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Long-term microplastic retention causes reduced body condition in the Langoustine, *Nephrops norvegicus*

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Abstract

Microplastic represents a rising proportion of marine litter and is widely distributed throughout a range of marine habitats. Correspondingly, the number of reports of species containing microplastics increases annually. *Nephrops norvegicus* in the Firth of Clyde have previously been shown to retain large aggregations of microplastic fibres. The potential for *N. norvegicus* to retain plastic over an extended time period increases the likelihood of any associated negative impacts to the individual. This study represents the longest observation of the impacts of microplastic retention in invertebrates. We exposed *N. norvegicus* to plastic over eight months to determine the impacts of extended exposure. Over this period we compared the feeding rate, body mass, and nutritional state of plastic-fed *N. norvegicus* to that of fed and starved control groups. Following the experimental period, the plastic-fed langoustine contained microplastic aggregations comparable to those of small individuals from the Clyde Sea Area. Comparisons between fed, unfed and plastic-fed individuals indicated a reduction in feeding rate, body mass, and metabolic rate as well as catabolism of stored lipids in plastic contaminated animals. We conclude that *N. norvegicus* exposed to high levels of environmental microplastic pollution may experience reduced nutrient availability. This can result in reduced population stability and may affect the viability of local fisheries.

Capsule: Long term retention of microplastic aggregations reduces the nutritional state of langoustine.

Keywords: Microplastic, Nutrition, *Nephrops norvegicus*, feeding rate

Introduction

Uptake of plastic debris has been recorded in numerous vertebrate taxa, including birds (Burger and Gochfeld, 2004; Ryan, 2008), fish (Lusher et al., 2013), and mammals (Baird and Hooker, 2000). However, as this large plastic debris breaks down it forms microplastic (<5mm), which represents an increasing proportion of global marine litter (Barnes et al., 2009). This increase has resulted in a rise in the number of records of plastic uptake by wild-caught invertebrates (Devriese et al., 2015; Mathalon and Hill, 2014; Murray and Cowie, 2011; Welden and Cowie, 2016). The uptake of microplastics may occur through a variety of routes, including active ingestion, a result of mistaking plastics for prey items; or passively, through contaminated prey and/or sediment. Both of these routes have been observed in laboratory experiments (Bern, 1990; Besseling et al., 2012; Farrell and Nelson, 2013; Frias et al., 2014). Uptake has even been observed through the gills during respiration (Watts et al., 2014b).

The Impacts of Plastic Ingestion

The ingestion of plastic by marine vertebrates has been seen to have a range of effects on the organism. One of the most apparent impacts is mortality due to gut damage as observed in turtles (Lutz, 1990; Tomás et al., 2002) and porpoises (Baird and Hooker, 2000), or starvation as a result of plastic taking up the space of food in the stomach. Damage to the gut may also result in reduced digestive efficiency and nutrient uptake, known as nutrient dilution (McCauley and Bjorndal, 1999). In birds, this has been linked to reduced body condition as contaminated individuals utilise the body's stores to bridge the energy gap (Connors and Smith, 1982; Pierce et al., 2004; Ryan, 1988). Finally, animals may also be subject to the transfer of hydrophobic contaminants, as previously observed in seabirds (Ryan et al., 1988). This is particularly concerning in areas where plastics exposed to sea water containing hydrophobic contaminants may develop loads much higher than that of the surrounding water (Endo et al., 2005; Teuten et al., 2009).

Microplastic and Invertebrates

Despite the previous work examining the impacts of plastic debris on both wild and captive vertebrates, the effect of microplastic ingestion by invertebrates have yet to be determined. Of those studies that address these impacts, most deal with short-term effects. For example, microplastic consumption has been seen to reduce feeding in *Arenicola marina* (Besseling et al., 2012; Wright et al., 2013) and shore crabs, *Carcinus maenas*, fed on chronic levels of plastic fibres over four week periods showed a dramatic reduction in both feeding and scope for growth (Watts et al., 2015). *A. marina* has also been seen to take up hydrophobic contaminants from plastics, which may result in harmful effects to the animal (Besseling et al., 2012). Responses have also

been observed in terrestrial invertebrates. Examination of the impacts of polyethylene powder (< 400μm) on the worm, *Lumbricus terrestris*, indicated reduced growth rate at all microplastic concentrations and increased mortality at concentrations over 28% (Huerta Lwanga et al., 2016).

