Culturing soles on ragworms: Growth and feeding behaviour

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Abstract

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Despite the high market demand and intensive research efforts since the 1960[s] commercial culture of common sole (*Solea solea* L.) has been unsuccessful. Problems related to availability and price of suitable raw materials (invertebrates) and the low tolerance to crowding have hampered the development of intensive sole culture. Alternative extensive pond cultures systems are currently explored where common sole can graze on natural food. The general aim of this study was to get insight into which factors limit growth of common sole foraging on ragworms in ponds. The results did not show any nutritional effects that may hamper the growth of common sole. At non-limiting conditions, i.e. when fed chopped ragworms and when housed in sediment free tanks, common sole showed higher food intake, higher growth rates and higher nutrient utilization efficiencies than when fed mussels or a formulated diet. The results however suggest that growth in a pond with ragworms was limited by reduced foraging capabilities of common sole. To explore this hypothesis, the effect of prey size, predator size and prey density were tested. Overall, intake of buried ragworms was reduced by more than half in contrast to intake of unburied ragworms. Intake of buried ragworms was reduced regardless of ragworm size or common sole size. Increasing ragworm density only resulted in satiation intake values in smaller common sole. Our results additionally indicate that the presence of common sole hampers ragworm performance in a pond. Ragworms reduced their feeding activities when receiving water from tanks which contained common sole and ragworms, i.e. when common sole could graze on ragworms. Results from this PhD study suggest that the growth of common sole in ponds is not limited by nutritional but by their foraging abilities. However, the results of this study are too incomplete to fully predict growth performance of common sole in a pond. Factors such as temperature, oxygen supply or feeding activity need to be investigated to make comprehensive growth predictions.

Contents

Chapter 1

General Introduction

Worldwide, aquaculture is one of the fastest growing animal food producing sectors (6.3% per year). It produces currently 47% of food fish for human consumption and is expected to continue to grow rapidly. This share is expected to rise to 62% by 2030 as catches from wild capture fisheries level off and seafood demand from an emerging global middle class substantially increases (FAO, 2014). About 40% of the total marine catch comes from only 23 major species such as anchovy, tuna or cod of which catches exceed half a million tons per year (Daniels, Watanabe, 2010). However, there is also a considerable market demand for flatfish species which are less exploited. Flatfishes are highly valued in the market due to their appealing firm texture along with a subtle mild seafood taste. Fisheries have been unable to meet the market demand for flatfish. This resulted in a rapid development of commercial farming of various flatfish species.

Currently flatfish aquaculture is dominated by the production of 69000 MT / year of lefteye flounder (*Arnoglossus* sp.) in China; 48000 MT / year of Bastard halibut (*Paralichthys olivaceus*) in Korea and Japan respectively, and 7000 MT / year of turbot (*Scophthalmus maximus*), mainly in Spain with minor volumes produced in Portugal and Netherland, United Kingdom, Germany and Denmark France (Daniels, Watanabe, 2010). Other farmed flatfish species of commercial importance include Atlantic halibut (*Hippoglossus hippoglossus*) mainly produced in Norway with some production in Iceland (1000 MT / year) (Daniels, Watanabe, 2010). Production of Sole (*Solea senegalensis* Kaup) in 2012 varied from 200 MT (France), 200 MT (Spain) to 100 MT (Portugal) (Morais et al., 2014). In contrast, common sole (*Solea solea* L.) were produced at minor production quantities in Spain (65 MT) and at small quantities in Italy, the Netherlands and Portugal (Bjørndal, Guillen, 2014). In contrast to other flatfish species the production of sole, in particular common sole, only marginally increased. Currently almost all marketed sole is wild. This is mainly explained by the fact that on-growing stages of common sole are difficult to feed. High quality fish meal is still the major protein source in aquaculture feeds. Fish are however not the natural prey of common sole and fish meal based diets are unattractive for this species (Reig et al., 2003). Including

invertebrate products, such as mussel meat, in the feed formulation, improves feed intake and growth (Day et al., 1997; Fonds et al., 1989a; Mackie et al., 1980). However, the limited availability and high price of invertebrate materials makes them an unsuitable supplement to fish meal based feeds. In addition to problems related to availability and price of suitable raw materials, the low tolerance to crowding in common sole is a problem towards profitable culture under intensive conditions. Alternative extensive pond cultures systems are currently explored where common sole can graze on natural food.

Life history traits of common sole

The flatfish family Soleidae, or true soles, inhabit salt and brackish water in the East Atlantic, Indian Ocean, and West and Central Pacific Ocean. Fresh water species are found in Africa, southern Asia, New Guinea and Australia. The Soleidae consist of 32 Genera with 175 Species (Eschmeyer, 1998).

At hatching, common sole is pelagic and displays the principal external morphological features of other teleost larvae. One eye is present on each side of the head and the mouth is horizontal or sub-horizontal. During metamorphosis one eye migrates to the opposite side of the head inducing morphological and functional asymmetries of the feeding apparatus (Wagemans, Vandewalle, 2001; Yazdani, 1969). The pelagic larvae feed on copepod nauplii (Russell, 1978). After metamorphosis common sole lie on the bottom with their blind side and adapt to a benthic feeding mode (Macquartmoulin et al., 1991). Common sole exhibit a slow moving search behavior and locates prey by olfactory and tactile cues (Appelbaum, Schemmel, 1983; Appelbaum et al., 1983; de Groot, 1969). The diet of metamorphosed common sole consists of benthic invertebrates living on and in the sediment such as polychaetes, bivalves and crustaceans (Braber, de Groot, 1973a; Braber, de Groot, 1973b; Cabral, Costa, 1999; de Groot, 1971; Heip, Herman, 1979; Molinero, Flos, 1991; 1992). The prey changes during lifetime; small common sole eat almost exclusively polychaetes whereas the diet of large common sole consists predominantly of crustaceans (Molinero, Flos, 1991).

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Common sole have a small mouth and a very small stomach and are therefore morphologically adapted to consume small individuals (de Groot, 1971; Piet et al., 1998). Common sole are typical "browser feeders" i.e. frequently eating a small amount of prey, and the morphology of the alimentary tract reflects this (Braber, de Groot, 1973a; de Groot, 1971). During feeding a considerable time is spent on searching for prey while the foraging sequences of detection-orientation-approach are strongly condensed (Holmes, Gibson, 1983). Common sole have 3 distinct feeding periods within the day: around sunset, midnight and before sunrise with moderate feeding activities in between (Kruuk, 1963; Lagardere, 1987).

According to the ICES fish map factsheet (2014), common sole reaches a maximum length of about 700 mm total length and a maximum age of about 70 years. The biogeographical range of common sole extends from the southern coast of Norway, Denmark (Skagerrak and Kattegat) down to the northwest African coast and the Mediterranean (Quéro et al., 1986; Wheeler, 1969). Younger common sole are found in very shallow coastal waters and estuaries. Older groups migrate to deeper waters. Common sole prefers sand or sandy/muddy habitats. Spawning occurs along the southern coast at five main spawning grounds: inner German bight, off the Belgian coast, in the eastern channel, in the Thames estuary and on the Norfolk Banks (Rijnsdorp et al., 1992; Russell, 1978). Most marine teleosts are broadcast spawners that release many, small pelagic eggs into the water column (Bruton, 1989). This is also generally true for flatfishes and has been noted also for common sole (Baynes et al., 1993). Fecundity varies between North sea stocks ranging from 400-900 eggs per gram body weight (Rijnsdorp et al., 1992). The pelagic eggs have a diameter of 1.0-1.6mm (Rijnsdorp, Vingerhoed, 1994; Rijnsdorp et al., 1992; Witthames, Greer Walker, 1995). Egg sizes ranges between 1.0 and 1.6 mm and showed a decrease in size during the spawning season (Baynes et al., 1993; Houghton et al., 1985). In contrast to most other flatfish species, common sole females grow faster than males and reach a larger size (Deniel, 1990). Females spawn in spring in the coastal waters. In species where the sex ratio is determined genetically, there is little evidence that the sex ratio

should vary from 1:1 (Krebs, Davies, 2009). It has been assumed that this is the case in species like common sole although skewed sex ratios suggest that the functional sex can be influenced by factors such as rearing temperature, stocking density, growth rate and water quality (Baynes, Hallam, 1999).

The history of common sole aquaculture

First attempts to rear common sole larvae (the synonym *Solea vulgaris* in older literature) in artificial conditions date back to the beginning of the last century (Fabre-Domergue, Biétrix, 1905). This early study was followed by Danneyi (1948) who successfully reared common sole larvae on oyster larvae, *Artemia* nauplii and natural plankton. Commercially viable culture techniques for this species were however only developed in the 1960s to the 1980s (Appelbaum, 1985; Flüchter, 1965; 1972; 1974; Shelbourne, 1964; Shelbourne, 1975). Common sole spawns naturally in captivity. The best hatching performance and minimum number of deformed common sole larvae was obtained at incubation temperatures between 10-16°C (Baynes, Howell, 1996; Devauchelle et al., 1987; Fonds, 1979). Similarly, Fonds (1979) reported highest survival and hatching performance at salinities between 20-40 ppm. The availability of viable eggs opened the opportunity for larval rearing trials. Successful larval rearing was achieved using rotifers (Howell, 1973), a combination of rotifers and *Artemia* nauplii (Fuchs, 1982b) or *Atermia* nauplii grown on yeast species enriched with fatty acids (Dendrinos, Thorpe, 1987). Common sole fed exclusively traditional feeds like rotifers or *Artemia* showed however signs of nutritional deficiencies such as poor health, anomalies in the development, reduced growth or malpigmentation. Improved performance and healthy metamorphosed fish were only obtained when adding organisms such as flagellates (*Cryptomonas* sp.) in addition to e.g. *Artemia* (Flüchter, 1974). Larval common sole use their visual sense for feeding though they are capable of feeding in the dark from the very early hatching stage onwards (Blaxter, 1969; de Groot, 1971). Feeding efficiency of larval, post larval and juvenile common sole improved when *Artemia* where stained compared to feeding efficiency of unstained *Artemia* (Dendrinos et al., 1984). Reports on the successful onset of weaning in common

sole differ among authors. Appelbaum (1985) reared common sole from first feeding to metamorphosis exclusively on an inert diet. Gatesoupe and Luquet (1982) began weaning of common sole at 10 days after hatch (dah), (Fuchs, 1982a) at 25 dah, Le-Ruyet et al. (1980) at 30 – 40 dah and Bromley (Bromley, 1977) at 25 – 40 dah. Problems related to the transfer from natural to artificial feeds during weaning have successfully been overcome by including invertebrate ingredients into weaning diets (Bromley, 1977; Gatesoupe, 1983; Girin et al., 1977). A major advance in the development of effective weaning diets however came with the identification of betaine and dimethylthetin as potent chemical feed stimulants (Appelbaum, Schemmel, 1983; Appelbaum et al., 1983; Bayer et al., 1980; Mackie, Mitchell, 1982; Mackie et al., 1980). Other studies have focused on the environmental and nutritional requirements of on-growing stages. Common sole fed the blue mussel *Mytilus edulis* showed better growth, intake and nutrient utilization rates than those fed formulated feeds (Fonds et al., 1989a). The optimum temperature for growth is between 20-25°C (Fonds, 1975; 1979; Howell, 1997; Irwin et al., 2002). A series of studies have contributed comprehensive knowledge on digestive capacities and nutrient metabolism in common sole (Clark et al., 1984a; Clark et al., 1985a; b; c; Clark et al., 1986; Clark et al., 1987; Clark et al., 1988). While a limited number of active carbohydrases was found in its intestine (Clark et al., 1984b) common sole possesses a full complement of protein degrading enzymes (Clark et al., 1985b). Common sole shows a 96 % digestibility of worm meal made from different oligochaetes (Hepher, 1988).

Although larvae culture techniques were in place, commercial farming of common sole did not develop really and remains challenging until today. In his re-appraisal on the potential of sole farming, Howell (1997) identified the improvement of diets for all life stages in conjunction with studies on the ontogenetic development of digestive capacities and costs for raw materials, feeding strategies and stock management practices as major bottlenecks towards commercial success. The author provided first evidence that nutritional challenges of larval feed can be overcome. High growth rates and negligible mortalities were obtained in common

sole larvae fed diets which contained high levels of betaine (feed attractant) and water soluble proteins. Small common soles up to 3cm could subsequently successfully be weaned on to commercially produced artificial diets (Aglonorse ®) with high survival rates and growth rates up to 80% of growth rates obtained with live feeds (Day et al., 1999; Howell, 1998). Attempts to improve on-growing diets have had less success. The inclusion of hydrolyzed fish protein concentrate improved survival during weaning in young common sole but did not improve growth in the subsequent on-growing stages (Day et al., 1997). Recent studies contributed to a more comprehensive knowledge on the nutritional requirements, feeding protocols or reproduction techniques. For example, highest growth rates in common sole were obtained at dietary crude protein levels of 57 – 58 % (Gatta et al., 2010). Piccinetti et al. (2012) showed that co-feeding of preserved copepods together with traditional diets such as rotifers and *Artemia* nauplii improved growth and survival, the tolerance to captive conditions and to thermal/density stress in common sole larvae. Mazzoni et al. (2015) worked on the taste perception in common sole. The authors suggested that taste-related molecules are regulated by changes in diet formulation in common sole aquaculture. Progress has also been made in reproduction techniques. Until recently the failure of the G1 generation to produce naturally fertilized eggs constituted a major bottleneck (Howell et al., 2011). In a recent study using a natural photothermal regime, the G1 generation of common sole successfully reproduced at large scale in captivity, both in outdoor pond tanks as in indoor RAS tanks (Palstra et al., 2014). In addition, a series of studies contributed to improve breeding programs (Blonk et al., 2010a; Blonk et al., 2009; Blonk et al., 2010b; Mas-Muñoz et al., 2011; Mas-Muñoz et al., 2013). Recent studies indicate that common sole has a low tolerance to crowding. Decreased growth rates are observed when fish densities exceed 7.4 kg/ m^2 (Schram et al., 2006). Similarly, Lund (2013) reports reduced feed intake in common sole reared at densities of 3.9 kg m^{-2} compared to those reared at 1.0 kg m^{-2}.

To summarize, commercial culture is still not achieved mainly because on-growing stages remain difficult to feed. High quality fish meal is still the major protein source in aquaculture feeds. However, fish are not the natural prey of common sole and fish meal based diets are unattractive for this species (Reig et al., 2003). It is known that the inclusion of invertebrate products such as mussel meat improves organoleptic characteristics of the fish meal based feeds and thus feed intake (Day et al., 1997; Fonds et al., 1989a; Mackie et al., 1980). However, the limited availability and high price of invertebrate materials makes them an unsuitable as a supplement to fish meal based feeds. In addition to problems related to the availability of suitable raw materials for cost- effective feeds the low tolerance to crowding in common sole is a problem towards profitable culture under intensive conditions.

Problem definition

Industrial scale reproduction and larval rearing methods have been developed for common sole. The lack of a cost effective diet for on-growing stages makes it however difficult to farm this species on industrial scale. The inclusion of invertebrate resources to fish meal based feeds is limited by their availability and price. Therefore an economically viable fish meal based feed which is accepted by this species is currently out of reach. In addition, the low tolerance to crowding makes this species not the ideal candidate for intensive culture.

An alternative concept might be an extensive farming system where common sole is raised in a semi-natural environment grazing on natural food. This technique is not new in sole farming. The related Senegalese sole (*Solea senegalensis* Kaup) has been traditionally cultured in ponds grazing on natural food in the south of Spain and the south of Portugal. Pond farming was done in extensive and semi-intensive land-based ponds in conjunction with other species like seabream to increase profit. This production type is however no longer used due to decreasing prices of target species such as sea bream and sea bass (Ferreira et al., 2010; Yufera, Arias, 2010). Common sole has never been commercially cultured in ponds

although possibilities to on-grow this species in ponds were explored in Italy (Palazzi et al., 2006). Recently an Integrated Multi Trophic Aquaculture approach (IMTA) was investigated in the Netherlands between 2007 and 2012 in the "Zeeuwse Tong (ZT)" project. The concept of an IMTA is that by-products (wastes) from one species (e.g. finfish/shrimps) are recycled and used as inputs (fertilizers, food and energy) for others (e.g. suspension feeders/deposit feeders/herbivorous fish) and inorganic extractive aquaculture species (e.g. seaweeds) (Chopin et al., 2010). In the ZT project the ragworm *Nereis virens* (Sars) was fed a commercial feed and constituted at the same time a natural food for common sole that was stocked in the same ponds. By-products from ragworms and common sole excretions and uneaten feed were used to grow mussels and algae. The ragworms were used because of their availability from a close by *Nereis* farm and the fact that nereidae are also a natural food of common sole. Therefore, in the current PhD project, the main aim was to determine which factors limit growth of common sole foraging on ragworms in ponds. The present study was financed by the project 'Zeeuwse Tong'.

Objectives of this study

The general aim of this study was to get insight into which factors limit growth of common sole foraging on ragworms in ponds. Growth is of major importance from an economic point of view. Growth may become limited when the feed is nutritionally imbalanced and/or cannot be consumed in adequate quantities. In nature common sole feeds on a wide range of different prey species of different composition. It is not clear if ragworms alone meet the nutritional needs of common sole. In addition, no information exists on whether sole has the foraging abilities to capture buried ragworms in a pond environment. I investigated intake of ragworms at non-limiting and limiting conditions. For the non-limiting conditions, common sole were fed with chopped ragworms in sediment free tanks to exclude feed intake limitations. Limited conditions were simulated by having common sole foraging on buried ragworms, i.e. when predator- prey interactions are present. Under these

conditions, factors which are relevant for pond conditions, were tested for their effects on the feed intake, growth and forgaging behaviour of the sole.

Chapters 2 investigated if growth and intake in common sole fed ragworm is comparable to those reported for common sole fed other natural foods, or whether signs of nutritional imbalances (excess/low intake, low growth) are observed. I used mussels as a reference food because common sole grow well on this natural food type. **Chapter 3** determined energy and nutrient utilization efficiencies in common sole fed ragworms. I used a regression method based on restricted feeding levels to eliminate the influence of feeding levels on utilization efficiencies present in chapter 2. Accurate data on nutrient utilization were needed also to identify nutritional imbalances. In both chapters I chopped the ragworms into small pieces and used sediment free tanks to exclude feed intake limiting factors such as handling and accessibility. In addition I included a commercial feed especially formulated to meet the nutritional requirements of flatfishes as a reference feed. **Chapters 4 and 5** investigated common sole's abilities to prey on buried ragworms. Here I looked at the impact of ragworm size, ragworm density and life history stage of common sole on ragworm intake. **Chapter 6** determines if the presence of common sole in a pond system has any influence on the behaviour of ragworms and their availability. The final **chapter 7** provides a short summary of my findings and a discussion of the outcomes. I discussed intake, utilization and growth results in relation to the suitability to serve of ragworms as an exclusive natural food source for common sole in an IMTA system.

Chapter 2

Feed intake, growth and nutrient retention of common sole (*Solea solea* L.) fed natural prey and an artificial feed.

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Abstract

This study compares growth, intake and retention efficiencies of nutrients and energy between common sole (*Solea solea* L.) fed ragworm (*Nereis virens,* Sars), blue mussel (*Mytilus edulis* L.) and an artificial (commercial) feed. Food types were fed to common sole (mean initial body weight: 44.9±2.3g) in excess three times a day over a 54-day-period. The growth rate in common sole fed the natural prey (8.5 g.kg$^{-0.8}$.d^{-1}) was significantly higher compared to the growth rate in fish fed the artificial feed (5.1 g.kg$^{-0.8}$.d^{-1}). Nutrient and energy intake was significantly lower in common sole fed the artificial feed than in fish fed natural prey. The only exception was fat intake which was higher in common sole fed the artificial feed in contrast to fish fed the natural prey. Nutrient and energy retention efficiencies were significantly lower in common sole fed the artificial feed than in fish fed the natural prey. In conclusion, the low growth in common sole fed the artificial feed was related to lower nutrient and energy intake as well as lower nutrient and energy retention efficiencies. It is suggested that reduced intake of the artificial feed might be related to the high dietary fat content of the artificial feed.

Introduction

Despite being considered a promising aquaculture species for more than three decades, the nutritional requirements of common sole (*Solea solea* L.) have received little attention. The absence of a suitable commercial diet remains a major constraint towards a viable production in Europe (Howell et al., 2009). Therefore, until now commercial turbot (*Scophthalmus maximus* L.) feeds were used in the Netherlands for the culture of common sole having a fat content above 15% (pers. comm. A. Kamstra, Wageningen Imares). High dietary fat levels (>12%) reduced nutrient and energy retention efficiencies and growth in Senegalese sole (*Solea senegalensis*, Kaup) (Borges et al., 2009), though this was not confirmed by Dias, Rueda-Jasso, Panserat, da Conceicao, Gomes and Dinis (2004). In common sole artificial feeds with a high protein and fat content resulted in considerably lower growth rates (about 30%) compared to common sole fed natural prey such as the oligochaete worm (*Lumbricillus rivalis*, Levinsen) (Gatta et al., 2010; Irvin, 1973).

