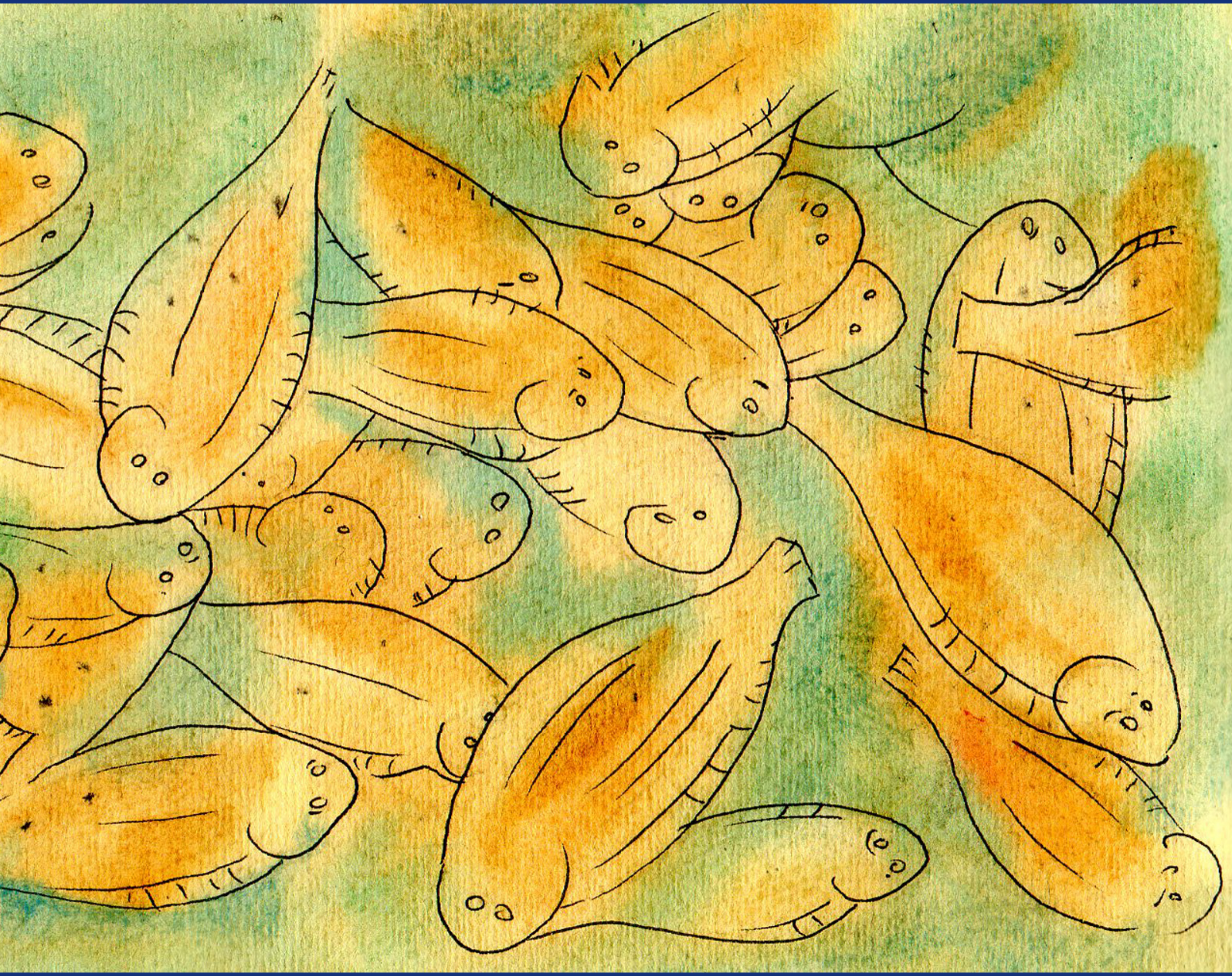
VARIATION IN BEHAVIOUR AND GROWTH OF COMMON SOLE

genetic and environmental influences



JULIA MAS-MUÑOZ

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genetic and environmental influences

Julia Mas-Muñoz

Thesis committee

Promotor

Prof. dr. ir. Johan Verreth
Professor of Aquaculture and Fisheries
Wageningen University

Co-promotor

Dr.ir. Johan W. Schrama
Associate professor, Aquaculture and Fisheries Group
Wageningen University

Dr.ir. Hans Komen
Associate professor, Animal Breeding and Genomics Centre
Wageningen University

Dr. ir. Robbert Blonk
Researcher, IMARES
Wageningen University and Research Centre

Other members

Prof. dr. ir. Marc Naguib, Wageningen University
Prof. dr. ir. Patrick Kestemont, FUNDP, University of Namur, Belgium
Dr. ir. Jan Ketelaars, Plant Research International, WUR
Dr.ir. Mathilde Dupont-Nivet, INRA, France

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VARIATION IN BEHAVIOUR AND GROWTH OF COMMON SOLE:
genetic and environmental influences

Julia Mas-Muñoz

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To Ewout and my family

“Would you tell me, please, which way I ought to go from here?"

"That depends a good deal on where you want to get to."

“I don't much care where...as long as I get somewhere"

"Oh, you’re sure to do that, if only you walk long enough.”

(Carroll, L. 1865, *Alice in Wonderland*)

ABSTRACT

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Common sole (*Solea solea*) has a high potential for commercial aquaculture because of its consumer popularity and high market values in Europe. However, a major economic constraint for the culture of sole is its slow and variable growth. The aim of this thesis was to investigate: 1) the importance of (non-) feeding behaviour of sole in relation to variation in growth; 2) the effect of (social and physical) environmental factors on behaviour, growth and the relation between them; 3) the existence of G×E interaction regarding growth. Feeding consistency, swimming activity in the tank, and boldness during (novel environment and light avoidance) behavioural tests explained variation in feed intake and thereby growth of individually housed sole. For communally housed sole, behavioural factors derived from individual behavioural tests and sex also explained variation in growth. The motivation to bury was negatively related to growth, whereas the motivation to explore a novel environment was positively related. Social interactions, both in quality (i.e., size hierarchies) and in quantity (i.e., stocking density), influenced (non-) feeding behaviour and growth of sole. High stocking density in sole reared without substrate results in more fish-fish interactions, which increases swimming activity, FCR and variation in growth. These conditions seem to induce social stress in sole, which is alleviated when sand is provided. Environmental factors which differ between nature and farming conditions, such as food type, sand and variability of environmental conditions, influenced individual behavioural responses of sole to a novel environment test but did not induce variation in growth. Results suggest that consistent relationships between behaviour and growth develop when fish are reared in stable barren environments but not when fish experience more variable, enriched/natural environments. The role of environmental factors in the relationship between (non)-feeding behaviour and growth was supported by strong genotype by environment interaction for growth of sole reared in a semi-natural or an intensive aquaculture environment. In conclusion, the effect of (non-) feeding behaviour on growth should be taken into account to foster progress in the farming of sole. Environmental factors (i.e., substrate, stocking density) that influence behaviour and growth should be used to optimize culture systems. Future genetic selection strategies should focus more on behavioural characteristics to select sole which will be able to cope and grow best in the different rearing conditions present in commercial aquaculture.

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Appendices

CHAPTER 1

General Introduction

History of sole farming

Common sole, *Solea solea* has a high potential for commercial aquaculture in Europe, because of its consumer popularity and high market value. In Europe marine aquaculture is mainly concentrated on a few established species such as Atlantic salmon, *Salmo salar*, turbot, *Psetta maxima*, European sea bass, *Dicentrarchus labrax*, and Gilthead seabream, *Sparus aurata* (Dinis et al., 1999; Howell, 1997; Imsland et al., 2003). Sole (*Solea solea* and *Solea senegalensis*) is considered to be an interesting species for the diversification of the existing marine fish farming industry.

The first attempts in developing rearing methods for sole date back to the beginning of the last century when eggs were artificially hatched for restocking purposes (Fabre-Domergue and Biétrix 1905, cited by Howell, 1997). The culture of sole became only realistic when the use of *Artemia* nauplii and/or rotifers as food allowed the rearing of large number of juveniles (Howell, 1973; Shelbourne, 1975). These and subsequent studies (Devauchelle et al., 1987; Fuchs, 1978; Fuchs, 1982; Ramos, 1977) proved that the culture of sole could be promising. However, sole did not become a commercial success, because the growth of juveniles turned out to be difficult and the occurrence of diseases was problematic. It was not until the end of the 90’s when advances in feed technology successfully improved the weaning and on-growing of sole, reviving the interest in sole as an aquaculture candidate (Dinis et al., 1999; Howell, 1997; Imsland et al., 2003; Reig et al., 2003). Nevertheless, the culture of sole is still in a developmental stage. Most production takes place in on-shore tanks, either in shallow raceways or conventional tanks, in flow-through or recirculating aquaculture systems. In Europe, the dominant cultured species is the Senegal sole, *Solea senegalensis*, which mainly takes place in Spain and Portugal. The production of common sole, *Solea solea* has been carried out in an intensive recirculation system farm (Solea B.V) in The Netherlands. In addition, production of *Solea solea* is also currently done in extensive pond culture in Italy and in extensive integrated aquaculture systems (“Zeeuwse Tong project”) in The Netherlands. Both species *S. solea* and *S. senegalensis*, are closely related and share a high degree of commonality in culture issues (Howell et al., 2011).

Currently, larvae of sole are produced by natural reproduction of mainly captured wild broodstock animals. As a result, undomesticated animals with a “wild’ genetic background are used for culturing sole. The major economic constraint is its slow growth and large variation in size, which is considered to be a consequence of individual differences in feed intake (feeding behaviour) (Exadactylos et al., 1999; Howell et al., 2011; Imsland et al., 2003). Common sole takes 2-3 years to reach a market weight of 250 grams. Specific growth rates (SGR) range from 0.4-0.7 %/d in sole of 40-75g (Schram et al., 2006) which are very low compared to other commercial species like turbot (*Scophthalmus maximus*) with SGR of 1.48-1.5 %/d in fish of 3-95 g (Sunde et al., 1998), Atlantic salmon (*Salmo salar*) with SGR of 0.9-3.4 %/d in fish of 25-70 g (Austreng et al., 1987) or Nile tilapia (*Oreochromis niloticus*) with SGR of 2.7-2.8 %/d in fish of 50-150 g (Tran-Duy et al., 2008). Next to this, there is a high individual variation in growth of sole, with coefficients of variation ranging between 30 and 50 % (Blonk et al., 2010; Day et al., 1997). As a consequence, juvenile quality is often low and may predispose the fish to diseases. Past and current research initiatives in sole have focused on improving growth and feed intake by increasing palatability of the food (i.e., applying attractants to pellets), improving feed quality (i.e., feed formulation), and by starting up controlled reproduction with selective breeding programs (reviewed by Howell et al., 2011).

Feeding behaviour of sole is primarily mediated by olfactory cues, although visual stimuli can also play a role (De Groot, 1969; De Groot, 1971). In its natural habitat sole is a nocturnal feeder and strictly feeds from the bottom (Kruuk, 1963; Lagardère, 1987). Moreover, the feeding regime of sole is based on the principle of “little and often” since they have a relative small stomach, with a long intestine and a high digestion rate (De Groot, 1971; Lagardère, 1987). Therefore, poor and often unpredictable growth rates are thought to be related to low and variable feed intake, as a consequence of the peculiar feeding behaviour of this species. However, initiatives looking at the role of feeding and non-feeding behaviour in the development of feed intake and determining its relation with growth in sole are still lacking.

Behavioural variation and its relation to growth

In nature, flatfish utilize a strategy to reduce predation risk by minimizing the chance to be detected by a predator: they cryptically match the colour of sediment (Healey, 1999), spend long times buried in it (Ellis et al., 1997), show low activity and nocturnal foraging (Burrows and Gibson, 1995). Under predation risk, the fish is exposed to a conflict between two competing motivations: hunger and fear. It is thought that feeding behaviour is the result of a trade-off between hiding motivation and explorative behaviour. This phenomenon has been explained by Sih et al. (2004b) as: 1) time budget conflicts, for instance, when an animal spends more time foraging, then less time can be used for hiding; or 2) covariance of behaviours across situations, for instance correlations in activity, when individuals spend more time foraging in the absence and in the presence of predators. Studies on foraging behaviour under predation risk have shown that within a population there is a continuum in the responses of prey species, ranging from fearless exploration (“boldness”) to complete predator avoidance (“shyness”) (Fraser and Huntingford, 1986). These consistent behavioural differences between individuals have been termed as differences in personality, temperament, coping styles, boldness, neophobia, or behavioural syndromes (Sih et al., 2004a). “Bold” animals are characterized by being more aggressive and more active in unfamiliar situations whereas “shy” animals are considered to be more fearful or timid and less active in the same situations (Wilson et al., 1994; Wilson et al., 1993).

This individual variation in temperament or personality can affect the animal’s behavioural reaction in a variety of contexts (such as novel situations, avoidance of predators, territorial aggression, foraging behaviour, investment in reproduction, etc.) resulting in correlated traits that are named “behavioural syndromes” (Sih et al., 2004a). Observations in African catfish (van de Nieuwegiessen et al., 2008) suggest that variation in anti-predator behaviour is related to differences in feed efficiency (residual feed intake). Moreover, Martins et al. (2005) found that individual differences in growth and feed efficiency are related to feeding motivation of African catfish. A study on halibut showed that individual variations in feeding behaviour were stable across time and situations and were related to feed intake and growth (Kristiansen and Fernö, 2007). Wilson et al. (1993) developed the shy-bold continuum for juvenile pumpkinseed sunfish with positive correlations between predator inspection, speed of acclimation to laboratory conditions, foraging behaviour and parasite infection. Boldness towards predators was also positively correlated with growth and dispersal in Trinidad killifish (Fraser et al., 2001) and activity, foraging and growth in larval salamanders (Sih et al., 2003). The rationale followed by most life-history studies, is that variation in (non-) feeding behaviour, influences phenotypic expression of fitness traits (i.e., growth, reproduction or survival under predation risk) (Biro and Stamps, 2008; Sih et al., 2004b; Stamps, 2007).

Therefore, it can be hypothesized that the slow growth and large inter-individual variability in growth of sole is related to individual variation in feeding behaviour (i.e.,feeding motivation, meal size and frequency) and non-feeding behaviour (i.e., anti-predator behaviour, behavioural coping strategy, activity levels).

Genetic variation in behaviour and growth

The phenotypic variation generally represents the sum of genetic effects, environmental effects, and interaction of genes within a range of environmental conditions (Falconer, 1990). Individual variation in behavioural traits often appears to be inherited and selection on this variation results in different phenotypes performing best in different conditions (Dingemanse et al., 2004). For instance, heritability of fearfulness has been reported in laboratory species (reviewed by Ramos and Mormède, 1998) and in farm animals (reviewed by Boissy et al., 2005). Heritable variation in sheltering behaviour, aggression levels and migration patterns have been documented in Atlantic salmon (reviewed by Garcia de Leaniz et al., 2007). Van Oers et al., (2004) found that both additive and dominant genetic effects were important determinants for phenotypic variation in exploratory behaviour and boldness of wild birds. In brown trout a correlation between boldness of fish under predation risk (presence of chemical cues) and intra-individual heterozygosity was found (Vilhunen et al., 2008)

Selection favouring different behavioural types could also vary between different environmental conditions. For instance, Álvarez and Bell, (2007) found consistent differences in the risk-taking behaviour (feeding behaviour under predation risk) of sticklebacks depending on the habitat of origin (stream vs. pond). Álvarez and Nicieza, (2005) showed that metabolic rate in brown trout was positively correlated with individual growth rate in captivity, but negative or no correlation was found in natural streams. Also in Atlantic salmon (*Salmo salar* L.) (reviewed by Garcia de Leaniz et al., 2007) and in Paradise fish (*Macropodus opercularis*) (Gerlai and Csányi, 1990), indications for the existence of genotype by environment interactions for some behavioural traits were revealed. Most studies in European sea bass (*Dicentrarchus Labrax* L.) (Dupont-Nivet et al., 2010; Saillant et al., 2006), gilthead seabream (*Sparus auratus* L.) and in rainbow trout (*Oncorhynchus mykiss*) (Fishback et al., 2002; McKay et al., 1984; Sylvén et al., 1991) show low genetic correlations for growth between families reared in different environments which implies that the magnitude for genetic by environment interactions (G×E) due to re-ranking of genotypes is high. Extensive rearing of flatfish is already taking place in Portugal, Spain and Italy. In the Netherlands integrated systems like “Zeeuwse Tong” might become more important for future coastal zone development. However, the only breeding program for sole is executed in an intensive rearing system. If there is a large degree of genotype by environment interaction, sole selected for high growth in intensive systems are not likely to perform well or as expected in extensive systems, and vice versa. Therefore, it is highly important to investigate the extend and causes of genotype by environment interactions in sole. In this study it is hypothesized that the potential role of feeding and non-feeding behaviour in growth of sole will strongly be influenced by environmental factors (i.e., food type, feeding method, presence of substrate to hide, light intensity) with a high potential for genotype by environment (G×E) interaction effects.

Hypothesis

The main hypotheses derived from literature were:

1. Feeding behaviour is the result of a trade-off between hiding motivation and explorative behaviour. Different animals will show different threshold levels for these two parameters, resulting in variation in feed intake and in growth.
2. Threshold levels for hiding motivation and explorative behaviour are context dependent. It is hypothesized that environmental factors (such as stocking density, presence of substrate, photoperiod, light intensity, temperature and feed type), are major determinants for these threshold levels and thereby influence feed intake and growth of sole.
3. The role of (non)-feeding behaviour in growth of sole reared under different environments is expected to result in genotype by environment interactions regarding growth.
4. It is hypothesized that genetic variation in feeding behaviour, partly determines the genetic expression of growth of sole reared in an environment (extensive/natural or in intensive environments), and can be utilized in (natural or artificial) selection.

Aim of the thesis

The overall aim of the thesis was to understand the role of (non-) feeding behavioural traits on growth variation in common sole (*Solea solea*). The following aspects were studied:

1. If feeding and non-feeding behavioural traits contribute to individual variation in feed intake and thereby growth.
2. The influence of rearing (social and physical) environmental factors on behaviour, growth and the relationship between behavioural traits and growth.
3. The existence of G×E interaction regarding growth.

Outline of the thesis

The thesis is composed of a general introduction (**Chapter 1**), five experimental chapters (**Chapter 2, 3, 4, 5, 6**) and a final general discussion (**Chapter 7**).

We first assessed if feeding and non-feeding behaviour contribute to individual variation in feed intake and thereby growth**.** In **Chapter 2**, the relationship between (non-) feeding behavioural traits measured in individually housed sole and feed intake, feed efficiency and growth of sole reared in captivity was assessed. Individual housing was used to measure (non-) feeding behavioural traits during rearing in the absence of social interactions. Moreover, two individual challenge tests (a novel environment and a light avoidance test) were developed to measure boldness of fish and its relationship with growth during rearing of individually housed sole. In **Chapter 3**, the consistency of individual differences in behavioural responses to four different tests (a novel environment, novel object, hiding motivation and net restrain tests), over time and across situations, and its relationship with growth during rearing of group housed sole was further investigated.

Next, the influence of environmental factors on behavioural traits and growth was assessed. In **Chapter 4**, the effect of social interactions, in quality (by sorting based on growth rate) and in quantity (by increasing stocking density), on (non-) feeding behaviour, feed intake, feed efficiency, and growth of sole in the absence and in the presence of sand (reducing potential fish-fish interactions) was assessed. In **Chapter 5**, the influence of physical environmental factors which are present differently in nature and captive conditions, such as food type, presence of sand and variability of environmental conditions, on individual behavioural responses to novelty, growth variation and the relationship between behavioural responses and growth were assessed.

In **Chapter 6**, the extent of genotype by environment interaction on growth of juvenile offspring of wild sole reared in intensive aquaculture systems and in semi-natural pond environments was addressed. Two environments, one indoor and barren, the other outdoor and semi-natural, were tested.

Finally in **Chapter 7**, the overall results of the various studies described in this thesis are discussed in a wider context. A critical reflexion of research done is presented. Besides, practical implications, future perspectives and concluding remarks are given.

CHAPTER 2

Feeding behaviour, swimming activity and boldness explain variation in feed intake and growth of sole *(Solea solea)* reared in captivity.