A small number of species have demonstrated minimal or no effects as the result of plastic ingestion. The larvae of the sea urchin *Tripneustes gratilla* were seen to readily ingest polyethylene spheres; however, this ingestion was not seen to significantly increase mortality, despite a slight reduction in survivorship in the highest concentration (Kaposi et al., 2014). The marine isopod *Idotea emarginata* also did not discriminate against plastics, ingesting beads, fragments, and fibres. The isopods were able to pass ingested microplastic, with no significant level of accumulation observed; as a result, it was determined that microplastic ingestion had no negative impact on the health of the organism (Hämer et al., 2014). Such variation in the impact of microplastic ingestion between species is to be expected. Biotic factors, such as microplastic uptake rate, residence time of microplastic in the gut, and tolerance to low nutrient conditions each influence the cumulative impact of exposure.

Microplastic Uptake and Retention by N. norvegicus

Nephrops norvegicus are decapod crustaceans found in fine sediments at depths between 20 and 800 meters across the Northeast Atlantic and in the Mediterranean. Their diet is mainly composed of bivalve molluscs, polychaetes, echinoderms, fish, and crustaceans including conspecifics (Aguzzi and Sardà, 2008). *N. norvegicus* recovered from the Clyde Sea Area have been seen to contain large aggregations of microplastic fibres (Murray and Cowie, 2011; Welden and Cowie, 2016), and may be at increased risk of the negative impacts of microplastic uptake. These large aggregations are believed to be the result of the complex gut morphology of *N.* norvegicus. The digestive system has three main portions, the foregut, mid gut and narrow hind gut, and a set of chitinous structures known as the gastric mill (Welden et al., 2015). Examination of wild caught *N. norvegicus* has shown microplastic aggregations directly anterior to the gastric mill and the narrowing at the entrance to the hind gut; it is though that these structures prevent some microplastics from leaving the digestive tract (Welden and Cowie, 2016).

It has been seen that *N. norvegicus* may lose aggregated microplastic during moult, as the foregut lining is shed (Welden and Cowie, 2016). *N. norvegicus* moult occurs biannually in male and immature individuals, and annually in mature females. This extended period over which they may retain microplastics suggests that the species are at increased risk of the negative impacts of plastic ingestion. For *N. norvegicus* populations in areas of high environmental microplastic contamination individuals may develop large aggregations of plastic fibres in the fore-gut, which increases the likelihood and magnitude of these effects (Welden and Cowie, 2016).

It is hypothesised that the level of microplastic contamination observed in *N. norvegicus* from the Clyde Sea Area may result in numerous impacts on the nutritional state of *N. norvegicus*, with the potential for false satiation and starvation in highly affected individuals. In this paper we aim to assess the impact of large fibre aggregations on nutritional health and mortality of this economically important population. In an eight month exposure trial, the impacts of long-term exposure to microplastics on food consumption and growth in male *N. norvegicus* was examined.

Methods

Male *N. norvegicus* were sampled from the Clyde Sea Area in early spring using 50 mm mesh otter trawls. Prior to the experiment, captured animals were kept in holding tanks (270 litres) supplied with recirculating sea water, and were fed on whole squid mantle that had been rinsed thoroughly with distilled water to remove any attached microplastic. After the group had moulted, 34 individuals were sacrificed for gut content analysis to confirm that the animals were free from plastics, and 36 were transferred to lidded individual tanks (10 litres). Each tank contained rock shelters, and was maintained at ambient temperature with a 12 hour light/dark regime.

A closed 68 litre header tank was used to circulate filtered seawater to prevent introduction of foreign microplastics. Water leaving tanks passed through a 0.2mm filter to prevent plastics from the treatment group being passed to control animals, and all water flowed through a secondary protein skimmer and filter before re-entering the header tank (Fig. 1). The system was reduced to 50% volume before being topped up with filtered seawater approximately every 2 weeks; more frequently during warmer weather. Water levels were monitored on a daily basis and ad hoc additions occasionally made to maintain constant volume. Air-stones and pumps were used to ensure sufficient oxygenation of the water and slate shelters were provided for cover.