These lower growth rates can be due to low nutrient and energy retention efficiencies as suggested for Senegalese sole (Borges et al., 2009). However, differences in feed intake may also be involved. Information on feed intake, nutrient and energy retention in common sole comparing natural prey versus artificial feeds is lacking. Polychaetes and bivalves are natural prey items for common sole (Lagardere, 1987; Molinero, Flos, 1991; 1992) and may therefore be good reference feeds. In the current study, feed intake, growth and retention efficiencies of nutrients and energy were compared between common sole fed natural prey, i.e. the ragworm (*Nereis virens*, Sars), and the blue mussel (*Mytilus edulis* L.) and an artificial (commercial) feed.

Material and Methods

This experiment was approved by the Ethical Committee for Animal Experiments and conducted at the facilities of Wageningen IMARES in Yerseke (The Netherlands) between March until May 2008.

Experimental feeds and feed preparation

Three feed types were compared: two natural preys (i.e. ragworms and mussels) and one artificial feed. The artificial feed was DAN- EX 1562 (DANA FEED A/S, Denmark, sinking pellet, 2 mm), a commercially available feed. We decided to use a 2mm pellet because this pellet size was readily ingested by common sole with no signs of handling problems. The macronutrient compositions of the feeds are shown in Table 1. The artificial feed was based on fish meal and fish oil without any ingredients of invertebrate origin (pers. comm. Dr. J. H. Bloom, BioMar A/S). Ragworms with individual weights ranging from 0.5g -2.0g were obtained twice a week from a commercial ragworm producer (Topsy Baits, Wilhelminadorp, Netherlands). Mussels were purchased twice a week from a local producer. Ragworms and mussels were kept, to a maximum of four days, in an outdoor flow through system using unfiltered seawater before being fed. Ragworms and mussels were chopped into small pieces (approx. <5mm) to avoid prey size effect on feed intake. Ragworms were rinsed with seawater for one minute and drip dried

for one minute. Mussels were opened, taken out of the shell, rinsed with seawater for 1 minute, and left to drip dry for 15 minutes following the referred protocol (Fonds et al., 1989b).

Table 2.1 Analysed proximate composition of the feeds: ragworm, mussel and artificial feed.

Diet	artificial feed	SD	mussel	SD	ragworm	SD
Dry matter (g.kg^{-1} WW)	930	0.1	191.3	0.1	183.3	0.1
Crude protein (g.kg^{-1} DM)	620.4	2.8	702.1	3.6	739.1	13.6
Crude fat (g.kg^{-1} DM)	177.1	2.5	69.7	1.7	82.7	4.18
Ash (g.kg^{-1} DM)	119.4	0.2	106.4	5.0	112.7	2.25
Gross energy (kJ.g^{-1} DM)[1]	23.1	0.1	21.4	0.1	21.8	0.17
NFE (g.kg^{-1} DM)[2]	83.1	5.0	121.8	7.4	65.5	14.6

[1] Calculated using the factors: carbohydrates, 17.2 kJ g^{-1}; protein, 23.6 kJ g^{-1} and lipids 39.5 kJ g^{-1} (Brett, Groves, 1979).

[2] NFE (nitrogen free extract) calculated subtracting the sum of crude protein, crude fat and ash from 1000 in g.kg^{-1} DM.

Fish and rearing conditions

Common sole (n = 200) were obtained from a commercial fish farm (Solea BV, IJmuiden, Netherlands), and transferred to the research facilities of Wageningen Imares in Yerseke, the Netherlands. The rearing system consisted of 10 rectangular glass tanks (0.4m^2 bottom area and a total water volume 120 L per tank). All tanks were connected to a recirculation system equipped with a sandfilter and a beadfilter. Conditions were kept constant throughout the experiment (photoperiod 12L:12D; water flow- 5-6 L.min^{-1}; DO > 7.9 mg.L^{-1}; T- 20.1 ± 0.75°C; salinity at 28.5 ± 2.5 g.L^{-1}; TAN< 1 mg.L^{-1}; NO$_2$-N < 0.063 mg.L^{-1} and NO$_3$-N < 54.2 mg.L^{-1}). The pH was kept above 6.14 except for one day when the pH dropped to 5.67. The pH was controlled by adding sodium bicarbonate to the system. Light cycle was reversed compared to natural circumstances and common sole were fed

analogous to their most active period which is at after sunset, midnight and before sunrise (Lagardere, 1987). During the dark period from 08:00 am until 20:00 pm a low radiation red light was used to provide sufficient light source for feeding.

Growth experiment and sampling procedures

Upon arrival all common sole were weighed individually to the nearest 0.1g. One hundred fish were selected based on body weight (average weight ±SD of 39.6g ± 5.8). The selected fish were randomly assigned to 9 experimental tanks at a density of 10 fish per tank. An additional tank was stocked with 10 fish and used for sampling of initial body composition at the beginning of the experimental period. After an acclimatization period of 15-d, the experimental period of 54-d started. During the acclimatization period, all fish were fed the artificial feed at a level of 5.5 $g.kg^{-0.8}.d^{-1}$.

At the start of the experimental period fish from the 9 experimental tanks were removed, individually weighed (mean initial body weight ± SD was 44.9 ± 2.3g) and returned to their tanks. The 9 tanks were randomly assigned to one of the three dietary treatments (three tanks per diet). All fish from the additional tank were removed. A subsample of 5 fish from the total 10 fish were randomly selected, sacrificed with an excess dose of anaesthesia (tricaine methane sulfonate, MS-222 Finquel®; Argent Chemical Laboratories) and stored at -20°C for later analyses of initial body composition.

During the experimental feeding period, fish were fed to apparent satiation during 3 meals per day (at 09:00, 12:00 and 17:00h). Each meal lasted for 70 min. During this period, feed was constantly provided to the tanks ad libitum. At the end of the meal, uneaten feed was siphoned out of the tanks and subsequently analysed for dry matter content. Feed samples were taken weekly (about 10g per sample, triplicate samples per feed type) and analysed for dry matter content to calculate feed intake on the basis of actual water contents (which may vary in natural prey). Dietary samples, corresponding to 10% of the daily amount of feed consumed per

tank, were taken daily stored at -20°C, and pooled after termination of the experiment per dietary feed type and analysed for proximate composition. No weighing and sampling of the fish was done during the 54-d experimental feeding period. At the end of the experimental period, fish were removed from the tanks and individually weighed. Five fish per tank were randomly selected and sacrificed with an overdose of MS 222, stored at -20°C and analysed for final body composition. Samples of the natural prey were taken every second week to determine their dry matter (DM) content.

Analytical methods and calculations

After grinding and homogenising, feeds and fish were sampled and analysed for: dry matter according to ISO 1442 (ISO, 1997); total nitrogen (protein is N*6.25) by the Kjeldahl method according to ISO 937 (ISO, 1978); ash by incineration at 550°C according to ISO 936 (ISO, 1998), and crude fat by extraction with petroleum ether according to ISO 1443 (ISO, 1973). Proximate analyses were performed by SGS (Spijkenisse, Netherlands). The dietary carbohydrate content (i.e., nitrogen free extract, NFE) was calculated subtracting the sum of crude protein, crude fat and ash from 1000 in $g.kg^{-1}$ DM. Gross energy (GE) of the feed types was calculated from the carbohydrate, protein and fat content by multiplying with the energy contents of carbohydrate, protein and fat of respectively, 17.2, 23.6 and 39.5 $kJ.g^{-1}$ (Brett, Groves, 1979).

We expressed performance data on the basis of metabolic body weight. The maintenance requirements are dependent on body weight raised to the power of 0.8. Data become independent of bodyweight and are better comparable between fish species as well as within fish species when expressed the basis of metabolic body weight (Lupatsch et al., 1998).

Mean initial body weight (IBW) and mean final body weight (FBW) were calculated per tank. The geometric mean body weight (W_G; in g) was calculated as *exp* ((*ln* (FBW) + *ln* (IBW)) / 2). The metabolic body weight of fish (MBW_G; in $kg^{0.8}$) was

calculated as (W_G) / $1000)^{0.8}$. Growth rate per metabolic weight (RGR; $g.kg^{0.8}.d^{-1}$) was calculated as (FBW – IBW)/ (MBW$_G$ x t) where t is the duration (days) of the growth study.

Daily feed intake (DFI; g DM fish^{-1}.d^{-1}) was calculated as TFI / ($t * n$), where TFI is the total FI per tank over the experimental days corrected for uneaten feed, t is the experimental period and n is the number of fish per tank. No mortalities occurred during the experiment hence calculations were based on 10 fish per tank. FI per metabolic body weight (FI$_{MBW}$; g DM kg$^{-0.8}$.d^{-1}) was calculated as DFI/ MBW$_G$. The crude protein, crude fat and ash intake per metabolic body weight was calculated as the product of FI (FI$_{MBW}$; g.kg$^{-0.8}$.d^{-1}) and the respective measured protein, fat or ash content of the feed (mg.g^{-1}). Gross energy intake per metabolic body weight (kJ.g$^{-0.8}$.d^{-1}) was calculated as FI$_{MBW}$ * gross energy content of the diet (kJ.g^{-1}). Retention of DM (RET$_{MBW}$) per metabolic body weight (g DM kg$^{-0.8}$.d^{-1}) was calculated as ((FBW * CF$_{DM}$ / 1000) - (IBW * CI$_{DM}$ / 1000)) / (t * MBW$_G$), where CF$_{DM}$ is the dry matter content of the final carcass and CI$_{DM}$ is the dry matter content of the initial carcass. Protein, ash, fat retention (g.kg$^{-0.8}$.d^{-1}) and energy retention (kJ.g$^{-0.8}$.d^{-1}) was calculated in a similar way but on the basis of final and the initial carcass protein, ash, fat and energy contents.

Relative retention of DM was calculated as (100 * RET$_{MBW}$ / FI$_{MBW}$). Relative protein, ash, fat and energy retention was calculated in a similar way but on the basis of protein, ash, fat and energy intake and retention. Feed conversion ratio (FCR) was calculated as FI$_{MBW}$ / RGR.

Statistical analysis
The tank was the experimental unit. Consequently, all calculations and statistical analysis were done at the tank level (i.e., mean value of the fish within a tank) and data are given as means± SD. The effect of dietary treatment was assessed by one-way ANOVA. In case of a significant effect of diet, Fisher's least significant difference (LSD) test was for pair wise comparison of dietary treatments. Statistical

significance was tested at the 0.05 probability level. All tests were performed using the statistical program SPSS 15.0 (Chicago, IL, USA). All data was tested for homogeneity in variance by Levene's test prior to ANOVA. Outlier tests were performed on the data prior to statistical evaluations.

Results

Performance data and nutrient intake are shown in Table 2.2. Final body weight (in g wet weight) was significantly affected by food types ($P<0.001$) and was higher in common sole fed the natural prey compared to common sole fed the artificial feed. Final body weight was also higher in common sole fed ragworms compared to common sole fed mussels. The relative growth rate (in $g.kg^{-0.8}.d^{-1}$) was significantly affected by food types ($P<0.001$) and was higher in common sole fed the natural prey compared to common sole fed the artificial feed. The relative growth rate was not different between common sole fed the natural prey. Dry matter, protein, fat and gross energy intake (in $g.kg^{-0.8}.d^{-1}$ or $kJ.g^{-0.8}.d^{-1}$) was significantly affected by food types ($P<0.001$) and was higher in common sole fed the natural prey compared to common sole fed the artificial feed. Dry matter and gross energy intake was also higher in common sole fed mussels compared to common sole fed ragworms, whereas protein and fat intake was not different between the natural prey.

Data on whole body composition, nutrient retention and nutrient retention efficiency are shown in Table 2.3. Dry matter, protein, fat and gross energy retention (in $g.kg^{-0.8}.d^{-1}$ or $kJ.g^{-0.8}.d^{-1}$) was significantly affected by food types ($P<0.05$). Dry matter, protein and gross energy retention differed between all food types being highest in common sole fed ragworms and lowest in common sole fed the artificial feed. Fat retention was not different between common sole fed mussels and those fed the artificial feed. Dry matter, protein, fat and gross energy retention efficiency (in %) was significantly affected by food types ($P<0.05$). Dry matter, protein and gross energy retention efficiency was higher in common sole fed ragworms compared to common sole fed mussels and the artificial feed. Dry matter, protein and gross energy retention efficiency was not different between common sole fed mussels

and those fed the artificial feed. Fat retention efficiency was higher in common sole fed the natural prey compared to common sole fed the artificial feed. Body moisture, ash, fat and energy contents (in % wet weight) were significantly affected by food types ($P<0.05$). Body protein contents (in % wet weight) were not affected by food types ($P=0.117$). Body fat content was similar in common sole fed ragworms and the artificial feed and higher compared to the body fat content in common sole fed mussels.

Table 2.2 Performance and nutrient intake of common sole fed artificial feed, mussel and ragworm

diet	artificial feed		mussel		ragworm		
	Mean	SD	Mean	SD	Mean	SD	P-value
Initial body weight (g)	43.3	0.91	44.7	3.07	46.7	1.33	0.208
Final body weight (g)	71.4[a]	5.57	96.2[b]	4.05	107.2[c]	0.36	<0.001
Growth (g.kg$^{-0.8}$.d^{-1})	5.1[a]	0.64	8.1[b]	0.2	8.8[b]	0.24	<0.001
FCR	1.11[a]	0.1	0.95[b]	0.01	0.78[c]	0.02	0.002
Daily nutrient intake (in g.kg$^{-0.8}$.d^{-1}or kJ.g$^{-0.8}$.d^{-1})							
Dry matter	5.62[a]	0.21	7.61[b]	0.27	6.87[c]	0.2	<0.001
Protein	3.49[a]	0.13	5.34[b]	0.19	5.08[b]	0.16	<0.001
Fat	1.00[a]	0.05	0.53[b]	0.02	0.57[b]	0.04	<0.001
Energy	129.69[a]	4.98	162.93[b]	6.41	149.97[c]	4.75	0.001

Values are means of triplicate tanks ± SD. Within a row, means without a common superscript letter differ significantly (P<0.05) as calculated by ANOVA and Fisher's least significant difference test.

Table 2.3 Whole body composition, retention and retention efficiency of common sole fed artificial feed, mussel and ragworm. Retention and retention efficiencies are expressed on dry matter basis.

diet	artificial feed		mussel		ragworm		
	Mean	SD	Mean	SD	Mean	SD	*P*-value
Final body composition							
Moisture (% wet weight)	74.33	0.64	74.67	0.81	73.3	0	0.071
Ash (% wet weight)	2.48^a	0.1	2.51^a	0.15	2.90^b	0.2	0.027
Protein (% wet weight)	16.83	0.31	16.83	0.42	17.4	0.2	0.117
Fat (% wet weight)	6.67^a	0.57	5.50^b	0.4	6.47^a	0.32	0.037
Energy (kJ/g wet weight)	6.61^a	0.26	6.15^b	0.24	6.66^a	0.1	0.047
Retention (in g.kg$^{-0.8}$.d^{-1} or kJ.g$^{-0.8}$.d^{-1})							
DM	1.54^a	0.24	2.22^b	0.08	2.63^c	0.06	<0.001
Protein	0.70^a	0.12	1.21^b	0.07	1.44^c	0.07	<0.001
Fat	0.65^a	0.11	0.63^a	0.05	0.82^b	0.04	0.037
Energy	41.97^a	7.21	53.36^b	3.06	66.22^c	0.32	0.002
Retention efficiency (%)							
DM	27.40^a	3.63	29.17^a	1.91	38.27^b	0.97	0.003
Protein	19.97^a	3.25	22.66^a	1.1	28.34^b	1.87	0.01
Fat	64.84^a	9.99	118.85^b	15.13	144.45^b	13.77	0.001
Energy	32.33^a	4.98	32.78^a	2.33	44.19^b	1.61	0.007

Values are means of triplicate tanks ± SD. Within a row, means without a common superscript letter differ significantly (P<0.05) as calculated by ANOVA and Fisher's least significant difference test.

Initial body composition was: moisture 77.2% wet weight, fat 2.85% wet weight, ash 1.32% wet weight, protein 18.90% wet weight, energy 5.59 kJ.g^{-1} wet weight.

Discussion

This study showed that common sole fed an artificial feed grew less (- 40%) than those fed natural prey, being in line with previous studies on common sole (Fonds, 1975; Gatta et al., 2010). Borges et al. (2009) suggested that the lower growth of Senegalese sole fed various artificial feeds was related to reduced nutrient and energy retention efficiencies. The current study shows that the low growth of common sole fed an artificial feed is due to both reduced nutrient and energy retention and reduced nutrient and energy intake. However, the contribution of intake and retention to the higher growth rate depended on the type of natural prey. Common sole fed mussels had a higher intake but lower retention efficiencies of nutrients and energy compared to common sole fed ragworms.

Feed intake in fish is dependent on environmental, dietary and animal related factors. In various fish species it is shown that FI is regulated to meet the demands for digestible energy (DE) for growth and maintenance requirements (Boujard, Médale, 1994; Lee, Putnam, 1973; Yamamoto et al., 2000; Yamamoto et al., 2001). We did not determine digestibilities firstly because faeces of common sole are very difficult to collect due to their loose structure. Secondly, the natural food lacked the presence of a good inert marker which is required for digestibility measurements. Present results nonetheless indicate that the low FI of the artificial feed was not related to demands for digestible energy. The GE contents of all feeds were similar (Table 2.1). However, natural prey generally have a higher digestibility than formulated feeds (Jobling, 1986). Therefore, if FI of the artificial feed was regulated by demands for digestible energy, the GE intake of the artificial feed should have been higher than the GE intake of the natural prey to compensate for lower digestibility. This was not the case, rather GE intake in common sole fed the artificial feed was considerably lower than in comparison to natural prey (Table 2.2, Fig. 1B). Also the dietary fat content may indirectly affect FI due to a negative feedback control of body fat content (Boujard, Médale, 1994; Heinsbroek et al., 2007; Johansen et al., 2002; Lee, Putnam, 1973; Yamamoto et al., 2001). The body fat content was similar in common sole fed the artificial feed and those fed

ragworms, but lower in common sole fed mussels. This might suggest an involvement of body fat content in FI regulation in common sole fed ragworms and the artificial feed. Previous studies have also demonstrated that FI is influenced when energy density of the diet is low, for example by a large concentration of dietary carbohydrate or water. In this case stomach fullness may limit FI (Vahl, 1979). The natural prey had the lowest energy content when expressed on wet weight basis but showed the highest FI. This indicates that FI of the natural prey might have been limited by stomach fullness.

Poor palatability might have also contributed to the observed low FI in common sole fed the artificial feed. The present artificial feed was based on fish meal and fish oil without any ingredients of invertebrate origin (pers. Comm. Dr. J. H. Bloom, BioMar A/S). Senegalese sole ate more pellets flavoured with a worm homogenate than those without this homogenate (Barata et al., 2009). The majority of animals including fish show a reduction of feed intake when fed diets of amino acid (AA) deficiency (D' Mello, 2003; De la Higuera, 2007; Fortes-Silva et al., 2012; Potier et al., 2009). The AA composition of the tested feed types was not analysed. Also the AA requirements of common sole are unknown. Therefore, the role of AA in control of FI in the present study remains unclear.

The observed differences in nutrient and energy retention efficiencies (Table 2.3) are likely due to the differences in macronutrient composition of the different feed types. Dietary crude protein requirements were estimated to be 57% in common sole (Gatta et al., 2010) and 53% in Senegalese sole (Rema et al., 2008) (on dry matter basis). Dietary crude protein contents of the present food types were at least 62% (on dry matter basis, Table 2.1) and the crude protein to gross energy ratios were 26.8, 32.8 and 33.9 mg.kJ^{-1} respectively for the artificial feed, mussels and ragworms. These data suggest an excess of protein of all food types, which might explain the observed relatively low protein retention efficiencies (<28%, Table 2.3). In many fish species it has been demonstrated that protein retention efficiency is reduced with increasing dietary protein to energy ratio (Hatlen et al.,

2007; Saravanan et al., 2012). With the natural prey (mussels and ragworms) the fat retention efficiency was larger than 100%, indicating that fat was synthesized de novo. This supports the suggested excess of dietary protein. However, in the current study an excess of protein cannot explain the observed differences in protein retention efficiency between the food types; Common sole fed ragworms, which had the highest crude protein content (on dry matter basis), had the highest protein retention efficiency. The major difference in macronutrient contents between the artificial feed and natural prey was the amount of fat (18% vs 7%, Table 2.1). Borges et al. (2009) found in Senegalese sole, that both growth and nutrient and energy retention efficiencies declined with increasing dietary fat content above 8%. Data of the current study suggest that common sole also have a low tolerance for dietary fat, being indicated by the lower protein as well as fat retention efficiency for common sole fed the artificial feed. However, Dias et al. (2004), did not observe such an impact of dietary fat content in Senegalese sole. Despite the very small differences in macronutrient composition between mussels and ragworms, nutrient and energy retention efficiencies were higher for common sole fed ragworms (Table 2.3). This suggests an involvement of other nutritional properties. One explanation is as high level of free amino acids in the body fluids of polychaetes providing a readily assimilated source of nutrients (Fyhn, 1989).