Julia Mas-Muñoz, Hans Komen, Oliver Schneider, Sander W. Visch, Johan W. Schrama

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Abstract

The major economic constraint for culturing sole (*Solea solea*) is its slow and variable growth. The objective was to study the relationship between feed intake/efficiency, growth, and (non-) feeding behaviour of sole. Sixteen juveniles with an average (SD) growth of 2.7 (1.9) $g/kg^{0.8}/d$ were selected on their growth during a 4-week period in which they were housed communally with 84 other fish. Selected fish were housed individually during a second 4-week period to measure individual feed intake, growth, and behaviour. Fish were hand-fed three times a day during the dark phase of the day until apparent satiation. During six different days, behaviour was recorded twice daily during 3 minutes by direct observations. Total swimming activity, frequency of burying and escapes were recorded. At the beginning and end of the growth period, two sequential behavioural tests were performed: "Novel Environment" and "Light Avoidance". Fish housed individually still exhibited pronounced variation in feed intake (CV= 23%), growth (CV= 25%) and behaviour (CV= 100%). Differences in feed intake account for 79% of the observed individual differences in growth of sole. Fish with higher variation in feed intake between days and between meals within days had significantly a lower total feed intake (r= -0.65 and r= -0.77) and growth. Active fish showed significantly higher feed intake (r= 0.66) and growth (r = 0.58). Boldness during both challenge tests was related to fast growth: 1) fish which reacted with a lower latency time to swim in a novel environment had significantly higher feed intake (r= -0.55) and growth (r= -0.66); 2) fish escaping during the light avoidance test tended to show higher feed intake and had higher growth ($P <0.05$). In conclusion, feeding consistency, swimming activity in the tank, and boldness during behavioural tests are related to feed intake and growth of sole in captivity.

Keywords: Solea solea, individual variation, feeding behaviour, feed intake, growth, activity, boldness

Introduction

Dover Sole (*Solea solea*) has a high potential for commercial aquaculture in Europe because of its consumer popularity and high market values (Howell, 1997; Imsland et al., 2003). Currently, larvae of cultured sole are produced by natural reproduction of captured wild broodstock. Despite attempts for selective breeding and optimization of diets attractiveness, the species is still in an early stage of domestication. Possibly this explains the variable and low growth of sole in culture conditions, which remains one of the most important economic constraints for commercial sole in aquaculture (Dinis et al., 1999; Exadactylos et al., 1998; Howell, 1997; Howell et al., 2009; Imsland et al., 2003).

Individual differences in growth are common in cultured animals, but fish generally show more pronounced variability than other livestock animals, with body weights ranging from 20-40% of the mean for most fish species (Gjedrem, 2000). Also in cultured sole, high growth variations have been reported: 30-50% for *Solea solea* (Blonk et al., 2010) and 24-29% for *Solea senegalensis* (Aragão et al., 2008).

Individual fish often show pronounced variation in both growth and behaviour within a group (Berghahn et al., 1995; Kristiansen and Fernö, 2007; Martins et al., 2005; Quian et al., 2002; Wang et al., 1998). Most studies on individual differences in growth have focused on social interactions in groups of fish with social hierarchies as a major cause for growth heterogeneity (Cutts et al., 1998; Jobling, 1985; Jobling and Baardvik, 1994; Jobling and Wandsvik, 1983; Koebele, 1985; Metcalfe, 1986; Ryer and Olla, 1996). Other studies have addressed the genetic component of growth rate distribution and the physiological mechanisms underlying growth variation of fish when held in isolation (Cui and Liu, 1990; Jobling and Baardvik, 1994; Martins et al., 2005; Quian et al., 2002). Heritability values for body weight in sole and other fish species have been estimated with values ranging from 0.2 to 0.4 (Blonk et al., 2010; Gjedrem, 2000). In the absence of competition, where no social interactions exists, the individual variation in growth would mainly indicate inherent inter- and intra-individual variability in feed intake, and feed efficiency (residual feed intake, RFI). Differences in residual feed intake are considered to be mainly due to differences in: basal metabolism and activity (maintenance costs), digestive efficiency (nutrient digestibility) and body composition (energy storage) (McCarthy et al., 1994). In fish, feed utilization efficiency has been proven to have significant genetic variability (Grima et al., 2010). Moreover, individual differences in feed consumption, can be caused by differences in feeding behaviour, such as day to day variation in feed intake or the feeding pattern within a day (Carter et al., 1992). Individual differences in feeding strategies have been studied in Salmonid fish (Bridcut and Giller, 1995) and in bluegill sunfish (Gotceitas, 1990) which have been explained in terms of the changing trade-off between foraging and predator avoidance in nature (Biro et al., 2006; Lima and Dill, 1990).

In nature, under predation risk, prey animals, such as young fish, face a conflict between two competing motivations: hunger and fear for predation. Studies on the foraging behaviour of prey species under predation risk show that individuals within a population show a continuum in their responses, from “bold” to “shy”, representing different strategies in terms of survival. “Bold” animals show active foraging behaviour regardless of predation risk, while “shy” animals try to limit predation risk at the expense of foraging (Fraser et al., 2001; Werner and Anholt, 1993). These coherent set of behavioural and physiological differences between individuals from the same population which are consistent over time and across situations are referred to as personality, coping styles, temperament or behavioural syndromes (Koolhaas et al., 1999; Sih et al., 2004a). “Bold or proactive” (active coping or fight-flight response) animals are often characterized by being more aggressive, explorative and more active in unfamiliar situations whereas “shy or reactive” (passive coping or conservation-withdrawal response) animals are considered to be more fearful or timid, and less active in the same situations (Wilson et al., 1993). These different “coping styles” result from genetic, environmental or ontogenetic factors and their interactions (Caro and Bateson, 1986; Gerlai et al., 1990; Gervai and Csányi, 1985). Previous studies have shown that innate behavioural and physiological traits represent different responses and adaptive strategies to environmental challenges (Kristiansen and Fernö, 2007). Proactive individuals have a tendency to dominate and outcompete reactive ones in a stable environment with feed in excess. Nevertheless, the latter appear to respond better in an unpredictable or variable environment (Huntingford, 2004; Sih et al., 2004a).

In nature, selection pressures on behaviour may vary across time as it depends on environmental circumstances which coping type will be in advantage, thus variation in behavioural strategies is maintained (Dingemanse and Réale, 2005). Farmed fish reared in captivity have no accessibility to shelter, are reared at high densities, with predictable food delivery, and in the absence of predators thus, it is suggested that bold individuals with high competitive ability, more active and with risk-prone feeding behaviour display higher growth rates (Huntingford, 2004; Huntingford and Adams, 2005). Previous studies have reported positive associations between boldness and growth in captive or domesticated animals (Biro et al., 2006; Biro and Stamps, 2008; Johnsson et al., 1996; Sundström et al., 2004).

Sole utilizes a detection minimization strategy to reduce predation risk: they match the colour of sediment (Ryer et al., 2008), spend long times buried in it (Ellis et al., 1997), show low activity levels (Burrows and Gibson, 1995) and nocturnal feeding (Lagardère, 1987). Therefore, it is hypothesized that individual variation in risk-prone feeding behaviour and activity (bold versus shy fish), may be related with the behavioural flexibility/capacity of fish to adapt to captive conditions and therefore explaining high individual differences in feed intake and thereby in growth of cultured sole.

This study aims to examine the inherent causes of individual variation in growth of sole *(Solea solea)*. The objective is to assess whether individual variation in feeding and non-feeding behaviour may explain differences in feed intake/efficiency and growth of sole reared in captivity.

Materials and methods

Ethics

All procedures involving animals were conducted in accordance with the Dutch law on experimental animals and were approved by the Ethical Committee for Animal Experiments (DEC) of Wageningen University.

Experimental animals and housing

Juvenile sole (*Solea solea, n*=100, not selected for sex) with an initial weight of 59.5 ± 6.5g were obtained from a local commercial farm (Solea BV, Ijmuiden, The Netherlands). Upon arrival fish were communally housed in one 400L black tank of 2 x 1 x 0.4m (L x W x H) connected to a RAS system. The RAS system consisted of two sludge settlers and one bio-filter containing lava rock filled with artificial sea water (25 ‰). Water temperature (17.8 ± 0.1 ∘C), pH (7.9- 8.2), dissolved O_2 (>7 mg/l), salinity (25 ± 0.1 ‰), NH_4^+ (<1 mg/ l) NO_2^- (<1 mg/ l) and NO_3^- (<50 mg/l) were monitored daily. Ammonia, nitrite, nitrate were maintained below this levels by exchanging sea water when necessary.

Fish were allowed to adapt to the experimental facilities for two weeks. After adaptation, they were individually weighted and PIT-tagged (Trovan ID100, DORSET GP, Aalten, The Netherlands) while anesthetized with 2-phenoxyethanol (1ml of solution/ litre of system water).

The experimental period consisted of two growth periods, each of 28 days duration. During the first growth period, the 100 fish were group housed in the same 400L tank. Based on the realized growth rate (GR, $g/kg^{0.8}/d$) of this period, fish were categorized into eight different growth classes (Table 1). Two fish from each growth class were randomly selected. The 16 selected fish were individually housed in 30L glass tanks (0.30 x 0.5 x 0.3 m) during a second growth period to measure individual feed intake and behaviour in the absence of social interactions. Tanks were connected to the same RAS systems as before and side walls were covered with black plastic to avoid any visual contact between fish. At the end of both growth periods fish were weighed and behavioural tests were performed.

A 12D:12L photoperiod was maintained using artificial fluorescent lights. As juvenile sole are nocturnal feeders (Lagardère, 1987) the light regime was reversed with lights on from 21:00h till 9:00h. During the dark period of the day red lights were used to provide sufficient light to feed and to perform video recordings.

Table 1. Classification of fish based on their growth in period 1[a].

Growth Class	**Growth Period 1 ($g/kg^{0.8}/d$)**	**Mean End BW Period 1 (g)[b]**	**Number of fish**
1	<0.00	57.2	5
2	0.00-0.90	61.8	10
3	0.90-1.50	64.3	11
4	1.51-2.50	64.6	21
5	2.51-3.50	70.1	23
6	3.51-4.30	69.6	21
7	4.31-5.30	76.9	6
8	>5.30	84.7	3

[a] Two random fish per growth class were selected

[b] BW=Body weight is averaged over the total number of fish categorized in each growth class

Feeding method

Fish were fed with a commercial feed diet, DAN-EX 1562 (DANA FEED A/S, Denmark, sinking pellet). Fish received 2 mm size pellets (61% protein , 20% fat and 24 kJ/g energy on dry matter basis) during period 1 and were switched to 3 mm pellets (63% protein, 19% fat and 23 kJ/g energy on dry matter basis) during period 2 when fish had grown bigger.

During period 1 group housed fish were fed in access (between 0.5-1% body weight d^{-1}) by an automated belt feeder, which distributed feed in two blocks of 3 hours. Feeding periods were from 9:00h till 12:00h and 13:00h till 16:00h. After each feeding all uneaten pellets were removed. To ensure feeding until apparent satiation daily rations were adjusted based on the feed intake of previous day.

The 16 individually housed fish in period 2 were hand fed three times a day at 8:00, 12:00 and 17:00h until apparent satiation. For all fish the feeding period started with a feed ration of 15 pellets (0.27g) and whenever pellets where eaten 5 extra pellets (0.09g) were added. Through this procedure there would always be at least 5 pellets of feed in each tank during the feeding time. Feeding continued after pellet addition for a maximum of 20 minutes and 5 minutes later remaining pellets were siphoned and counted.

Live behavioural observations

The behaviour of the 16 fish housed individually was recorded by direct observations twice a day in between meals at Days 8, 10, 13, 15, 16 and 24 of period 2. In the morning observations were made between 10:30h-11:00h and in the afternoon between 15:00h-16:00h. Each fish was observed throughout a three minutes period during which total swimming activity (% of observation time), frequency of burying attempts on the bare bottom (#/3min) and frequency of escapes (#/3min) were recorded following the ethogram presented in Table 2. In total 12 observations per fish were made.

Behavioural Tests

At the end of each growth period two sequential behavioural tests: “Novel Environment Test” and “Light avoidance Test”, were performed to each fish individually during the dark phase of the day. Red lights were used as illumination to allow video recording. Twenty-four hours prior to the behavioural tests fish were not fed to increase their potential activity.

The testing was performed in two successive rounds. During each round eight random fish were screened individually in eight 120L (0.6 x 0.5 x 0.4m) glass barren-bottom tanks at the same time for the conduction of the tests. The test-tanks were filled up to 20cm with water from the RAS system and were refreshed completely at the end of each testing round to avoid chemical cues to interfere in the behavioural response. The test-tanks were visually isolated from each other by black acrylic sheets covering three sides of the tank. Each test-tank was divided into two equal sections (section A and B) by a plastic lid. Section A was open on the top and had a fluorescent light above, while section B was covered with a plastic lid on the top and was in complete darkness. Behavioural responses were recorded with two video cameras, one above and one on the side in section A of each tank.

During the “Novel Environment Test”, fish were restricted to section A. The test started with the introduction of the fish into the test-tank after which fish were monitored for 15 min. The reaction of each fish to this new environment was analysed following the ethogram in Table 2.

The second test, the “Light avoidance test”, started 45 minutes after the introduction of the fish into the test-tank. The test started with the opening of the connection to section B by lifting the plastic lid 12-15cm and simultaneously increasing the light intensity in section A (approx. 600 Lux), whereas section B stayed dark (0 Lux). The behavioural response was recorded using the ethogram (Table 2) for a maximum of 15 minutes.

For each behavioural test the activity patterns were expressed as the percentage of total observation time. Burying and escapes bouts (frequency) were recorded during each test. Latency time to swim during the novel environment test and latency time to move towards section B during light avoidance test was measured as elapsed time in seconds from the time the test started. When no activity was performed at all during the 15 minutes of test, the fish was given as latency time a score of 15 minutes for statistical convenience. Total activity time was calculated as 100-Time resting (%).

Each test was performed twice with each fish, at the start and at the end of period 2. Due to technical problems (short-circuit) videos from 8 fish of the second testing day (end of period 2) were damaged and thus excluded from the analysis. Therefore, the data used for the behavioural analysis was the mean of all observations per fish. Video recordings from the behavioural tests were analysed using the “The Observer XT 9.0” software package (Noldus, Wageningen, The Netherlands).

Table 2. Ethogram used for behavioural observations.

Behavioural element	**Description**	**Live Observations**	**Novel Environment**	**Light Avoidance**
Resting	Lying motionless on the bottom or against the side of the tank without performing any other described behaviour (state event)	x	x	x
Swimming	Displacement of the body using body or fin movement as propulsion (state event)	x	x	x
Small Movement	Fish moves slowly with no real displacement of the body, maximum distance covered is <half of fish length (state event)	x	x	x
Burying	Fish makes an attempt to bury by performing quick wave movements with its whole body (point event)	x	x	x
Escape	Fish moves its body straight up in the water column and is pushing its head out of the water surface (point event)	x	x	x
Activity	The total observation time minus the time spent resting	x	x	x
latency time to swim	Time elapsed from the time the fish went to rest for the first time until it performs any other active behaviour	-	x	-
Latency to go to dark	Time elapsed until the fish moves from Section A to Section B	-	-	x
Time in dark	Time the fish stays in the dark, section B	-	-	x

Data analysis

In the present study, fish were considered as experimental unit. Growth rate (GR) and feed intake (FI) were expressed per metabolic body weight as units of g BW (kg)$^{-0.8}$ d^{-1}. This was done to correct for the variation in fish size as it is known that larger fish have a greater absolute metabolic requirement of feed compared to smaller fish (Hepher, 1988). BW is the geometric mean of the weight calculated as:

$BW = \exp\left\{ \frac{1}{2} \left(\ln(W_1) + \ln(W_2) \right) \right\}$, where W_1 is the initial weight (g) at the beginning of each growth period and W_2 the end weight at the end of each growth period. Feed conversion ratio (FCR) was calculated by dividing total feed intake by weight gain during the period.

Feed efficiency was analysed using RFI (g/kg$^{0.8}$/d). RFI was calculated as the difference between feed consumed by an animal and its consumption as predicted from a linear regression model involving the maintenance requirements and growth as independent variables FI=M +βGR +ε, where FI is the feed intake (g/kg$^{0.8}$/d), M is the maintenance (g/kg$^{0.8}$/d) and GR the growth (g/kg$^{0.8}$/d) (Luiting and Urff, 1991). Animals with a low RFI (i.e. negative RFI) are assumed to be more feed efficient than animals with a high RFI (i.e. positive RFI).

Coefficient of variation (CV, %) was calculated as CV=$\left(\frac{\sigma}{\mu}\right)*100$, where σ is the standard deviation and μ the observation mean. The CV of feed intake between days (FIdays, %) was calculated using the standard deviation of FI between days and the average FI per day. The CV of feed intake between meals within days (FImeals, %) was calculated using the average standard deviation between meals in the day and the average FI per meal.

During the experiment one individually housed fish did not eat during period 2 (28 days starving) and was considered an outlier thus only data from 15 fish were included in the analysis.

Statistical analyses were performed using SAS system (SAS, 2002). Data was analysed using linear regression models and performing Pearson's correlations between quantitative traits or if qualitative traits were defined significant effects were analysed using one-way analysis of variance (ANOVA) followed by the Turkey's HSD post-hoc test. The error terms of these analyses were tested for homogeneity of variances and normality, using the Levene's test and the Shapiro-Wilk test, respectively. Behavioural data was squared rooted (frequencies) or log transformed (latencies) when necessary. Results were considered statistically significant when P-values were below 0.05. Data is reported as mean ± SE.

Results

Growth during group housing conditions (Period 1)

The average growth of all the fish when group housed was 2.55 ± 0.15 g/kg$^{0.8}$/d (*n*= 100) and of the selected fish was 2.70 ± 0.49 g/kg$^{0.8}$/d (*n*= 15), displaying a wide range in growth (CV = 70%) during period 1. Body weight of selected sole at the end of period 1 was of 69.29 ± 3.03 g (CV = 17%).

Growth and feed intake/efficiency of individually housed sole (Period 2)

The average growth of the 15 individually housed fish was 5.2 ± 0.3 g/kg$^{0.8}$/d. Feed intake and FCR were of 4.3 ± 0.3 and 0.84 ± 0.03 g/kg$^{0.8}$/d, respectively (mean ± SE, Table 3). No significant correlation was found between growth of individually housed sole and initial body weight (r= 0.17, P>0.1). The fish still exhibited pronounced variation in growth (CV= 25%) and feed intake (CV= 23%) during period 2.

The growth (GR, in g/kg$^{0.8}$/d) of sole juveniles individually housed was strongly correlated to individual differences in feed intake (FI, g/kg$^{0.8}$/d) and was described through the regression equation FI= $\mu + \beta$*GR + ε (μ= 0.79 ± 0.52; β= 0.68 ± 0.09; R^2= 0.79; P<0.001, Fig. 1). According to the estimated linear regression on average 79% of the individual variation in feed intake was explained by variation in growth. The remaining 21% of variation in feed intake is the residual feed intake (RFI, g/kg$^{0.8}$/d) which represents individual differences in feed efficiency and measuring errors. The average maintenance ration (μ), feed intake at which growth is zero obtained from the regression line, was 0.79 ± 0.52 g/kg$^{0.8}$/d.

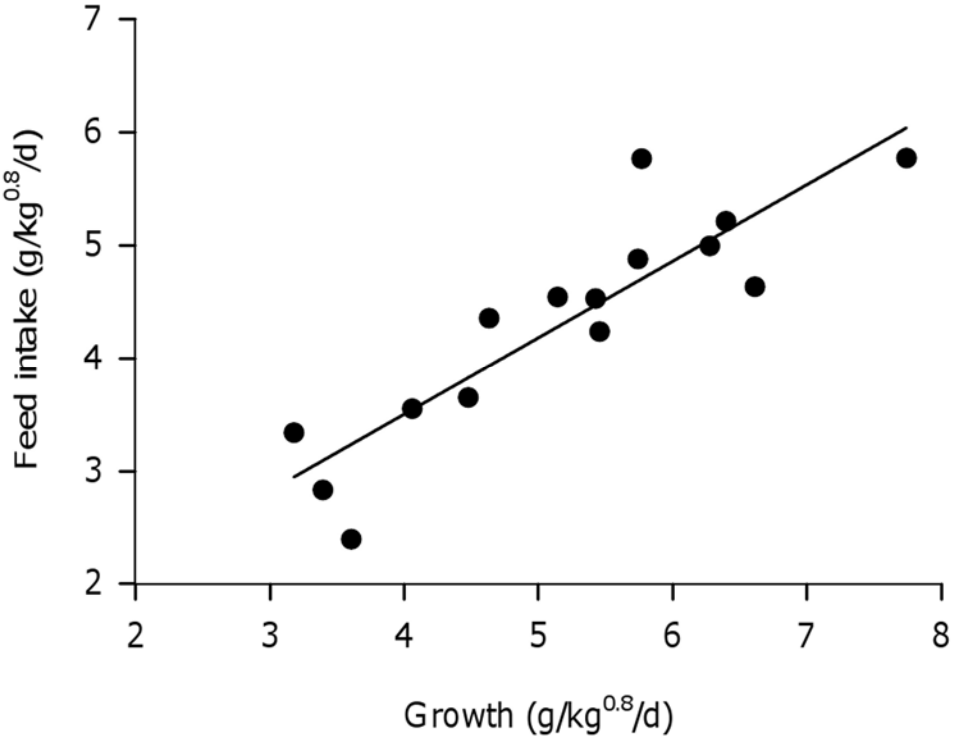

Fig. 1. Relationship between feed intake and growth (g/kg$^{0.8}$/d) of 15 individually housed sole (FI= 0.79 + 0.68*GR, R^2=0.79, P<0.001).