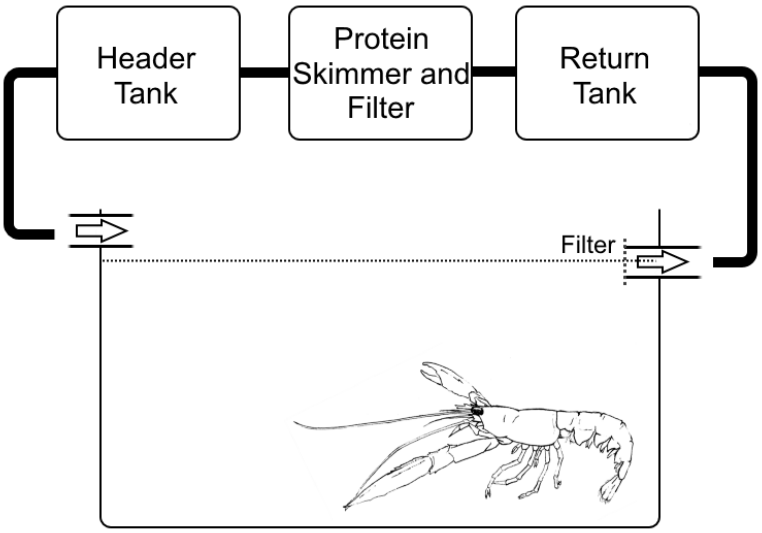

Fig. 1. Individual tank set up with model of sea water circulation system

After a month-long acclimatisation period in the experimental tanks, the carapace length and body mass of each individual was recorded. The average carapace length at day 0 was 31.357 mm, and the average mass was 19.28 g. Total haemolymph protein was also determined using a Bradford assay, described in full below.

The 36 individuals were then divided into three groups (12 individuals per group): the treatment group, fed a 1.5 g squid mantle seeded with five polypropylene fibres; the fed control group, fed with 1.5 g of squid mantle only; and the starved control group. Fibres were chosen for this experiment as they were the most abundant category of microplastic recovered in previous studies of ingestion by *N. norvegicus*. Similarly, they are the dominant microplastic category observed in environmental sampling.

Polypropylene rope was chosen owing to its widespread use in the fishing industry and prevalence in previously studied *N. norvegicus* from the Clyde Sea Area (Welden and Cowie, 2016). Fibres were removed from twisted split film polypropylene rope supplied by Gaelforce. Individual fibres measured between 3 mm and 5 mm in length and were approximately 0.2 mm in diameter. *N. norvegicus* in the Clyde Sea Area are known to be subject to high aggregations of microplastic. Five fibres per feeding would expose the plastic-fed group to 360 fibres over the experimental period, although not all were expected to be ingested and it is uncertain what proportion of ingested plastic is retained in the foregut.

Previous observations of nutritional state in *N. norvegicus* have indicated that animals can survive for long periods without food (Watts et al., 2014a). The starved control was used to provide a baseline for reduced nutritional health. Over the eight month experimental period the treatment

group and fed control were fed every two days, and feeding rate in both groups was determined by weighing the un-eaten food. After eight months, a second haemolymph sample was taken from each animal, after which the animal was killed and immediately dissected; the stomach was removed and transferred directly into 80% ethanol, and the hepatopancreas removed, weighed, and frozen at -80˚C.

Foregut Microplastics

Microplastic retention was determined by examining the foregut of each individual under stereo microscope, as outlined in Murray and Cowie (2011) and Welden and Cowie (2016). Plastic recovered were visually examined to determine that they originated from the experimental condition rather than prior exposure. The weight of retained microplastic was recorded using a Mettler MX5 balance (Mettler-Toledo international Inc., Columbus, USA).

Bradford Assay

Changes in the composition of the haemolymph and the level of stored lipids were used as indicators of nutritional state. Reduction in haemolymph protein has been linked to metabolic depression (Watts et al., 2014a). The results of the Bradford assay obtained following the acclimatisation period were compared to a second assay carried out on haemolymph samples taken at the end of the experimental period. The method followed that outlined by Hagerman (1983), with 10 µl of haemolymph diluted with 990 µl of deionised water. 50 µl of the diluted sample was then added to 950 µl of coomassie blue. The absorbance of the resulting solution was determined at 562 nm using a spectrophotometer, calibrated using standardised solutions of bovine serum albumen (BSA)(Hagerman, 1983).

Copper in the Hepatopancreas

The breakdown of haemocyanin, the protein responsible for oxygen transport, results in an increase in copper levels in the hepatopancreas. To determine the level of copper in the hepatopancreas, dehydrated tissue samples were subjected to atomic absorption spectrometry (AAS). Hepatopancreas samples were freeze dried over five days and the dry weight recorded. 100 mg of the dry tissue was pre-digested at 95˚C using 8 ml of nitric acid. The resulting material was allowed to cool for 10 minutes, following which 3 ml of hydrogen peroxide were added. After a minimum of 8 hours, the samples were made up to 10 ml with distilled water, and analysed using an AA Analyst400 (Perkin Elmer Ltd, Cambridge, UK). Results were compared to standards of copper nitrate (Sigma Aldrich) diluted to concentrations of 15, 10, 5, 2.5 and 1.25 ppm and a distilled water blank (Watts et al., 2014a).

