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SHORT COMMUNICATION

Mussel (*Mytilus edulis* L.) and ragworm (*Nereis virens*, Sars) both alleviate anaemia in common sole (*Solea solea* L.)

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Common sole (*Solea solea* L.) is a polychaete-mollusc feeder (de Groot 1971). Based on the feeding ecology of sole, we assume that both ragworm (*Nereis virens*, Sars) and mussel (*Mytilus edulis* L.) reflect the dietary requirements of common sole.

Common sole grows approximately 70% faster on ragworm than on commercial pellets (Ende, Kroeckel, Schrama, Schneider & Verreth 2014), the latter causing them to suffer from nutritional anaemia (Kals, Blonk, Palstra, Sobotta, Mongile, Schneider, Planas, Schrama & Verreth submitted). Changing the diet from pellet to ragworm alleviates this nutritional anaemia (Kals, Blonk, van der Mheen, Schrama & Verreth 2015). This suggests a mismatch between the feeding requirements of sole and the dietary composition of the commercial pellets.

Fonds, Drinkwaard, Resink, Eysink and Toet (1989) and Ende *et al.* (2014) showed that the growth of sole fed mussel is comparable to the growth of sole fed ragworm. We, therefore, expect that also mussel will alleviate anaemia in sole, consequently showing comparable levels of haematocrit (Hct) and haemoglobin (Hb) for sole fed mussel or ragworm, but higher for sole fed pellet.

Apart from trying to find evidence for the foregoing hypothesis, we wanted to gain insight into which dietary components could be important to alleviate nutritional anaemia in sole.

This experiment was approved by the Ethical Committee for Animal Experiments and conducted at IMARES, Yerseke, the Netherlands. The experimental set-up contained three diets; raw mussel meat (mussel), fresh ragworm (ragworm) and a commercial pellet (pellet) (crude protein 63%, ether extract 14%, ash 12%). The diets were tested in triplicate for a period of 23 days with 10 fish per tank, or 90 fish in total. Mussels were collected at the Oosterschelde, the Netherlands. The meat was separated from their shell and stored at −80°C. The frozen mussel was thawed in a fridge the day before feeding. Ragworms were delivered by Topsy Baits, Wilhelminadorp, the Netherlands.

Sole (220 ± 44 g) reared on pellet and naïve to ragworm and mussel were randomly accommodated in 9 tanks (0.4 m^2, 130 L), in a flow through system using sand filtered seawater. Husbandry conditions were as follows: photoperiod 12L:12D, light intensity 11–15 lux, temperature 16.9 ± 1.3°C, oxygen 8.3 ± 0.5 mg L^{-1}, pH 8.1 ± 0.1, TAN 0.3 ± 0.2 mg L^{-1}, NO_2^-, 0.01 ± 0 mg L^{-1}, salinity 25–30 g L^{-1} and flow 4.5 ± 0.5 L min^{-1} kept within pre-set limits. Temperature and oxygen were measured daily; flow, pH, TAN, NO_2^- weekly. Fish were fed by hand twice a day (8:30 and 16:30 hours). During acclimatization, all fish were fed the pellet. The dry matter content of the diets was analysed to enable equal feeding levels (restricted to 0.54 g dm per fish per day) for all diets. One hour after feeding, tanks were checked if all feed was consumed. Both, ragworm and mussel, were cut and sieved for one minute to drain excess fluids prior to weighing. Daily samples, equal to the amount of feed given, were taken and stored at -20°C, and at the end of the experiment pooled per treatment and analysed for dry matter content, proximate composition, iron and vitamin B_{12}. The proximate composition and B_{12} were analysed by Nutrilab, Rijswijk, the Netherlands. The iron content was analysed by the Chemical Biological Soil Laboratory, Wageningen, the Netherlands. The experiment consisted of a 7-day acclimatization and a 23-day experimental period. Fish were sacrificed, using an overdose of phenoxyethanol (1:1000) before sampling. Blood was sampled by caudal venous puncture, using a heparinized syringe (0.6 mm/30 mm needle) at the start (30 fish) and at the end (10 fish per tank) of the experiment. The samples were stored in tubes on ice and processed within 15 min. Haematocrit was determined by centrifuging blood samples for five minutes at 5000 *g* (SpinCrit, Brown, Indianapolis, IN, USA). and Hb was determined according to van Kampen and Zijlstra (1961). Data were analysed using one-way ANOVA to test for diet effects. Homogeneity of variance was tested using Levene's test. For all tests, a probability $P < 0.05$ is considered significant. When significant, means were compared using the Fisher's Least Significant Difference (LSD) test.

No mortality occurred during the experiment. No feed refusal or spillage was observed. The proximate composition, iron and B_{12} content of the diets are shown in Table 1. As both iron and B_{12} are playing a role in erythropoiesis (Koury & Ponka 2004), we focused on those two. The high levels of B_{12} and iron in both mussel and ragworm are prominent when compared to the respective levels in pellets.

At the start of the experiment, sole reared on pellet had an average Hct and Hb level of 12.5% and 19.6 g L^{-1} respectively. At the end, both levels were affected by diet ($P < 0.05$, Table 2). Haematocrit levels of sole fed mussel or ragworm increased by 39.7% and 51.8%, respectively, and are not different ($P > 0.05$), while Hb levels increased by 36.5% and 74.5% respectively. The average Hb level of sole fed pellet (18.9 g L^{-1}) is significantly lower compared to the levels of sole fed mussel ($P < 0.05$) or ragworm ($P < 0.01$), which are comparable ($P > 0.05$).