Relationship between feeding behaviour and feed intake/efficiency and growth

The feed intake of individually housed sole showed high variation between days and between meals within days with CV= 55% and 27 %, respectively (Table 3). Differences in day to day variation in feed intake ranged from 14-85 %; variation in feed intake between meals within a day varied from 38-75%.

The CV of feed intake between days (FIdays, %) and between meals within days (FImeals, %) was negatively correlated with feed intake (g/kg$^{0.8}$/d) of sole (FI= 5.49-0.04*FIdays, R^2= 0.43; $P<0.01$ and FI= 7.17-0.06*FImeals, R^2= 0.60; $P<0.001$, Fig. 2). Correspondingly a significant negative correlation was found between the CV of feed intake and growth (g/kg$^{0.8}$/d) (Pearson's correlations with FIdays and FImeals of r= -0.52 and r= -0.64, $P<0.05$, Table 3). However, no significant correlations were found with feed efficiency (RFI, g/kg$^{0.8}$/d) ($P>0.1$, Table 3).

The feeding pattern within day showed that during the three meals given at 9:00, 12:00 and 17:00h, sole consumed on average 38.1 ± 1.2, 27.1 ± 1.0 and 34.8 ± 1.1 % of their total FI respectively (means ± SE, Table 3). Fish which showed a higher percentage of feeding at 17.00h had higher feed intake (r=0.71, $P<0.01$) and growth rate (r= 0.58, $P<0.05$) during period 2. Whereas fish which showed high feeding levels during the first meal of the day (9:00h) tended to have a lower total feed intake and growth (r= -0.46, $P<0.1$, Table 3). The percentage of FI during the midday meal (12:00h) was significantly lower than the other two meals ($P<0.05$) and showed no significant relationship with feed intake or growth.

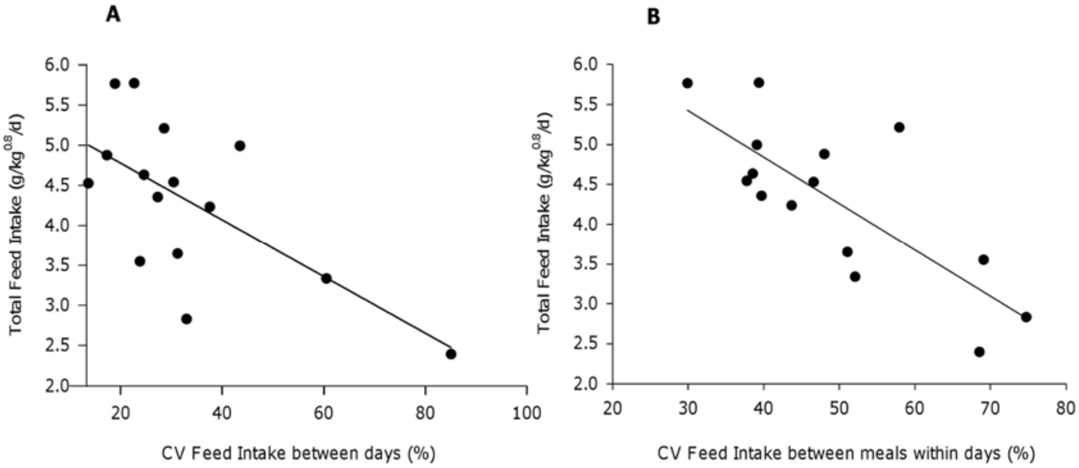

Fig. 2. Relationship between total feed intake (g/kg$^{0.8}$/d) and the CV of feed intake between days (A) and between meals within days (B). Regression equations are A) y= 5.49 - 0.04x (R^2 = 0.43, $P<0.01$) and B) y=7.17- 0.06x (R^2 = 0.60, $P<0.001$).

Relationship between swimming activity and feed intake/efficiency and growth

Feed intake was positively correlated with the average swimming time (SWIM, in %) during live observations in the tank (in between feeding periods) (FI= 3.63+0.12*SWIM, R^2= 0.44, $P<0.05$). Moreover, feed efficiency was not affected by differences in swimming activity (RFI= -0.15+0.03*SWIM, R^2= 0.1, $P>0.1$, Fig. 3). Correspondingly, a positive correlation was found with growth (r= 0.58, $P<0.05$, Table 3). Active swimmers were also feeding more consistently with a significantly lower CV of feed intake between meals within days (r=-0.61, $P<0.05$) and a trend for lower CV of feed intake between days (r= -0.47, $P<0.1$, Table 3).

Sole that were escaping during live observations (n=10 fish) had higher feed intake than fish which did not (n=5 fish) (FI: 4.7 ± 0.3 versus 3.6 ± 0.4 g/kg$^{0.8}$/d, $P<0.05$, Table 4). However, fish escaping during observations also tended to be less feed efficient compared to those that were not seen displaying this behaviour (RFI : 0.1 ± 0.1 vs. -0.3 ± 0.2 g/kg$^{0.8}$/d, $P<0.1$, Table 4) but no significant differences were found regarding growth ($P>0.1$, Table 4).

The frequency of burying in the barren tank during live observations was positively correlated with growth but not with feed intake (r= 0.55, $P<0.05$ and r= 0.37, $P>0.1$, Table 3).

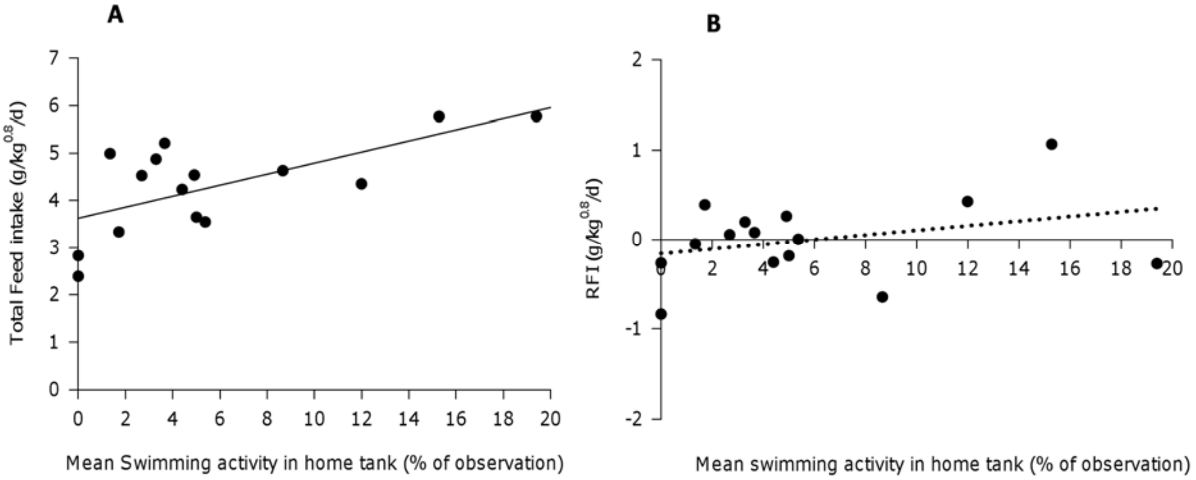

Fig. 3. Relationship between swimming activity (%) in the home tank and A) total feed intake (g/kg$^{0.8}$/d) and B) residual feed intake (g/kg$^{0.8}$/d) of 15 individually housed sole. Regression equations are A) FI=3.63+0.12*SWIM (R^2= 0.44, $P<0.05$) for feed intake and B) RFI= -0.15+ 0.03*SWIM (R^2=0.10, $P>0.1$) for residual feed intake.

Boldness during behavioural tests and its relationship with feed intake/efficiency and growth

Results from the challenge behavioural tests show high individual variation in behavioural responses, CV of behavioural traits ranging from 36-170 % (Table 3). Behavioural tests were relatively consistent in time with Pearson's correlation of individual behavioural responses between both testing periods ranging from 0.4-0.7. Individual's responses to novelty and to light showed to be related with their feed intake and growth in captivity.

1. *Novel environment test*

Sole responded to a new environment with a swimming activity of 10.2 ± 2.1 % of observation, with on average of 6.0 ± 2.7 escapes and 6.1 ± 4.7of burying attempts (Table 3). The average latency time to swim and to start exploring the new environment (as a measure of boldness) was 189 ± 69 sec, and was negatively correlated with total feed intake ($r = -0.55$, $P<0.05$) and growth ($r = -0.66$, $P<0.01$, Table 3). Sole which responded with escaping ($n=10$ fish) did not show a significance difference in growth compared to sole which did not escape ($n=5$ fish) ($P>0.1$, Table 4).

2. *Light avoidance test*

Sole subjected to the "Light avoidance test" showed in the illuminated area an average activity of 8.3 ± 2.1 % of observation, and displayed on average 0.9 ± 0.4 escapes and 4.2 ±1.5 burying attempts. The average latency to go to the dark section of the tank (section B) was 720.7 ± 68.5 seconds and the total time in the dark was of 16.4 ± 5.3 % (Table 3). Sole showed two opposite coping styles when exposed to a high light intensity: 1) Proactive fish which escaped and, 2) Reactive fish which remained in the bottom. The frequency of escaping during the light test tended to be positively correlated with feed intake ($P<0.1$, Table 3) and significantly with growth ($P<0.05$, Table 3). However, no significant relationship was found with feed efficiency ($P>0.1$, Table 3). Sole which responded with escaping ($n=5$ fish) had a higher growth compared to sole which did not escape ($n=10$ fish) (6.1 ± 0.5 vs. 4.7 ± 0.4 $g/kg^{0.8}/d$, $P<0.05$, Table 4).

Table 3. Pearson's correlations between growth, feed intake, feed efficiency (RFI), and behaviour of individually housed sole (n=15)[a].

			Pearson's correlations (r)		
Variable	Mean ± SE	CV (%)	Feed intake ($g/kg^{0.8}/d$)	Growth ($g/kg^{0.8}/d$)	RFI ($g/kg^{0.8}/d$)
Performance					
Initial body weight (g)	69.8 ± 3.1	17	0.24	0.17	0.20
Weight gain (g)	19.1 ± 1.6	32	0.83***	0.89***	0.09
Growth P2 ($g/kg^{0.8}/d$)	5.2 ± 0.3	25	0.89***	1	0.00
Feed intake ($g/kg^{0.8}/d$)	4.3 ± 0.3	23	1	0.89	0.46^{+}
FCR (g/g)	0.8 ± 0.0	12	0.12	-0.33	0.90***
Feeding behaviour					
CV FI btw days (%)	33.2 ± 4.8	55	-0.65**	-0.52*	-0.41
CV FI btw meals (%)	49.1 ± 3.4	27	-0.77***	-0.64*	-0.45^{+}
FI morning (% of daily FI)	38.1 ± 1.2	12	-0.46^{+}	-0.46^{+}	-0.12
FI midday (% of daily FI)	27.1 ± 1.0	14	-0.26	-0.12	-0.33
FI afternoon (% of daily FI)	34.8 ± 1.1	13	0.71**	0.58*	0.42
Activity home tank					
Activity (%)	5.9 ± 1.5	97	0.66**	0.58*	0.32
Escapes (#/3min)	0.5 ± 0.1	102	0.34	0.22	0.31
Bury (#/3min)	0.2 ± 0.1	124	0.37	0.55*	-0.25
Novel Environment test					
Activity (%)	10.2 ± 2.1	80	0.31	0.41	-0.11
Escapes (#/15min)	6.0 ± 2.7	172	0.33	0.24	0.26
Bury (#/15min)	6.1 ± 4.7	76	0.18	0.40	-0.39
latency time to swim (s)	189.3 ± 68.8	141	-0.55*	-0.66**	0.08
Light avoidance test					
Activity (%)	8.3 ± 2.1	97	0.44	0.23	-0.34
Escapes (#/15min)	0.9 ± 0.4	170	0.46^{+}	0.56*	-0.08
Bury (#/15min)	4.2 ± 1.5	137	-0.13	0.00	-0.26
Latency to move to dark (s)	720.7 ± 68.5	37	0.06	-0.03	0.19
Time dark (%)	16.4 ± 68.5	125	0.23	0.22	0.09

Significant differences are indicated by ***$p<0.001$; **$p<0.01$; *$p<0.05$; $^{+}p<0.1$

[a] CV=Coefficient of variation, FI=Feed intake , FCR= feed conversion ratio, RFI=Residual feed intake/feed efficiency

Table 4. Comparison of growth, feed intake and feed efficiency (RFI) between fish displaying escape behaviour (present vs. absent)[b].

Variable	Home tank observations			Novel environment test			Light avoidance test		
	Escape (*n*=10)	**No Escape (*n*=5)**	***P***	**Escape (*n*=10)**	**No Escape (*n*=5)**	***P***	**Escape (*n*=5)**	**No Escape (*n*=10)**	***P***
Growth P2 (g/kg$^{0.8}$/d)	5.5±0.4	4.6±0.6	*ns*	5.4±0.4	4.7±0.6	*ns*	6.1±0.5	4.7±0.4	*
Feed intake (g/kg$^{0.8}$/d)	4.7±0.3	3.6±0.4	*	4.5±0.3	4.0±0.5	*ns*	4.9±0.4	4.0±0.3	*ns*
RFI[a] (g/kg$^{0.8}$/d)	0.1±0.1	-0.3±0.2	+	-0.01±0.15	0.02±0.21	*ns*	-0.06±0.21	0.03±0.15	*ns*

Values are means ±SE. Significant differences are indicated by;*$p<0.05$; $^{+}p<0.1$; *ns*=not significant

[a] RFI= Residual feed intake/feed efficiency

[b] Classification of the fish differs between observations in the home tank, the Novel environment test and the Light avoidance test.

Discussion

The present study showed that sole *(Solea solea)* housed individually, in the absence of social interactions, still exhibits high individual differences in feed intake, growth and behaviour (on average CV of 23, 25 and 100% respectively) , which has also been observed in other species when held in isolation (Martins et al., 2005; Quian et al., 2002; Ragland and Carter, 2004; Wang et al., 1998). Growth variation of sole housed individually was lower than when communally held in a group of 100 fish (CV was 25 % in period 2 compared to 70% in period 1, *P*<0.05). This results are in line with studies in other fish species were the reported variation in feed consumption within grouped fish showed a marked increase compared to variation in feed intake when fish were held in isolation, on average 60-100% versus 25-40 % (Carter et al., 1992; Jobling and Baardvik, 1994; Jobling et al., 1989; McCarthy et al., 1992). There are three main causes of phenotypic variation among individuals in a population: 1) genetic; 2) environmental; 3) interaction between genetic and environmental factors. In this study, individual differences were measured in the absence of social interactions and with equal and predominantly constant environmental conditions, which suggests that differences in feed intake, growth and behaviour have a genetic basis.

This study showed that under ad libitum conditions and individual housing, differences in feed intake account for 79% of the observed individual differences in the growth of sole. These results are in agreement with other studies in fish showing that the variation in the growth of fish is mainly due to variation in feed intake (Carter et al., 1992; Jobling and Baardvik, 1994; Koebele, 1985; Martins et al., 2011). Data on individual feed intake of sole on dry feed is limited in literature and generally difficult to compare as feed intake depends on the respective diet nutrients, feeding protocol, size of the fish, temperature and origin of the fish. However, our results (4.3 g/kg$^{0.8}$/d or 0.87 %/d) are comparable to feed intake of grouped housed *Solea solea* of similar weight class from studies of S. Ende et al., 2009 (Personal communication) and (Fonds et al., 1989). Mean growth rate in this study (5.2 g/kg$^{0.8}$/d or 0.86 %/d) is within the higher level of displayed growth of grouped housed *Solea solea* with values in literature ranging from 0.86-0.3 %/d (Fonds et al., 1989; Overton et al., 2010; Schram et al., 2006).

Feeding behaviour was expressed as individual differences in feeding consistency over time and the daily feeding pattern: the coefficient of variation of intra-individual feed intake between days and between meals in the day was measured. A low CV indicated that the meal size or feed intake of an individual fish was similar from day to day and/or between the daily meals whilst a high CV indicated a more varied feed intake. Variation in feed intake between days is caused by a combination of endogenous and exogenous factors which can influence appetite and it appears to be a common feature of feeding in fish (Carter et al., 1992; Smagula and Adelman, 1982). The observed individual variations in CV of feed intake between days (14-85%) in sole were quite high compared to studies in other species held in isolation such as carp, with ranging values of 16-22% (Carter et al., 1992) or with minnows with values ranging from 21-27 % (Cui and Wootton, 1988). Moreover, the present results show that fish which feed more consistently over time (within day and over days), show higher feed intake and growth but also tend to be less feed efficient. The influence that the regularity of feeding has on growth and feed efficiency is yet not well understood. However, it has been reported that the rate of protein synthesis is correlated with growth, which accounts for large proportion of total energy costs in fish and thus contributing to individual variations in growth efficiency (Carter and Brafield, 1991; McCarthy et al., 1994). These findings are in accordance with a study in grass carp where fish with larger variability in feed intake had lower growth rates and hence lower rates of protein synthesis (Carter et al., 1992). Recent studies also reported that differences in feed efficiency (residual feed intake) were related to the feeding motivation in African catfish (Martins et al., 2005) and to feeding activity in Nile Tilapia (Martins et al., 2011). Additionally, we found that the daily feeding pattern also explained variation in feed consumption and growth of sole. Fish which showed a higher percentage of feeding during the last meal of the day (afternoon meal at the end of dark period, 17.00h) had higher feed intake and growth. In line with our results, studies on other flatfish showed that individual variations in the feeding behaviour of halibut were stable across time and situations and were related to feed intake and growth (Kristiansen and Fernö, 2007). The feeding rhythm of sole can be described by two major meals: one in the morning and one in the afternoon (at the beginning and the end of the dark period), where feed intake was significantly higher than during the midday meal. These results agree with other studies in which juvenile sole were found to have two main activity/feeding peaks during the night, one at sunset and another shortly before dawn (De Groot, 1971; Lagardère, 1987). Other species, such as Atlantic salmon, also show feed intake peaks during the early morning and late afternoon (Kadri et al., 1997).

Results suggests that for sole endogenous factors already explain high individual differences in food consumption, which indicate consistent differences in feeding strategies between individuals. Individual differences in feeding behaviour could be related to differences in the behavioural flexibility (or adaptive capacity) between fish to feed and grow in captivity, where coping styles might play an important role, as bold or active fish were also found to feed more consistently.

Active sole had significantly higher feed intake and growth, which agrees with results on Chinese sturgeon (Quian et al., 2002). Activity time was not correlated with feed efficiency (RFI), thus individual differences in maintenance costs due to different activity levels in sole seem to have a relative small effect on RFI. In accordance, other studies highlighted that flatfish probably spend relatively less energy in swimming and allocate more food energy on growth than (pelagic) round fish (Fonds et al., 1992). The high feed intake of active fish might be due to the fact that individuals that spend more time swimming have higher appetite and increase their feed intake which may overcompensate differences in maintenance costs. Another explanation for this can be that more active individuals are often seen as better competitors, expropriating resources from less active individuals (Werner and Anholt, 1993). Fish displaying escaping behaviour at the water surface also showed a higher feed intake, however tended to be less feed efficient and no differences in growth were found. This type of behaviour is considered to be indicative for abnormal or stereotypic behaviour in flatfish (reflecting a stressed state of the fish). Contrary to this, findings in Atlantic halibut showed that surface swimming was an indicator for low growth rate (Kristiansen et al., 2004). However, surface swimming in this case was a combination of escapes and swimming close to water surface as it was measured with a pit antenna, thus the behaviour measured is a different behavioural trait. Furthermore, halibut were group housed, so this behaviour could have also been triggered by social interactions. Both behaviour and housing conditions were different, thus the comparison between results from both studies is difficult.