Mass and Lipid Content of the Hepatopancreas

Reduction of energy stores is an indication that the level of starvation exceeds that which can be managed by metabolic depression alone. In *N. norvegicus*, the greatest concentration of stored lipids is found in the hepatopancreas; as these are catabolised they are replaced with water to maintain tissue volume. In a study of the nutritional value of pelleted and natural food sources carried out by Mente (2010), the starved control group exhibited a reduction in lipid concentration of 12.16% in over 8 months. Two indices were used as indicators of catabolism of stored lipids. Hepatosomatic Index (HSI), which is the mass of the hepatopancreas in relation to overall body mass (Mayrand and Dutil, 2008), and the percentage of water in the hepatopancreas (% H_20 HP). HSI was calculated using the mass of the hepatopancreas recorded at dissection and the final body weight of the animal. The percentage water of the hepatopancreas was calculated from the final weight of the hepatopancreas and the weight of the freeze dried hepatopancreas samples.

Statistical Analysis

Statistical analysis was carried out using Minitab15. Differences in food consumption between the plastic-fed treatment group and the fed control group were examined at each month using a Mann-Whitney U analysis. Comparisons of the aggregation of gut plastic, variation in body mass, and the various indices of nutritional state between the three treatment groups at month eight were conducted using a Kruskall-Wallis test. In the event of a significant result, the relationship was explored using post hoc Mann-Whitney tests to determine the treatment group responsible for the response.

Results and Discussion

Analysis of the gut content of plastic-fed individuals revealed aggregations weighing between 0.41 – 3.49 mg, (average 1.5 mg). One of the control animals was also observed to have plastic in the foregut. This was a single pink fibre, distinctly different to those provided to the treatment group. Despite this, there remained a highly significant difference in the level of contamination between the three groups ($H = 16.77$, d.f. = 2, $P < 0.001$). The levels of plastic observed in the treatment group at the end of the exposure period are comparable to aggregations found in highly contaminated individuals from the Clyde Sea Area (Welden and Cowie, 2016). This comparable level of plastic retention indicates that the effects outlined below are representative of those experienced by animals in areas of high microplastic contamination.

Survivorship varied between treatments groups, with the starved condition displaying the highest mortality (58.3%), followed by plastic fed (41.6%), and then fed individuals (33.2%). The mortality rate of plastic fed animals fell between the two control conditions, indicating that *N. Norvegicus* were weakened by the presence of plastic in the diet. Mortality in the fed control was higher than that which might be expected. It may be that *N. norvegicus* are less able to cope with starvation under ambient temperature conditions than under a lower temperature regime, or that the group were subject to an unidentified stressor, such as the presence of a pathogen.

Ingestion of large plastic debris has previously been identified as a cause of increased mortality in both turtles (Lutz, 1990; Tomás et al., 2002) and cetaceans (Baird and Hooker, 2000), as a result of blockage of the gut. Similarly, worms that were fed microplastics have also demonstrated an increased mortality rate (Huerta Lwanga et al., 2016). The increased mortality in the plastic-fed condition may be the result of starvation caused by reduced nutrient availability, or of direct damage to the organism. However, as part of its natural diet *N. norvegicus* regularly ingest items that are of irregular size and shape, which have equal potential to damage the animal's digestive tract. As a result, reduced nutrient availability is considered the most likely factor influencing the observed increase in mortality of the plastic fed animals.

The effect of microplastic ingestion on growth

Over the eight month survey period significant differences were observed in the body mass of the three treatment groups, and statistical analysis revealed a significant difference between the conditions ($H = 13.78$, d.f. = 2, $P < 0.001$) (Fig. 2). The normally fed control exhibited a mean increase in body mass of 0.0795% per day, while starved individuals demonstrated a mean decrease in body mass of -0.0303% per day. Animals fed microplastics showed a decrease in mean body mass of -0.0189% per day, falling between the two control treatments.

As scope for growth is determined by food availability it is assumed that the decrease in body mass of the plastic fed individuals is the result of lowered nutrient uptake. Reduction in the body mass of both the starved and plastic fed groups occur as a result of the body's stores being metabolised in the absence of nutrition from food sources (Connors and Smith, 1982). The mean reduction in body mass recorded in plastic-fed individuals was not as marked as that in the starved control, suggesting that a proportion of the nutrients consumed by the treatment group were successfully absorbed. No significant correlation was found between the mass of plastic in the gut and the reduction in body mass, suggesting that there is not a direct relationship between the two; however, the sample size in this study is small, and a relationship may become apparent in a larger experiment using more *N. norvegicus*.