In conclusion, growth of common sole fed natural prey is higher compared to growth in common sole fed artificial feed. The current study demonstrated that this is due to both a higher nutrient and energy intake and improved nutrient and energy retention efficiencies. Our results suggest that the low protein efficiency observed in common sole fed the artificial feed is not only related to an excess of dietary protein of the artificial feed. Results further suggest that the reduced feed intake observed in common sole fed the artificial feed is not related to the dietary energy content of the artificial feed. It is hypothesized that both reduced intake and reduced retention efficiencies of nutrients and energy in common sole fed the artificial feed are related to an excess in dietary fat content.

Acknowledgements

**The effect of food type (artificial diet vs. natural food) and fish size on feed
utilization in common sole, *Solea solea*.**

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Abstract

This study compares the effect of food type (formulated diet vs. natural food) and fish size on protein and energy utilization efficiencies for growth in common sole (*Solea solea*). Replicate groups of common sole (mean initial body weight ±SD was 45.7g ± 2.1 and 111.2g ± 4.2) received the diets at 5 (natural feed) or 4 (formulated diet) feeding levels. The protein utilization efficiency for growth (k_{gCP}) was higher (P >0.001) in common sole fed ragworms than in common sole fed the formulated diet (respectively, 0.40 and 0.31). Likewise, the energy utilization efficiency for growth (k_{gGE}) was higher (P =0.001) in common sole fed ragworms than in common sole fed the formulated diet (respectively, 0.57 and 0.33). The protein maintenance requirement was not different between food types (P =0.64) or fish size (P =0.41) being on average 0.82 g kg$^{-0.8}$.d^{-1}. The energy maintenance requirement was not different between food type (P =0.390) but differed between fish size (P =0.036). The gross energy maintenance requirement of small common sole was 35 kJ g$^{-0.8}$.d^{-1}. The gross energy maintenance requirement of large common sole was 25 kJ g$^{-0.8}$.d^{-1}. In conclusion, the low growth of common sole fed formulated diets was related to reduced feed utilization.

Introduction

Common sole (*Solea solea*) grows less on formulated diets than on natural food (Fonds et al., 1989a; Gatta et al., 2010; Irvin, 1973). This lower growth in common sole seems partly related to lower feed intake in common sole fed formulated diets (Ende et al., 2014). Previous studies suggest also that formulated diets are less efficiently utilized than the natural foods. Feed utilization efficiencies are however difficult to compare due to the differences in feed intake which may influence this measure of nutrient utilization efficiency. One way of comparing nutrient utilization efficiencies is to use a bioenergetic approach where regression values of protein and energy utilization efficiency for growth at increasing feeding levels are compared. The advantage of this bioenergetic approach is that by comparing regressed utilization values, effects of variable intake affecting utilization efficiencies are removed and considerable experimental power is gained (Searcy-Bernal, 1994). This method has been used to compare diets of different macronutrient content in several fish species such as rainbow trout (*Oncorhynchus mykiss*) (Glencross et al., 2007), barramundi (*Lates calcarifer*) (Glencross, 2008), yellowtail kingfish (*Seriola lalandi*) (Booth et al., 2010) and Atlantic cod (*Gadus morhua*) (Hatlen et al., 2007). To our knowledge, the bioenergetic approach has so far not been used for comparisons of feed utilization in common sole fed feeds that result in considerable growth differences, i.e. natural vs. formulated feeds. The present study compares the influence of feed type (formulated vs. natural) and fish size on protein and energy utilization efficiencies for growth in common sole using increasing feeding levels.

Material and Methods

This experiment was approved by the Ethical Committee for Animal Experiments and conducted at the facilities of Wageningen IMARES in Yerseke (The Netherlands) between October until December 2008.

Experimental diets and diet preparation

The proximate composition of the two food types, i.e. ragworm (*Nereis virens*) and the formulated diet (DAN- EX 1562, Denmark, sinking pellet, 2 mm, BioMar A/S, formerly DANA FEED A/S), are given in Table 3.1. The formulated diet was based on fish meal and fish oil without any ingredients of invertebrate origin (Joost Blom, BioMar A/S, pers. comm.). Food types differed mainly in dietary fat level, which was almost 50% higher in the formulated diet than in ragworms. Ragworms with individual weights ranging from 0.5g -2.0g were obtained twice a week from a commercial producer (Topsy Baits, Wilhelminadorp, Netherlands). Ragworms were stored for a maximum of four days, in an outdoor flow through system using unfiltered seawater before being fed. Ragworms were prepared by chopping them into small pieces (approx. <5mm) to avoid prey size effect on feed intake. Subsequently ragworms were rinsed with seawater for one minute and excess water was shaken off for one minute in a sieve.

Table 3.1 Analysed proximate composition of the diets: formulated diet and ragworm.

Diet	formulated diet	ragworm
Dry matter (g.kg^{-1} WW)	916	190
Crude protein (g.kg^{-1} DM)	622	737
Crude fat (g.kg^{-1} DM)	189	100
Ash (g.kg^{-1} DM)	114	92
Gross energy (kJ g^{-1} DM)[1]	23.4	22.6
NFE (g.kg^{-1} DM)[2]	75	71

[1] Calculated using the factors: carbohydrates, 17.2 kJ g^{-1}; protein, 23.6 kJ g^{-1} and lipids 39.5 kJ g^{-1} (New, 1987).

[2] NFE (nitrogen free extract) calculated subtracting the sum of crude protein, crude fat and ash from 1000 in g.kg^{-1} DM.

Fish and rearing conditions

Sole (n = 600) were obtained from a commercial fish farm (Solea BV, IJmuiden, Netherlands), and transferred to the research facilities of Wageningen Imares in Yerseke, the Netherlands. The rearing system consisted of 28 rectangular glass

tanks (0.4m^2 bottom area and a total water volume 120 L per tank) and 19 square plastic tanks (1m^2 bottom area and a total water volume 300 L per tank). All tanks were connected to a recirculation system equipped with a sandfilter and a beadfilter. Conditions were kept constant throughout the experiment (photoperiod 12L:12D; water flow 5-6 L.min^{-1}; DO > 7.3mg.L^{-1}; T 20.0 ± 0.3°C; salinity at 28.0 ± 1.2g.L^{-1}; TAN< 1.27mg.L^{-1}; NO_2-N < 0.67mg.L^{-1} and NO_3-N < 19.5mg.L^{-1}; pH > 6.78). The pH was controlled by adding sodium bicarbonate to the system. Light cycle was reversed compared to natural circumstances and sole were fed analogous to their most active period which is at after sunset and before sunrise (Lagardere, 1987). During the dark period from 08:00 am until 20:00 pm a low radiation red light was used to provide sufficient light source for providing feed.

Growth experiment and sampling procedures
Upon arrival all sole were weighed individually to the nearest of 0.1g. Four hundred and seventy fish, 280 small and 190 large sole were selected based on body weight (mean weight small sole ±SD of 45.9g ± 6.3, mean weight large sole ±SD of 111.9g ± 15.3). Small sole were randomly distributed over 28 small experimental tanks at a density of 10 fish per tank. Large sole were randomly distributed over 19 large experimental tanks at a density of 10 fish per tank. Tanks were randomly assigned to treatments. One tank out of 28 (small) and 19 (large) was randomly selected as a sampling tank for initial proximate body composition. Initial stocking densities of small and large sole were similar (1.1 kg.m^{-2}). After an acclimatization period of 8-d, the 54-d experimental period started. During the acclimatization period, all fish were fed the formulated diet at a level of 5.5 g kg$^{-0.8}$.d^{-1}. At the start of the experimental period, 5 small and 5 large common sole were randomly selected from the sampling tank, sacrificed with an overdose of Tricaine methanesulfonate (TMS 222), stored at -20°C for later analyses of initial body composition. Samples of the polychaetes were taken every second week to determine their DM content. Feeding levels aimed to be maintained during the experimental period were 0.2, 0.4, 0.6, 0.8 or 1.0 % of the daily average individual geometric body weight (ABW^{-1} d^{-1}). The daily amount of food that had to be fed to

each fish to reach the aimed relative feeding level was calculated based on existing feed conversion ratios for the present feeds (Ende et al., 2014) and the calculated daily weight gain. The feeding level of 1.0 % $ABW^{-1}.d^{-1}$ of the formulated diet appeared above satiation and was subsequently excluded from data analysis.

At the start of the experimental period, fish were weighed individually to the nearest of 0.1g (mean initial body weight ±SD was 45.7g ± 2.1 and 111.2g ± 15.7) and randomly assigned to tanks. Tanks were randomly assigned to a diet and feeding level combination. Feed was supplied within 1 or 2 meals depending on feeding level at 09:00h or at 09:00h and 17:00h. Each meal lasted for 70 min. At the end of the meal, uneaten feed was siphoned out of the tanks and subsequently analysed for dry matter content. Dietary samples, relative to the daily amount of feed consumed per tank, were taken daily, stored at -20°C, and pooled after termination of the experiment and analysed for proximate composition. At the end of the experimental period, fish were removed from the tanks and individually weighed. Five fish per tank were randomly selected and sacrificed with an overdose of TMS 222, stored at -20°C and analysed for final body composition.

Analytical methods and calculations
Sampled feed and fish were ground, homogenised and analysed for dry matter according to ISO standard 1442 (ISO, 1997), total nitrogen (protein is N*6.25) was determined by the Kjeldahl method according to ISO 937 (ISO, 1978), ash (gravimetrically 550°C incineration) according to ISO 936 (ISO, 1998) and crude fat (gravimetrically after extraction with petroleum ether) according to ISO 1443 (ISO, 1973). Proximate analysis was done by SGS (Spijkenisse, Netherlands). The dietary carbohydrate content (i.e., nitrogen free extract, NFE) was calculated as the sum of crude protein subtracting crude fat and ash from 1000 in $g.kg^{-1}$ DM. Gross energy (GE) of the feeds was calculated by multiplying the carbohydrate, protein and fat content with their energy equivalents (respectively, 17.2, 23.6 and 39.5 kJ g^{-1}) (New, 1987). The energy content of the fish was calculated by multiplying the protein and fat content with their energy equivalents.

In the present study we compared performance data between different body fish weights. Therefore we expressed growth, intake and utilization data on the basis of metabolic body weight. The maintenance requirements are dependent on body weight raised to the power of 0.8. By expressing the data on the basis of metabolic body weight data become independent of bodyweight and are better comparable between fish species as well as within fish species (Lupatsch et al., 1998).

From weight measurements, mean initial body weight (IBW) and mean final body weight (FBW) were calculated per tank. The metabolic body weight of fish (MBW$_G$; in kg$^{0.8}$) was calculated as $MBW_G = (W_G) \div 1000)^{0.8}$, W$_G$ is the geometric mean body weight (in g) which was calculated as $W_G = exp((ln(FBW) + ln(IBW)) \div 2)$. Growth rate per metabolic weight (RGR; g.kg$^{0.8}$.d^{-1}) was calculated as $RGR = (FBW - IBW) \div (MBW_G \times t)$ where t is the duration (days) of the growth study. FI of dry matter (DM) per metabolic body weight (FI$_{MBW}$; g DM kg$^{-0.8}$.d^{-1}) was calculated as $FI_{MBW} = DFI \div MBW_G$, where the daily feed intake (DFI; g DM fish^{-1}.d^{-1}) was calculated as the total FI per tank over the experimental days corrected for uneaten pellets and divided by the number of fish per tank. No mortalities occurred during the experimental period; therefore calculations were based on 10 fish per tank. Intake of crude protein, crude fat and ash per metabolic body weight was calculated as the product of FI (FI$_{MBW}$; g.kg$^{-0.8}$.d^{-1}) and the respective measured protein, fat or ash content of the feed (mg.g^{-1}). Gross energy intake per metabolic body weight (kJ.g$^{-0.8}$.d^{-1}) was calculated as FI$_{MBW}$ multiplied with the gross energy content of the diet (kJ.g^{-1}). Dry matter retention per metabolic body weight (RET$_{MBW}$ in g.kg$^{-0.8}$.d^{-1}) was calculated as $RET_{MBW} = ((FBW \times CF_{DM} \div 1000) - (IBW \times CI_{DM} \div 1000)) \div (t \times MBW_G)$, where CF$_{DM}$ is the DM content of the final carcass and CI$_{DM}$ is the DM content of the initial carcass. Retention of protein, ash, fat per metabolic body weight (in g.kg$^{-0.8}$.d^{-1}) and energy retention (in kJ.g$^{-0.8}$.d^{-1}) was calculated in a similar way but on

the basis of final and the initial carcass protein, ash, fat and energy contents. Feed conversion ratio (FCR) was calculated as $FCR = FI_{MBW} \div RGR$.

Crude protein and gross energy utilization efficiency for growth (respectively, k_{gCP} and k_{gGE}) was determined by linear regression of crude protein intake against protein retention as

$$P_{retention} = intercept + k_{gCP} \times CP_{intake} (for\ gross\ energy\ E_{retention} = intercept + k_{gGE} \times GE_{intake}).$$

Crude protein and gross energy maintenance requirements (respectively, CP_m and GE_m) were calculated by making intake the response variable. Digestibilties were not determined because of the extremely low cohesion of faecal material in common sole and technical difficulties using inert markers in natural food. Therefore, in contrast to previous studies on other fish species, protein and energy utilization efficiencies for growth were expressed on crude protein and gross energy basis.

Statistical analysis

Data were analysed using the Statistical Analysis Systems statistical software package version 9.2 (SAS Institute, Inc., Cary, NC, USA). Mean variables like body weight, feed intake, FCR, nutrient retention and body composition data were subjected to a one-way analysis of variance (ANOVA) using the procedure general linear model (GLM) followed by multiple comparison of means using Tukey's multiple range test. The effect of nutrient intake, food type and fish size on k_{gCP}, k_{gGE}, GE_m and CP_m was determined by a three-way analysis of variance (ANOVA) using the procedure general linear model (GLM). In all tests, the statistical significant difference between groups were considered when $P<0.05$.

Results

Performance data, nutrient intake, nutrient retention and whole body composition are shown in Table, 3.2, 3.3, 3.4 and 3.5. Growth, nutrient intake and nutrient retention variables were all significantly affected by feeding levels except only a trend towards a significant increase was observed for fat retention in large sole fed

ragworms (P =0.093). Only in small common sole fed with ragworms body fat content significantly increased with feeding level (Table 3.3). In contrast body fat contents were not influenced by feeding levels in small sole fed the formulated diet (Table 3.2). In large common sole, body fat contents were not influenced by food types (Table 3.4 and 3.5).

Table 3.2 Performance, nutrient intake, nutrient retention and whole body composition (on fresh weight basis) of small sole fed the artificial diet.

diet/ fish size	artificial diet/small sole								
feeding level	0.2		0.4		0.6		0.8		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P-value
Growth									
Final body weight (g)	47.3 [a]	2.44	56.6 [b]	3.14	64.5 [b,c]	3.39	70.9 [c]	3.4	<0.001
RGR (g kg$^{-0.8}$ d^{-1})	0.37 [a]	0.2	1.95 [b]	0.2	3.36 [c]	0.13	4.59 [d]	0.09	<0.001
Nutrient Intake									
DM (g kg$^{-0.8}$ d^{-1})	1.12 [a]	0.0	2.23 [b]	0.04	3.22 [c]	0.14	4.29 [d]	0.11	<0.001
Protein (g kg$^{-0.8}$ d^{-1})	0.7 [a]	0.0	1.39 [b]	0.03	2.0 [c]	0.08	2.67 [d]	0.07	<0.001
Fat (g kg$^{-0.8}$ d^{-1})	0.21 [a]	0.0	0.42 [b]	0.01	0.61 [c]	0.03	0.81 [d]	0.02	<0.001
Energy (kJ kg$^{-0.8}$ d^{-1})	26.26 [a]	0.1	52.23 [b]	0.99	75.41 [c]	3.17	100.49 [d]	2.52	<0.001
Nutrient retention									
DM (g kg$^{-0.8}$ d^{-1})	-0.04 [a]	0.07	0.43 [b]	0.08	0.76 [c]	0.1	1.09 [d]	0.07	<0.001
Protein (g kg$^{-0.8}$ d^{-1})	-0.06 [a]	0.04	0.24 [b]	0.03	0.43 [c]	0.02	0.63 [d]	0.01	<0.001
Fat (g kg$^{-0.8}$ d^{-1})	-0.06 [a]	0.02	0.1 [a,b]	0.03	0.16 [b]	0.11	0.27 [b]	0.08	0.002
Energy (kJ kg$^{-0.8}$ d^{-1})	-3.78 [a]	1.49	9.55 [b]	1.81	16.63 [b]	4.42	25.73 [c]	3.3	<0.001
Whole body composition									
Moisture (g kg^{-1})	767	0.21	759	0.32	760	0.65	757	0.7	0.146
Ash (g kg^{-1})	29	0.27	28	0.12	28	0.31	27	0.04	0.837
Protein (g kg^{-1})	159 [a]	0.1	163 [b]	0.17	159 [a]	0.1	159 [a]	0.1	0.009
Fat (g kg^{-1})	40 [a]	0.12	48 [a,b]	0.23	48 [a,b]	0.85	52 [b]	0.59	0.123
Energy (kJ g^{-1})	5.4	0.04	5.8	0.06	5.7	0.31	5.8	0.23	0.083

Values are means of triplicate tanks ± SD. Within a row, means without a common superscript letter differ significantly (P<0.05) as calculated by ANOVA and Tukey's multiple range test. Initial body composition was: moisture 754 g kg^{-1}, fat 48 g kg^{-1}, ash 36 g kg^{-1}, protein 171 g kg^{-1}, energy 5.9 kJ g^{-1}.

Table 3.3 Performance, nutrient intake, nutrient retention and whole body composition (on fresh weight basis) of small sole fed ragworm.

diet/ fish size	ragworm/small sole										
feeding level	0.2		0.4		0.6		0.8		1		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P-value
Growth											
Final body weight (g)	49.4 [a]	1.49	58.6 [a,b]	5.21	72.1 [c]	1.64	82.8 [c,d]	2.13	93.7 [d]	4.68	<0.001
RGR (g kg$^{-0.8}$ d^{-1})	0.59 [a]	0.03	2.69 [b]	0.12	4.59 [c]	0.07	6.32 [d]	0.2	7.89 [e]	0.23	<0.001
DM (g kg$^{-0.8}$ d^{-1})	1.14 [a]	0	2.18 [b]	0.03	3.25 [c]	0.05	4.22 [d]	0.12	5.13 [e]	0.08	<0.001
Protein (g kg$^{-0.8}$ d^{-1})	0.84 [a]	0	1.6 [b]	0.02	2.4 [c]	0.03	3.11 [d]	0.09	3.78 [e]	0.06	<0.001
Fat (g kg$^{-0.8}$ d^{-1})	0.11 [a]	0	0.22 [b]	0	0.33 [c]	0	0.42 [d]	0.01	0.51 [e]	0.01	<0.001
Energy (kJ kg$^{-0.8}$ d^{-1})	25.73 [a]	0.03	49.1 [b]	0.69	73.4 [c]	1.07	95.32 [d]	2.81	115.71 [e]	1.79	<0.001
Nutrient retention											
DM (g kg$^{-0.8}$ d^{-1})	-0.08 [a]	0.14	0.48 [b]	0.09	0.98 [c]	0.0	1.48 [d]	0.07	1.91 [e]	0.04	<0.001
Protein (g kg$^{-0.8}$ d^{-1})	-0.03 [a]	0.05	0.33 [b]	0.03	0.7 [c]	0.03	1 [d]	0.04	1.23 [e]	0.02	<0.001
Fat (g kg$^{-0.8}$ d^{-1})	-0.18 [a]	0.03	-0.04 [a,b]	0.09	0.06 [b]	0.03	0.26 [c]	0.08	0.43 [c]	0.06	<0.001
Energy (kJ kg$^{-0.8}$ d^{-1})	-7.57 [a]	1.11	6.14 [b]	4.1	18.95 [c]	1.66	33.66 [d]	2.85	46.02 [e]	2.15	<0.001
Whole body composition											
Moisture (g kg^{-1})	776	1.44	770	0.7	766	0.12	760	0.58	756	0.21	0.057
Ash (g kg^{-1})	30	0.32	30	0.19	33	0.11	28	0.07	28	0.17	0.056
Protein (g kg^{-1})	159	0.5	160	0.12	164	0.31	165	0.1	163	0.15	0.067
Fat (g kg^{-1})	28 [a]	0.24	33 [a,b]	0.76	36 [a,b]	0.25	45 [b,c]	0.61	51 [c]	0.49	0.002
Energy (kJ g^{-1})	4.9 [a]	0.11	5.1 [a,b]	0.33	5.3 [a,b,c]	0.16	5.7 [b,c]	0.25	5.9 [c]	0.23	0.002

Values are means of triplicate tanks ± SD. Within a row, means without a common superscript letter differ significantly (P<0.05) as calculated by ANOVA and Tukey's multiple range test. Initial body composition was: moisture 754 g kg^{-1}, fat 48 g kg^{-1}, ash 36 g kg^{-1}, protein 171 g kg^{-1}, energy 5.9 kJ g^{-1}.

