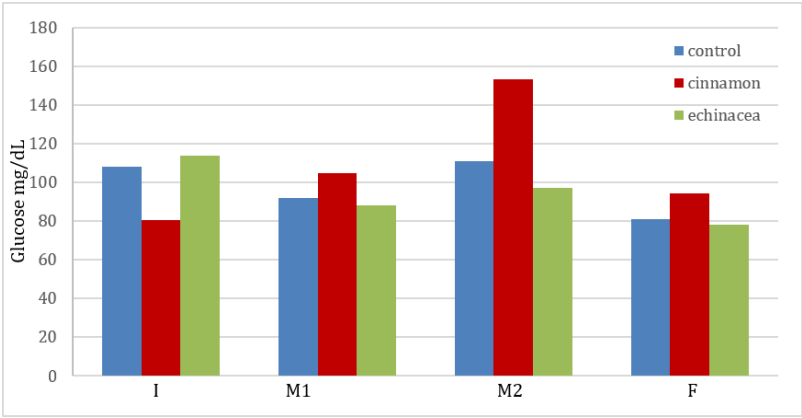

Figure 8- Levels of glucose measured weekly. I= Day 1; M1= Day 8, M2 = Day 15 and F= Day 22.

Overall, cinnamon showed a clear potential to treat a parasitosis with *S. pancerii* when administered orally to juvenile meagre. Cinnamon also showed immunostimulant properties. Unfortunately, there was no opportunity to test cinnamon EO in vitro and this will be done during 2018, together with other EOs.

Deviations:

No deviations were made to the proposed task.

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Co-funded by the Seventh Framework Programme of the European Union