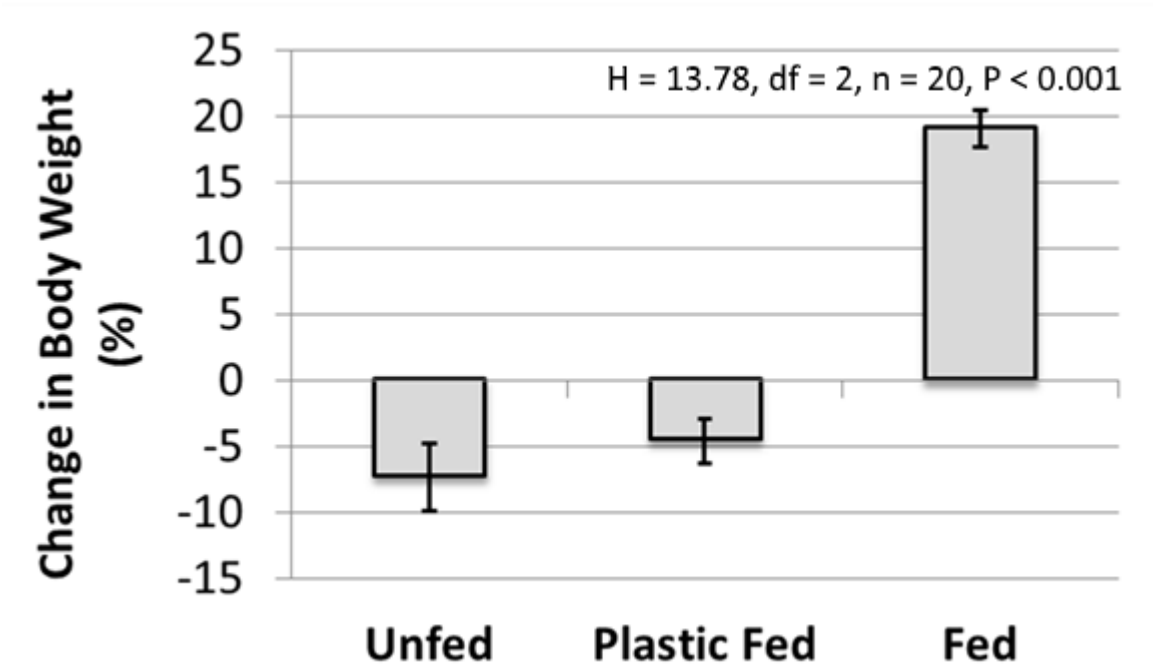

Fig. 2. Mean change in body mass of animals from the three experimental groups observed at month eight (bars indicate standard error)

Variation in Feeding Rate

During the experimental period, the feeding rates of both the fed and the plastic fed groups were seen to vary on a monthly basis; however, the mass of food consumed by plastic-fed animals decreased in relation to that of the fed control. Over the 8-month experimental period, a decline in the mass of consumed food could be seen in the plastic-fed group (Fig. 3), indicating reduced feeding in plastic-fed individuals. However, this effect was not seen to be significant. ($W = 44.0$, $P < 0.1824$).

These results support previous observations of reduced feeding in microplastic-fed *C. maenas* reported by Watts et al., (2015), and microsphere-fed *A. marina* (Besseling et al., 2012) and *D. manga* (Besseling et al., 2014). The reduction in feeding rate is believed to be the result of false satiation, as plastic aggregations take up an increasing volume in the stomach. This effect has been observed in a number of vertebrate species, particularly birds (Azzarello and Fleet, 1987; Connors and Smith, 1982; Pierce et al., 2004; Ryan and Jackson, 1987).

Sustained reduction in feeding is known to result in reduced body condition (Watts et al., 2014a). However, it is not clear whether the reduced growth rate and increased mortality described above are solely the result of the decreased feeding rate or whether plastic in the gut also reduces

nutrient uptake by the digestive tract. Damage to or irritation of the gut membrane may reduce the efficiency with which digested food is absorbed, reducing the nutritive value of the food that is ingested.

For some species, microplastic ingestion may not result in false satiation. Organisms that regularly take in indigestible material may possess compensatory mechanisms. Oysters exposed to microspheres were seen to dramatically increase their food uptake, apparently in an attempt to compensate for the ingestion of plastic (Sussarellu et al., 2014).