Table 3.4 Performance, nutrient intake, nutrient retention and whole body composition (on fresh weight basis) of large sole fed the artificial diet.

diet/ fish size	artificial diet/large sole								
feeding level	0.2		0.4		0.6		0.8		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P-value
Growth									
Final body weight (g)	118.7 [a]	2.89	133.9 [a]	7.62	149.5 [a]	10.59	149.5 [a]	1.74	0.027
RGR (g kg$^{-0.8}$ d^{-1})	0.35 [a]	0.5	2.06 [b]	0.32	3.5 [b,c]	0.2	3.91 [c]	0.27	0.002
Nutrient Intake									
DM (g kg$^{-0.8}$ d^{-1})	1.34 [a]	0.04	2.69 [b]	0.02	3.91 [c]	0.1	5.26 [d]	0.03	<0.001
Protein (g kg$^{-0.8}$ d^{-1})	0.84 [a]	0.02	1.67 [b]	0.01	2.43 [c]	0.06	3.27 [d]	0.02	<0.001
Fat (g kg$^{-0.8}$ d^{-1})	0.25 [a]	0.01	0.51 [b]	0.0	0.74 [c]	0.02	0.99 [d]	0.01	<0.001
Energy (kJ kg$^{-0.8}$ d^{-1})	31.48 [a]	0.87	63.05 [b]	0.49	91.68 [c]	2.23	122.96 [d]	0.65	<0.001
Nutrient retention									
DM (g kg$^{-0.8}$ d^{-1})	-0.02 [a]	0.14	0.59 [b]	0.16	0.97 [b]	0.02	1.06 [b]	0.13	0.003
Protein (g kg$^{-0.8}$ d^{-1})	0.01 [a]	0.02	0.31 [b]	0.11	0.56 [b,c]	0.04	0.69 [c]	0.01	0.001
Fat (g kg$^{-0.8}$ d^{-1})	0.06 [a]	0.14	0.28 [a,b]	0.02	0.44 [b]	0.02	0.25 [a,b]	0.04	0.027
Energy (kJ kg$^{-0.8}$ d^{-1})	2.84 [a]	6.08	18.4 [a,b]	1.69	30.49 [b]	1.46	26.19 [b]	1.21	0.003
Whole body composition									
Moisture (g kg^{-1})	763	0.14	748	0.64	747	0.21	747	0.42	0.037
Ash (g kg^{-1})	33	0.2	32	0.54	28	0.22	31	0.09	0.583
Protein (g kg^{-1})	162	0.49	163	0.42	164	0.49	169	0.21	0.468
Fat (g kg^{-1})	43	0.99	54	0.29	61	0.21	46	0.4	0.119
Energy (kJ g^{-1})	5.5	0.27	6	0.01	6.3	0.2	5.8	0.21	0.041

Values are means of duplicate tanks ± SD. Within a row, means without a common superscript letter differ significantly (P<0.05) as calculated by ANOVA and Tukey's multiple range test. Initial body composition was: moisture 754 g kg^{-1}, fat 39 g kg^{-1}, ash 37 g kg^{-1}, protein 165 g kg^{-1}, energy 5.4 kJ g^{-1}.

Table 3.5 Performance, nutrient intake, nutrient retention and whole body composition (on fresh weight basis) of large sole fed ragworm.

diet/ fish size	ragworm/large sole										
feeding level	0.2		0.4		0.6		0.8		1		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P-value
Growth											
Final body weight (g)	120.5 [a]	8.05	145.4 [b]	1.21	153.2 [b,c]	1.05	186.6 [d]	2.14	190.7 [d,e]	3.25	<0.001
RGR (g kg$^{-0.8}$ d^{-1})	1.12 [a]	0.01	2.94 [b]	0.28	4.63 [c]	0.01	6.54 [d]	0.01	6.6 [d]	0.39	<0.001
Nutrient Intake											
DM (g kg$^{-0.8}$ d^{-1})	1.51 [a]	0.23	2.63 [a]	0.0	4.04 [b]	0.35	4.76 [b,c]	0.32	5.34 [c]	0.07	<0.001
Protein (g kg$^{-0.8}$ d^{-1})	1.11 [a]	0.17	1.94 [a]	0.0	2.97 [b]	0.26	3.51 [b,c]	0.24	3.93 [c]	0.05	<0.001
Fat (g kg$^{-0.8}$ d^{-1})	0.15 [a]	0.02	0.26 [a]	0.0	0.4 [b]	0.04	0.48 [b,c]	0.03	0.53 [c]	0.01	<0.001
Energy (kJ kg$^{-0.8}$ d^{-1})	34.08 [a]	5.15	59.31 [a]	0.02	91.04 [b]	7.91	107.42 [b]	7.23	114.56 [b]	1.49	<0.001
Nutrient retention											
DM (g kg$^{-0.8}$ d^{-1})	0.16 [a]	0.12	0.44 [a]	0.31	1.25 [a,b]	0.03	1.7 [b]	0.06	1.93 [b]	0.4	0.002
Protein (g kg$^{-0.8}$ d^{-1})	0.15 [a]	0.01	0.48 [a,b]	0.03	0.81 [b,c]	0.06	1.13 [c]	0.01	1.18 [c]	0.19	<0.001
Fat (g kg$^{-0.8}$ d^{-1})	0.07 [a]	0.12	0.25 [a]	0.08	0.38 [a,b]	0.09	0.43 [a,b]	0.12	0.66 [b]	0.3	0.093
Energy (kJ kg$^{-0.8}$ d^{-1})	6.32 [a]	5.2	21.14 [a,b]	3.75	34.19 [a,b]	2.32	43.74 [a,b]	4.31	53.82 [b]	16.19	0.012
Whole body											
Moisture (g kg^{-1})	764	0.92	776	1.77	747	0.21	748	0.42	735	1.84	0.118
Ash (g kg^{-1})	30	0.21	30	0.08	33	0.3	27	0.28	29	0.13	0.287
Protein (g kg^{-1})	163	0.07	165	0.57	168	0.42	169	0.07	171	0.78	0.532
Fat (g kg^{-1})	41	0.99	49	0.64	53	0.64	50	0.74	64	1.71	0.378
Energy (kJ g^{-1})	5.5	0.41	5.8	0.38	6.1	0.15	6.0	0.27	6.6	0.86	0.361

Values are means of duplicate tanks ± SD. Within a row, means without a common superscript letter differ significantly (P<0.05) as calculated by ANOVA and Tukey's multiple range test. Initial body composition was: moisture 754 g kg^{-1}, fat 39 g kg^{-1}, ash 37 g kg^{-1}, protein 165 g kg^{-1}, energy 5.4 kJ g^{-1}.

The linear relationships between protein retention and protein intake and between energy retention and energy intake are depicted in Fig. 3.1. The k_{gCP} was higher in common sole fed ragworms than the k_{gCP} obtained with the formulated diet (P >0.001, Table 3.6, Figs. 3.2b and 3.2d). The k_{gCP} was higher in small common sole than in large common sole (P =0.009, Table 3.6). For the k_{gCP} no interaction between food type and fish size was observed (P =0.963, Table 3.7). The protein maintenance requirement (Table 3.6), i.e. zero protein retention, was not different between food types (P =0.64) and fish size (P =0.41) and no interaction of food type with fish size was observed (P = 0.93) (Table 3.7). The pooled (diet and fish size) protein maintenance requirement of common sole was 0.82 g kg$^{-0.8}$ d^{-1}. The k_{gGE} was higher in common sole fed ragworms than the k_{gGE} obtained the formulated diet (P >0.001, Table 3.6, Figs. 3.2a and 3.2c). The k_{gGE} was higher in small common sole than in large common sole (P =0.037, Table 6). For the k_{gGE} no interaction between food type and fish size was observed (P =0.561, Table 3.7). The energy maintenance requirement (Table 3.6), i.e. zero energy retention, was not different between food type (P =0.390) but differed between fish size (P =0.036). No interaction of food type with fish size was observed (P = 0.78) (Table 3.7). The gross energy maintenance requirement of small common sole was 35 kJ.g$^{-0.8}$.d^{-1}. The gross energy maintenance requirement of large common sole was 25 kJ.g$^{-0.8}$.d^{-1}.
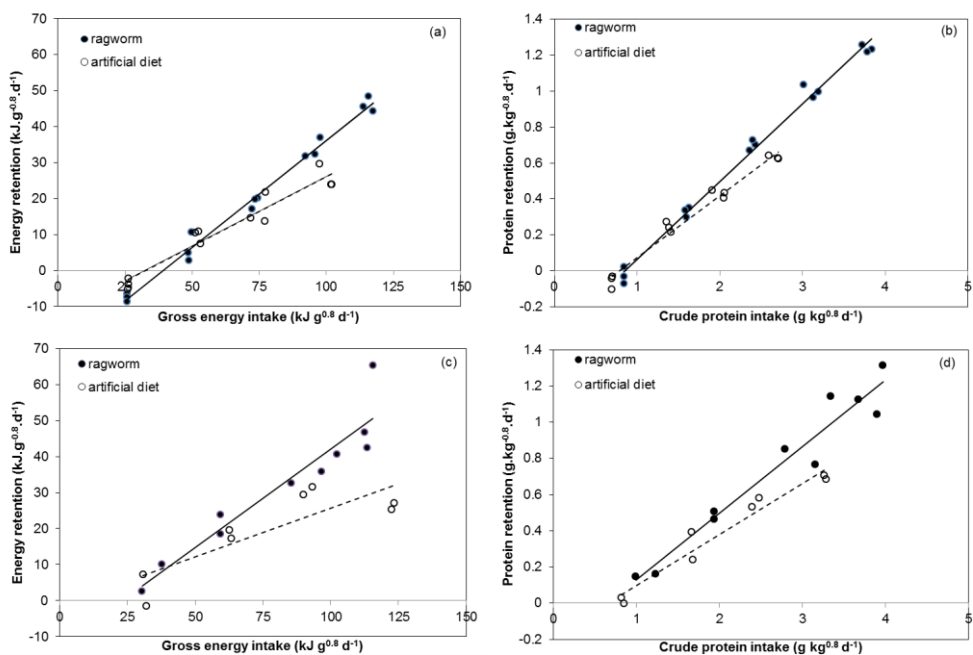
Figure 3.1 Linear relationship between gross energy intake and energy retention (a and c) and crude protein intake and protein retention (b and d) in small common sole (top) and large common sole (bottom) fed ragworm and an artificial diet.

All relationships between protein retention and protein intake and between energy retention and energy intake were checked for nonlinearity. The relationship between crude protein intake (CP) and protein retention (RP) in small common sole fed the formulated diet was non-linear (P =0.038) according to the equation $RP = -0.402 + 0.539 \times CP - 0.058 \times CP^2$ (R^2 98.5%). The relationship between CP and RP in small common sole fed ragworm was non-linear (P =0.030) according to the equation $RP = -0.485 + 0.561 \times CP - 0.028 \times CP^2$ (R^2 99.4%). The relationship between gross energy intake (GE) and energy retention (RE) in large common sole fed the formulated diet was non-linear (P =0.016) according to the equation $RE = -26.121 + 1.064 \times GE - 0.005 \times GE^2$ (R^2 92.1%).

Table 3.6 Protein and energy utilization efficiency for growth (respectively, k_{gCP} and k_{gGE}) and energy maintenance requirement (GE_m) ± standard error in g kg$^{-0.8}$ d^{-1} or kJ g$^{-0.8}$ d^{-1}.

fish	Food type	CP_m	GE_m	k_{gCP}	R^2 (%)	k_{gGE}	R^2 (%)
Small	ragworm	0.86 ± 0.08	39.55 ± 4.0	0.43 ± 0.02	99	0.6 ± 0.04	99
Small	artificial diet	0.81 ± 0.08	34.65 ± 4.60	0.34 ± 0.03	98	0.39 ± 0.05	93
Large	ragworm	0.77 ± 0.13	27.64 ± 7.03	0.37 ± 0.02	94	0.53 ± 0.04	93
Large	artificial diet	0.73 ± 0.12	5.14 ± 7.8	0.28 ± 0.03	95	0.27 ± 0.05	72

Table 3.7 Three-way analysis of variance on the effect of food type, fish size and interaction of food type and fish size on protein and energy utilization efficiency for growth (respectively, k_{gCP} and k_{gGE}) and energy maintenance requirement (GE_m).

	P value GE_m				P value k_g		
	diet	fish size	diet*fish size	FI_{MBW}	FI_{MBW}*diet	FI_{MBW}*fish size	FI_{MBW}*diet*fish size
CP	0.64	0.41	0.93	>0.001	>0.001	>0.001	0.963
GE	0.39	0.036	0.78	>0.001	>0.001	0.037	0.561

Discussion

Our results showed that the lower growth previously observed in common sole fed formulated diets of similar dietary macronutrient composition (Fonds et al., 1989a; Gatta et al., 2010; Irvin, 1973) is not only related to a lower dry matter intake of the formulated diet, but also to lower protein and energy utilization efficiencies for growth.

To our knowledge the present study is the first comparison of protein and energy utilization efficiencies for growth between a natural food and a formulated diet using a bioenergetic approach in common sole. Similar differences in protein utilization efficiencies for growth (22%) were found between formulated diets in Atlantic cod and gilthead sea bream (*Sparus aurata*), though on digestible protein (DP) basis (Hatlen et al., 2007; Lupatsch et al., 2001). Present considerable differences in energy utilization efficiencies for growth (42%) between the formulated diet and ragworm have, to our knowledge, never been reported in comparisons between formulated diets. This seems however not related to high

utilization efficiencies obtained with the present natural feed. Present k_{gCP} (on average 0.40) and k_{gGE} (on average 0.57) values in common sole fed ragworms were within the lower range of values previously reported for other species such as gilthead sea bream, European sea bass (*Dicentrarchus labrax*) and white grouper (*Epinephelus aeneus*) Atlantic salmon (*Salmo salar*) Atlantic cod, barramundi, rainbow trout and yellowtail kingfish on DP basis (k_{gDP} 0.33–0.83 (Booth et al., 2010; Bureau et al., 2006; Glencross, 2008; 2009; Hatlen et al., 2007; Helland et al., 2010; Lupatsch, Kissil, 2005; Lupatsch et al., 2003; Lupatsch et al., 1998)). Present values for k_{gGE} (on average 0.57) in common sole fed ragworms were also within the lower range of values previously reported for other species such Tra Catfish (*Pangasianodon hypothalamus*) barramundi, rainbow trout, yellowtail kingfish, Atlantic cod, Atlantic salmon, gilthead sea bream, European sea bass and white grouper on DE basis (0.57-0.82) (Booth et al., 2010; Bureau et al., 2006; Glencross et al., 2010; Glencross et al., 2007; Glencross et al., 2008; Glencross, 2008; Hatlen et al., 2007; Helland et al., 2010; Lupatsch et al., 2003; Lupatsch et al., 2001; Pirozzi et al., 2010). The large differences in utilization efficiencies were rather related to the low utilization efficiencies obtained with the formulated diet. Lower values than those obtained in common sole fed the formulated diet (k_{gCP} on average 0.31 and k_{gGE} on average 0.33) have, to our knowledge, never been reported.

We did not determine digestibilities of the present feed types because the loose structure of the faeces of common sole requires faeces to be collected by the detrimental method of posterior intestine dissection. Next to this, the ash content of the natural feed did not provide sufficient material to be used as a natural marker for digestibility. A previous study using the dissection method reported a protein digestibility of about 96% and an energy digestibility of about 94% in Senegalese sole (*Solea Senegalensis*) fed fish meal fish oil based formulated diets (Dias et al., 2010). This suggests that the low k_{gCP} and k_{gGE} values obtained in the current study with the formulated diet were not related to a poor digestibility of this diet. This assumption is supported by similar and lower protein and energy maintenance

requirements obtained in the present study with the formulated diet and ragworms (Table 3.6). Based on the linear regression equations obtained with the formulated diet and ragworm and the common relation of gross energy to digestible energy (or respectively crude protein to digestible protein) the following equation was obtained; $GEm = DEm \times ADC_{GE}$, were ADC_{GE} is the apparent digestibility coefficient of gross energy (ragworm digestibility was assumed to be 100%, i.e. GE equals DE and CP equals DP). According to this equation, a low digestibility should be reflected in higher protein and energy maintenance requirements (on CP and GE basis). This was not the case in the present study.

The influence of dietary macronutrient composition on k_{gDP} and k_{gDE} has been confirmed for many fish species including Tra Catfish, barramundi, rainbow trout, yellowtail kingfish, Atlantic cod, Atlantic salmon, gilthead sea bream, European sea bass and white grouper (Booth et al., 2010; Bureau et al., 2006; Glencross et al., 2010; Glencross et al., 2007; Glencross et al., 2008; Glencross, 2008; Hatlen et al., 2007; Helland et al., 2010; Lupatsch et al., 2003; Lupatsch et al., 2001; Pirozzi et al., 2010). Based on the large amount of existing literature, Schrama et al. (2011) established regression relations between k_{gDE} and dietary protein, fat and NFE levels. Present k_{gGE} values in common sole fed the formulated diet differ substantially from k_{gDE} values obtained with these regression relations. Similarly, present k_{gCP} values in common sole fed the formulated diet differ substantially to k_{gDP} values obtained in gilthead sea bream fed an formulated diet of similar dietary macronutrient composition (Lupatsch et al., 2001). Assuming a high digestibility of the present formulated diet (see earlier discussion), the large differences between present values and those reported for other species cannot be explained by different units alone. Present utilization efficiencies could however be hampered by the considerable excess in fat of the present formulated diet (19% fat). In Senegalese sole a low tolerance to dietary fat levels in excess of 12% was reported (Borges et al., 2009). Negative effects of high dietary fat levels on k_{gDP} or k_{gDE} have not been reported elsewhere. This may however be explained by the fact that diets used in previous studies were within the species dietary fat requirements.

Large differences in k_{gGE} in large common sole fed the formulated diet compared to the k_{gGE} in large common sole fed ragworms can at least partly be related to k_{gGE} estimated by linear regression. A curve-linear relation for gross energy intake and energy retention was observed in small common sole fed the formulated diet and ragworms and in large common sole fed the formulated diet which showed the most pronounced curvature. Curve-linear relationships have also better described for the relation of DE and RE, e.g. in rainbow trout (Glencross et al., 2007; Glencross et al., 2008) barramundi (Glencross, 2008) and Tra catfish (Glencross et al., 2010). It has been suggested that the variability in the relationship between DE intake and RE (linear vs. curvilinear) may partly explain the variability in k_{gDE} estimated by linear regression found in literature (Schrama et al., 2011).

The low k_{gCP} and k_{gGE} values in common sole fed the formulated diet could also be related to the lack of bioactive compounds, inadequate dietary water levels or an inadequate amino acid (AA) profile. Unfortunately no information exists on the role of bioactive compounds in nutrient utilization efficiencies in fish. The importance of dietary water content for nutrient efficiencies on the other hand is controversial. Bromley (1980) reported no influence of dietary water content on nutrient efficiencies in Turbot (*Scophthalmus maximus*) whereas Grove et al. (2001) reported higher feed efficiencies in Turbot when fed moist squid than when fed dry squid. The influence of the AA profile on utilization efficiencies is well documented in fish (Helland et al., 2010; Rodehutscord et al., 1997). However, fish meal has a well-balanced AA profile in contrast to invertebrates which may contain low levels of certain amino acids (Molinero et al., 1994). Therefore, a negative influence would be expected in common sole fed ragworms of a less balanced AA profile than in those fish fed the formulated diet with a balanced AA profile.

The lower k_{gCP} and k_{gGE} values obtained in large common sole fed the formulated diet seem in line with low growth rates observed during the on-growing stage (Howell et al., 2009; Howell et al., 2011). Differences k_{gCP} and k_{gGE} values may be related to a higher feed intake obtained in large common sole compared to small

common sole at the highest feeding level. Feed levels were calculated based on the estimated weight gain which was overestimated in large and underestimated in small common sole fed the formulated diet. As a result, feed intake (in $g.kg^{-0.8}.d^{-1}$) at the highest feeding level (theoretically 0.8% dry matter per ABW for both fish size classes) was in reality 18% higher in large common sole compared to small common sole fed the formulated diet.