Moreover, boldness of sole measured as the reaction to an unknown/novel environment and to a sudden increase in light intensity proved to be related to feed intake and growth but not with feed efficiency (RFI). Sole which resume activity earlier in a novel environment and those that reacted escaping when confronted with a light stimulus had higher feed intake and growth. These results suggest that individual differences in behaviour when confronted to environmental challenges explain individual variations in feeding behaviour and growth, where proactive sole seem to be more successful in their feeding behaviour and thus display higher growth under captive conditions. Accordingly, animal personality traits, such as boldness, activity and aggressiveness have been reported in many species and have been found to be also positively correlated with feed intake or growth in captivity: Wilson et al.(1993) developed the shy-bold continuum for juvenile pumpkinseed sunfish with positive correlations between predator inspection, speed acclimation to the laboratory, foraging behaviour and parasitic infection. Boldness towards predators was also positively correlated with growth and dispersal in killifish (Fraser et al., 2001) and activity, foraging and growth in larval salamanders (Sih et al., 2003). Salmonid fish also show individual variation in behaviours such as space use (Lahti et al., 2001), boldness (Sundström et al., 2004), and aggressiveness (Armstrong et al., 1997) where behavioural characteristics proved to be related with growth differences (Johnsson et al., 1996; Sundström et al., 2004) . Studies on Paradise fish, found that behavioural responses to a Novel environment were highly inherited (Gerlai et al., 1990; Gervai and Csányi, 1985). Thus, as coping styles seem to have a genetic base (Koolhaas et al., 1999) these results suggest that selecting for growth in fish under such conditions will promote risk-prone feeding behaviour and high activity in tanks.

Conclusions

The wide inherent individual variations in behaviour, feed intake and growth of sole suggest scope for improvement in sole aquaculture. Individual differences in feeding consistency, swimming activity and behavioural reactions under challenging situations (novel environment; increased light intensity) explain variations in feed intake and growth. Both feeding consistency and escaping behaviour also tended to explain differences in feed efficiency (RFI). These results suggest the existence of coping styles in sole which can influence their adaptive capacity to farming conditions: Proactive fish seem to have a more successful feeding strategy in captivity, displaying higher feed intake and growth. Therefore, behavioural traits may be of interest to have into account for selection in breeding programs. Additionally, high feed intake was related with the presence of more escaping behaviour which has been considered to be stereotypical behaviour in flatfish (reflecting a stressed state of the fish) which might be of importance when considering welfare and performance of fish in captivity.

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CHAPTER 3

Consistency of behavioural traits and its relationship with growth in sole (*Solea solea*)

Julia Mas-Muñoz, Hans Komen, Robbert Blonk, Johan W. Schrama

Submitted

Abstract

Consistency of individual differences in behavioural traits over time and across situations, and their relationship with variation in growth were investigated in sole (*Solea solea*). Individual growth rate of communally raised sole was assessed during four consecutive 16-d periods. At the end of each growth period, either a “novel environment test” followed by a “novel object test” or a “hiding motivation test” followed by a “net restrain test” were performed individually to each fish. Each test was performed twice with an elapse time of 40 days, termed as trial A and B, respectively for the first and second time each test was done. Behavioural traits were consistent over time, indicated by the spearman rank correlations between trials ranging from 0.27 to 0.56 for measured behavioural traits. A principal components analysis (PCA) on all behavioural variables measured in the tests within trials yielded six factors, which accounted for 78% of the total variation between individuals. These factors represented respectively: hiding motivation (23%); exploration in novelty (16%); novel object (anti-predator) escape (13%); novel object (anti-predator) bury (10%); freeze and bury in novelty (9 %); and escapes during net restrain (8%). PCA indicated that consistency of behavioural traits across situations (i.e., tests) was absent to very low in sole. Traits related to anti-predator behaviour, such as burying seemed to be important innate behaviour, indicated by the highest consistency over time and its slight consistency across situations. However alterations in the strength of the flexibility in burying behaviour responses during the first and second time of exposure (trial A vs. B) may reflect learning or habituation. PCA behavioural factors derived from trial A and sex explained 23% of the variation in growth, whereas PCA behavioural factors of trial B did not. For trial A, the motivation to bury was negatively related to growth, whereas the motivation to explore a novel environment was positively related. Female sole that were less reactive upon an artificial predator threat grow faster, whereas in males this factor had no impact.

Keywords: flatfish, personality, anti-predator, burying behaviour, coping styles

Introduction

Behavioural differences between individuals from the same population have been termed as differences in animal personality (Gosling, 2001; Dingemanse and Réale, 2005), temperament (Réale et al., 2007), coping styles/strategies (Koolhaas et al., 1999) or boldness (Wilson et al., 1994). Personality traits can been divided in five main categories: activity; boldness or willingness to take risks; exploration-avoidance to novel situations; aggressiveness; and sociability (Réale et al., 2007). The personality of an individual is commonly presumed to be consistent over time and/or across situations. This is reflected in how animals react or behave in a variety of contexts such as novel situations, avoidance of predators, territorial aggression, foraging behaviour, etc.; resulting in correlated behavioural traits named as "behavioural syndromes" (Sih et al., 2004b). For instance, "bold" animals are characterized by being more aggressive, more active or explorative in unfamiliar situations whereas "shy" animals are considered to be more fearful or timid, less active or immobile in the same situations (Sih et al., 2004b; Wilson et al., 1994). The presence of personality types implies limited behavioural plasticity, however, behavioural correlations are not universal and can also be context specific (Coleman and Wilson, 1998; Dingemanse et al., 2007 , 2010). There is evidence that personality traits can be plastic and can change with experience by learning or habituation (Sih et al., 2004b; Stamps and Groothuis, 2010a , 2010b). For instance, bold rainbow trout (*Oncorhynchus mykiss*) reduced its boldness after losing a fight or watching shy demonstrators responding to novelty (Frost et al., 2007), foraging activity increased with experience in brown trout (*Salmo trutta*) (Adriaenssens and Johnsson, 2011) and in great tits the latency to explore a novel environment decreased when exposed to the same environment a second time (Dingemanse et al., 2002). Numerous studies assessed the existence of personality traits in fish, however only few studies have reported consistency or stability (i.e., repeatability) of personality traits over time (Bell et al., 2009; Conrad et al., 2011).

Personality traits are considered a strong mediating force in trade-offs between productivity (growth or fecundity) and predation risk. Active individuals forage more and grow at higher rates, and/(or) mature earlier, but expose themselves to a higher predation risk and thus have a lower adult survivorship (Wolf et al., 2007; Biro and Stamps, 2008; Dammhahn, 2012). Common sole (*Solea solea*) is a flatfish species, which displays nocturnal feeding and is predominantly buried in sediment during daytime as a strategy to avoid predation (Kruuk, 1963). It is hypothesized that conflicts between foraging activity and hiding motivation to avoid predation might explain the high individual variation in growth of sole. Few studies have addressed the mechanisms responsible for the existence of variation in behavioural traits and its relation to fitness (Conrad et al., 2011). For instance, boldness towards predators was positively correlated with: growth and dispersal in killifish (Fraser et al., 2001); activity, foraging and growth in larval salamanders (Sih et al., 2003); exploration, aggressiveness and growth in brown trout (Johnsson et al., 1996; Sundström et al., 2004; Adriaenssens and Johnsson, 2011); and growth in three-spined sticklebacks (Ward et al., 2004). Recently in common sole positive correlations were found between variability in feed intake, swimming activity, boldness towards novelty and feed intake/growth of fish individually housed (Mas-Muñoz et al., 2011).

This study examined individual variation in behavioural traits of group raised soles individually exposed to various challenge tests such as: 1) explorative behaviour in a novel environment; 2) behavioural response towards a novel object; 3) hiding motivation in a sandy environment; and 4) reaction to a net restraint test. Each test was performed twice (trial A and B). Activity patterns (i.e., swimming) in a novel or in a sandy environment and avoidance behaviours (i.e. burying and escape) towards a novel object or a restraint situation were expected to reflect individual differences in personality or coping styles. It was studied if such individual differences in personality traits in sole are: 1) consistent over time (i.e., trials); 2) consistent across different situations (i.e., tests); 3) and to what extent they are related to individual variations in growth.

Materials and methods

All procedures involving animals were conducted in accordance with the Dutch law on experimental animals, which complies with the ETS123 (council of Europe 1985) and the 86/609/EEC directive. The Ethical Committee for Animal Experiments (DEC) of Wageningen University approved the experiment.

Fish and holding conditions

Juvenile sole (*Solea solea*, *n*=96; 79♂:17♀) of approximately 1.5 years old with an initial weight (±S.D.) of 63 ± 7 g were used in this study. These fish were offspring of wild broodstock and were produced at a commercial local farm (Solea BV, Ijmuiden, the Netherlands). Fish were transported in an oxygenated 400 L tank filled with system water from the farm. Upon arrival at the experimental facilities (De Haar Vissen, Wageningen University, The Netherlands), fish were individually weighed and subcutaneously PIT-tagged using a syringe retractor solution (1M-100USL/LAN-100) with a micro transponder (Trovan ID100A: 2.12x11.5mm, DORSET GP, Aalten, The Netherlands). Weighing and tagging were done while being anesthetized with 2-phenoxyethanol (1 ml/L of system water for minimal 0.5 min) to avoid stress and potential pain. Fish recovered quickly from the anaesthesia showing normal feeding behaviour immediately after recovery. After arrival, fish were allowed to accommodate to experimental facilities for three weeks. At the end of the experiment, fish were sacrificed by an overdose of 2-phenoxyethanol (4 ml/L water for minimal 10 min), which rapidly induced loss of consciousness and hypoxia following ethical protocols for euthanasia in fish (Ross and Ross, 1999). After euthanasia the fish were decapitated and gender was determined by dissection.

Fish were group housed in one of two 400 L tanks (48 fish/tank), which were connected to one recirculating water system (RAS) filled with artificial sea water (25‰). Water flow rate over tanks was set at 9 liter/minute. Water temperature (18.0 ± 0.1∘C), pH (7.94 -7.55), dissolved O_2 (>7 mg/L), salinity (25 ± 0.1‰), NH_4^+ (<1 mg/L) NO_2^- (<1 mg/L) and NO_3^- (<50 mg/L) were daily monitored.

A 12D:12L photoperiod was maintained using artificial fluorescent lights. As sole are nocturnal feeders (Lagardère, 1987), the light regime was reversed with lights being on from 21:00 h till 09:00 h. During the dark phase of the day, red lights (0 LUX) were used to provide sufficient light for feeding and video recordings.

Fish were fed with a commercial diet, Weanex-3000 (BioMar, Nersac, France) 3mm sinking pellet (70% protein, 14% fat and 23 kJ/g energy on dry matter basis). Feed was continuously distributed by an automated belt feeder from 09:00 h till 17:00 h. At 17:00 h, uneaten pellets were removed from the tanks. To ensure that fish were fed to apparent satiation, the feeding ration was daily adjusted based on the observed amount of uneaten pellet. The feeding ration ranged between 0.5-1% body weight d^{-1}.

Experimental procedure

Individual growth of fish was assessed during four consecutive 16-d periods. Fish were weighed at the start and end of each period: day 0-17, 21-37, 41-57, and 61-77. At the end of each growth period, each fish was exposed to two consecutive performed behavioural tests. A “novel environment test” followed by a “novel object test” were done at the end of period 1 and 3 and a “hiding motivation test” followed by a “net restrain test” at the end of period 2 and 4. Thus each test was done twice within a 40-d interval, termed as trial A and B, respectively for the first and second time a test was done. The tests were conducted in eight identical 120 L (0.6x0.5x0.4 m) glass tanks. These tanks contained 20 cm of water from the RAS. After testing an individual fish, the water from these tanks was refreshed in order to avoid the of accumulation chemical cues interfering with the behavioural response of the next fish tested. The experimental tanks were visually isolated by covering all sides with black acrylic sheets except the front. Behavioural responses were recorded with two video cameras mounted above the tank. The behavioural tests were conducted during the dark phase of the day. Twenty-four hours prior to the behavioural tests, fish were not fed in order to increase their potential activity. Video recordings from the behavioural tests were analysed using the "The Observer XT 9.0" software package (Noldus, Wageningen, The Netherlands).

Behavioural Tests

1. *Novel environment test*

This test started with the introduction of a single fish into a barren-bottom experimental tank, thereafter fish were monitored for 10 min. The following behavioural traits were recorded: 1) latency to rest (immobile at the bottom of the tank) for the first time after its introduction to a new environment (s); 2) duration of first resting bout (s); 3) total swimming activity (s), being the time spent swimming during the test; 4) mean duration of a swimming bout (s) (calculated as total swimming activity divided by the number of swimming bouts); and 5) number of bury attempts (quick wave moments with whole body) (#).

2. *Novel object test*

The novel object test was done 25 min after the introduction of the fish into the experimental tank for the novel environment test. All the sides of the experimental tank were marked with grid lines (every centimeter) for measuring distances in the tank. The novel object consisted of an 8 cm diameter purple ball, which was presented and moved towards the head of the fish at approximately 5 cm/s. The ball was removed from the tank at the moment the sole showed a response or when the ball could touch its head. This artificial model, originally developed by Ellis et al. (1997), is thought to mimic an visual predator stimulus. The ball evokes a flight response in sole, being similar to the flight response triggered by a natural predator (Ellis et al., 1997). The following behavioural traits of the sole were recorded during 5 min after ball exposure: 1) latency time for fish to show any reaction (swimming away or bury) after the ball was introduced into the tank (s); 2) reactive distance, the smallest distance between ball surface and fish's head at the moment of first reaction (cm); 3) escape distance from starting position of fish's head to its end position after the first activity bout in response to the ball exposure (cm); and 4) number of bury attempts during the 5 min after the ball was presented (#). When fish showed no responses to the visual stimulus during the 5 min of observation, latency time for a reaction was given the maximal score of 5 min for statistical convenience.

3. *Hiding motivation test*

Fish were gently introduced singly into a 120 L experimental tank provided with 3 cm layer of coarse sand (grain size 1-2 mm) and were video recorded during 25 min. Burial coverage of each individual fish was scored at four moments after its introduction into the experimental tanks (1, 5, 15,and 25 min) on a scale from 0 (not buried) to 5 (completely covered) according to the method of Gibson and Robb (1992). The following behavioural traits were analyzed: 1) latency time to bury in sand (s); 2) total time buried (s); 3) number of burying bouts (#); 4) mean hiding (burial) coverage (0-5); and 5) total swimming activity of fish in tank (s). When fish did not bury throughout the 25 min of observation, latency time to bury was given a max score of 1500 s for statistical convenience.

4. *Net restrain test*

A net restrain test was done directly after the hiding motivation test (i.e., 25 min after the introduction of the fish into the sandy bottom tank). The restraint stress test consisted of holding each fish individually in a net partially immersed in water during 1 min. Direct observations were made on the number of struggles (termed as escapes, i.e., body displacements) that fish displayed inside the net.

Data calculations

Growth data from 85 fish (70 males and 15 females) of the 96 fish being tagged, were available due to technical reasons and natural mortality. Moreover, behavioural videos from 12 fish during different behavioural tests were damaged and excluded from the analysis.

In the present study, fish were considered as experimental unit. Growth rate (GR) was expressed as metabolic body weight (in g/kg$^{0.8}$/d^{1}), to correct for the variation in fish size as it is known that larger fish have a relative greater absolute metabolic requirement of feed compared to smaller fish (Hepher, 1988). GR was then calculated as:

$$GR = \frac{\dfrac{(W2-W1)}{T}}{\left(\dfrac{(W1/1000)^{0.8}+(W2/1000)^{0.8}}{2}\right)}$$

, where W1 is the weight (g) at the beginning of each growth period, W2 the weight (g) at the end of each growth period and T is the duration in days of the growth period. Pearson's correlations of individual growth between consecutive periods were 0.6-0.7. For comparison with behavioural measures mean growth rate was calculated over the whole experimental period as the average over the four growth periods.

Principal component analysis

Behavioural measurements were standardized using the mean as the location measure and the standard deviation as the scale measure. A principal component analysis (PCA) was used to summarize variation of behavioural traits measured in the four tests within each trial: Trial A and B being respectively the first and second time sole were subjected to each specific test. Thus the PCA was done separately per trial. PCA was used to identify relationships among all behavioural traits in all tests, separating individuals in a sample in terms of a reduced number of principal components. An eigenvalue of minimally 0.8 was set as the criterion for selecting components (Kaiser-Guttmann criterion). The loading of each behavioural trait measured on the principal component represents the correlation between the extracted component and the original measure. Therefore behavioural traits with high loadings on the same principal component are correlated and thus are expected to summarize the same underlying behavioural mechanism (i.e., behavioural syndrome or boldness) (Budaev, 2010). The principal components extracted from PCA's were rotated for improving interpretation using the orthogonal solution (Varimax rotation) to produce factors statistically independent from each other, which enables their use in a multiple regression analysis of growth.

Statistical analysis

Statistical analyses were performed using SAS system (SAS, 2002). To assess the consistency of behaviour over time, Spearman's correlations (r_s) of behavioural traits between trials and of identical PCA factors between trials were calculated. The consistency of behaviour across tests was assessed by comparing the loadings of behavioural traits from the different tests in the extracted PCA factors.

To test the overall effect of trial and gender, a repeated measures analysis of variance (ANOVA) with gender as the main fixed effect followed by the Tukey's HSD post-hoc test was done for each behavioural trait measured. The error terms were tested for homogeneity of variances and normality with the Shapiro-Wilk test. Behavioural traits, which did not comply with homogeneity of variance and normality, were analyzed by Kruskal-Wallis test followed by pair wise comparisons using the Mann-Whitney U-test.

Behavioural factors extracted from PCA done on data of trial A (first time of exposure to the tests) as well as on data for trial B were related with mean growth using multiple linear regression analysis using the following model:

$$GR_{ij} = \mu + G_i + ß_1PC1_j + ß_2PC2_j + \ldots + ß_nPCn_j + e_{ij}$$

where GR_{ij} is the response variable for growth for an individual (j = number of individuals), μ is the overall mean, G_i is the fixed effect of gender (i = male, female), $PC1_j$-PCn_j are the principal components extracted from the PCA for an animal j modeled as covariables so with regression coefficients $ß_1$-$ß_n$, and e_{ij} is a random residual effect associated with individual j.

Results

Growth performance

At the start of the experiment, average body weight of sole was of 63 ± 0.7 g (CV= 11%, Table 1) and did not differ between males and females. Sex ratio was 5.5:1 (males:females), with 18% females. Sole displayed a wide range in growth rate (CV = 33%, Table 1). Males had an average growth rate 4.19 g/kg$^{0.8}$/d (*n* = 70) and females 4.62 g/kg$^{0.8}$/d (*n* = 15) (Table 1).

Table 1. Mean (± SE) and coefficient of variation (CV) for body weight and growth of sole.

	Males		**Females**		
Variable	**Mean**	***n***	**Mean**	***n***	**CV (%)**
Initial BW[a] (g)	62.85 ± 0.81	79	62.53 ± 1.78	17	11
End BW (g)	89.19 ± 1.90	70	92.34 ± 4.10	15	16
GR[a] (g/kg$^{0.8}$/d)	4.19 ± 0.17	70	4.62 ± 0.37	15	35

[a] BW = Body weight; GR = Metabolic Growth rate;

Sex and trial effect on average behavioural responses

We found a significant sex effect on burying behaviour during the hiding motivation test. Females buried quicker (204 ± 110 vs. 515 ± 51 s), more often (1.77 ± 0.20 vs. 1.30 ± 0.10 s), and for a longer time (1295 ± 110 vs. 927 ± 51 s) compared to males ($P < 0.05$, Table 2). For all other behavioural traits, no significant sex effect was present ($P > 0.10$, Table 2).

The hiding motivation test showed that sole buried quicker, for a longer period and with a higher proportion of coverage during the second time they were presented with sand, trial B ($P < 0.05$, Table 2). The percentage of sole buried by the end of the 25-min observation period was 75% and 84% during trial A and B, respectively. During the first trial (A), 50% of the fish buried themselves in sand within 114 s, whereas during the second trial (B) this happened within 43 s (Fig. 1). Overall for the different behavioural tests, sole decreased latency to bury and increased the number of burying bouts between trial A and B ($P < 0.05$, Table 2). However, no significant differences between trials were found for other behavioural traits measured, such as exploration in novelty, novel object escape or net restrain escapes ($P > 0.10$, Table 2).