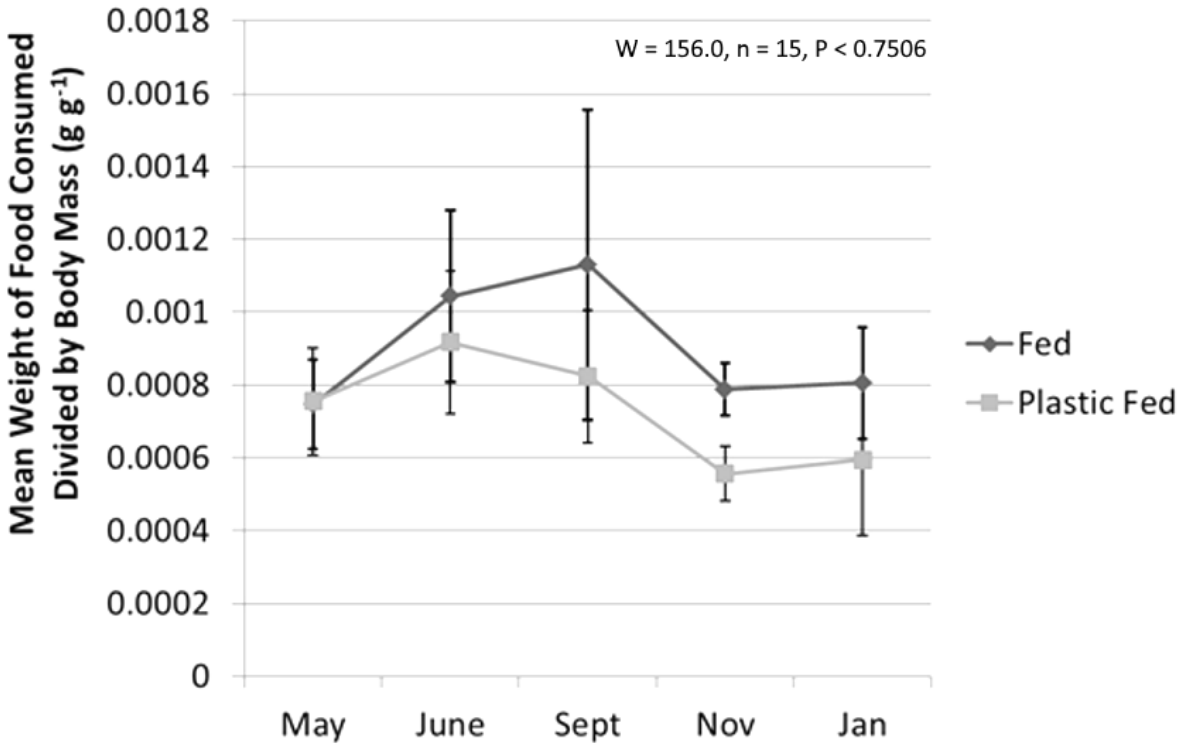

Fig. 3. Mean weight of squid mantle consumed by the treatment group and fed control over the experimental period (bars indicate standard error)

Metabolic Depression

Analysis of the measures of metabolic depression indicated differences between the plastic-fed, fed, and starved conditions. The level of protein in the haemolymph showed apparent variation between the three groups (Fig. 4); however, this was not found to be significant when analysed staistically ($H = 4.96$, d.f. = 2, $P < 0.084$). The minimal variation observed was predominantly driven by the fed and starved control groups as indicated by post hoc Mann-Whitney analysis (W

= 21.0, P < 0.05), comparisons between the plastic-fed condition and the two control groups did not reveal significant variation.

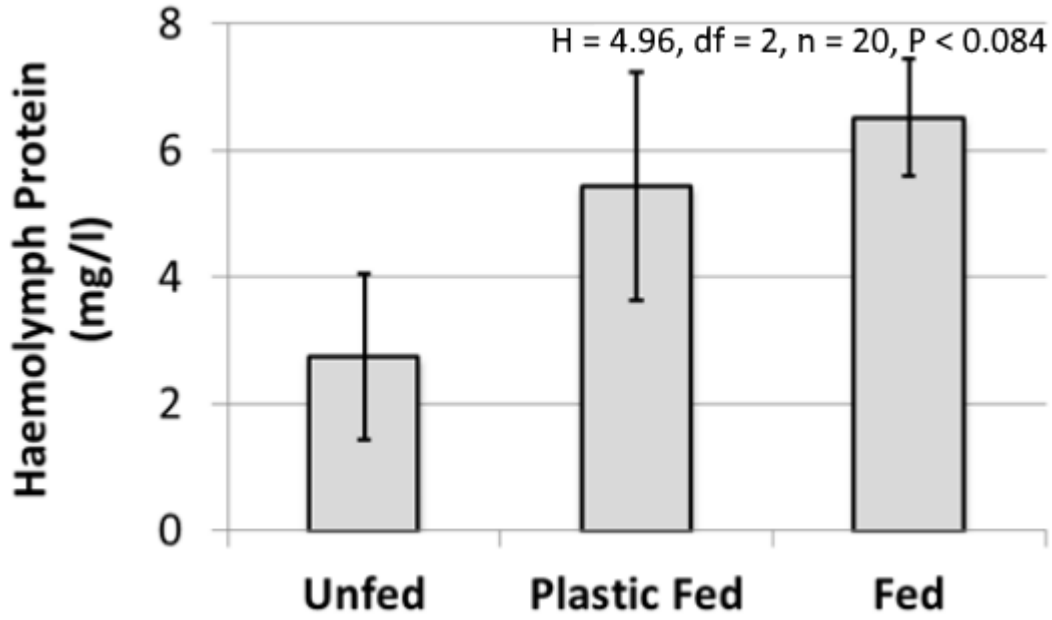

Fig. 4 Mean level of haemolymph protein of animals from the three experimental groups observed at month eight (bars indicate standard error)