In conclusion, present results show that the lower growth as observed previously in common sole fed formulated diets compared to common sole fed natural feeds is partly related to lower protein and energy utilization efficiencies for growth. It is hypothesised that the low k_{gCP} and k_{gGE} value in common sole fed the formulated diet may be related to an excess in dietary fat (i.e., a dietary fat level above the requirement of common sole).

Acknowledgement

The influence of prey size, sediment thickness and fish size on consumption in common sole (*Solea solea* L.)

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Abstract

The present study examined the ability of common sole (*Solea solea* L.) to forage on buried prey (*Nereis virens* (Sars)). In the first experiment we determined the relation of prey size and prey burial depth. Prey burial depth increased with increasing prey size ($P> 0.01$) ranging from a mean depth of 7.7cm (0-0.5g) to a mean depth of 13.3cm (4-5g). In the second experiment we determined prey consumption of common sole as a function of prey size, sediment thickness and fish size. Factors and levels were: prey size (0-0.5, 1-1.5, 2-2.5 and 4-5g); sediment thickness (20cm and 2cm) and fish size (50g, 125g or 300g). Prey consumption (in numbers of prey eaten per fish per day) was reduced with increasing prey size and sediment thickness, and was increased with increasing fish size ($P>0.001$ for all factors). All 3 factors showed significant two way interactions ($P>0.001$), when expressed in numbers of prey eaten. Prey consumption decreased with prey size when prey could not escape by burying (i.e. in tanks filled with 2cm of sediment) irrespective of fish size. The reduction in prey consumption with increasing prey size was not related to satiation as consumption also decreased when expressed in grams of prey eaten. This suggests that increasing effort to ingest and handle larger prey played a role. Prey consumption increased with fish size when prey could not bury (in tanks with 2cm of sediment). However, when prey was able to bury, i.e. at 20cm sediment thickness, prey consumption was similar irrespective of fish size ($P>0.001$ for interaction fish size x sediment). This interaction suggests that with increasing fish size there is an increasing mismatch between foraging adaptation and prey burial depth. This may explain the dominance of crustaceans in the diet of adult common sole in nature, despite the high abundance of polychaetes.

Introduction

The nature of prey selection in fishes is often not an active choice of the fish but simply related to the relative vulnerability of the food items (Ivlev, 1962; Menge, 1974; Ware, 1972; 1973). One major factor influencing vulnerability of a prey is its size. Prey size vulnerability curves, i.e. numbers of prey consumed as a function of prey size, are usually dome shaped (Bailey, Houde, 1989; Lundvall et al., 1999; Manderson et al., 2000; Manderson et al., 1999; Pastorok, 1981; Rice et al., 1993). The decrease in number of large prey eaten when approaching the upper prey size limit is related to increasing difficulties of ingesting and handling prey (Anderson, 1988; Sissenwine, 1984; Sogard, 1997). The decrease in number of small prey eaten when approaching the lower prey size limit on the other hand is explained by increasing difficulties to detect and retain small prey (Breck, Gitter, 1983; Howick, O'Brien, 1983; Persson, 1987).

Prey selection also depends on the ability of the fish to adapt its foraging behavior to the escape strategies of the prey. Prey use structures such as sediment or vegetation to minimize the risk of predation (Persson, Eklov, 1995; Turner, Mittelbach, 1990). For example, the proportion of Eurasian perch (*Perca fluviatilis*) young of the year that was successfully attacked by Eurasian perch was about 70% lower in tanks where refuge (vegetation) was available compared to the success rate in tanks without refuge available (Lundvall et al., 1999). Though prey of the most vulnerable size may be highly abundant, it may not be selected due to efficient escape strategies. Therefore, when conducting experimental studies on prey vulnerability as a function of size, prey's anti-predator strategies must also be considered.

Common sole (*Solea solea* L.), feeds on benthic invertebrates such as polychaetes, bivalves and crustaceans (Braber, de Groot, 1973a; Braber, de Groot, 1973b; Cabral, Costa, 1999; de Groot, 1971; Molinero, Flos, 1991; 1992). Feeding grounds of common sole are often dominated by ragworms (polychaetes) species such as *Hediste diversicolor* (Müller, 1776) or *Nereis virens* (Sars) which contribute

to the diet of common sole (Cabral, Costa, 1999; Heip, Herman, 1979). The feeding behavior of common sole however seems not well adapted to the anti-predator defenses used by these species. Common sole exhibits a slow moving search behavior and locates prey by olfactory and tactile cues (Appelbaum, Schemmel, 1983; Appelbaum et al., 1983; de Groot, 1969). Prey recognition by tactile cues requires physical contact with the prey. Physical contact is possible with immobile or slow moving prey. Species such as *N. virens* rapidly however retreat into their burrows when sensing predation risk. Even small individuals of *N. virens* or *N. diversicolor* are found at depth more than 5cm (Caron et al., 1996; Esselink, Zwarts, 1989), a depth which is considered a safe refuge to most benthic fish (Esselink, Zwarts, 1989). To our knowledge, no studies have been conducted so far on the abilities of common sole foraging on buried polychaetes. The present study examines the influence of prey size, sediment thickness and fish size on prey consumption in common sole.

Material and Methods

Common sole were obtained from a commercial fish farm (Solea BV, IJmuiden, Netherlands). The benthic polychaete, *N. virens* (subsequently referred to as 'prey') was used as prey and was obtained from a commercial producer (Topsy Baits, Wilhelminadorp, Netherlands). Experiments were conducted at the research facilities of Wageningen Imares in Yerseke, the Netherlands.

General experimental conditions

We conducted 2 consecutive experiments using the same experimental system. This system consisted of 18 square plastic tanks ($1m^2$ bottom area and a total water volume 300 L per tank) connected to a recirculation system equipped with a beadfilter. Outflow pipes were covered with screens to prevent prey from escaping experimental tanks. Seven days prior to the experimental period tanks were filled with pre-washed Metsel sand (De Houtkaai, zand en grindhandel, The Netherlands) to either 20cm (experiment 1) or 20cm and 2cm (experiment 2). Four prey sizes were used (0-0.5, 1-1.5, 2-2.5 and 4-4.5g). Conditions were kept

constant throughout the experimental periods (photoperiod 12L:12D; water flow- 5-6 L min^{-1}; T- 21.0 ± 1.2°C; salinity at 29.5 ± 3.0; TAN< 0.73 mg L^{-1}; NO$_2$-N < 0.55 mg L^{-1} and NO$_3$-N < 5.5 mg L^{-1}, pH > 7.03). Oxygen (DO) was kept above > 7.0 mg L^{-1} except for one day were DO was 3.42 mg L^{-1} due to a power failure.

Experiment 1

In experiment 1 we determined the inflection point, i.e. the time point after which no further increase in prey depth occurred. This time point was used in the subsequent main experiment as the latency time between introduction of prey and introduction of common sole to the tanks. The second aim of experiment 1 was to determine if prey burial depth increased with increasing prey size. The relation of prey size and prey burial depth was used for interpretation of results from experiment 2. All tanks in experiment 1 were filled with 20cm of sediment. We considered 20cm of sediment sufficient depth to perform natural burying behaviour.

Each tank (n=18) was divided into 4 compartments by plastic boards giving a total of 72 experimental units (bottom area of 0.25m^2). By dividing tanks into smaller compartments two compartments were available per prey size and time. Each compartment was then stocked with a single prey size at a density of 1.5kg m^{-2}. Two core samples (7.5cm diameter) were taken per compartment at a random place. Areas bordering walls were excluded from sampling. The core sampler was forced into the sediment until the tank bottom was reached. A plug was inserted from above and the core sampler was removed. After removal the sediment sample was pushed out of the core from below into an extension and sliced into 4cm pieces to get core samples of 0-4, 4-8, 8-12, 12-16 and 16-20cm. Each sample was sieved on a 0.1cm sieve. Prey recovered for each sample was weighed individually to the nearest of 0.01g. When disturbed at or near the surface, ragworms retreat rapidly to the bottom of its burrow (Vader, 1964). Therefore, we assume that prey was recovered at the deepest burial depth.

Experiment 2

In Experiment 2 we examined prey consumption as a function of prey size, sediment thickness and fish size. The influence of prey size on common sole predation was studied using a thin sediment layer of 2cm. A minimum of sediment was necessary because common sole struggled to catch the prey on a plastic tank floor when no sediment was available (pers. Obs.). We used only 2cm of sediment to avoid that capture effort by the fish would be influenced by other factors than prey size. The influence of capture effort related to prey depth was studied using 20cm of sediment thickness. We considered 20cm of sediment sufficient depth to perform natural burying behaviour.

Prior to starting experiment 2, common sole were adapted for 21-d to the present prey type either restricted in burying (2cm sediment thickness) or unrestricted in burying (20cm sediment thickness). This adaptation was necessary as fish were exclusively fed pelleted feed before. Fish were adapted in a separate system (not the experimental system) consisting of 2 shallow raceways and 6 square plastic tanks connected to a recirculation system equipped with a drumfilter, trickling filter, ozone and UV. Conditions, i.e. sediment type, sediment depth, sediment settling time and water quality were the same as in the experimental system. During this adaptation period, prey of unsorted size was used. As a result, all common sole had access to all possible prey sizes. During the adaptation period, prey densities declined due to consumption. To maintain a theoretical density of 1.5kg m^{-2}, prey was re-stocked once a week, always during daytime. No feeding activity of common sole was observed in response to newly introduced prey. This was important as active feeding on unburied prey would have interfered with the desired adaptation of common sole to forage on buried prey.

In experiment 2, a 4 x 2 x 3 factorial design was used including the following factors and levels; prey size (mean initial body weight ± SD was 0.7 ± 0.2g, 1.5 ± 0.2g, 2.4 ± 0.2g and 4.1 ± 0.3g), sediment thickness (20cm and 2cm) and fish size (mean initial body weight ± SD of the 3 fish size classes was 51.8 ± 4.2g, 125.8 ±

8.8g and 300.9 ± 20.9g). Each treatment was done in duplicate. It was not possible to study all treatment combinations at once. Therefore, the experiment was conducted in 3 series, starting with small sole, followed by medium and large soles. Prey was stocked at densities of 0.2kg m^{-2} with one size class per tank. An additional 100 prey individuals were weighed to determine the mean initial body weight. Results from experiment 1 (conducted prior to experiment 2) showed that the inflection point, i.e. time point after which no further increase in prey depth occurred, did not vary between prey size classes and varied from 15 to 23h. Therefore we stocked prey 24h before introducing common sole. Common sole of one size class were individually weighed and introduced to the tanks at densities of 4 fish tank^{-1}. Common sole were starved for 24-h before being introduced to their respective tanks. The feeding period lasted for 48-h after which common sole were removed. Remaining prey was recovered from the sediment by sucking water, sediment and prey out of the tanks and separating prey by running the homogenate through a rotating drum. Prey was counted, and total biomass and individual weight of 100 individuals was recorded.

Calculations and statistical analysis
Consumption in grams of prey fish^{-1} d^{-1} was calculated per tank as initial biomass – final biomass – prey mortality and divided by the number of experimental days. Consumption in numbers of prey fish^{-1} d^{-1} was calculated by dividing grams of prey eaten by average prey weight.

Data were analysed using the Statistical Analysis Systems statistical software package version 9.2 (SAS Institute, Inc., Cary, NC, USA). Prey size to prey depth data were analysed with a one-way analysis of variance (ANOVA). Data from the experiment were subjected to a three-way analysis of variance (ANOVA). In all tests the general linear model (GLM) procedure was used followed by multiple comparisons of means using Tukey's multiple range test. In all tests, the statistical significant difference between groups were considered when $P<0.05$.

Results

Experiment 1

Mean prey burial depth (at 24-112h after stocking) increased with increasing prey size as shown in Fig. 4.1. The equation for the observed linear regression was y (depth) = 1.78x (worm size) + 6.40, R^2 = 0.95; *P*> 0.001, Fig. 4.1). Prey depth (mean ± SEM) ranged from 7.7cm ± 0.34 (prey of 0-0.5g) to 13.3cm ± 0.34 (prey of 4-5g).
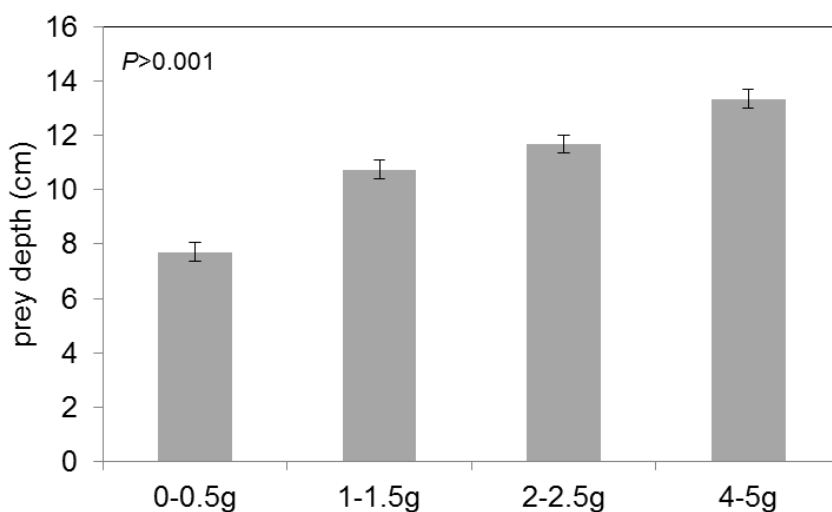

Figure 4.1 The relationship of prey size (in g, x- axis) to prey depth (in cm, y- axis). Error bars show the standard error of means (SEM).

Experiment 2

The three-way interaction of sediment thickness, fish size and prey size was significant for numbers of prey eaten (*P*>0.001), but not for grams of prey eaten (*P*=0.39). All 2 Way interactions were significant for numbers of prey eaten (Fig. 4.2A, 4.2C and 4.2E), whereas only the interaction of sediment thickness with fish size was significant in grams of prey eaten (Fig. 4.2F). All main effects were significant both in numbers and grams of prey eaten.

Prey consumption decreased with prey size (Fig. 4.2A and 4.2B); however an interaction between sediment thickness and prey size was observed for the numbers of prey eaten (Fig. 4.2A). This interaction was related to a less pronounced decrease in prey consumption with sediment thickness for the largest prey size.

Prey consumption increased with fish size (Fig. 4.2C and 4.2D); an interaction between prey size and fish size was present for the numbers of prey eaten (Fig. 4.2C). This interaction was caused by the fact that prey consumption increased with fish size for small and medium sized prey but not for largest prey.

Prey consumption (both in grams and number) was influenced by an interaction between fish size with sediment thickness (Fig. 4.2E and 4.2F). Prey consumption increased with fish size when prey could not escape by burying (in tanks with 2cm of sediment). When prey was able to escape by burying, i.e. in tanks filled with 20cm of sediment consumption was similar irrespective of fish size.
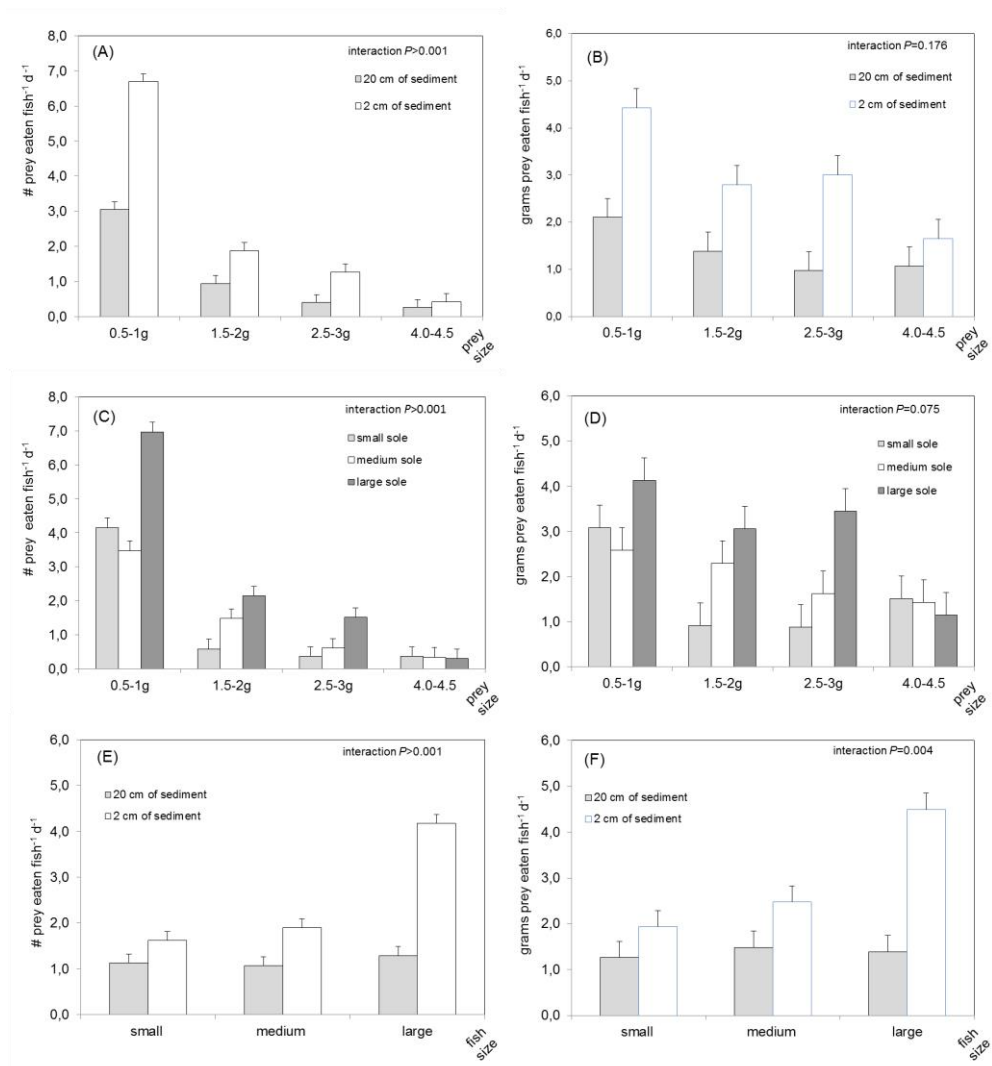
Figure 4.2 Numbers of prey eaten as affected by prey size x sediment thickness (A), prey size x fish size (C) and sediment thickness x fish size (E). Grams of prey eaten as affected by prey size x sediment thickness (B), prey size x fish size (D) and sediment thickness x fish size (F). The x- axis in Fig. A-D shows prey size. The x- axis in Fig. E-F shows fish size. The y- axis in Fig. A, C and E shows the numbers of prey eaten per fish per day. The y- axis in Fig. B, D and F shows the grams of prey eaten per fish per day. Values are least square means of duplicate tanks. Error bars show the standard error of means (SEM).

Discussion

Our results showed that prey consumption clearly decreased with prey size when prey could not escape by burying (i.e. in tanks filled with 2cm of sediment) irrespective of fish size (Fig. 4.2A – 4.2D). We assume that the observed relation of prey consumption and prey size in 2cm is only influenced by handling and ingestion effort (see section 2. for details). A reduction in prey consumption with increasing prey size can occur when satiation is reached with fewer prey of larger size (Gill, Hart, 1994; Hambright, 1991; Hart, Hamrin, 1988; Hart, Connellan, 1984; Hart, Gill, 1992). The observed decline also in grams of prey eaten (in addition to decline in numbers of pre eaten) with increasing prey size (Fig. 4.2B) however suggests that satiation was not the explanation for the observed reduction in consumption in the present study. A reduction in prey consumption with increasing prey size is commonly associated with increasing efforts to ingest and handle prey approaching upper prey size limits (Anderson, 1988; Sissenwine, 1984; Sogard, 1997). The observed decline in prey consumption with increasing prey size for all size classes of common sole suggests that all size classes of common sole approached upper prey size limits. This assumption is supported by the predator-prey mass ratio's (PPMR) obtained for a related tongue sole (*Cynoglossus* sp.), showing a similar diet specialization. The PPMR reflects the order of magnitude by which a fish is larger than its common prey. Prey above this ratio is not selected due to increasing handling and ingestion effort. The PPMR for *Cynoglossus* sp. is about 3.1 (Kondoh, 2011). Applying this ratio to present common sole size classes would result in considerably smaller preferred prey sizes of approximately 0.04, 0.1 and 0.24g for small, medium and large common sole respectively.