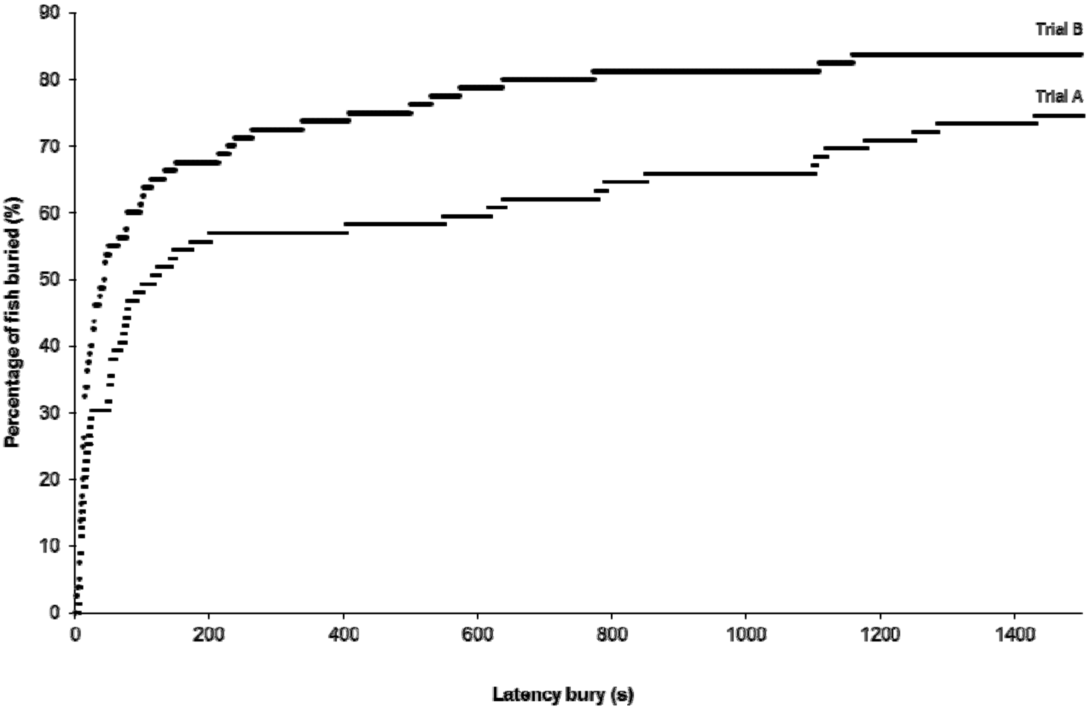

Fig. 1. Cumulative percentage of fish burying for the first time over the observation time period (1500 s) during the hiding motivation test in trial A and B (i.e., the first and second time the test was done).

Table 2. Means (± SD) during trial A and B (i.e., the first and second time a test was performed), effect of trial and sex on behavioural traits and Spearman's correlation of behavioural traits between trials (r_s).

	Trial A		Trial B		Effects[a]		Correlation[a]
	Mean ± SD	n	Mean ± SD	n	Trial	Sex	r_s
Novel environment							
Latency to rest (s)	11.5 ± 15	81	10.1 ± 8.8	85	ns	ns	0.27*
First resting time (s)	117 ± 196	81	171 ± 232	85	ns	ns	0.29**
Mean time swim (s)	9.1 ± 4.5	81	8.8 ± 8.4	85	ns	ns	0.32**
Total time active (s)	69 ± 81	81	74 ± 94	85	ns	ns	0.42***
Number of bury	3.3 ± 2.9	81	4.8 ± 4.3	85	*	ns	0.28*
Novel object							
Escape distance (cm)	24 ± 33	81	21 ± 32	85	ns	ns	0.56***
Reactive distance to head (cm)	2.1 ± 3.8	81	1.5 ± 2.8	85	ns	ns	0.48***
Latency for reaction (s)	69 ± 134	81	30 ± 72	85	*	ns	0.36**
Number of bury	1.3 ± 1.5	81	2.1 ± 1.6	85	**	#	0.33**
Hiding motivation							
Latency to bury (s)	586 ± 651	77	341 ± 542	85	*	*	0.46***
Total time buried (s)	842 ± 647	77	1133 ± 553	85	**	**	0.46***
Number of bury	1.2 ± 1	77	1.5 ± 1.1	85	#	*	0.34**
Hiding coverage (0-5)	1.9 ± 1.5	77	2.4 ± 1.4	85	*	#	0.36**
Total time active (s)	129 ± 211	77	65 ± 157	85	*	ns	0.39**
Net restrain							
Number of escapes	3.6 ± 4.3	82	2.8 ± 2.3	85	ns	ns	0.29**

[a] Significant differences are indicated by ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; $^{\#}P < 0.10$; *ns* not significant.

Consistency of individual behavioural traits over time and across tests

All measured behavioural traits showed a moderate positive correlation between trials (r_s, $P< 0.05$, Table 2). Correlations for activity and bury attempts in a novel environment test ranged between 0.27-0.42 and correlations for behavioural reactions to a novel object were slightly higher 0.33-0.56. For behavioural responses during the hiding motivation test correlations ranged from 0.34 to 0.46, while for the number of escape attempts in a net restrain test the correlation was 0.29.

For both trials the PCA yielded six behavioural components with Eigenvalues larger than one (Table 3 and 4). Percentage of variance explained by the factors extracted was similar for both trials. In trial A and B these six factors accounted for 78% and 77% respectively of the total variance of all behavioural traits measured during the four tests. The first factor with the largest amount of variance explained, represented hiding motivation in sand (23-24% of variance); the second factor represented the exploration activity in a novel environment (14-16% of variance); the third factor was related to the escape response to a novel object (13% of variance); the fourth factor combined the avoidance (burying) reaction to a novel object (10% of variance); the fifth factor was loaded by the avoidance reaction (first freeze time and bury) in a novel environment (9% variance); and the sixth factor represented the number of escapes in the net restraint test (8% of variance) for trial A and the latency time to rest in the novel environment test for trial B. The loadings of the different behavioural traits into PCA factors were very similar in trials A and B, except for factor 6 (Table 3 and 4). Except for factor 6, spearman correlation coefficients for these similar PCA factors between trials were significant and ranged 0.2-0.4, (Table 5).

Behavioural traits measured within a test generally clustered together into one or two PCA factors. In general the PCA did not cluster behavioural traits measured in different tests within the same factor (Table 3 and 4), with the exception of burying responses towards a novel object which was clustered with swimming activity in hiding motivation test during trial A and with the escape attempts in a net restrain test during trial B.

Relationship between behavioural factors and growth

Behavioural factors extracted from the PCAs were rotated (orthogonal rotation) to produce uncorrelated factors to assess their relationship with growth in a linear model. The multiple regression analysis showed that 23% of the variation in growth was explained by six behavioural factors extracted from PCA in trial A and sex ($R^2 = 22.84$, $P < 0.05$, Table 6). Mean growth of sole was explained by hiding motivation ($P< 0.1$, Table 6) and swimming activity in a novel environment ($P< 0.05$, Table 6). Regression coefficients were -0.28 (±0.16) and 0.31 (±0.16) respectively, which means that sole which buried less when sand was present and were more explorative in a novel environment grew more. Moreover there was an interaction effect between anti-predator escape and sex, with female growth being negatively correlated with the escape reaction towards a novel object ($P< 0.05$, Table 6). None of the models tested to relate individual growth of sole to the behavioural factors extracted from the PCA during trial B (second exposure to a test) were significant.

Table 3. PCA rotated factor loadings[a] for all individual behavioural traits measured during trial A (*n*=74).

Variables trial A	**Factors**					
	1	**2**	**3**	**4**	**5**	**6**
Novel environment Test						
Latency to rest (s)	0.11	**0.71**	0.22	-0.13	-0.05	-0.09
First resting time (s)	0.14	**-0.45**	-0.06	-0.10	**0.66**	0.21
Mean time swimming (s)	0.08	**0.85**	0.05	-0.02	-0.02	0.04
Total time active (s)	-0.14	**0.82**	-0.05	0.25	0.00	0.30
Number of bury	0.25	-0.31	-0.12	0.09	**-0.77**	-0.01
Novel Object Test						
Escape distance (cm)	-0.05	0.10	**0.90**	-0.12	-0.04	-0.06
Reactive distance to head (cm)	-0.15	0.12	**0.86**	-0.08	-0.08	0.04
Latency for reaction (s)	-0.16	0.04	**-0.58**	**-0.55**	-0.32	-0.18
Number of bury	0.12	-0.07	-0.16	**0.81**	0.18	-0.12
Hiding motivation Test						
Latency to bury (s)	**-0.95**	-0.09	0.05	0.02	-0.01	-0.07
Number of bury	**0.77**	0.05	-0.13	0.11	0.19	-0.11
Total time buried (s)	**0.94**	0.03	-0.02	-0.13	0.03	0.06
Hiding mean coverage (0-4)	**0.81**	-0.08	-0.01	0.07	-0.04	-0.13
Total time active (s)	**-0.45**	0.32	-0.09	**0.63**	-0.24	-0.05
Net restrain Test						
Number of struggles	-0.07	0.11	0.03	-0.09	-0.11	**0.95**
Eigenvalue	3.44	2.35	2.00	1.51	1.27	1.14
Percentage of total variance	22.93	15.67	13.33	10.07	8.47	7.60
Percentage of cumulative variance	22.93	38.60	51.93	62.00	70.47	78.07

[a] Factors with loadings above |0.40| are presented in bold.

Table 4. PCA rotated factor loadings[a] for all individual behavioural traits measured during trial B (*n*=85).

Variables trial B	**Factors**					
	1	**2**	**3**	**4**	**5**	**6**
Novel environment Test						
Latency to rest (s)	-0.12	0.22	-0.02	-0.01	-0.10	**0.87**
First resting time (s)	-0.09	**-0.80**	-0.08	-0.11	0.27	-0.09
Mean time swimming (s)	-0.17	**0.82**	0.00	-0.02	0.12	0.08
Total time active (s)	-0.17	**0.69**	0.01	0.25	**0.47**	0.19
Number of bury	0.25	-0.03	-0.18	0.02	**-0.84**	0.15
Novel Object Test						
Escape distance (cm)	-0.30	-0.04	**0.83**	-0.13	-0.02	-0.11
Reactive distance to head (cm)	-0.16	0.10	**0.86**	0.05	-0.16	0.08
Latency for reaction (s)	0.18	-0.22	-0.37	**-0.54**	-0.35	0.22
Number of bury	0.12	0.24	-0.36	**0.63**	0.23	-0.24
Hiding motivation Test						
Latency to bury (s)	**-0.87**	0.10	0.18	0.05	0.12	0.17
Number of bury	**0.79**	0.03	-0.10	-0.07	-0.03	0.04
Total time buried (s)	**0.91**	-0.07	-0.18	0.01	-0.13	-0.14
Hiding mean coverage (0-4)	**0.91**	-0.06	-0.10	0.15	-0.06	0.09
Total time active (s)	**-0.50**	0.23	0.29	-0.00	0.32	0.30
Net restrain Test						
Mean number of struggles	-0.03	0.20	0.01	**-0.79**	-0.25	-0.24
Eigenvalue	3.57	2.07	1.90	1.44	1.42	1.17
Percentage of total variance	23.82	13.77	12.70	9.59	9.49	7.77
Percentage of cumulative variance	23.82	37.59	50.29	59.88	69.37	77.14

[a] Factors with loadings above |0.40| are presented in bold.

Table 5. Spearman's correlation coefficient (r_s) between trials of behavioural PCA factors.

Factors	**Label**	**r_s**
1	Hiding motivation	0.31**
2	Exploration novelty	0.35**
3	Novel object escape	0.42***
4	Novel object bury	0.23*
5	Freeze & bury in novelty	0.34**
6	Net restrain /latency rest	-0.04

Significant differences are indicated by ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; #$P < 0.1$; *ns* not significant.

Table 6 . Association between PCA behavioural factors in trial A and growth in captivity (n = 74)

Dependent factor	**Variables**[a]	**P-value**	**Estimate (SE)**	**R^2 (%)**
Mean GR (g/kg$^{0.8}$/d)	Model	0.02		**22.84**
	Intercept	0.0001	4.02 (0.18)	
	Sex (female)	**0.02**	**1.01 (0.43)**	
	Hiding motivation	**0.09**	**-0.28 (0.16)**	
	Exploration novelty	**0.05**	**0.31 (0.16)**	
	Novel object escape female	**0.01**	**-0.78 (0.29)**	
	Novel object escape male	0.41	-0.16 (0.19)	
	Novel object bury	0.44	0.12 (0.16)	
	Freeze & bury in novelty	0.46	-0.12 (0.16)	
	Net restrain	0.50	0.11 (0.16)	

[a] Behavioural factors explaining growth (GR) ($P<0.1$) are presented in bold.

Discussion

Behavioural consistency over time

This study investigated the consistency of individual differences in personality traits over time in sole (*Solea solea*). All behavioural traits, measured in four different tests, were consistent over time; indicated by the low to intermediate positive Spearman correlations between trial A and B (ranging 0.27-0.56, Table 2). This constancy over time was confirmed by the positive correlations between the PCA behavioural factors between trial A and B, which ranged from 0.22 to 0.42 (Table 5). These estimates are well within the range of repeatability measures for personality (behavioural) traits reported in other species (Bergmuller and Taborsky, 2007; Bell et al., 2009). Lower repeatability measures for personality traits observed in the current study can be due to different levels of behavioural flexibility between individuals (Dingemanse et al., 2012), or to micro-environmental effects between the replicated measures (Réale et al., 2007), since time interval between trials (i.e., the first and second time each test was done) was 40 days, thereby this could change the expression of the behaviour.

Although the novel environment and open-field test are one of the most widely used behavioural tests in all taxa to study personality traits such as the exploratory activity (Walsh and Cummin, 1976), we did not find high correlations in behavioural traits measured between repeated trials. Learning and habituation can affect the novelty aspect of the novel environment behavioural test when replicated (Réale et al., 2007). For example great tits explore a novel environment quicker when confronted with it for a second time (Dingemanse et al., 2002). However in our study, repeating the test did not have an effect on the latency to rest or to explore the environment.

The escape response to a novel object (which represented an artificial predator) had the highest correlation coefficient between trials ($r = 0.56$, $P < 0.001$). Sole shows a flight response to an artificial predator which is suggested to be similar to the response sole display towards a natural predator (De Groot, 1969). The stress response towards a novelty or a potential threat is largely influenced by the individual's appraisal of the environmental change in combination with the individual's capacity to cope (Koolhaas et al., 1999). It appears that fish show two opposite coping strategies upon exposure to an artificial predator model: the first type the active response corresponds to fish showing a flight or escape response, and the second type the passive response, characterized by immobility or burying. Anti-predator behaviour is considered to be one of the most important fitness traits in nature (Fuiman and Magurran, 1994). This explains why sole display various behavioural responses to avoid predation risk in nature: burying itself in sand, cryptically matching the skin color to that of the substrate, showing low activity levels and nocturnal foraging, and fleeing only when predators get really close (Ellis et al., 1997; Ryer et al., 2008). Behavioural responses towards predators seem to be an innate behavioural response as the sole used in the current study were reared in captivity without substrate and predators and thus naive individuals. Studies on three-spine sticklebacks (*Gasterousteus aculeatus*) (Giles and Huntingford, 1984), Trinidad guppy (*Poecilia reticulata*) (Magurran and Seghers, 1990), European minnow (*Phoxinus phoxinus*) (Magurran, 1990), Atlantic Salmon (*Salmo salar*) (Hawkins et al., 2008), Paradise Fish (*Macropodus opercularis*) (Miklosi et al., 1997), and Artic charr (*Salvelinus alpinus*) (Vilhunen and Hirvonen, 2003) also suggested a genetic variation for anti-predator responses. However, innate anti-predator responses can also be modified by social learning, experience or habituation and by environmental circumstances (Huntingford, 1982; Magurran and Girling, 1986; Magurran, 1990; Jarvi and Uglem, 1993; Griffin and Galef, 2005).

Repeated burial attempts in tanks with barren bottoms and the short latency times to bury in sand of sole, being naive to substrate, seem to indicate that burying is an innate behaviour of sole, which may lead to stereotype behaviour in a barren environment. Ellis et al. (1997) showed that both reared and wild sole attempted to bury as frequently in a barren bottom. However, reared sole were suggested to have lower burying abilities by showing a lower percentage of coverage compared to experienced wild sole when presented with sand for the first time (Ellis et al., 1997). We found a significant trial effect on hiding behaviour in all tests. Fish resumed burying quicker, buried more time and covered themselves with more sand when tested for a second time. Sole responds earlier with burying and has a shorter immobility time after they have already experienced the same environment before, which likely reflects cognitive processes such as habituation or learning (Kieffer and Colgan, 1992). Therefore innate burying responses, which show behavioural plasticity for learning may enable adaptation to changes in environmental conditions (Kieffer and Colgan, 1992).

Behavioural correlations across situations

Individual differences in suites of inter-correlated behaviours such as exploratory, anti-predator, risk- taking and aggressive behaviour, also named as “behavioural syndromes”, have received increased attention (Sih et al., 2004b; Dingemanse and Réale, 2005; Bell, 2007; Huntingford et al., 2010). The existence of “behavioural syndromes’ would imply that behavioural traits may not evolve independently, but that a trait may be correlated with some other behavioural trait(s) resulting in the evolution of multiple traits simultaneously (Van Oers et al., 2004). However, in our study we could not find strong indications for the existence of "behavioural syndromes", as indicated by the performed PCA on all behavioural traits measured over the four different tests for each trial. In general terms, the loadings of the factors in these PCA did not combine behavioural traits of different tests in a single factor. We only found correlations between burying after presenting an artificial predator threat and exploration activity in a sandy environment during trial A and with escape attempts in a net restrain situation during trial B. Sole is a flatfish and uses burying behaviour to reduce predation risk in nature, which might explain this consistency across different "risk-taking" situations. Correspondingly, other studies also showed that "behavioural syndromes" might be context-specific and thus behavioural traits do not always correlate in time and in space (van Oers et al., 2005; Dingemanse et al., 2007; Sinn et al., 2010).

Relationship between behaviour and growth

In the present study, sex and behavioural traits measured for the first time in laboratory conditions (trial A) such as hiding motivation, anti-predator behaviour and exploration in novelty explained 23% of the variation in growth. Therefore behavioural traits measured when fish were exposed to the tests for the first time, might reflect differences in personality or coping styles, and thus can be predictive to how an animal will react to stimuli or perform in a specific environment. This would imply that "risk-taking" behaviours may trade-off with other fitness related behaviours such as foraging, resulting in consistent individual differences in growth (Stamps, 2007; Biro and Stamps, 2008). Theoretically the maintenance of individual differences in growth can be explained if individuals with different growth rates end up with comparable fitness in nature as a result of growth-mortality trade-offs (Stamps, 2007). Therefore, bold individuals with risk-taking behavioural characteristics, such as being more active and explorative, display more foraging and show fast growth, but at the costs of a higher predation risk (Sih et al., 2004a; Biro and Stamps, 2008). In a previous study with individually housed sole *(Solea solea)* we also found positive relationships between feeding consistency, swimming activity and boldness in a novel environment and growth (Mas-Muñoz et al., 2011). In other species positive associations between boldness, foraging behaviour (Wilson et al., 1993; Huntingford et al., 2010) and growth in captivity were also found (Johnsson et al., 1996; Lahti et al., 2001; Sundström et al., 2004; Huntingford and Adams, 2005; Biro and Stamps, 2008). However, some studies found a negative relationship between exploration and growth in the wild of brown trout (*Salmo trutta*) (Adriaenssens and Johnsson, 2011) or between surface swimming and growth of Atlantic halibut (*Hippoglossus hippoglossus L.*) (Kristiansen et al., 2004).

Furthermore, the fact that the association between behavioural traits measured and growth was absent when fish were tested for a second time (trial B), suggests that this association might be context dependent. Other studies also suggested more dynamic associations between personality and growth depending on age and on environmental conditions (Adriaenssens and Johnsson, 2009; 2013). It should also be noted that our behavioural tests where done in environments where there was no real risk involved and moreover, the sole were naïve, having no previous experience to predators. Therefore, fish could have behaved differently during the second trial due to learning or habituation.

Sole is a dimorphic species, with female sole growing faster and to a bigger size compared to males. We found an interaction effect between anti-predator escape behaviour and sex, with females growing slower when showing a stronger flight reaction to an artificial predator. Moreover females also seem to show a higher efficiency in burying behaviour during the hiding motivation test, which might explain different survival strategies between females and males in nature. However, in our study we had a disproportional sex ratio which is typical for hatchery reared sole, with more males than females, which could also bias these results. However one hypothesis behind this is if predation risk varies as a function of body size, animals with different body sizes will also consistently differ in their risk-taking behaviour (McElreath and Strimling, 2006). Moreover in adult females, reproductive output or fecundity is size and age related, where some individuals consistently grow faster and have a higher age or size-specific fecundity than others (Beverton, 1992). This reflects clearly different fitness strategies: risk-taking individuals who grow faster may reproduce at an early age but at the risk of lower adult survival. Less bold individuals on the other hand will reproduce later in life, but generally will have higher fecundity (Giesel, 1976; Wolf et al., 2007; Biro and Stamps, 2008) .