The Hct and Hb values of sole fed mussel or ragworm rose strongly. In contrast, the Hct and Hb values of sole fed pellet stayed anaemic. This confirms earlier findings for sole fed ragworm (Kals *et al.* 2015, submitted) showing that mussel and ragworm both alleviate anaemia in common sole.

In anaemic states, the maximum oxygen consumption of fish falls sharply (Gallaugher & Farrell 1998) and hampers metabolic performance, including growth (Wang, Lefevre, Huong, Cong & Bayley 2009). Mas-Muñoz (2013) found that the positive effect of ragworm on growth of sole disappeared at lower water temperatures, while Hct values of these sole were up to 98% higher compared to sole fed pellet (Kals *et al.* 2015). We infer that the slow growth of sole fed pellet, kept at 'optimal' culture temperatures, is due to their nutritional anaemia, which hampers oxygen uptake.

We believe that higher levels of Hct and Hb improve the ability to take up oxygen, as maximum oxygen consumption is highly dependent on these levels and suppose that iron and B_{12} are key elements in alleviating anaemia in common sole.

The iron concentration, of major importance for the synthesis of Hb, is comparable in mussel and ragworm and higher than in the pellet (Table 1). However, also in the pellet, the iron level was above the requirement as known for fish in general (Table 1). Nevertheless, sole fed pellet stayed

Table 1 The proximate composition, iron and vitamin B_{12} content of the different diets and requirements according to (NRC 2011)*

Diet	Unit	Ragworm	Mussel	Pellet (CPEL)	NRC (2011)
Dry matter	g kg^{-1}	181	252	894	NA
Crude protein	g kg^{-1} dm	685	575	670	NA
Ether extract	g kg^{-1} dm	138	103	177	NA
Fe	mg kg^{-1} dm	352	372	277	30–150*
B_{12}	μg kg^{-1} dm	1602	1671	338	20–50*

*Minimum and maximum values of different species as no values of sole are given. NA is not applicable.

Table 2 Hct level in per cent and Hb level in g L^{-1} with standard deviations of sole fed ragworm, mussel and pellets

Diet	Day	Hct (%)	Hb (g L^{-1})
–	0	12.5 ± 3.17^{a}	19.6 ± 8.10^{a}
Pellets	23	13.1 ± 1.16^{a}	18.9 ± 3.28^{a}
Mussel	23	17.4 ± 1.95^{b}	26.7 ± 4.38^{b}
Ragworm	23	19.0 ± 1.42^{b}	34.1 ± 5.13^{b}
P-value	–	<0.01	0.01

[ab]Means within columns with a common superscript are not significantly different using the one- or two-sided Fisher LSD post hoc test depending on the hypothesis.
Hct, haematocrit; Hb, haemoglobin.

anaemic. Moreover, in J. Kals, R.J.W. Blonk, A.P. Palstra, T.K. Sobotta, F. Mongile, O. Schneider, J.V. Planas, J.W. Schrama & J.A.J. Verreth (unpublished data), we showed that, although the dietary iron intake of sole fed treated pellets versus sole fed boiled ragworm was not significantly different, the Hct level of sole fed boiled ragworm was significantly higher. Therefore, not the intake of iron, but the absorption of iron might explain the anaemia in sole fed pellet. This might be related to the source of iron in the diets. In mussel and ragworm, this source is mostly haeme iron in its native form of which the structure is comparable (Vinogradov 1985). The iron in the pellet consists of inorganic iron from the premix and/or damaged haeme due to processing. The absorption of haeme iron, which is independent of pH, may therefore be essential for sole to achieve normal Hct and Hb levels. An additional factor that could be responsible for different Hct and Hb levels between sole fed pellet, ragworm or mussel, is the difference in the dietary level of B_{12} between these diets. B_{12} is essential for erythropoiesis (Koury & Ponka 2004). The higher levels of B_{12} in ragworm and mussel may explain the higher Hct and Hb levels of sole fed these diets. In ragworm, we found levels of B_{12} from 1134 to 3033 μg kg^{-1} dm and in mussel a level of 1671 μg kg^{-1} dm. These high levels of B_{12} are comparable, yet four times higher than the levels in the pellet, and 20–100 times higher than the amount of B_{12} in a general premix or mentioned as requirement for fish, in NRC (2011). In most fish species, B_{12} requirements are low or not required due to sufficient production by the intestinal flora (NRC 2011). Yet, B_{12} absorption depends on a low pH and pepsin like activity in the stomach (Nielsen, Rasmussen, Andersen, Nexø & Moestrup 2012; Pawlak, James, Raj, Cullum-Dugan & Lucus 2012). Sole lacks this pre-digestion in the stomach, has a low pepsin like activity and its intestine has an alkaline character (Clark, MacDonald & Stark 1985; Lagardere 1987; Yúfera & Darías 2007). In conclusion, we infer that B_{12} absorption in sole is limited and suggest that sole depends on the inactive process of B_{12} diffusion. As only 1% of the available B_{12} is absorbed through diffusion (Pawlak *et al.* 2012), sole may depend on high dietary levels of B_{12}.

Mussel and ragworm both alleviate anaemia in common sole. We cannot exclude that also other factors could explain a comparable effect of mussel and ragworm on Hct, Hb levels and growth of sole. But looking at the current data, we suggest that the ability of mussel and ragworm to alleviate anaemia in sole can be explained by a combination of haeme and high B_{12} levels.

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