Prey consumption was reduced when prey could escape by burying (i.e. in tanks with 20cm of sediment), irrespective of prey size (Fig. 4.2A) and fish size (Fig. 4.2E). However, the highest numerical reduction was not observed for the deepest prey but for prey found at the lowest depth as can be seen by the interaction between prey size and sediment thickness. On average, consumption of the smallest prey found at the lowest depth was reduced by 3.6 individuals, whereas

consumption of large prey was only reduced by 0.2 individuals. This suggests high capture effort for smallest prey size. Though smallest prey were found at lower depth than preceding prey size classes, they were not located within the first few centimeters of sediment. Common sole is a benthic feeder and known to rely mainly on olfactory and tactile cues to search and detect prey (Appelbaum, Schemmel, 1983; Appelbaum et al., 1983). Olfactory and tactile cues may be foraging strategies not adapted for prey which bury in excess of the first few centimetres (Geluso, 2005; Reichman, Oberstein, 1977). However, we would have expected that capture efforts further increase with increasing burial depth. The absence of such an effect may partly be related to the fact that consumption of large prey was already limited by the low numbers of prey eaten (on average only 0.4 individuals) in tanks filled with 2cm of sediment (prey size effect). Also sediment characteristics may have played a role by limiting large prey to escape predation. In the present study large *N. virens* were mainly found within the depth range of 12-14cm (Fig. 4.1), whereas in nature larger *N. virens* (>1g) are found in excess of this depth (Caron et al., 1996). The lower depth relative to *N. virens* burying potential may be related to prey not being able to stabilize deeper burrows in the coarse sediment which we used.

Largest common sole ate the highest numbers of prey when prey could not escape by burying (e.g. in tanks with 2cm of sediment). However, this was not the case when prey was able to escape by burying, i.e. in tanks filled with 20cm of sediment. In this condition similar numbers of prey were eaten irrespective of fish size which is shown by the significant interaction (Fig. 4.2E and 4.2F). This finding appears not in line with the major ecological concept that foraging abilities improve with fish size (Chattopadhyay, Baumiller, 2009). The interaction of fish size x sediment thickness found in the present study suggests that with fish size there is an increasing mismatch between foraging adaptation and the prey burial depth. As a consequence, in our study, larger common sole could possibly not benefit from better foraging abilities.

In conclusion, the observed reduction of prey consumption with increasing sediment thickness (i.e. prey burial depth) suggests that the foraging behaviour of common sole is less well adapted to this prey type. This poor adaptation particularly affects large common sole which were not able to consume high numbers of buried prey. Present results may explain why in nature common sole progressively substitute polychaetes by crustaceans as they grow.

Acknowledgements

The influence of prey density and fish size on prey consumption in common sole (*Solea solea* L.)

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Abstract

We examined the influence of prey density and fish size on prey consumption in common sole (*Solea solea* L.) foraging on buried ragworm (*Nereis virens,* Sars). The tested prey densities of 0.8, 2.2, 4.3 and 6.5 $No.dm^{-2}$ were exposed to common soles of either 100g or 300g. At each prey density common sole foraged for 48h. At both common sole classes studied, a positive correlation between prey consumption and prey density was observed ($P>0.001$). Relationships however differed between 100g and 300g common sole. In 300g common sole the relationship between prey consumption and prey density was linear ($P>0.001$), whereas in 100g common sole the relationship between prey density and prey eaten was polynomial ($P = 0.018$). Small common reached satiety prey consumption rates at nearly every prey density while large common sole did not reach satiation rates even at highest prey densities. The data suggest that in nature, polychaetes such as *N. virens* may contribute to the diet of small common sole even when they are only moderately abundant. In contrast, polychaetes may not be an ideal prey for larger common sole as indicated by the absence of satiety regardless of prey density.

Introduction

In common sole (*Solea solea* L.) no information is available on the relation between prey consumption and prey density for any naturally exploited prey. In fishes foraging on exposed prey (e.g. free swimming prey) the consumption of prey is positively correlated with prey density (Rice, Cochran, 1984). However, common sole, being a benthic feeder, mainly encounters prey which bury themselves into the sediment to reduce predation risk. Some species such as the polychaete *Arenicola marina* (L.), *Nereis diversicolor* (Müller) or *Nereis virens* (Sars) bury even deeper than the first few centimeters (Caron et al., 1996; Kristensen, 1984; Zwarts, Wanink, 1993) and maintain their burial depth even at high intraspecific densities (Duport et al., 2006). A burial depth in excess of the first few centimeters is considered a safe refuge from most predators including flatfish (Esselink, Zwarts, 1989). This suggests that prey density may have little influence on prey consumption in common sole foraging on species like *N. virens* or *A. marina*. However *A. marina* for example are frequently found in the diet of small common sole while they are absent in the diet of larger common sole (Braber, de Groot, 1973a). This raises the question whether the contribution of such prey in small common sole is related to prey density and also why differences in the contribution of this prey type are observed in small and large common sole. Therefore, in the present study we investigated prey consumption in small and large common sole in response to increasing densities of buried ragworms (*Nereis virens*, Sars).

Material and Methods

Common sole were obtained from a commercial fish farm (Solea BV, IJmuiden, Netherlands). The benthic polychaete, *N. virens* (subsequently referred to as 'prey') was used as prey and was obtained from a commercial producer (Topsy Baits, Wilhelminadorp, Netherlands). Experiments were conducted at the research facilities of Wageningen Imares in Yerseke, the Netherlands.

General experimental conditions

In this experiment, 16 square plastic tanks (1m^2 bottom area and a total water volume 300 L per tank) were used. These experimental tanks were connected to one recirculation system, which was equipped with a beadfilter. Outflow pipes of the tanks were covered with screens to prevent prey from escaping. Seven days prior to the experimental period tanks were filled with pre-washed Metsel sand to a depth of 20cm. One prey size was used (mean initial body weight ± SD 2.4 ±0.29g). Conditions were kept constant throughout the experiment (photoperiod 12L:12D; water flow 5-6 L min^{-1}; temperature 18.6 ± 0.7°C; salinity 32.3 ± 0.9; TAN< 0.8 mg L^{-1}; NO_2-N < 0.55 mg L^{-1} and NO_3-N < 17.1 mg L^{-1}, pH > 7.7). Oxygen (DO) was kept above > 7.8 mg L^{-1}.

Experimental design and procedures

Prior to as well as after arrival at the experimental facilities, the common sole used in this study were exclusively fed with a commercial pelleted feed, thus being naïve to eating live prey. Therefore, prior to starting the experiment common sole were adapted for 7-d to accept the live prey type (e.g., polychaetes) and to a system in which prey were enabled to bury freely (20cm sediment thickness). During this adaptation, prey of mixed size was used. As a result of consumption prey densities declined and were re-stocked once during the adaptation phase to maintain a theoretical density of 1.5kg m^{-2}. Prey was re-stocked during day-time when no feeding activity of common sole was observed. Avoiding active feeding on unburied prey was important to assure adaptation of common sole to forage on buried prey. Fish were adapted in a separate system (not the experimental system) consisting of 6 square plastic tanks connected to a recirculation system equipped with a drumfilter, trickling filter, ozone and UV. Conditions, i.e. sediment type, sediment depth, sediment settling time and water quality were the same as in the experimental system.

In the experiment the following factors and levels were used: prey density (0.8, 2.2, 4.3 and 6.5 prey per dm²); common sole size (mean initial body weight ± SD of the

2 common sole sizes was 111.3 ± 15.6g and 315.8 ± 57.4g, subsequently referred to as 100g vs. 300g). In total, 32 common sole (16 per weight class) were distributed over 8 groups with each group consisting of 4 fish. Each group of common sole was used four times, once at each of the four prey densities, thus four groups at each prey density. After being starved for 24h, each group of fish was introduced at random to the next prey density treatment. This procedure was repeated until each group of fish was subjected to all prey density treatments once.

The experimental system consisted of 16 square plastic tanks (1m^2 bottom area and a water volume of 300 L per tank). One density of prey was stocked per tank. An additional 100 individuals were weighed individually to determine the mean initial body weight. Earlier observations showed that prey needed 20 to 23h to reach their maximum burying depth. Therefore, common sole was introduced to the tanks 24-h after stocking the prey. Common sole of one size class were individually weighed and stocked at densities of 4 fish tank $^{-1}$. Common sole were starved for 24-h before being introduced to their respective tanks. The feeding period lasted 48-h after which common sole were removed. During this period unburied prey was removed, weighed and counted during working hours between 09h and 17h. After 48h, remaining prey was recovered from the sediment by sucking water, sediment and prey out of the tanks and separating prey by running the homogenate through a rotating drum. Prey was counted, and total biomass and individual weight of 100 individuals was recorded. The time required to empty a tank was about 1-h for each experimental tank. Emptying all 16 tanks at once would have resulted in significant differences in the experimental feeding periods, e.g. the first tank emptied after 48h, the last tank emptied after 66h. Therefore, experimental feeding periods started at different dates so that only 4 tanks needed to be emptied per day.

Calculations and statistical analysis

Number of prey consumed per fish^{-1} d^{-1} was calculated per tank as the initial number of prey – final number of prey – numbers of unburied prey divided by the number of fish per tank divided by the number of experimental days.

Statistical evaluations of data were performed using the Statistical Analysis Systems statistical software package version 9.2 (SAS Institute, Inc., Cary, NC, USA). In order to test if the linear relationship between prey consumption and prey density was different between the two common sole size classes the following model was used:

$$Y_{ijk} = \mu + S_i + e_{1ij} + \ss X_k + \ss_i X_k + e_{2ijk}$$

where Y_{ijk} = number of prey eaten by common sole size class i in group j at prey density k; μ = overall mean; S_i = the effect of common sole size class i (i=1, 2); e_{1ij} = error term 1, which represents the random effect of group j within common sole size class I (j= 1,..,4); X_k = the measured prey density at the start of the feeding period within group j (k=1,..4); $\ss$ = overall regression coefficient of Y on X; $\ss_i$ = regression coefficient of Y on X within common sole size class i, which represents the interaction effect between common sole size and prey density; and e_{2ijk} = error term 2. The effect of common sole size was tested against error term 1 and the other effects against error term 2. Moreover, within common sole size class, it was tested if the relationship between prey consumption and prey density was polynomial. In all tests, the statistical significant difference between groups were considered when *P*<0.05.

Results

At both common sole classes studied, a positive correlation between prey consumption and prey density was present (*P*>0.001; Figure 5.1). The linear relationship between prey density (x, in No. dm^{-2}) and prey eaten (y, No. fish^{-1} d^{-1}) was

   y = 1.71 (SE 0.68) + 0.91 (SE 0.17) x (for 300g common sole),
   y = 2.20 (SE 0.68) + 0.32 (SE 0.16) x (for 100g common sole),

with a combined R^2 of 69.1%. The intercepts did not differ between the two common sole size classes (P>0.05), but the regression coefficient was higher for the 300g than for the 100g common sole ($P = 0.02$). At low prey densities (0.8 and 2.2 prey dm^{-2}) worm intake was similar between common sole sizes, but at higher prey densities the intake of 300g common sole was larger than that of 100g common sole. Within the 300g common sole class, the relationship between prey consumption and prey density was linear within the measured prey density range (P>0.001). However, within the 100g common sole class, the quadratic function between prey density (x, in No. dm^{-2}) and prey eaten (y, No. fish^{-1} d^{-1}) was significant ($P = 0.018$) being:

$$y = 1.2 \text{ (SE 0.43)} - 11.2 \text{ (SE 0.30) } x - 0.116 \text{ (SE 0.04) } x^2$$

with a R^2 of 99%. This curvilinearity indicated that above prey density of 2.2 worms per cm^2 the prey intake of 100g common sole levelled off at about 3.7 prey eaten per fish per day (Figure 5.1).
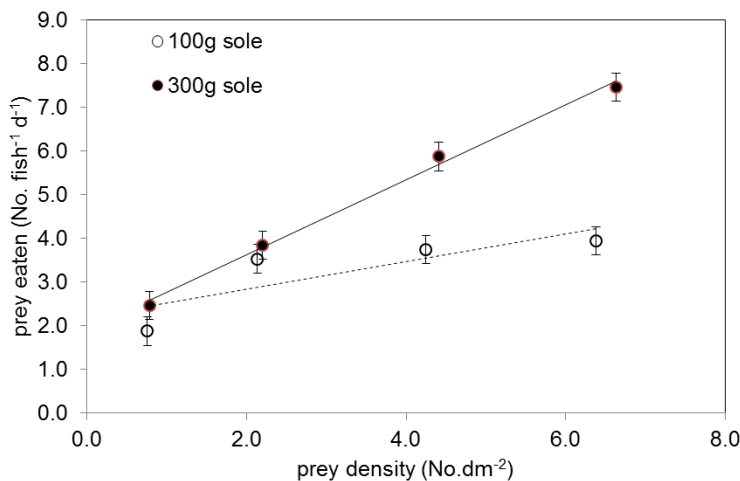
Figure 5.1 Linear relationship of prey density (No. dm^{-2}, x-axis) and numbers of prey eaten (No. fish^{-1} d^{-1}, y-axis) in 100g (broken line, empty circles) and 300g common sole (solid line, solid circles). Presented values are means (n=4) with error bars showing the standard error of mean.

The number of unburied prey increased with prey density ($P > 0.001$, data not shown). However, when expressed as percentage of the number of prey stocked, the fraction of unburied prey did not alter with increasing prey density ($P=0.30$; Fig. 5.2). However the percentage of unburied prey was higher in tanks stocked with 100g common sole compared to those containing 300g common sole, being 1.15% versus 0.22% ($P > 0.001$).
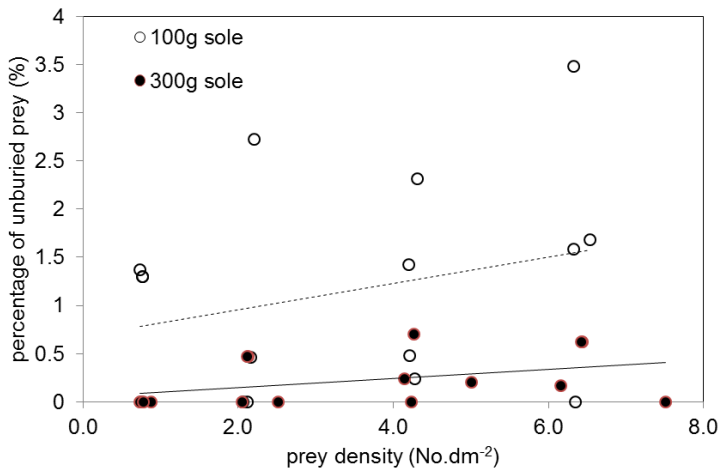

Figure 5.2 The relationship of prey density (in No. dm^{-2}, x- axis) and numbers of unburied prey expressed as percentage of intial prey stocked (%, y-axis) in 100g (broken line, empty circles) and 300g common sole (solid line, solid circles).

Discussion

This study assessed the prey consumption of two common sole size classes (100g vs 300g) in relation to the density of a burying prey (ragworms, *Nereis virens*, Sars). Ragworms bury deeper than the top sediment layer, the first few centimeters (Caron et al., 1996; Kristensen, 1984; Zwarts, Wanink, 1993). Such a depth is considered a safe refuge from most predators including flatfish (Esselink, Zwarts, 1989). Therefore it was expected that prey density of ragworms would have minor impact on the prey consumption in common sole. However, the prey (i.e., ragworms) density influenced prey consumption of common sole (Fig. 5.1). The

current finding is paralleling the observed positive correlation between prey consumption and prey density in foraging on exposed prey (e.g. free swimming prey) (Rice, Cochran, 1984).

At both common sole classes studied, a positive correlation between prey consumption and prey density was present, but the relationships differed between 100g and 300g common sole. The straight line response in 300g common sole suggests that prey consumption was limited by all prey densities; the polynomial response in 100g common sole in contrast suggests that at higher prey densities prey consumption was limited by other factors than prey density.

The differences in prey consumption between predator size in response to prey density could be related to differences in physiological (satiation), physical (handling of prey size) or ecological (access to prey) capacities of the fish. The levelled-off prey consumption in 100g common sole indicates that at the plateau-level, prey consumption in 100g common sole was limited by satiation. On average, 100g common sole ate 1.4% dry matter/ unit body weight (dm/BW, dry matter content of prey was 0.18 g/g wet weight (Ende et al., 2014)). This value is higher than consumption values previously reported in unrestrictedly fed common sole ((1.1% dm/BW (Ende et al., 2014)). This suggests that consumption of prey in 100g common sole was limited by satiation at prey densities higher than 2.2 prey per dm². In contrast, the straight line response in prey consumption in 300g common sole suggests that 300g common sole did not reach satiation. Even at the highest prey density prey consumption (0.9% dm/BW) remained below the value of 1.1% dm/BW previously reported for this species. The different influence of satiation between the two fish sizes is further supported by the differences in percentage of unburied prey found in the two fish size treatments. The lower percentage of unburied prey found in tanks with 300g common sole (0.22%) compared to 1.15% of unburied prey found in tanks with 100g common sole suggests that 300g common sole ate relatively more unburied prey than the 100g common sole that was presumably satiated already.

Besides the physiological capacity (satiation), ecological or morphological factors may constrain prey consumption differently between the two predator sizes. For example, the time required to ingest and handle prey increases when prey size approaches the fish's physical capacity limits (Gill, Hart, 1994; Hoyle, Keast, 1987; Kislalioglu, Gibson, 1976; Werner, 1974). Capacities to ingest and handle prey generally increase with increasing predator size (Chattopadhyay, Baumiller, 2009). Therefore smaller fishes require more time to ingest and handle prey than larger fishes which may be reflected by a lower prey consumption in smaller fish. However, our results suggest that differences in prey consumption between the two common sole sizes were not related to limitations in overall time budget. If time would have limited prey consumption in 100g common sole prey consumption would have been lower than in 300g common sole at all prey densities. In contrast, prey consumption was identical for 100g and 300g common sole at the lower densities.

In conclusion, other than expected prey density influenced prey consumption in common sole. Small common reached satiety prey consumption rates at nearly every prey density while large common sole did not reach satiety prey consumption rates even at highest prey densities exceeding those in nature. Results suggest that polychaetes such as *N. virens* can contribute to the diet of small common sole even at low/moderate densities. In contrast, the absence of satiety prey consumption in large common sole indicates that polychaetes such as *N. virens* are not an ideal prey type for larger individuals. These assumptions are in line with ecological data showing that polychaetes are an important prey type in the diet of small common sole but nearly absent in the diet of large individuals.

Acknowledgements

A matter of perception: the influence of waterborne signals from fish and conspecifics on the feeding related behavior of *Nereis virens* (Sars)

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Abstract

The present study compares the influence of signals released from fish (common sole, *Solea solea* L.) and conspecifics on the feeding behavior of the ragworm *Nereis virens* (Sars). Experimental tanks stocked with ragworms were connected to a donor tank by a tube to allow the transfer of water born signal molecules. The donor tanks were stocked with sole and ragworms to create 4 different treatments: „control (blank)", „ragworms", „sole" and „sole+ragworms". All behavioral recordings were made within the first 19 days of a total of 31 feeding days. Video recordings were made each time for 30 minutes starting when first feed entered the tank. All behavioral responses, i.e. latency, numbers and length of ragworms out of burrow were negatively affected when ragworms were exposed to water from "sole+ragworms" donor tanks. When exposed to water from "ragworms" and "sole" donor tanks, the experimental ragworms also reduced their behavioral responses but to a lesser extent than when exposed to the combination of "sole+ragworm" donor tanks. Out-of-burrow activities remained hampered in ragworms exposed to water coming from "sole" and "sole+ragworms" donor tanks at all observation days. Results indicate that signals released by damaged conspecifics and to a lesser extent fish borne cues play an important role in anti-predatory behavior of *N. virens*.

Introduction

In natural marine environments polychaetes such as nereids are almost constantly subjected to the risk of predation (Clark, 1960; Evans, 1969). As a response to this risk nereids withdraw into their burrows (Clark, 1960). However, the time spent in the burrow is lost for feeding. Therefore from an energetic point of view it is important to be able to distinguish between harmless and harmful threats. Evans (1969) demonstrated that *Hediste* (*Nereis*) *diversicolor* becomes habituated to changes in illumination, shadowing, mechanical shock and touch. However, out of burrow activities remained reduced over days when exposed to fish borne cues coming from flounder and flounder mucous (Schaum et al., 2013). For *Nereis virens* (Sars) less information is available. This species reduces its' out of burrow

activities when exposed to conspecific whole-body extracts. Also the number of individuals that was actively feeding decreased in comparison to a seawater control (Watson et al., 2005). To our knowledge it is not known how *N. virens* responds towards fish borne cues or to long term exposure to signals of conspecifics under predation pressure. Such information is of particular importance since this species dominates benthic communities in large parts of the North Sea and plays a central role in marine food webs. The present study compares the influence of waterborne signals from fish and conspecifics on the feeding related behavior of *N. virens*.