Conclusions

This study showed that individual behavioural traits in sole measured by challenge tests are relatively consistent over time. Anti-predator behaviours such as burying seem to be an important innate behaviour, indicated by the highest consistency over time and its slight consistency across situations. However alterations in the strength of the burying behaviour responses during the first and second time of exposure may reflect a learning or habituation effect. Individual behavioural traits measured by different tests, clustered into factors using PCA, are predictive in explaining part of the variation in growth of sole. In line with the growth-mortality trade-off theory, the motivation to bury is negatively related to growth, whereas the motivation to explore a novel environment is positively related. Female sole that are less reactive upon an artificial predator threat grow faster, whereas in males this factor has no impact.

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CHAPTER 4

Effect of sorting and stocking density on behaviour and growth variation of sole (*Solea solea*) reared with or without substrate

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Julia Mas-Muñoz, Sara Leigo, Robbert Blonk, Hans Komen, Johan W. Schrama

Submitted

Abstract

A major economic constraint for culturing sole is its low and variable growth. The objective was to study the effect of sorting by growth rate ("fast", "medium" or "slow") and of stocking density ("high" versus "low") on behaviour, feed intake and growth of sole. This was assessed in the presence and in the absence of sand. At the start of the experiment, fish of 125 ± 32g were weighed and categorized based on their growth rate during a 3 month period in three different classes: "fast" (3.7 $g/kg^{0.8}/d$), "medium" (2.5 $g/kg^{0.8}/d$) or "slow" (1.5 $g/kg^{0.8}/d$) growers. Fish of each growth class were reared at either a "high" (1.3 kg/m^2) or "low" (3.4 kg/m^2) stocking density. There was a significant effect of sorting on the growth of sole when compared to their previous performance in an unsorted group. "Fast" class sole showed a 41% decrease in growth compared to the previous period. In contrast, "slow" sole, increased their growth 53% when compared to the previous period and no changes in growth rate of "medium" growers were found. Stocking density had a significant effect on FCR and on growth variation; both being lower at "low" stocking density (1.6 vs. 1.7; and 28.4% vs. 49.6%, respectively). At the "low" stocking density burying behaviour (1.7 vs. 6.7; #/fish) and swimming activity (0.3 vs. 0.4; % fish) were lower than at "high" stocking density. However, when sand was provided, no differences in behaviour and growth variation between stocking densities were observed. In conclusion, increasing stocking density in tanks without substrate promotes fish-fish interactions, which increases activity and variation in growth of sole. This condition seems to induce social stress in sole, which is alleviated when sand is provided. Sorting sole by growth rate, improves growth of "slow" sole and reduces growth of "fast" sole, which is suggested to relate to behavioural coping style and indicates for the presence of hierarchical structures in sole.

Keywords: Flatfish, grading, stocking density, size dispersal, environmental enrichment, welfare

Introduction

Sole (*Solea solea and Solea senegalensis*) is an important fish species for commercial aquaculture in Europe because of its consumer popularity and high market value. This explains the continuing interest in developing methods for the culture of this species (Howell, 1997; Imsland et al., 2003). Sole is however still in an early stage of domestication and its culture is not yet mastered. A major economic constraint for culturing sole is its slow and variable growth (Howell, 1997; Imsland et al., 2003).

Variation in growth between individuals is common in fish populations, and for cultured *Solea solea*, coefficient of variation in body weight ranges between 30 and 50% (Blonk et al., 2010a). Little attention has been directed towards the mechanisms explaining intraspecific growth variation in sole. It is known that part of the variation in growth is genetically determined with heritability values for mean body weight between 0.2 and 0.3 (Blonk et al., 2010a). However, genetic influences on size variation in sole seems to be limited (Panagiotaki and Geffen, 1992). Even in genetically identical cloned individuals variability in growth still exists (Komen et al., 1993; Nakanishi and Onozato, 1987; Umino et al., 1997). In fish, social interactions and associated hierarchies are regarded as one of the main reasons for growth variation, which is greatly influenced by stocking density (Barbosa and Volpato, 2007; Jobling and Wandsvik, 1983; Koebele, 1985; Martins et al., 2005; Volpato and Fernandes, 1994). Yamagishi (1969) showed species related differences and suggested that growth variation is related to the social habit of the species (i.e. higher in non-shoaling than in shoaling species).

In commercial aquaculture, sole is reared at high densities, with 100 to 300% of the bottom area covered by fish and in the absence of sand (Schram et al., 2006). There is evidence that sole in the absence of sand increases its resting metabolic rate, which possibly indicates a physiological stress situation for sole (Howell and Canario, 1987). Moreover, high stocking densities in bare bottom tanks increase the possibilities for social interactions and therefore of disproportional food acquisition due to: food competition, appetite suppression and/or increased energy expenditure as a consequence of social stress (Barbosa and Volpato, 2007; Jobling and Baardvik, 1994; Jobling and Wandsvik, 1983; Jørgensen et al., 1993; Koebele, 1985; Volpato and Fernandes, 1994). Cultured fish have to interact with each other to acquire food and thus individual behavioural differences in the level of tolerance to crowding or grouping (i.e. different coping styles “bold” vs. “shy” personalities) might explain variation in growth of fish (Kristiansen and Fernö, 2007; Metcalfe, 1986; Ruzzante and Doyle, 1990; Ruzzante and Doyle, 1992). This attribute, as well as age, sex, physical size and physiology of species under study, can determine the social status of an individual in a social hierarchical structure. Dominant fish can supress growth of subordinate fish by either actively dominating food access or passively reducing their feed intake/efficiency due to social stress (Abbott and Dill, 1989; Ang and Manica, 2010; Barbosa and Volpato, 2007; Chase et al., 2002; Jobling and Wandsvik, 1983; Koebele, 1985; McCarthy et al., 1992; Volpato and Fernandes, 1994).

The effect of stocking density on growth has been reported for diverse flatfish species. In Atlantic halibut, *Hippoglossus hippoglossus*, high stocking densities increased swimming activity while feeding, decreased mean growth and increased size variation (Kristiansen et al., 2004). Similar findings were reported in studies with California halibut, *Paralichthys californicus* (Merino et al., 2007) and turbot, *Scophthalmus maximus* (Irwin et al., 1999). Studies on Common sole (*Solea solea)* have reported a negative relationship between stocking density and growth, with fish showing less favourable feed conversion ratio at high densities (Howell, 1997, 1998; Piccolo et al., 2008; Schram et al., 2006). Moreover, an increase in growth variation was found for *Solea solea* when grouped housed compared to when sole were kept in isolation (Mas-Muñoz et al., 2011). This suggests that part of the variation observed in grouped sole arises from the interaction between fish. For Senegalese sole (*Solea senegalensis*) contradictory results have been reported: Salas-Leiton et al., (2010b) found no negatives effects of stocking density on growth, but did find a significant effect on plasma cortisol levels which suggests that crowding stress occurred. Sánchez et al. (2010) found that individually tagged sole, grew slower at high stocking densities during the first 61 days of culture, but after 134 days this difference disappeared. The shape of Senegalese sole (*Solea senegalensis*) seems to be affected by stocking density (Ambrosio et al., 2008). However to our knowledge no studies reported the differential effect of stocking density when sand is provided.

In addition to this, many fish farms practice size-grading to reduce size variation and improve overall feed efficiency and growth. It is expected that the growth of small fish will improve when separated from large fish (Barki et al., 2000; Overton et al., 2010). However, changes in the population structure may disrupt hierarchies, resulting in high levels of interactions between individuals of a similar size that can affect growth rate and size distribution increase again (Baardvik and Jobling, 1990; Overton et al., 2010; Salas-Leiton et al., 2010b). The effect of sorting on larger fish can be detrimental in some cases (Jobling and Reinsnes, 1987).

In nature, sole is known to be predominantly a solitary fish and presumably rarely in contact with other sole (Frimodt, 1995). Like other flatfish, sole is a bottom-dwelling species, which spends long time buried in the sediment and shows low activity during the day, probably to reduce predation risk (De Groot, 1971; Kruuk, 1963). Therefore it is hypothesized that sole when reared at high stocking density exhibit an increased competition for food and activity during feeding. Together, this results in a disproportional food acquisition and thereby high individual variation in growth. Growth variation might reflect individual differences in behaviour or coping styles of sole with different tolerance levels to social stress (i.e. bold-dominant fish will be fast growers whereas shy-subordinate fish will be slow growers). Moreover, the effect of fish-fish interactions might be context dependant and thus influenced by the absence or presence of substrate.

Therefore, the objective of the current experiment was to study the effect of sorting by growth rate (“fast”, “medium” and “slow”) and of stocking density (“high” versus “low”) on (non-) feeding behaviour, feed intake/feed efficiency and variation in growth of sole in the presence and in the absence of sand.

Materials and methods

Ethics

All procedures involving animals were conducted in accordance with the Dutch law on experimental animals, which complies with the ETS123 (council of Europe 1985) and the 86/609/EEC directive. The Ethical Committee for Animal Experiments (DEC) of Wageningen University approved the experiment (2010033.b).

Fish, adaptation period and experimental conditions

Mixed sex, juvenile Common sole (*Solea solea, n*=90), weighing 125 ± 32g (mean ± SD) originating from a local commercial farm (Solea BV, IJmuiden, the Netherlands) were used in this study. Prior to the experiment, all fish were individually weighed and PIT-tagged subcutaneously with a micro transponder (Trovan ID100A: 2.12x11.5mm, DORSET GP, Aalten, the Netherlands) while anesthetized with 2-phenoxyethanol to avoid any stress or potential pain (1ml of solution /litre of system water for 0.5 minutes). Thereafter fish were housed in a 400 L tank (2 x 1 x 0.4m; L x W x H) during a pre-experimental period of 3 months at the fish experimental facilities (De Haar Vissen, Wageningen University, the Netherlands) for adaptation to the recirculating water system (RAS).

At the start of the experiment, all fish were weighed while anesthetized and categorized based on their growth rate during the 3 month period prior to experiment in which fish remained in an heterogeneous (not sorted) group. Three different classes (*n*=30) were created, containing fish which had expressed "fast" (3.7 ± 0.6 g/kg$^{0.8}$/d), "medium" (2.5 ± 0.5 g/kg$^{0.8}$/d) or "slow" growth rate (1.5 ± 0.3 g/kg$^{0.8}$/d) (mean ± SE) in the preceding period (Table 1).

Table 1. Classification of fish in different growth classes based on weight, standard length and metabolic growth rate (GR) (means ± SD) over a 3 month period prior the experiment.

Growth class	**Weight (g)**	**Length (cm)**	**GR (g/kg$^{0.8}$/d)**
Fast	143 ± 25	25 ± 1.3	3.7 ± 0.6
Medium	112 ± 22	23 ± 1.5	2.5 ± 0.3
Slow	98 ± 17	22 ± 1.3	1.5 ± 0.5

The experimental design was a 3 x 2 factorial arrangement of growth class by density treatments. Fish categorized in each growth class (*n*=30) were randomly assigned to one of two stocking densities ("high" or "low") (*n*=45). The different stocking densities were provided by using two types of glass tanks with different volumes/bottom area (altering the degree of fish-fish interactions). Fish at the "low" stocking density were housed in 9 glass tanks of 120L (90x45x45cm; 0.08 m^2/fish or 15% initial bottom coverage) and fish at the "high" stocking density were housed in 9 glass tanks of 30L (50x30x30cm; 0.03 m^2/fish or 40% initial bottom coverage) (Table 2). The side walls of every tank were covered with black plastic to avoid any visual contact between fish of different tanks. Tank was the experimental unit, with all tanks containing 5 fish each (equal number of potential fish-fish interactions). Each treatment (growth by density combination) was studied in triplicate.

Table 2. Experimental design with initial body weight (mean ± SD) and initial stocking density (fish/m^2, kg/m^2 and percentage of bottom coverage) for all treatments at the start of the experiment.

Growth class	Density	Initial BW (g)	Fish/m^2	Kg/m^2	Bottom coverage[a] (%)
Fast	High	155 ± 28	33.3	4.4	47.4
Fast	Low	156 ± 27	12.5	1.6	17.5
Medium	High	119 ± 21	33.3	3.4	40.5
Medium	Low	124 ± 24	12.5	1.3	15.2
Slow	High	99 ± 24	33.3	3.0	37.3
Slow	Low	100 ± 15	12.5	1.1	13.8

[a] Calculated using the surface area of *Solea solea* in $cm^2=6.0487*W(g)^{0.6467}$ (Schram et al., 2006)

After sorting, all fish were allowed to adapt to the new experimental conditions during a 2-week adaptation period. The experiment consisted of two consecutive growth periods of 30 days duration. At the end of each growth period all fish were individually weighed while anesthetized with 2-phenoxyethanol to avoid handling stress (1ml of solution /litre of system water for 0.5 minutes). During the first growth period fish were reared without substrate while during the second period all aquaria were provided with a 3cm thick layer of coarse sand (grain size 1-2 mm).

During the pre-experimental, adaptation and experimental period, all tanks were connected to a recirculating water system (RAS) which consisted of two sludge settlers and one bio-filter containing lava rock filled with UV-treated artificial sea water (25 ‰). Water flow rate over tanks was set at 9 liter/minute. Tanks were provided with air stones for extra aeration. Water temperature (17.4 ± 0.1 ∘C), pH (7.6 -7.8), dissolved O_2 (>7 mg/l), salinity (25 ± 0.3 ‰), NH_4^+ (<1 mg/ l) NO_2^- (<1 mg/ l) and NO_3^- (<50 mg/l) were monitored daily.

A 12D:12L photoperiod was maintained using artificial fluorescent lights. As juvenile sole are nocturnal feeders, the light regime was reversed with lights on from 21:00h till 9:00h. During the dark period of the day red lights were used to provide sufficient light for feeding and video recordings.

Feeding method

Fish were fed a commercial diet, Weanex-3000 3 mm sinking pellet (BioMar, Nersac, France) (60% protein, 20% fat and 24 kJ/g energy on dry matter basis).

During the pre-experimental period (3 months), the unsorted fish were fed in excess (between 0.5-1% body weight/day) by an automated belt feeder, which distributed feed in two blocks of 3 hours. Feeding periods were from 9:00h till 12:00h and 13:00h till 16:00h. After each feeding uneaten pellets were measured and removed. To ensure feeding until apparent satiation, daily rations were adjusted based on the feed intake of previous day.

During the adaptation period and the experimental period (for both period 1 and 2) fish were hand fed two times a day at 9.00 and 16.00h until apparent satiation. For all tanks, the feeding period started with a feed ration of 20 pellets (0.7 g) and whenever pellets where eaten, 10 extra pellets (0.35g) were added. Through this procedure there would always be at least 10 pellets of feed in each tank during the feeding period. Feeding continued for a maximum of 40 minutes and 10 minutes later remaining pellets were siphoned and counted.

Feeding behavioural observations

Once a week live observations were made both during the morning and the afternoon feeding periods. For all tanks the mean latency to eat the first pellet (sec) of each fish and the proportion of fish eating in each tank was recorded during the first 7 minutes of the feeding period using a stopwatch.

Non-Feeding behavioural observations

Live observations on the non- feeding behaviour of sole were performed twice a week in between feeding meals (12.00-13.30h). Each tank was observed individually for 10 minutes. The following events were recorded for each fish within a tank: frequency of take-offs (#/10 min), frequency of burying attempts on the bare bottom (#/10 min), frequency of escapes (#/10 min) and total time of surface swimming (min) during the observation. During period 2 when substrate was provided the total time a fish was buried in sand (min) during the observation was assessed. The behavioural observations were analysed following the ethogram adapted from Kristiansen et al. (2004) and presented in Table 3. The mean number of times each behavioural trait was performed per fish during the observation was calculated. Moreover the proportion of fish in each tank (0 if no fish to 1 when all fish) performing the indicated behaviour was also assessed. Video recordings were performed twice a week during

24h/day to complement the live behavioural observations (backup). Video analysis showed similar findings to live observations therefore data are not presented.

Table 3. Ethogram used for non-feeding behavioural observations.

Behavioural element	**Description**
Take-offs	Fish leaves the bottom and produces a displacement of the body using body or fin movement as propulsion swimming >2 sec
Burying	Fish attempt to bury by performing quick wave movements with its whole body
Escape	Fish moves its body straight up in the water column and is pushing its head out of the water surface
Surface swimming	The total observation time of fish swimming close (<3 cm) to water column
Time buried	The total observation time of fish buried in the substrate (>50% body coverage) during period 2

Data analysis

In the present study, tank was considered the experimental unit so data were calculated as the means per tank. Growth rate (GR) was expressed per metabolic body weight as units of g BW $(\text{kg})^{-0.8}$ d^{-1}. This was done to correct for the variation in fish size between growth classes (size scaling) as it is known that larger fish have a greater absolute metabolic requirement of feed compared to smaller fish (Hepher, 1988). GR was calculated as:

$$GR = \left. \frac{(W2-W1)}{T} \middle/ \left(\frac{(W1/1000)^{0.8} + (W2/1000)^{0.8}}{2} \right) \right.$$

, where W1 is the initial weight (g) at the beginning of each growth period, W2 the end weight (g) at the end of each growth period and T is the duration in days of the growth period. Feed intake was also expressed as a mean of the metabolic body weight (FI, $\text{g/kg}^{0.8}$/day) per tank. Feed efficiency was analysed using the feed conversion ratio (FCR) per tank which was calculated as feed intake (FI, $\text{g/kg}^{0.8}$/day) divided by growth ($\text{g/kg}^{0.8}$/day).

Coefficient of variation (CV, %) in growth was calculated as $CV = \left(\frac{\sigma}{\mu}\right) * 100$, where σ is the standard deviation of all fish within tank and μ the observation mean in each tank.

Fish were classified/sorted in significant different growth classes ("fast", "medium" and "slow") based on their growth in an heterogeneous group prior to experiment (Table 1). The effect of sorting on growth was expressed as the change in growth (%) of fish between their growth in an homogeneous group (G_{hom}) during the experimental period compared to the growth of fish in an heterogeneous group prior to the experiment (G_{het}), as following: $\left(\frac{G_{het} - G_{\text{hom}}}{G_{\text{hom}}}\right) * 100$.

Statistical analyses were performed using SAS system (SAS, 2002). A multivariate analysis of variance (ANOVA's) with "growth class" and "density" as the main fixed effects and their interaction and followed by the Tukey's HSD post-hoc test was done for each growth period. The error terms were tested for homogeneity of variances and normality with the Shapiro-Wilk test. Behavioural data were squared root (frequencies) or log transformed (latencies) when necessary. Results were considered statistically significant when P-values were below 0.05. Data are reported as mean ± SE.

Results

In the absence of substrate (period 1)

1. *Effect of growth class and stocking density on feed intake, feed efficiency and growth*

In the absence of sand, no differences were found in mean growth and feed intake (g/kg$^{0.8}$/d) between the three growth classes of sole: "fast", "medium" and "slow" reared at "high" or "low" stocking densities ($P > 0.1$, Table 4). Stocking density had a significant effect on FCR ($P < 0.05$) and on the coefficient of variation in growth ($P < 0.01$) (Table 4). FCR (1.04 vs. 1.12) and variation in growth (28.4% vs. 49.6%) were lower when sole were reared at the "low" stocking density compared to the "high" stocking density.

Table 4. Effect of growth class ("fast", "medium" and "slow"), density ("high" vs. "low") and their interaction on the performance of sole (*Solea solea*) when housed in barren bottom tanks (no sand).

Density	HIGH			LOW				P-value[b]		
Growth class	Fast	Medium	Slow	Fast	Medium	Slow	SEM	GC	D	GCxD
Initial BW[a] (g)	153.7	119.4	99.0	156.5	124.2	100.2	3.9	***	ns	ns
Final BW[a] (g)	173.4	138.3	116.0	183.5	148.6	114.4	5.1	***	ns	ns
FI[a] (g/fish/d)	0.73	0.67	0.58	0.93	0.81	0.50	0.07	**	ns	ns
FI[a] (g/kg$^{0.8}$/d)	3.1	3.5	3.5	3.9	4.0	3.0	0.3	ns	ns	ns
FCR[a]	1.13	1.07	1.17	1.07	0.98	1.07	0.04	ns	*	ns
GR[a] (g/kg$^{0.8}$/d)	2.8	3.3	3.0	3.7	4.1	2.8	0.4	ns	ns	ns
CV-GR[a] (%)	59.2	33.1	56.5	31.1	25.4	28.7	7.9	ns	**	ns

Values are given as means ± SEM.

[a] BW=Body weight; FI=Feed intake; FCR= Feed conversion ratio; GR= growth; CV-GR= coefficient of variation in growth within tank.

[b] Effects of growth class (GC), stocking density (D) and their interaction (GCxD) are indicated: ***P<0.001; **P<0.01; *P<0.05; ns not significant.