The breakdown of the main haemolymph protein, haemocyanin, releases two copper atoms which build-up in the hepatopancreas. Examination of the level of copper in the hepatopancreas revealed significant variation between the groups (H = 7.96, d.f. = 2, P < 0.019), although this was driven by extraordinarily high levels of copper in two plastic-containing individuals. Mann-Whitney analysis revealed differences between plastic-fed individuals and both controls; however, these were only to 90 to 95% confidence (plastic-fed/starved: W = 42.0, P < 0.0128; plastic-fed/fed: W = 20.0, P < 0.0513).

While the reduction in haemolymph protein in the plastic-fed individuals is not as marked as that in the starved condition, it is clear that there is reduced nutrient uptake in *N. norvegicus* contaminated with plastic, causing metabolic depression. A previous evaluation of starvation in *N. norvegicus* carried out by Watts et al. (2014a) revealed that copper levels above 350.19 μg g^{-1} were indicative of starvation. The mean copper level in each group was above this threshold; however, those of the plastic-fed groups were markedly higher, indicating that *N. norvegicus* in the treatment group experienced metabolic depression.

Use of Stored Lipids

Metabolic depression is only effective for limited periods, prolonged episodes of insufficient nutrients force *N. norvegicus* to utilise energy stores; first glycogen, and then lipids. Prolonged starvation has previously been seen to result in a reduction in lipid in both the hepatopancreas and tail (Barden, 1994).

All three treatment groups showed significant variation in nutritional state after eight months, with an obvious reduction in the body condition of individuals exposed to microplastic. The average water content of the hepatopancreas varied between the three conditions; 85.3% in the starved condition, 79.9% in the plastic-fed condition, and 71% the fed condition (Fig. 5). This variation was seen to be significant ($H = 12.70$, d.f. = 2, $P < 0.002$). Post hoc Mann-Whitney tests revealed significant differences of at least 95% confidence between the three groups. The relatively high water content in the starved and plastic condition indicates that these animals have experienced a reduction in stored lipids which were then replaced by water.

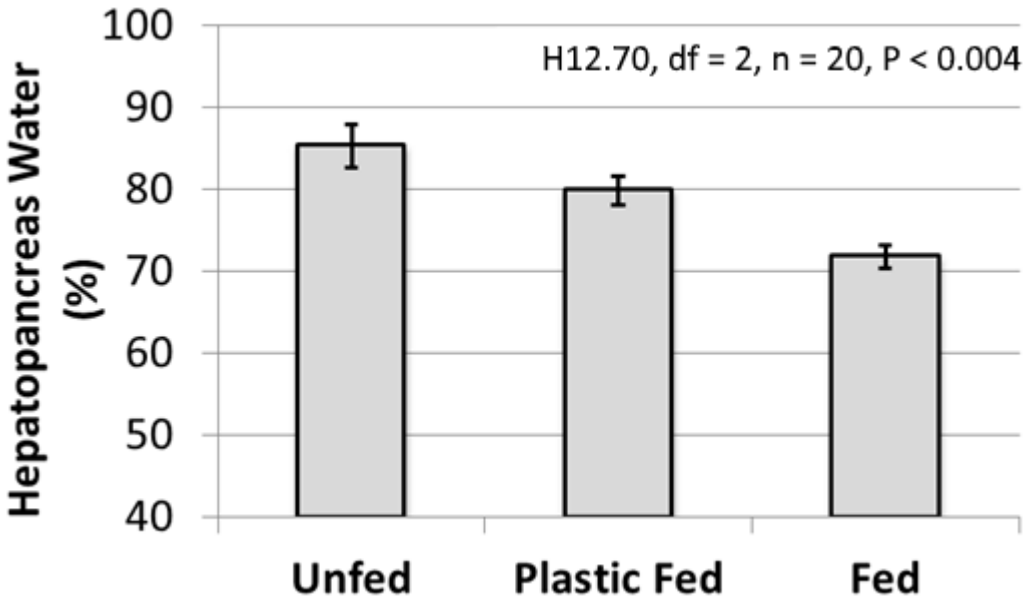

Fig. 5. The mean level of water in the hepatopancreas (%) of animals from the three experimental groups as observed at month eight (bars indicate standard error)

Correspondingly, the HSI of the plastic-fed condition was between that of the control groups. The hepatosomatic index was seen to vary significantly between the three groups, ranging from 0.8% in the starved condition to 4.5% in the fed condition ($H = 10.98$, d.f. = 2, $P < 0.004$) (Fig. 6). Post hoc Mann-Whitney tests revealed differences between individuals in the fed and starved controls

(W = 76.0, P < 0.0043), and plastic-fed treatment and starved control (W= 86.0, P < 0.0128). The lower HSI in the starved and plastic-fed conditions indicate that these animals have experienced a reduction in available nutrients greater than that which could be mediated by metabolic depression.