Material and methods

Animal origin and ethical notes

Experiments were conducted at the research facilities of Wageningen Imares in Yerseke, the Netherlands between 13.08.2008 and 08.04.2008. Soles originated from a commercial fish farm (Solea BV, IJmuiden, Netherlands). Ragworms were obtained from a commercial producer (Topsy Baits, Wilhelminadorp, Netherlands). Soles were supposed to graze on ragworms in donor tanks containing soles and ragworms. Because the soles were exclusively fed with a commercial pelleted feed prior to their arrival in the research facilities, they were not adapted to foraging on ragworms. However, previous unpublished observations showed that naïve soles successfully forage on ragworms. In addition, the ragworms came directly from the commercial producer and were thus naïve to predators and to fish borne alarm cues. All procedures involving animals were conducted in accordance with the Dutch law on experimental animals.

General experimental conditions and design

The experiment consisted of 8 experimental tanks (bottom area 0.030 m²). Each experimental tank was considered one experimental unit. The experimental tanks were stocked with ragworms at mean initial stocking densities of 1.2 kg m⁻² (mean initial body weight 2.6g ± 0.23 (SD)). Cameras were placed above the experimental tanks to record ragworm behaviour. Each experimental tank was connected to a donor tank (each donor tank with a bottom area 0.24m²) by a tube to allow the

transfer of water born cues produced by soles or ragworms in the donor tanks. The donor tanks were stocked with sole and ragworms according to a 2 by 2 factorial design. To test the "sole"- effect donor tanks were stocked with 0 or 2 soles (mean initial body weight 120g). To test the "ragworm"- effect donor tanks were stocked with 0 or 1.5 kg m^{-2} of ragworms (mean initial body weight 2.6g ± 0.23 (SD)). This resulted in the following 4 treatments: "control", "ragworms", "sole" and "sole+ragworms".

The water used in this study was pre-filtered (sandfilter Triton TR60) seawater from the Oosterscheldt estuary. The water was heated (15ºC, SF Profi Heater 3 kw,) and pumped into a reservoir tank from which it flowed continuously (flow rates of 34.3 ± 14.1 l.h^{-1} (SD)) to donor tanks and from the donor tanks to the experimental tanks by gravity. All tanks were filled with pre-washed construction sand to a depth of 20cm prior to the start of the experiment. Conditions were kept constant throughout the experiment (natural photoperiod; temperature 16.8 ± 0.8°C; pH > 7.8; Dissolved oxygen > 59%).

At the beginning of the experiment, donor tanks were stocked randomly according to the treatments (in duplicates). The experimental tanks were stocked with ragworms. These ragworms were fed each day at the beginning and at the end of the day during daylight hours. Ragworms were fed commercial feed pellets (Dragonfeed polymeal, composition on www.dragonfeeds.com). The sole in the "sole+ragworms" donor tanks were supposed to feed on the ragworms. Soles in the "sole" donor tank were fed a commercial feed (Turbot Extruded Grower Feed Dan-Ex 1562, Dana-feed, Denmark). The cameras recorded half an hour after feeding each day (after Evans (1963). Animals in the donor tanks were only fed after videotaping animals in the experimental tanks to avoid feeding related signals on the behavior of ragworms. In total 6 and 9 behavioral recordings (for latency and numbers and length of ragworms out of burrow respectively) were made within a period of 31 days. At the end of the experiment the final weight of the individual

sole and the total weight (biomass) and number of ragworms of each donor and experimental tank was recorded.

Measurements and video recordings

Daily feed intake of ragworms (DFI; g DM ragworm^{-1}.d^{-1}) was calculated as TFI / (t * n), where TFI is the total FI per tank over the experimental days corrected for uneaten feed, t is the experimental period and n is the number of ragworms per tank. All behavioral recordings were made within the first 19 days of a total of 31 feeding days. Video recordings were made each time for 30 minutes starting when first feed entered the experimental tank. The following behavioral responses were measured: The latency time, i.e. the time after which the first animal came out of the burrow (in min.); the number and length of ragworms out of the burrow (during 30 min after being fed). From the 30 minutes of film, frames were taken out at 5 minutes intervals at time points 5, 10, 15, 20, 25 and 30 minutes. The images (frames) obtained were scored for the number of ragworms out of burrow and length of ragworms out of burrow. The latency time was determined by watching the total 30 minutes frames. Latency time was considered the time elapsed from the moment feed entered the tank until the first worm came out of its burrow. The length of the worms was measured using the ImageJ software (NIH). A 10 cm stick was placed on the sediment for length reference.

Statistics

Data were analyzed using the Statistical Analysis Systems statistical software package version 9.2 (SAS Institute, Inc., Cary, NC, USA). Growth and feed intake of ragworms were tested for the effect of "ragworms" and "sole" (being present in the inflowing water) as well as their interaction effect using a 2-way ANOVA. The latency time to respond to feeding was assessed for the effect of rageworm, sole, time (i.e., day after the start of the trial) and all interaction effects by repeated measurement analysis (PROC GLM). In this analysis the effect of ragworm and sole were tested against the between tank variation and time and time interaction against the within tank variation. Because of non-normal distributions, parameter

free testing by Wilcoxon exact test was done for the effects of time as well as for the effects of treatments on the number of worms emerging and the length of emerged worms. In this parameter free testing, 2 by 2 factorial testing was not possible therefore all 4 experimental treatments were tested. Within each of the four treatments, it was tested if the number of worms emerged was different between day numbers (by parameter free test). In all tests, the statistical significant difference between groups were considered when $P<0.05$.

Results

Latency

Averaged over all treatments and observation days, the latency time between the start of feeding and the first ragworm coming out of its burrow was 1.3 min. This latency time was dependent on whether the water supply was coming from tanks with or without ragworms ("ragworms" effect; $P<0.001$) as well as from tanks with or without sole ("sole" effect; $P<0.01$). However both these main effects did also interact with each other ($P<0.01$; Fig. 6.1A). The latency time for the "control", "ragworms" and "sole" treatment were equal (being on average 0.4 min), whereas in the "sole+ragworms" treatment the latency time was about ten times higher (4.1 min; Fig. 6.1A). The pattern in the effect of "ragworms" and "sole" was consistent during the various observation days in the experiment. In Fig. 6.1B the latency time averaged over treatments during the different observation days are given. A significant time effect (i.e., observation day; $P<0.001$) was present, which was fully due to an about 2.5 times higher latency time during day 6 of the experiment. When day 6 was excluded in the statistical analyses, there was no significant time effect anymore, nor interaction with time. No clear reason was noted for this increased latency time on day 6.
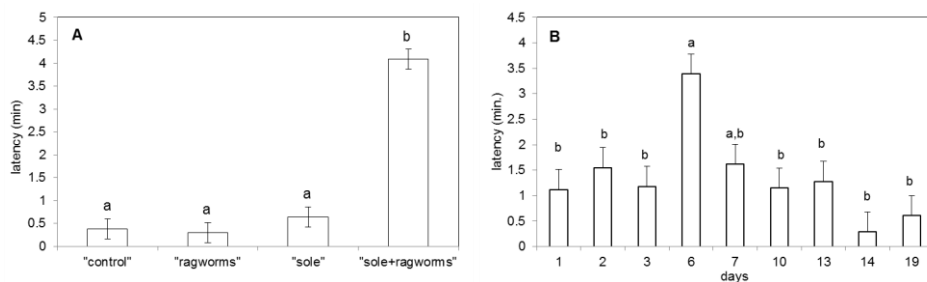
Figure 6.1 The latency time between the start of feeding and the first responding ragworm to the presence of food by coming out of its' burrow as affected by the differently conditioned water: with or without the presence of common sole and with or without the presence of ragworm (A); and by day number in the experiment averaged over treatments (B). Displayed are least square means and the standard error of means (SEM). Mean without a common letter differ significantly (P<0.05).

Numbers and length of ragworms out of their burrow

During 30 min after the start of feeding, the number of ragworms being out of their burrows was counted at the following time points: 5, 10, 15, 20, 25 and 30 min. The mean number of ragworms out of their burrows at these time points was different between the 4 experimental treatments ($P<0.01$; Fig. 6.2A). The ragworms receiving water from the "sole+ragworms" tanks, did not come out of there burrow within 30 min post feeding; averaged over all days 0.09 individuals emerged (Fig. 6.2A). The highest number of ragworms emerging in 30 min was at the "control" treatment (3.7 Individuals) and the "ragworms" and "sole" treatments were intermediate. The treatments also affected the length of the ragworms coming out of their burrows ($P<0.05$, Fig. 6.2B). Ragworms receiving water from "sole+ragworms" donor tanks hardly exposed themselves out of the burrow (1.6 mm), whereas ragworms receiving water from the "ragworms" or "sole" donor tank only had a small reduction in exposure length compared to the "control" treatment (Fig. 6.2B).
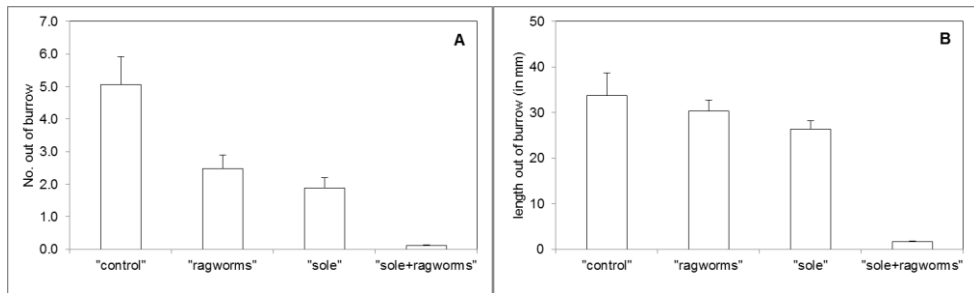
Figure 6.2 The number of ragworms out of the burrow (at time points 5, 10, 15, 20, 25 and 30min during 30 min after being fed (A) and the length of worms out of the burrow averaged over the first 30 min after feeding (B) as affected by: the differently conditioned water, with or without the presence of sole and with or without the presence of ragworms Displayed are means and the standard deviation (SD).

The observed differences between treatments in number and length of ragworms out of their burrow, for each of the 5 min observation moments after the start of feeding was similar to the total sum as depicted in Fig. 6.3. Regarding the number of ragworms, at all moments there was a treatment effect ($P<0.05$) but for the length of ragworms out of their burrow the differences were not significant (data not shown). The number and length of ragworms out of their burrow did not change with time after the start of the feeding when averaged over treatments ($P>0.05$; Fig 6.3A and 6.3B).
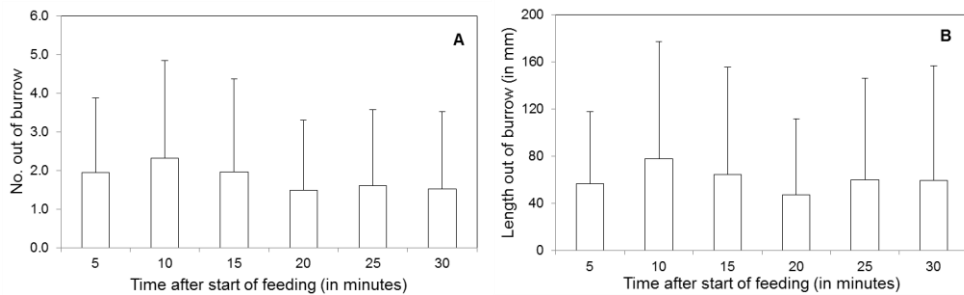
Figure 6.3 The number of ragworms out of their burrows (A) and the mean length of ragworms out of their burrows (B) at different intervals after the start of feeding. The values are the average and standard deviation (SD) over all 4 experimental treatments.

Figure 6.4 shows how the total sum of ragworms out of burrow activities vary over time. The number of ragworms out of the burrow increased with time in the "control" and "ragworms" treatment ($P<0.05$). In contrast, the number of ragworms out of the burrow did not increase in ragworms receiving water from the "sole" and "sole+ragworms" donor tanks.
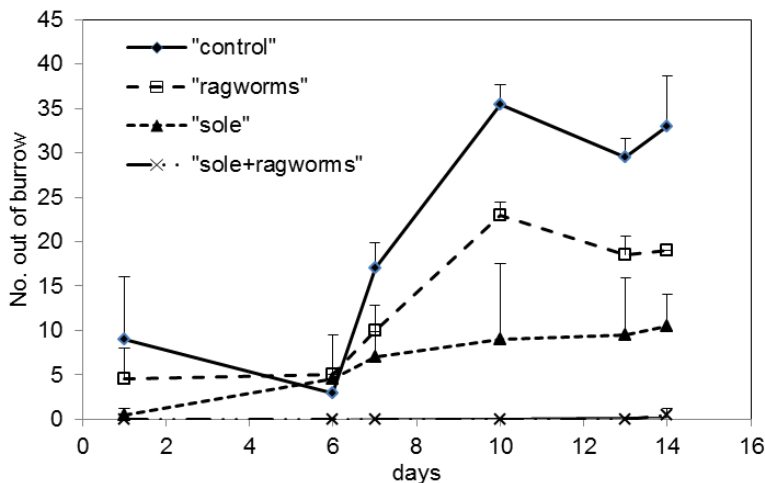

Figure 6.4 The number of ragworms out of their burrows at different days. The values are the average and standard deviation (SD) averaged over time points 5, 10, 15, 20, 25 and 30 minutes.

Feed intake and SGR

The feed intake of ragworms was influenced by the interaction effect between "sole" and "ragworm" being present in the receiving water (P<0.05, Fig 6.5A). Ragworms receiving water from the "sole+ragworms" donor tanks had a 49% lower feed intake compared to the other treatments (*P*<0.05, Fig 6.5A). Between the "control", "sole" and "ragworms" treatments feed intake was similar. Specific growth rate (SGR) was reduced when the incoming water came from donor tanks stocked with sole (main effect of "sole" *P*<0.05). Also the main effect of "ragworms" resulted in a lower SGR ("ragworm" effect *P*<0.001). But at the treatment "sole+ragworms" both main effects seems to enhance the reduction in SGR, (interaction effect *P*=0.08; Fig 6.5B). This tendency for an interaction effect between "sole" and "ragworms" is also reflected in the Tukey pairwise comparisons. At the "sole+ragworms" treatment SGR was significantly lower compared to all other treatments (Fig 6.5B).
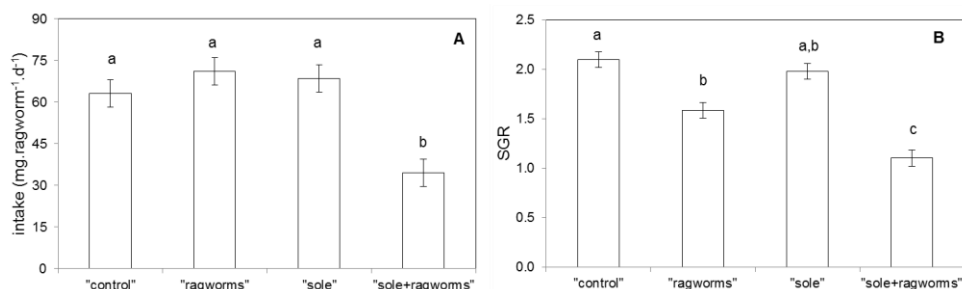

Figure 6.5 Ragworm feed intake (A, in $mg.ragworm^{-1}.d^{-1}$) and ragworm specific growth rates (B) as affected by the differently conditioned water: with or without the presence of common sole and with or without the presence of ragworm. Displayed are least square means and the standard error of means (SEM). Mean without a common letter differ significantly (P<0.05).

Discussion

The most striking behavioral response was observed in ragworms receiving water from the "sole+ragworms" donor tanks; all behavioral responses, i.e. latency, numbers and length of emerges were negatively affected (Figs. 6.1-6.4).

Behavioral responses did not improve with time suggesting that ragworms did not get adapted to the stimuli in the water coming from "sole+ragworms" tanks. We could additionally demonstrate that these reduced "out of burrow" activities of ragworms also reduced their food intake and growth (Fig. 6.5). Experimental tanks had only indirect contact to donor tanks by receiving their water. Therefore results demonstrate that *N. virens* was responsive to waterborne chemical cues released by common sole, ragworms or both.

We assume ragworms were responsive towards cues of damaged conspecifics, either directly at capture or indirectly at excretion by the fish. Additional data (not presented) showed that common sole extensively grazed on ragworms in "sole+ragworms" donor tanks. This suggests that signals of damaged conspecifics were involved. It is known that ragworms (*N. virens*) reduce their out of burrow activity in response to exposure to damaged conspecifics (Watson et al., 2005). The strong predation pressure and/or damage cues from conspecifics may have also created a positive feedback loop such that conspecifics in the donor tank picked up damage signals and additionally released stress cues. Results further indicate that fish borne cues (e.g. skin particles, mucous or faeces) also induced negative behavioral responses in ragworms. Ragworms exposed to water from "sole" donor tanks considerably reduced the number and length of ragworms out of burrow. Behavioral responses in *N. virens* towards fishborne cues have also been observed in other nereids such as *H. diversicolor* (Schaum et al., 2013). Substances originating from fish could be skin particles, mucous or faeces (Brown, 2003; Chivers et al., 2007; Forward Jr, Rittschof, 2000; Nunes et al., 2013). Our results also indicate that ragworms don't adapt to the fishborne cues. The number of ragworms out of burrow did not increase over time in ragworms exposed to water coming from "sole" donor tanks (Fig. 6.4). This result is in line with observations in *H. diversicolor* showing no signs of habituation to fish alarm cues in a seven day trial (Schaum et al., 2013). In contrast, habituation to fish alarm cues has been demonstrated in the freshwater isopod *Lirceus fontinalis*, and it was suggested that the cue is only significant in eliciting short-term predator avoidance

activity (Holomuzki, Hatchett, 1994). Our results indicate that ragworms (*N. virens*) are also responsive towards cues originating from conspecifics which are not under predation pressure. The number of ragworms out of their burrow decreased in ragworms receiving water from "ragworms" donor tanks. Other behavioral responses, i.e. latency and length of ragworms out of burrow were not affected. However, the number of ragworms out of burrow increased over time in ragworms exposed to water coming from "ragworms" donor tanks demonstrating habituation (Fig. 6.4). Interestingly, we found that ragworms exposed to water from "ragworms" donor tanks had a relatively high FCR compared to the control treatment (1.2 and 0.8 respectively). This fits to the observed behavioral response towards conspecifics and indicates that the presence of conspecifics induces density stress. Stress is associated with metabolic costs, as it is an energy demanding process (Barton, Iwama, 1991; Mommsen et al., 1999).

In conclusion, ragworms showed a strong behavioral response when receiving water from "sole+ragworms" donor tanks and, to a lesser extent, also responded when exposed to water from "ragworms" and "sole" donor tanks. Results indicate that signals released by damaged conspecifics play an important role in anti-predatory behavior.

Chapter 7

General discussion

The general aim of this study was to get insight into which factors may limit growth of common sole foraging on ragworms in ponds. Growth may become limited when the feed is nutritionally imbalanced and/or cannot be consumed in adequate quantities. In nature common sole feeds on a wide range of different prey species of different composition. It is not clear if ragworms alone meet the nutritional needs of common sole. In addition, no information exists on whether sole has the foraging abilities to capture buried ragworms in a pond environment. This chapter reflects on the results of the previous chapters and discusses the suitability of ragworms as an exclusive food source for common sole in a pond system.

Performance at non-limiting feeding conditions

The first aim of this study was to test if common sole grows well when fed exclusively with ragworms at non-limiting conditions, i.e. using a feeding protocol that allows maximum daily food intake by providing chopped ragworms and sediment free tanks. I used mussels as a control feed because highest growth rates reported so far for common sole were obtained with mussels (Fonds et al., 1989a). The fact that growth rates were even higher in common sole fed with ragworms than with mussels demonstrates the nutritional suitability of this food type for common sole (growth rates reported in Chapter 2 were 8.8, 5.1 and 8.1 g.kg$^{-0.8}$.d^{-1} for ragworms, formulated feed and mussels respectively). The formulated protein rich feed was used because it was specifically formulated to meet the nutritional requirements of flatfish. However, the low growth in common sole fed the formulated feed suggests that the present flatfish feed was not suited for common sole. A theory for the poor performance is discussed at the end of this section. The growth rates reported in Chapter 2 for common sole, fed with mussels were lower than those reported by Fonds et al. (1989a) (also fed with mussels). This demonstrates that growth data cannot be compared between the two studies. One explanation for observed differences could be differences in nutritional history of the fish used in the present study and those used by Fonds. Fishes in the present study came from a farm and some individuals showed signs of nutritional

deficiencies (malpigmentation). In contrast, fishes used in the study by Fonds where obtained from the wild.