2. *Effect of sorting on growth*

The effect of sorting on growth was expressed as the change in mean growth rate (%) between the growth of fish during the experiment in a homogeneous sorted group and their previous growth in an heterogeneous group. This change in growth was not affected by stocking density (P > 0.05) but was influenced by growth class (P<0.001, Fig. 1). "Fast" class sole showed a -41±12% decrease in growth after sorting. In contrast, "slow" class sole, showed an increase in growth of 53±12% improvement after sorting. Growth of sole classified as "medium" growers showed an intermediate growth with no change in their growth after sorting.

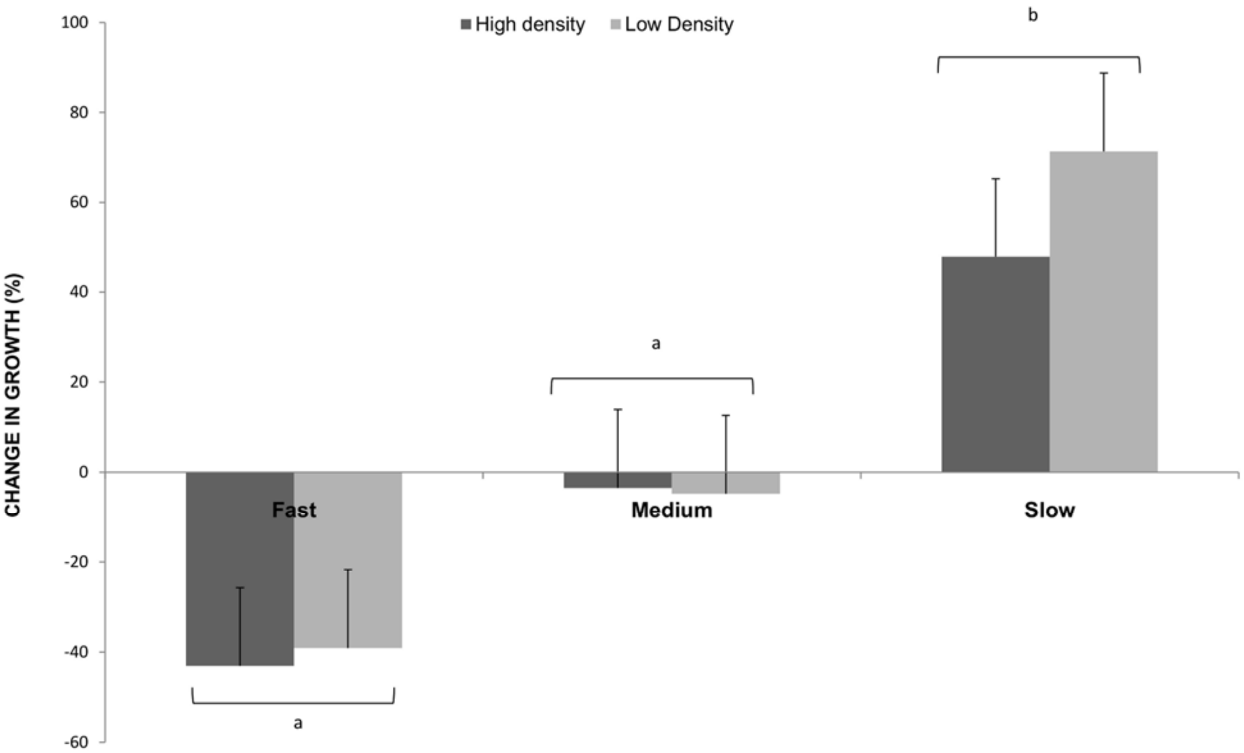

Fig. 1. Change in growth (%) after sorting of sole housed in barren bottom tanks (no sand). Expressed as the difference in growth between the growth of fish housed in homogenous groups in tanks without sand, and their previous growth when kept in a heterogeneous group. Means lacking a common script (a, b) differ significantly ($P<0.05$).

3. *Effect of growth class and stocking density on feeding behaviour*

The average latency to feed the first pellet for sole housed in barren bottom tanks was affected by growth class ($P<0.01$, Fig. 2) but not by stocking density ($P>0.05$, Fig. 2). “Fast” growth class of sole showed a higher latency to start feeding (2.8±0.15 min) compared to “medium” (2.1±0.15 min) and “slow” class sole (2.1±0.15 min). However, no differences were found in the proportion of fish eating (data not shown) during the first 7 minutes of observation between “fast”, “medium” and “slow” class (0.66, 0.75 and 0.65, respectively; $P>0.05$) or between “low” and “high” density (0.66 ± 0.04 vs. 0.71 ± 0.04, respectively; $P>0.05$).

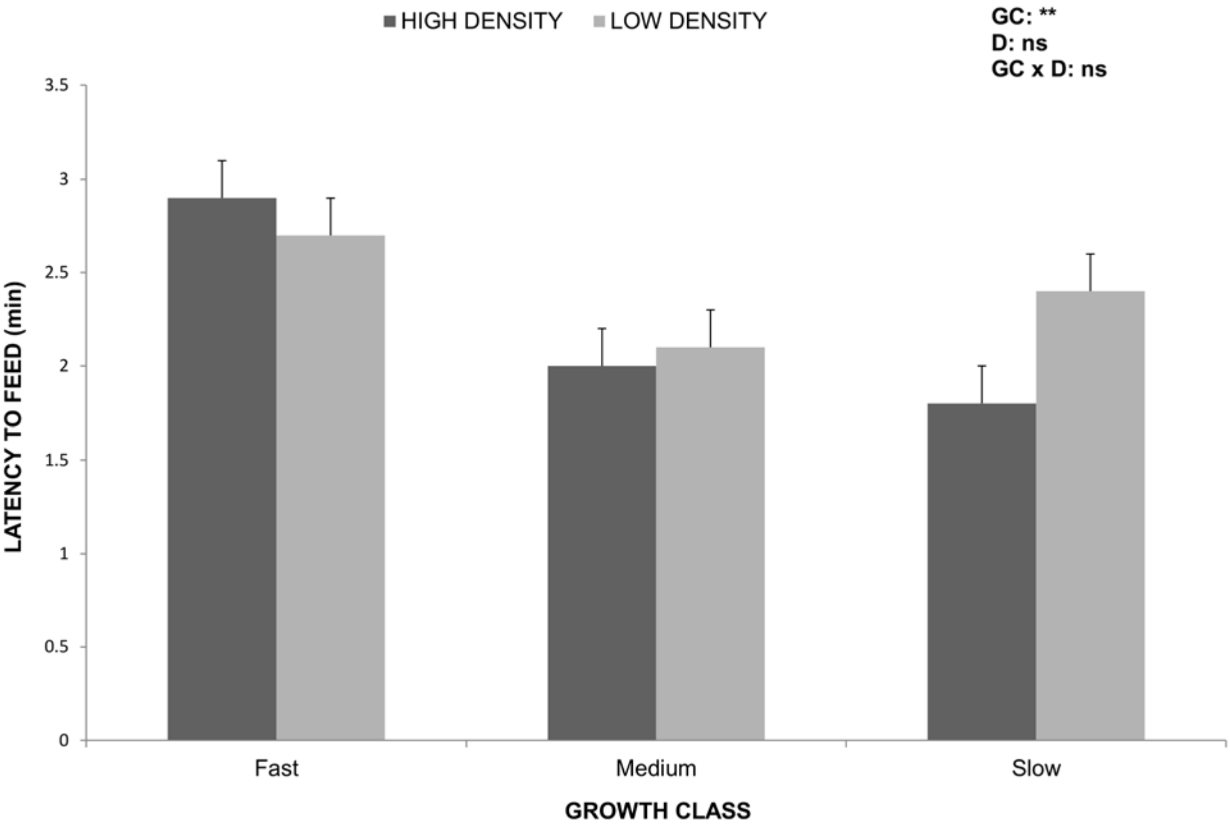

Fig. 2. Latency to feed (min) of the different growth classes of sole ("fast", "medium" and "slow") stocked at a "high" or "low" density and housed in barren bottom tanks (no sand). Effects of growth class (GC), stocking density (D) and their interaction (GCxD) are indicated: ***P*<0.01; **P*<0.05; ns not significant; and error bars represent SEM.

4. *Effect of growth class and stocking density on non- feeding behaviour*

The activity of sole housed in barren bottom tanks was lower at the "low" stocking density compared to the "high" stocking density ($P<0.05$, Table 5). Both the proportion of fish taking off' (0.3 vs. 0.4) and the proportion of fish burying (0.6 vs. 0.8) were lower at the "low" stocking density than at the "high" stocking density. Moreover we found an interaction effect between stocking density and growth class in the mean number of burying attempts per fish ($P<0.05$, Table 5). "Slow" class sole when stocked at the "low" density displayed a lower number of bury attempts per fish than when stocked at the "high" density (1.7 vs. 6.4).

Table 5. Effect of growth class (“fast”, “medium” and “slow”), density (“high” vs. “low”) and their interaction on non-feeding behaviour of sole (*Solea solea)* when housed in barren bottom tanks (no sand).

Density	HIGH			LOW				P-value[a]		
Growth class	Fast	Medium	Slow	Fast	Medium	Slow	SEM	GC	D	GCxD
No bury /fish (#)	2.8	4.5	6.4	3.6	2.5	1.7	0.8	ns	**	*
Prop fish burying	0.7	0.8	0.9	0.7	0.6	0.6	0.1	ns	*	ns
No take offs/fish (#)	1.7	2.0	1.9	1.0	1.2	1.7	0.4	ns	ns	ns
Prop fish take offs	0.4	0.4	0.5	0.3	0.3	0.3	0.1	ns	*	ns
Surf. swim/ fish (min)	1.6	2.7	2.6	1.5	2.0	1.1	1.1	ns	ns	ns
Prop fish surf. swim.	0.2	0.2	0.2	0.2	0.2	0.1	0.1	ns	ns	ns

Values are given as means ± SEM.

[a] Effects of growth class (GC), stocking density (D) and their interaction (GCxD) are indicated: ****P*<0.001; ***P*<0.01; **P*<0.05; *ns*=not significant.

In the presence of substrate (period 2)

1. *Effect of growth class and stocking density on feed intake, feed efficiency and growth*

No differences were found in mean growth and feed intake (g/kg$^{0.8}$/d) between three growth classes of sole: “fast”, “medium” and “slow” during period 2 when sole were housed in tanks with substrate (*P*<0.1, Table 6). The effect of stocking density on FCR and on individual variation in growth was not significant when sand was present (*P*>0.1, Table 6). However, growth class did have a significant effect on the coefficient of variation in growth within tank when sand was present (*P*<0.05, Table 6). “Slow” growth class fish showed a higher variation in growth compared to “medium and “fast” growers (82 % versus 41 %, Table 6).

Table 6. Effect of growth class ("fast", "medium" and "slow"), density ("high" vs. "low") and their interaction on performance of sole (*Solea solea*) when housed in tanks provided with substrate.

Density	HIGH			LOW				P-value[b]		
Growth class	Fast	Medium	Slow	Fast	Medium	Slow	SEM	GC	D	GCxD
Initial BW[a] (g)	172.0	136.8	111.8	181.0	147.0	113.4	5.8	***	ns	ns
Final BW[a] (g)	186.8	152.5	123.2	203.4	170.9	124.8	7.6	***	ns	ns
FI[a] (g/fish/d)	0.70	0.66	0.53	0.86	0.82	0.45	0.08	**	ns	ns
FI[a] (g/kg$^{0.8}$/d)	2.8	3.1	2.9	3.2	3.6	2.5	0.3	ns	ns	ns
FCR[a]	1.5	1.3	2.1	1.2	1.0	1.2	0.4	ns	ns	ns
GR[a] (g/kg$^{0.8}$/d)	2.8	3.1	2.9	3.2	3.6	2.5	0.4	ns	ns	ns
CV-GR[a] (%)	46.2	46.6	99.1	36.4	36.2	64.6	13.5	*	ns	ns

Values are given as means ± SEM.

[a] BW=Body weight; FI=Feed intake; FCR= Feed conversion ratio; GR= growth; CV-GR= coefficient of variation in growth within tank.

[b] Effects of growth class (GC), stocking density (D) and their interaction (GCxD) are indicated: ***P<0.001; **P<0.01; *P<0.05; ns not significant.

2. *Effect of sorting on growth*

The change in growth (%) after sorting was unaffected by stocking density (P > 0.05) but was affected by growth class (P<0.05, Fig. 3), during the period when sand was present. "Fast", "medium" and "slow" class sole showed a change in growth of respectively, -33±23%, 17±23% and 76±23%, when housed in tanks with substrate.

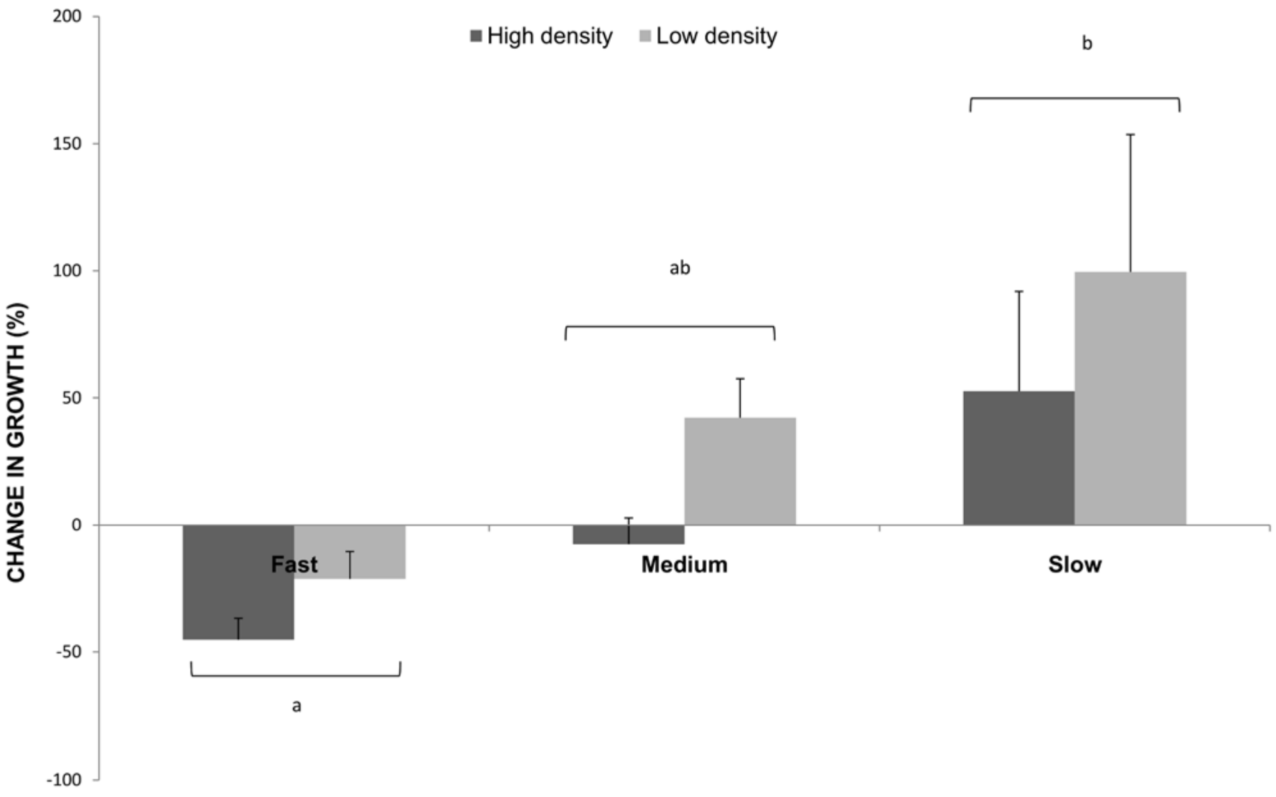

Fig. 3. Change in growth (%) after sorting of sole housed in tanks provided with substrate. Expressed as the difference in growth between the growth of fish housed in homogenous groups in tanks with sand, and their previous growth when kept in a heterogeneous group. Means lacking a common script (a, b) differ significantly (*P*<0.05).

3. *Effect of growth class and stocking density on feeding behaviour*

Contrary to what was found when sole were deprived of substrate (Fig. 2), the average latency to feed the first pellet for sole housed in tanks with substrate was affected by stocking density (*P*<0.01) but not by growth class (*P*>0.05, Fig. 4). Fish at the “low” stocking density showed a higher latency to start feeding (2.8 ± 0.17 min) compared to the “high” stocking density (2.0 ± 0.17 min). No differences were found in the proportion of fish eating (data not shown) during the first 7 minutes of observation between the “low” and “high” density (0.57 ± 0.05 vs. 0.46 ± 0.05, respectively; *P*>0.05) or between “fast”, “medium” and “slow” class of sole (0.60, 0.51 and 0.42, respectively; *P*>0.05).

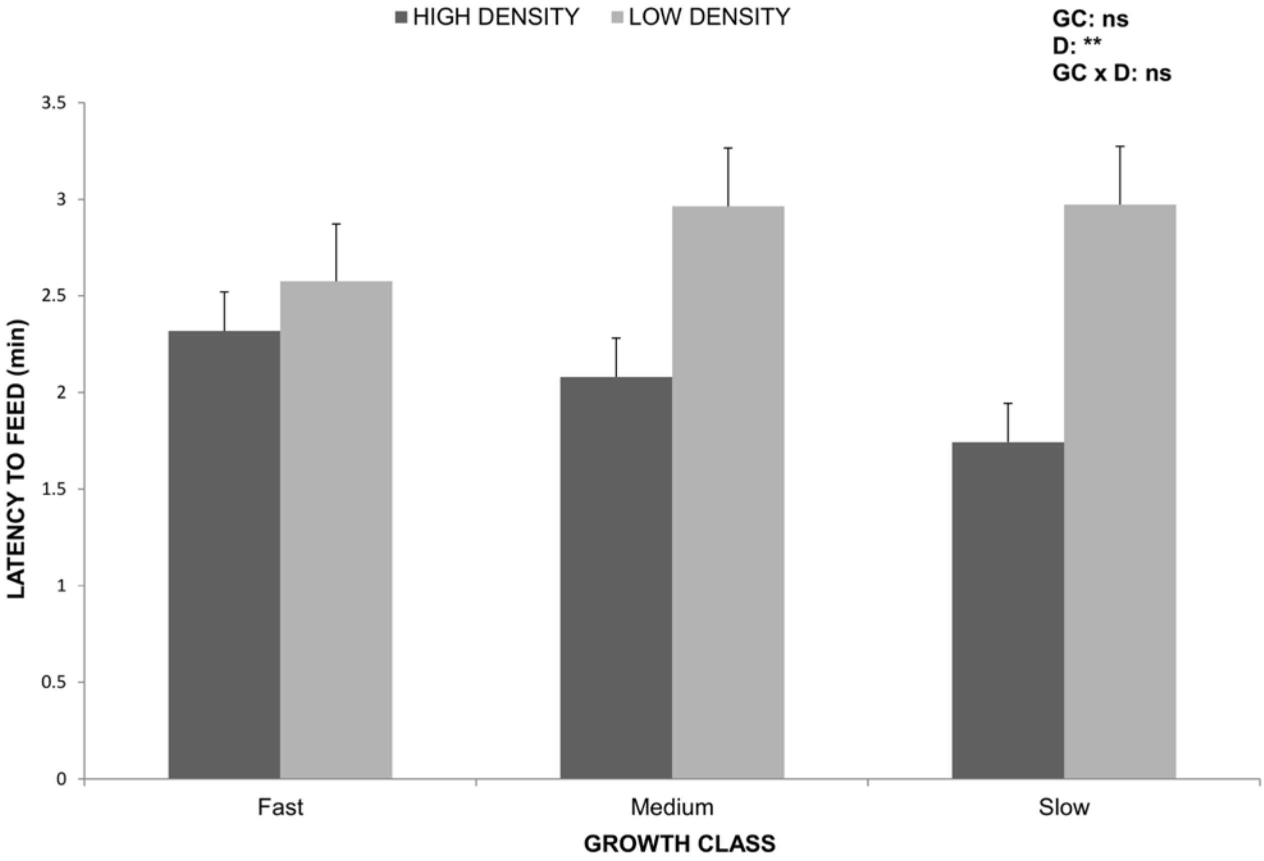

Fig. 4. Latency to feed (min) of the different growth classes of sole ("fast", "medium" and "slow") stocked at a "high" or "low" density and housed in tanks provided with substrate. Effects of growth class (GC), stocking density (D) and their interaction (GCxD) are indicated: $^{**}P<0.01$; ns not significant; and error bars represent SEM.