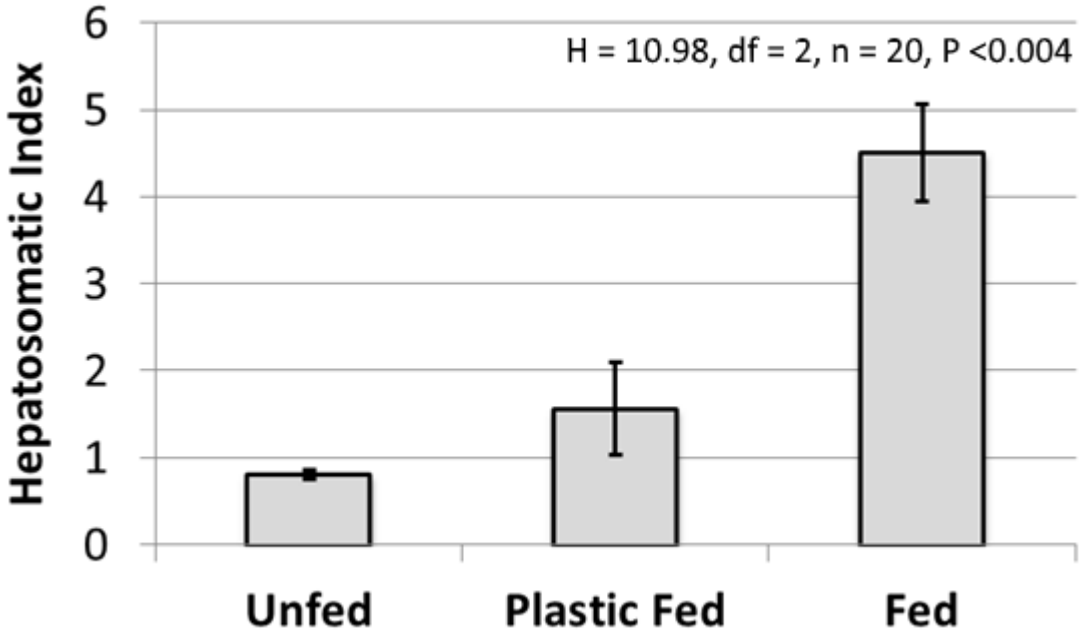

Fig. 6. The mean hepatosomatic index of animals from the three experimental groups observed at month eight (bars indicate standard error)

Further Impacts of Long Term Plastic Retention

The results described above indicate that microplastic retention by *N. norvegicus* is linked to reduced nutritional state; however, the prolonged exposure period experienced by *N. norvegicus* may lead to secondary impacts of plastic ingestion beyond those of reduced feeding. The intermoult period of *N. norvegicus* varies between the sexes; occurring every six months in males and twelve months in ovigerous females (Farmer, 1973). As a result, females retain their aggregations for longer periods and are at increased risk of developing large microplastic aggregations as documented in Welden and Cowie (2016).

In addition to the impacts of low nutrient availability on metabolism and lipid stores discussed above, starvation in crustaceans may cause reduced fecundity (Abellô and Sardá, 1982; Beyers and Goosen, 1987; Hines, 1991; Lizárraga-Cubedo et al., 2003), limiting egg production and, therefore, population growth. Such reduced fecundity has previously been observed in oysters

exposed to polystyrene microspheres (Sussarellu et al., 2014). This potential effect should be of high concern in an economically important species such as *N. norvegicus*, landings of which are regularly in the UK's top five highest grossing fisheries.

Retention of microplastic for long periods will also influence the uptake of additives and adsorbed pollutants. Investigations of partitioning of hydrophobic contaminants have shown transfer between gut plastic and animal tissues (Farrell and Nelson, 2013). In many of these species the residence time of ingested plastics is believed to range from a number of hours (in marine worms), to a number of days (in birds). The ability of *N. norvegicus* to retain plastic for a number of months indicates an extended period over which hydrophobic contaminants may be transferred to the organism and potentially contribute to deleterious population effects.

Conclusions

The results presented above indicate that microplastic aggregations reduce the nutritional health of *N. norvegicus*. The reduction in mean body mass of the plastic-fed individuals indicates that retention of microplastic results in lower growth rates. In wild populations experiencing additional pressures, retention of microplastic may result in increased mortality and decreased fecundity.

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