Whether common sole will achieve their growth potential when fed with ragworms depends on whether ragworms are consumed sufficiently or whether intakes get limited e.g. by nutritional imbalances. The most striking indication of an imbalance is observed for protein. The protein content of ragworms was 74% (chapter 2), far in excess of the known dietary protein requirements of common sole which is around 57% (Gatta et al., 2010). Fish regulate feed intake based on the demand for oxygen needed to metabolize the ingested diet (Saravanan et al., 2012). Excess dietary protein increases the dietary oxygen demand and intake will become regulated by a lack of oxygen. Our data suggest that the high protein content in ragworms increased the dietary oxygen demand because fat was synthesized from protein. The fat retention efficiency was larger than 100%, indicating that fat was synthesized de novo (chapter 3). However, the increased dietary oxygen demand may have been compensated by a simultaneous increase in blood haematocrit and blood haemoglobin increasing oxygen availability. An increase in blood haematocrit and blood haemoglobin with the intake of ragworms in common sole was recently reported by (Kals et al., 2015). The increase in blood haematocrit and blood haemoglobin with ragworm intake may be related to high levels of vitamins and/or iron in ragworms (e.g. iron content in ragworm is 490mg.kg^{-1}) (Kals et al., 2015).

A diet which contains protein higher than the species requirements is less efficiently utilized. This is because protein is less efficiently used for de novo fat synthesis than fat or carbohydrates. This was however not the case in the present study. Despite excess protein, common sole fed ragworms showed higher protein, fat and energy utilization efficiencies than animals fed mussels or the formulated feed (chapter 3). Values obtained in common sole fed ragworms were comparable to values reported in the literature for other commercial fish species fed formulated, balanced feeds (see chapter 3). This can be related to suitable physical and

nutritional properties of ragworms, e.g. bioactive compounds, water content, free amino acids (AA) but also a suitable amino acid profile and ratio of essential to non-essential amino acids (EAA:NEAA). With regard to the first possible explanatory factor, e.g., bioactive compounds, not much can be said. Unfortunately no information exists on the role of bioactive compounds in nutrient utilization efficiencies in fish. The same counts for the importance of dietary water content for nutrient efficiencies. Its role is not clear. Grove et al. (2001) reported higher feed efficiencies in Turbot (*Scophthalmus maximus)* when fed moist squid than when fed dry squid*.* In contrast, Bromley (1980) reported no influence of dietary water content on nutrient efficiencies in Turbot. Finally, the significant role of the AA profile on utilization efficiencies is well documented (Helland et al., 2010; Rodehutscord et al., 1997). Therefore high nutrient utilization in common sole fed ragworms may be explained by a suitable a AA profile of ragworms. Results suggest that common sole has adapted to efficiently utilize invertebrates which naturally contain high levels of protein and amino acids profiles which are less balanced than those in fish meal.

A striking outcome of this study was the poor performance in common sole fed the formulated feed (chapter 2). The high dietary fat content of the formulated feed did not result in excess fat deposition (chapter 2). Figure 7.1 shows that at higher feed intake levels about 70% of the energy consumed was used as an energy source rather than being deposited. The utilization of fat as an energy source usually results in a protein sparing effect enhancing growth. This was however not observed in the present study. In addition growth was impaired by the low intake of the formulated feed compared to intake values obtained with the natural foods (chapter 2). I discussed a possible involvement of body fat, palatability and AA balance as factors explaining the low intake of the formulated feed (chapter 2). Further studies will be needed as none of these factors could directly be related to the low feed intake.
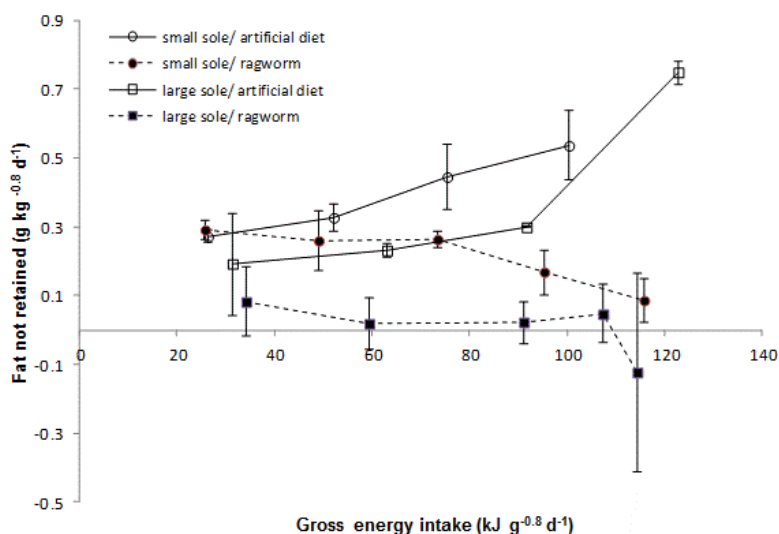
Figure 7.1 The relationship of fat not retained (in $g.kg^{-0.8}.d^{-1}$) in small and large sole fed ragworms and the artificial diet.

To summarize present results demonstrate high, feed intake and nutrient utilization efficiencies in common sole fed ragworms. Comparisons to literature suggest that the full growth potential of common sole fed ragworms may have not been obtained in this study. This could be related to a poor nutritional history of the present animals or suboptimal rearing conditions.

Performance at limiting feeding conditions

My second aim was to test if common sole is able to capture buried ragworms and to test the behavioural interactions when both species are kept in one system (chapter 4, 5 and 6). The main conclusion of this part of the study is that common sole is not well adapted to forage on buried ragworms. On average, the intake of buried ragworms was reduced by more than half in contrast to intake of unburied ragworms (at 20cm and 2cm sediment thickness respectively), regardless of ragworm size (chapter 4). In addition, none of the common sole size classes tested was able to reach satiation when foraging on buried ragworms. Intake of buried compared to unburied ragworms was most strongly reduced in the largest tested

sizes of common sole (300g) (chapter 4 and 5). Results showed that increasing ragworm density increased ragworm intake. However, only smaller common sole reached satiation values with ragworm densities above 2.2 No.dm^{-2} (chapter 5). A detailed description of foraging tactics of common sole and anti-predatory behaviour of ragworms explaining present results is provided in chapters 4 and 5.

In the following text I will discuss the possible impact of experimental conditions on ragworm availability and intake. I have conducted experiments under pre-determined laboratory conditions (temperature, tank design, water temperature and sediment thickness and type). All of these factors may have influenced the availability of buried ragworms in a positive or negative way and different results may be obtained at different conditions present in ponds.

Sediment thickness and sediment type
My choice for coarse sediment and limited sediment thickness may have improved the availability of ragworms. I used a sediment thickness of 20cm in the experimental tanks. This sediment thickness may have limited ragworms to migrate deeper and to further reduce their availability to common sole. Ragworm depths in the experimental conditions was generally much lower than ragworm depths observed in the field for this species (Caron et al., 1996). In addition, the choice for coarse sediment may have facilitated the availability of ragworms. Ragworms like *N. virens* are considered generalists accepting a wide range of sediment grain sizes to build their burrows. However, burrows of benthic invertebrates are less deep in sandier substrate with higher water contents than in muddy sides (Morrisey et al., 1999). In addition, in coarse sand, animals tend to remain within the sand whereas in muddy sands ragworms tend to accumulate at the tank bottom, i.e. at the deepest, least accessible point (Dantu, 1957; Dorgan et al., 2006).

Temperature and ragworm condition

Experiments were conducted at a constant temperature of around 20°C. In addition, ragworms were not fed during acclimation and during the experimental period. Both factors may have facilitated the availability of buried ragworms in the present study. Firstly, ragworms are found closer to the burrow opening at a temperature of 20°C and migrate deeper as temperatures fall (Esselink, Zwarts, 1989). In addition, ragworms of poor condition reduce their burial depth (Esselink, Zwarts, 1989). Although I did not find any increase in mortalities of ragworms during this period I recorded a general weight loss of ragworms indicating a loss of condition. In addition, the feeding activity of larger common sole may have been compromised by the present temperature. Larger common sole reduce their intake at temperatures above 15°C as they suffer from respiratory stress (Fonds, 1979). Smaller common sole in contrast show highest intake rates between 20-25°C (Fonds, 1979). These differences in temperature preference are explained by the live history of common sole. Common sole leave shallow warmer coastal areas and migrate into deeper cooler waters as they grow (Rijnsdorp, Vingerhoed, 1994).

Foraging experience of experimental animals

Present experimental animals were adapted to capture buried ragworms prior to each experimental period. This adaptation was necessary because common sole used in our study had been exclusively fed with a commercial pelleted feed before arriving at the experimental facility, thus being naïve to eating live prey. Although I observed that soles were capable to consume ragworms immediately upon arrival it is not clear if an adaptation period of only 7 days (as in chapter 5) is sufficient to gain full experience in capturing buried prey. Ragworm intake may be higher when using animals which come from the wild or are adapted to capture buried prey for several months.

Ragworm harvesting

The recovery of buried ragworms from large amounts of sediment posed a risk of false measurements. I however assume that 100% of buried ragworms were

recovered after the feeding trials. This assumption is based on a pre-trial which I conducted showing 100% ragworm recovery irrespective of ragworm size.

Ragworm behaviour in response to common sole

In ponds ragworms may respond towards to presence of a predator like common sole by increasing their anti-predatory response, i.e. reduced outside burrow activity and deeper migration into their burrow. These responses may additionally constrain their capture. The present study showed that all behavioral responses, i.e. latency, numbers and length of ragworms out of their burrow were negatively affected in ragworms exposed to water from the "sole+ragworms" donor tanks with no signs of adaptation (see chapter 6 for detailed discussion). As a result intake in experimental ragworms was reduced by 44% when receiving water from the "sole+ragworms" donor tanks compared to intake in ragworms receiving water from the "control" donor tank (35 and 63 mg.ragworm^{-1}.d^{-1}, respectively). Results indicate that signals released by damaged conspecifics play an important role in anti-predatory behaviour.

A reduction of feed intake in ragworms by 44% will certainly have a great negative impact on overall pond productivity. It is however possible that signal strength in ponds may be diluted simply by larger water volumes in ponds. In *N. diversicolor* a negative behavioral response was only observed at and above fish mucous concentrations of 0.4 µg.ml^{-1} (Schaum et al., 2013). In addition it may be possible to reduce signal strength in ponds e.g. by reducing common sole density or increasing flow rates. However, these options may conflict with economic viabilities. Soles were stocked at densities of 1kg.m^{-2} which can already be considered a low density. Increasing flow rates to wash out signals will increase energy demands of the system. One possibility to increase ragworm feeding despite soles grazing on conspecifics in the same system may by adjusting feeding protocols. Diurnal feeding of ragworms as observed in the present study has to our knowledge not been reported so far. However, this flexibility may offer the

opportunity to feed ragworms at times when signal strength and feeding activities of sole are lowest, presumably around midday.

Implications and recommendations for common sole pond culture

Present results showed that at non-limiting conditions, growth, intake and nutrient utilization efficiencies are higher in common sole fed ragworms than in those fed mussels and formulated diets (chapter 2 and 3). Present growth rates may be below the species potential not because of a nutritional imbalance of ragworms but due to suboptimal experimental conditions or inadequate nutritional history of the experimental animals. Intake values obtained when common sole had to capture buried ragworms (chapters 4 and 5) cast some doubt on whether ragworms will be consumed in sufficient quantities to support high sole growth in a pond; Intake of all ragworm sizes (when buried) was limited either by numbers (as for small ragworms) or by additional handling costs (for larger ragworms). Increasing ragworm densities improved intake of ragworms though only smaller common sole reached satiation at higher densities. In addition, present results also indicate negative interspecific interactions in a pond, i.e. that ragworms will reduce their feeding activities at the presence of common sole grazing on conspecifics (chapter 6). This will negatively affect overall pond productivity.

Certainly the present results are too incomplete to enable and fully predict intake and growth performance of common sole in a pond. Factors such as temperature, oxygen supply or feeding activity may additionally have positive or negative influences on ragworm availability in a pond. For example, ragworms were starved in this study which may have facilitated their availability. Fed ragworms in a pond will be fitter and may be deeper unless depth will be restricted by lowering the sediment thickness. I also assume that the present temperature has improved ragworm availability. In a pond the temperature will be lower than 20°C most of the year decreasing ragworm availability (unpubl. data of the Zeeuwse Tong Project).

Present results suggest that the key to common sole growth on natural foods in a pond is the prey burial depth. It is not clear if pond conditions can be altered in a way to overcome this problem of deep dwelling of ragworms by manipulating e.g. sediment thickness without reducing the overall productivity of the pond. Future research efforts should therefore explore the suitability of alternative prey species such as the polychaete *Captiella capitella*. This species is also commercially cultured, and may be easier available to common sole due to its lower burial depth.

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Appendices

Samenvatting (Dutch)

Acknowledgements

About the Author

Publications

Training and Supervision Plan

Samenvatting (Dutch)

Ondanks de grote vraag vanuit de markt en intensief onderzoek sinds de jaren 1960 is de commerciële kweek van tong en met name *Solea solea* L. tot nu toe weinig succesvol geweest. Problemen met betrekking tot de beschikbaarheid en de prijs van geschikte grondstoffen (invertebraten) en het feit dat tongen niet goed in grote groepen kunnen worden gehouden, verhinderen de ontwikkeling van intensieve tongteelt. Op dit moment wordt onderzoek gedaan naar extensieve teelt van tong in vijvers met natuurlijk voer.

Het algemene doel van dit onderzoek was om inzicht te krijgen in de factoren die een negatieve invloed hebben op de groei van zagers etende tong in vijvers. Uit de resultaten bleken geen nutritionele effecten die de groei van zagers etende tong in vijvers kunnen verhinderen. Onder niet-beperkende omstandigheden, dat wil zeggen op een dieet van stukjes zagers en in een tank zonder sediment, aten de vissen meer, groeiden ze beter en benutten ze de voedingsstoffen beter dan tongen die mossels of een samengesteld dieet kregen. Uit de resultaten komt echter naar voren dat de groei van tong in een vijver met zagers wordt beperkt door verminderde foerageeractiviteit van de tong. Er werd gekeken naar de volgende factoren die die het meest relevant zijn in een vijver: de grootte van de prooi, de grootte van het roofdier en de prooidichtheid. Over het algemeen aten de vissen ruim de helft minder zagers als de zagers in de bodem werden gestopt dan wanneer dat niet zo was. De grootte van de zagers en de grootte van de tong hadden hier geen invloed op. Het verhogen van het aantal zagers leidde alleen tot oververzadiging van de kleinere tongen. Onze resultaten wijzen er tevens op dat de aanwezigheid van tong het functioneren van zagers in een vijver negatief beïnvloedt. Zagers aten minder als ze water kregen uit tanks met zowel tongen als zagers en waar tongen op zagers konden jagen.

Uit de resultaten van dit PhD-onderzoek blijkt dat de groei van tong in vijvers niet zo zeer beperkt wordt door de voedingstoestand, maar eerder door hun foerageergedrag. De resultaten van dit onderzoek zijn echter te onvolledig om de

groei van tong in een vijver volledig te kunnen voorspellen. Er moet nader onderzoek worden gedaan naar factoren als temperatuur, zuurstoftoevoer en voedingsactiviteit om uitgebreide groeivoorspellingen te kunnen doen.

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About the Author

Stephan Siegfried Werner Ende was born in Gifhorn, Germany, on April 11th, 1975. After finishing secondary modern school in 1991 he started a vocational training at Volkswagen AG (Wolfsburg, Germany) and became an industrial mechanic. He continued to working for VW for another year before he went back to school and received the University entrance qualification 1996. His subsequent academic carrer was driven by his interst in marine life. After having received a Diploma degree in Environmental Biology from the University of Applied Sciencies Bremen (Germany) he continued with an MSc Thesis at Rhodes University (Grahamstown, South Africa). From 1th October 2007 he was employed as a research associate at the Aquaculture and Fisheries Group (Wageningen University, Wageningen, The Netherlands). During this time he worked on aspects of growth retardation in fish in Recirculating aquaculture Systems (RAS). A few month later we started with the present PhD study at Wageningen University.

Publications

Peer- Reviewed Papers

Ende, S.S.W., Kroeckel, S., Schrama, J.W., Schneider, O., Verreth, J.A.J., 2014. Feed intake, growth and nutrient retention of common sole (*Solea solea* L.) fed natural prey and an artificial feed. Aquaculture Research.

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Conference Proceedings

Ende, S.S.W., Thiele, R., Schrama, J.W., Blonk, R.J.W., Verreth, J.A.J. 2011. The influence of ragworm, Nereis virens, (Nereidae) density on intake by sole, *Solea solea*. Aquaculture Europe 2011, 18-21 October, Rhodes, Greece.

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Thompson, E.; **Ende, S.S.W.**; Markovina, M.; Hecht, T.; Kaiser, H., 2007: On the mariculture potential of the white-margined sole. *Dagetichthys marginatus*. J. World Aquacult. 39, 37–41.

Training and Supervision Plan

Training and Supervision Plan		Graduate School WIAS
Name PhD student	Stephan Siegfried Werner Ende	
Project title	Culturing soles on ragworms: Growth and feeding behaviour	
Group	Aquaculture and Culture and Fisheries Group	
Daily supervisor(s)	Dr. Johan Schrama	
Supervisor(s)	Prof. Dr. Johan Verreth	
Project term	from 15.10.2007	until 18.08.2012
Submitted	31.07.2015	first plan / midterm / **certificate**

The Basic Package (*minimum 3 cp*)	year	credits *
WIAS Introduction Course (mandatory, 1.5 cp)	2009	1.5
- Course on philosophy of science and/or ethics (mandatory, 1.5 cp)	2009	1.5
- Introduction interview with WIAS scientific director and secretary: 06.12.2007	2007	-
- Introduction interview with WIAS education coordinator: 11.12.2007	2007	-
- Introduction interview with WIAS PhD students confidant: 06.12.2007	2007	-
Subtotal Basic Package		**3**
Scientific Exposure (*minimum 8 cp*)	year	credits
International conferences (*minimum 3 cp*)		
- The cultivation of soles, Faro, Portugal	2008	1.3
- Aquaculture Europe, Trondheim, Norway, Portugal	2009	1.3
- Aquaculture Europe, Porto, Portugal		1.3
- Aquaculture Europe, Rhodos, Greece	2011	1.3
Seminars and workshops		
- WIAS Science Day	2009	0.3
- WIAS Science Day	2010	0.3
Presentations (*min. 4 orig. presentations of at least 1 oral, 1 cp each*)		
- Oral presentation, The Cultivation of Soles IV, Faro, Portugal	2008	1.0
- Oral presentation, Aquaculture Europe, Trondheim Norway	2009	1.0
- Poster presentation, Aquaculture Europe, Porto, Portugal	2010	1.0
- Poster presentation, Aquaculture Europe, Rhodos, Greece	2011	1.0
- Oral or poster presentation at the WIAS Science Day	2009-2010	2.0
Subtotal Scientific Exposure		**12**
In-Depth Studies (minimum 6 cp, of which minimum 4 at PhD level)	year	credits
Disciplinary and interdisciplinary courses		
- Aquaculture Nutrition September 22-26, 2008 Bergen, Norway	2008	3

- New developments and Perspectives in Aquaculture, Rostock, Germany	2010	6
Advanced statistics courses *(optional)*		
Linear models	2010	0.9
MSc level courses *(only in case of deficiencies)*		
Subtotal In-Depth Studies		**10**
Statutory Courses	**year**	**credits**
Use of Laboratory Animals (mandatory when working with animals)	2007	3
Subtotal Statutory Courses		**3**
Professional Skills Support Courses (minimum 3 cp)	**year**	**credits**
Course Techniques for Scientific Writing	2009	1.2
Workshop Markterkundung (09.07.)	2015	0.15
Beratung Ideenschutz (19.08.)	2015	0.15
Workshop Finanzplan (26.08.)	2015	0.15
AlgaePARC open workshop (Wageningen)	2014	0.15
Fachvortrag Rechtsformen (27.08.)	2015	0.15
Absicherung betrieblich (24.09.)	2015	0.15
Carrer Assessment	2010	0.3
Subtotal Professional Skills Support Courses		**3**
Research Skills Training (optional)	**year**	**credits**
Preparing own PhD research proposal (maximum 6 credits)	2008	3.0
Subtotal Research Skills Training		**3**
Didactic Skills Training (optional)	**year**	**credits**
Lecturing *(real time including preparation)*		
Supervising practicals and excursions *(real time)*		
Supervising theses *(max 2 cp MSc major, 1.5 cp MSc minor, 1 cp BSc)*		
MSc major	2008	2.0
BSc major	2010	1.0
Tutorship *(real time)*		
Preparing course material *(real time)*		
Subtotal Didactic Skills Training		**3**
Management Skills Training (optional)	**year**	**credits**
Organisation of seminars and courses		
Membership of boards and committees		
Subtotal Management Skills Training		**0**
Education and Training Total (minimum 30, maximum 60 credits)		**36**
* one ECTS credit equals a study load of approximately 28 hours		