4. *Effect of growth class and stocking density on non- feeding behaviour*

No significant differences between treatments were found for the activity of sole housed in the tanks provided with substrate: number of take offs, time buried in sand or surface swimming ($P>0.1$, Table 7). Stocking density only had a significant effect on the proportion of fish burying ($P<0.05$, Table 7), which was higher at the "high" stocking density compared to the "low" stocking density (0.5 vs. 0.3, respectively) when substrate was present.

Table 7. Effect of growth class ("fast", "medium" and "slow"), density ("high" vs. "low") and their interaction on non-feeding behaviour of sole (*Solea solea*) when housed in tanks provided with substrate.

Density	HIGH			LOW				P-value[a]		
Growth class	Fast	Medium	Slow	Fast	Medium	Slow	SEM	GC	D	GCxD
No bury /fish (#)	4.5	2.9	3.3	1.5	0.9	0.9	1.4	ns	ns	ns
Prop fish burying	0.5	0.4	0.5	0.3	0.3	0.2	0.1	ns	*	ns
Time buried (min)	3.9	5.4	4.0	5.7	4.6	6.9	1.4	ns	ns	ns
No take offs/fish (#)	1.7	1.1	1.5	1.3	1.4	1.3	0.4	ns	ns	ns
Prop fish take offs	0.4	0.3	0.4	0.3	0.3	0.3	0.1	ns	ns	ns
Surf. swim/ fish (min)	0.7	0.6	0.8	0.5	0.3	0.2	0.3	ns	ns	ns
Prop fish surf. swim.	0.1	0.1	0.1	0.1	0.03	0.03	0.03	ns	ns	ns

Values are given as means ± SEM.

[a] Effects of growth class (GC), stocking density (D) and their interaction (GCxD) are indicated: ****P*<0.001; ***P*<0.01; **P*<0.05; ns not significant.

Discussion

This study shows that social interactions are an important factor influencing the inter-individual variation in growth of sole (*Solea Solea*). Growth variation increased with stocking density when sole were deprived of substrate, indicating that behavioural interactions at high stocking densities influence growth. However, mean feed intake (in g/d and in g/kg$^{0.8}$/d) and growth (g/kg$^{0.8}$/d) of sorted fish were not significantly different between "low" (1.3 kg/m^2 or 15 % initial bottom coverage) and "high" (3.4 kg/m^2 or 40 % initial bottom coverage) stocking density. Other studies in *Solea solea* also found an effect on growth variation (Howell, 1998; Schram et al., 2006) and on mean growth (%BW/d) with increasing stocking densities from 0.5-12 kg/m^2 (Schram et al., 2006) and 1.3-2.3 kg/m^2 (Piccolo et al., 2008). However mean growth and size dispersal of *Solea senegalensis* was not significantly different for stocking densities up to 45 kg/m^2 (Salas-Leiton et al., 2008; Sánchez et al., 2010). Studies in other flatfish species, like Atlantic halibut, *Hippoglossus hippoglussus* L. (Kristiansen et al., 2004) , California halibut, *Paralichthys californicus* and turbot, *Scophthalmus maximus* (Irwin et al., 1999) and *Psetta maxima* (Aksungur et al., 2007) also found that high stocking densities can negatively affect mean growth and/or size variation of fish. In pelagic species, such as Atlantic cod, *Gadus morhua* (Lambert and Dutil, 2001) and rainbow trout, *Oncorrhynchus mykiss* (Boujard et al., 2002; Holm et al., 1990) size dispersal also increases with stocking density.

The swimming activity and bury attempts of sole housed in the absence of substrate, increase when reared at a “high” compared to a “low” stocking density. Howell and Canario (1987) also reported higher activity and burying attempts in sole kept without substrate versus with substrate, although data were not shown. Studies in Altantic halibut (Kristiansen et al., 2004) and in burbot (*Lota lota*) (Wocher et al., 2011) also observed an increase in swimming activity at high stocking densities. Therefore behavioural changes related to group density might express a physical or social stress state of the fish, and thus impaired welfare. This hypothesis is corroborated by the high variation in plasma cortisol levels and in free amino acids (FAAs) found in *Solea senegalensis* exposed to high stocking densities (Costas et al., 2008; Salas-Leiton et al., 2010a). Therefore it seems that high stocking density can generate chronic stress in fish (Pickering, 1993). Further, the high levels of social interactions may increase energetic costs due to higher maintenance levels used in locomotion and/or related to social stress (Jobling and Wandsvik, 1983; Koebele, 1985; Volpato and Fernandes, 1994). This might explain the higher FCR found for sole reared at the “high” stocking density compared to the “low” stocking density (in the absence of sand). The results of Salas-Leiton et al. (2008) who found higher levels of oxygen consumption with increasing density in *Solea senegalensis* and of Piccolo et al. (2008) who found less favourable FCR for *Solea Solea* stocked at high densities, support the current data.

Social interactions through competition for food and/or space can increase feeding disturbances and/or competitive relationships between sole when stocked at high stocking densities (Howell, 1998; Schram et al., 2006). In social hierarchical structures, prominent dominance-subordinate relationships may lead to disproportional food acquisition. Therefore higher inter-individual variation in growth might be the result of growth suppression of subordinate individuals by larger conspecifics (Barki et al., 2000; Koebele, 1985). In the present study we found that “slow” growth class of sole increased 53% of its mean growth from 1.5 to 2.9 (g/kg$^{0.8}$/d) after sorting. In contrast “fast” class of sole decreased 41 % their growth from 3.7 to 3.2 (g/kg$^{0.8}$/d), whereas “medium” growers did not show a significant change in growth after sorting. Therefore it seems that individual growth is being regulated by the population structure. For example, Mas-Muñoz et al. (2011) reported that when *Solea solea* were taken from a large population and held in isolation “slow” growers showed a marked growth recovery which decreased growth variation. Moreover, similar results were found in *Solea senegalensis* (Salas-Leiton et al., 2011) and in *Solea solea* (Overton et al., 2010) with similar mean growth (% BW/d) between the different size/growth sorted classes ("large", "medium" and "small") and where overall growth was not improved when compared to an unsorted group of sole. Sorting had similar effects on Arctic char (Jobling and Reinsnes, 1987), turbot (Strand and Oiestad, 1997), silver perch (Barki et al., 2000), brown trout (Brown, 1946) and Atlantic salmon (Maclean and Metcalfe, 2001). In line with this, we found that "slow" class sole in the absence of "fast" growers showed a shorter latency to eat compared to the "fast" growth class of sole. This suggests that "slow" fish might occupy a subordinate position in the hierarchical structure and thus exhibit improved growth once hierarchies are disrupted and new interactions appear between them. Whereas "fast" sole might be dominant over food supply and/or suppress appetite of subordinates, discouraging feeding behaviour of other conspecifics (Koebele, 1985). Moreover, we observed a significant interaction effect between stocking density and growth class in the mean number of burying attempts per fish. "Slow" class sole increased their number of bury attempts in barren bottom tanks at high stocking densities. However no differences in burying behaviour were found for "medium" and "fast" class sole for the different stocking densities. This seems to suggest that the "slow" growth class of sole have a lower tolerance for social interactions and thereby grow slower in a heterogeneous group. Individual behavioural differences or coping styles ("bold" vs. "shy") differ in their tolerance for crowding and for social interactions and thus may determine their social status in a hierarchical structure ("dominant" vs. "subordinate") , which can influence their feed intake and growth (Kristiansen and Fernö, 2007; Metcalfe, 1986; Ruzzante and Doyle, 1990; Ruzzante and Doyle, 1992). Accordingly in other studies with *Solea solea,* we found that "shy" fish being less active and showing longer latency time to explore a novel environment grew slower (Mas-Muñoz et al., 2011). In *Solea senegalensis* "shy" fish had higher cortisol levels after an acute net stressor and displayed longer latencies to feed (Silva et al., 2010). In halibut, *Hippoglossus hippoglussus* L. "shy" fish performed more surface swimming (stereotypic behaviour) and reduced feed intake, showing less tolerance to changes in feeding strategies and in stocking densities (Kristiansen and Fernö, 2007; Kristiansen et al., 2004).

The presence of substrate in flatfish, reduces the chances for social interactions and/or stress related with crowding as fish can bury themselves in sand. This environmental feature influences swimming activity and feeding behaviour of sole (*Solea solea*). Fish in the presence of substrate spent most of their time buried and thus no differences in swimming activity were found between stocking densities. Although, sole displayed longer feeding latencies at the “high” stocking density compared to the “low” stocking density, no differences in mean growth or variation in growth were found. A recent study also found that shelter availability did not affect growth, but did influence swimming activity and feeding behaviour of burbot (*Lota lota*) (Wocher et al., 2011). We also found a significant effect of growth class on the variation in growth when sole were provided with substrate. “Slow” class sole showed a larger variation in growth compared to “medium” and “fast” growers. This seems to suggest that “slow” growers with a “shy” coping style display a higher motivation to bury in sand which might result in less feeding behaviour. Thereby “slow” class of sole displayed a higher variation in growth compared to “medium” or “large” sole. In a previous study we also showed that “shy” sole with a higher motivation to bury grow slower (Mas-Muñoz et al., submitted).

Conclusions

This study showed that social interactions and/or associated hierarchies are important factors influencing behaviour and growth variation of sole (*Solea solea).* Sorting sole by growth rate, improves growth of “slow” sole and reduces growth of “fast” sole, which is suggested to relate to behavioural coping style and indicates the presence of hierarchical structures in sole. High stocking density in tanks without substrate promotes fish-fish interactions which increases swimming activity, FCR and variation in growth of sole. Therefore high stocking density in barren conditions seem to induce social stress in sole, which is alleviated when sand is provided.

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CHAPTER 5

Rearing environment influences behaviour and its relation with growth in sole (*Solea solea*)

Julia Mas-Muñoz, Hans Komen, Robbert Blonk, Johan Verreth, Johan W. Schrama

Submitted

Abstract

Consistent individual variation in behavioural traits have been explained by associations with growth-mortality trade-offs. However, it remains unclear how behavioural traits are affected by rearing environment, and if associations between behavioural traits and growth vary between environments. In this study, the influence of rearing environment on behavioural responses to novelty and growth and the relationship between behavioural responses and growth in offspring originating from wild sole (*Solea solea*) was assessed. Environmental factors which differ between nature and commercial farming conditions, such as food type (live versus dry food), sand (presence versus absence) and variability of environment (indoor vs. outdoor) were studied. All fish originated from a commercial farm and were kept indoor in barren tanks and fed with pellets prior to the experiment. A novel behavioural test was done individually to all fish before and after being reared in different environments for a period of 6 weeks. Environmental conditions (i.e., light, photoperiod and temperature) present in an outdoor system compared to a controlled indoor system influenced mean growth (3.7 vs. 5.2 g/kg$^{0.8}$/d, respectively). Moreover, this effect was more pronounced when fish were fed with live ragworms, indicated by the interaction effect between feed type and variability in environmental conditions. Regardless, none of the studied environmental rearing factors affected individual variation in growth of sole (CV, %). Swimming activity of sole in a novelty test was influenced by rearing environment. Rearing fish with sand or outdoor variable environmental conditions changes behavioural responses to a novel environment test. Consistent associations between individual behavioural responses to novelty and growth were only present for fish reared in barren and controlled conditions. These results suggest that stable relationships between behaviour and growth can develop when fish are reared in homogeneous or plain environments, but not when fish experience more heterogeneous or natural environments.

Keywords: Environment, enrichment, captivity, nature, novel environment, activity, boldness, personality, behavioural flexibility, fitness, trade-offs, behaviour-growth association

Introduction

In a wide variety of animal taxa, variation between individuals in personality or boldness (i.e., risk-taking behaviour and activity towards novelty or challenge situations) has been reported (Bell and Sih, 2007; Dingemanse et al., 2012; Gosling, 2001 ; Koolhaas et al., 1999; Wilson et al., 1994a; Wilson et al., 1993). Sole (*Solea solea*) is a flatfish species which spends long times buried in the sediment, shows nocturnal foraging and a low diurnal activity level, probably to reduce predation risk (Burrows and Gibson, 1995; De Groot, 1971; Kruuk, 1963). The potential role of risk-taking behaviour on feeding behaviour and thus growth suggests an influence of environmental rearing factors. In flatfish, it is known that physical variables, especially light, temperature, sediment, feeding conditions and feed type, can markedly modify their behaviour and growth (De Groot, 1969; De Groot, 1971; Gibson, 1973, 1997; Imsland et al., 2003; Kristiansen et al., 2004; Reig et al., 2010). Literature on the development of animal personality suggests that individual behavioural responses are plastic and thus can be affected by prior experiences and environmental conditions at any stage in life (Braithwaite and Salvanes, 2005; Frost et al., 2007; Stamps and Groothuis, 2010). For instance, Alvarez and Bell (2007) found differences in the risk-taking behaviour (feeding behaviour under predation risk) of sticklebacks depending on the habitat of origin (stream vs. pond). In line with this, correlations between boldness and foraging behaviour in squid seem to differ over time and between different demographic populations (Sinn et al., 2010). Alvarez and Nicieza (2005) showed that the metabolic rate in brown trout (*Salmo trutta*) was positively correlated with individual growth rate in captivity, but negative or no correlation was found in natural streams. Similarly, Adriaenssens and Johnsson (2009, 2011, 2013) suggested that correlations between personality traits and growth in brown trout may differ between environments, being more stable in captivity but more variable in the wild.

Fish in captivity are reared under very different environmental conditions compared to nature. For instance, the physical environment in captivity is plain and available resources such as shelter, space are absent/restricted and food type is different and ready available. Moreover, social environment is altered by: the absence of predation pressure; the presence of relatively high fish densities which lead to increased social (fish-fish) interactions; and feeding schemes that promote competition (i.e., localized and predictable food delivery) (Huntingford, 2004). Since culture conditions in farms are more homogeneous or stable, more stable correlations between behavioural strategies and growth may emerge (Huntingford, 2004).

In contrast, in the wild, heterogeneous or variable environmental experiences, may generate behavioural flexibility and thus result in complex interactions with different behavioural strategies and growth payoffs (Adriaenssens and Johnsson, 2009; Braithwaite and Salvanes, 2005; Huntingford, 2004; Magnhagen and Borcherding, 2008). It is hypothesized that individual differences in risk-taking behaviour and activity of sole may determine individual variation in feed intake and thereby in growth, depending on the predictability/variability of the environment. Therefore, the current study assessed the influence of environmental rearing factors, which differ between natural and extensive or intensive culture systems (such as food type, live vs. dry food; sand, presence vs. absence; and variability of environmental conditions, variable vs. constant light and temperature), on individual behavioural responses to novelty, on growth and on the relationship between individual behavioural responses and growth in common sole.

Materials and methods

Ethics

This study was performed from October to December 2010. All procedures involving animals were conducted in accordance with the Dutch law on experimental animals, which complies with the ETS123 (council of Europe 1985) and the 86/609/EEC directive. The Animal Care and Use Committee for Animal Experiments (DEC) of Lelystad in The Netherlands approved the experiment (2010085.b).

Animals

Juvenile sole (*Solea solea, n*=480, not selected for sex) with an initial weight of 54 ± 9g (mean ± SD) were obtained from a commercial farm (Solea BV, IJmuiden, The Netherlands). Fish were produced by natural mating of wild broodstock and were reared in captivity under intensive recirculating aquaculture conditions prior to the experiment. Upon arrival at the experimental facilities all fish were treated with formaline (100 ppm). One week later, fish were individually tagged (Trovan ID100A: 2.12x11.5mm, DORSET GP, Aalten, The Netherlands), and weighed while anesthetized using a 2-phenoxyethanol solution (1ml /l of system water for minimally 0.5 min). After tagging, fish were allowed to adapt to the indoor experimental facilities for another two weeks.

Experimental design

The following environmental factors were assessed: 1) substrate, a barren bottom (B) or a sandy bottom (S); 2) feed type, live ragworms (R) or dry pelleted feed (P); 3) variability in environmental conditions, outdoor/natural or indoor/constant rearing conditions. The experiment was set up as an incomplete 2 by 2 by 2 factorial design (Fig. 1). Sole are limited in their ability to capture and swallow ragworm on a smooth surface (i.e., barren bottom) and ragworms in a tank without sand cluster together in one large ball (personal observations). It was therefore expected that feed intake would be strongly reduced at the treatments BR (sole fed live ragworms in tank with a barren bottom). These were therefore excluded (both indoor as outdoor). Moreover, BR conditions are less relevant in nature and extensive culture conditions, where sole predominantly captures live prey, which is being buried in the sand.

The experiment was run with four replicate tanks per treatment. Each tank contained 20 fish. To ensure that tanks at all treatments at the start of the experiment had the same body weight distribution (similar mean and within tank variation, CV 17%), fish were classified into four different weight classes. Fish within weight classes were then randomly assigned to one of the 6 combinations of treatments studied: SR, SP, BP in outdoor and SR, SP, BP in indoor conditions (Fig. 1). The total duration of the experiment was 6 weeks (growth period of 40 days). Individual fish growth and the coefficient of variation of growth within tank was assessed.

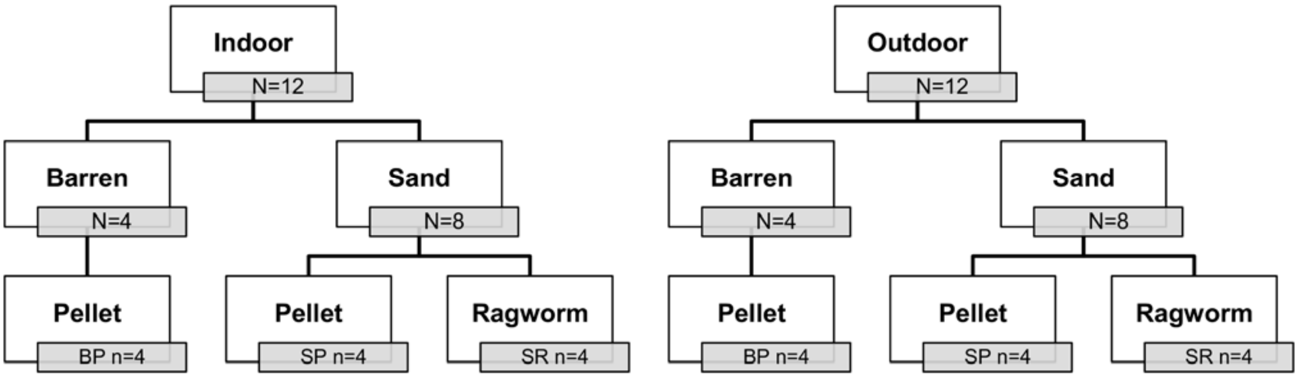

Fig. 1. Experimental setup. Treatments: BP= sole kept in barren tanks and fed with pellets; SP= sole kept in tanks enriched with sand and fed with pellets; SR= sole kept in tanks enriched with sand and fed live ragworms. Sole were reared in either an outdoor or an indoor system (i.e., variable vs. controlled regimes in light, photoperiod and temperature). *n*=number of experimental units (4 tanks per treatment).

Rearing environments

1. *Environmental conditions*

At the farm of origin (prior to the experiment), fish were reared indoors under intensive recirculating aquaculture conditions (RAS) in barren shallow raceway tanks, with constant temperature (18-23°C) and photoperiod (24L:0D, 50 LUX). At this farm, fish were fed the same commercial diet as during the experiment (i.e., Weanex-3000; 3 mm dry pellets).

For the experiment, two identical RAS systems were used: one for fish reared indoors and one for fish reared outdoors. Each system consisted of: 12 fish tanks; a buffer tank (565L); a water treatment unit including mechanical filtration (sand filter), biological filtration (trickling filter) and bacterial disinfection (UV) unit. Tanks dimensions were 120 x 80 x 85 cm (L x W x H) filled with 565 litres of system water. Water flow rate over each tank was set at 5 l/min. All tanks were provided with one air stone for extra aeration. Water pH (7.5 -8.4), dissolved O_2 (>7 mg/l), salinity (32 ± 0.3 ‰), NH_4^+ (<1 mg/ l) NO_2^- (<1 mg/ l) and NO_3^- (<50 mg/l) were monitored daily.

Outdoor tanks were exposed to natural variations of light and temperature. Temperatures were 15.9 ± 1.5 °C (mean ± SD), ranging from 18°C to 9 °C. To prevent fish from not feeding, water temperature was kept above 8°C by a heating system. Indoor tanks were kept under controlled environmental conditions using artificial fluorescent lights, constant photoperiod 12D:12L (with lights on from 08.00h to 20.00h) and constant temperatures of 19.2 ± 0.5 °C (mean ± SD), ranging from 20 to 17 °C.

2. *Substrate*

Tanks had either a barren bottom with no substrate (BP tanks) or a layer of 7 cm sand (1.25-2 mm grain size) (SP and SR tanks).

3. *Feeding regimes*

Fish were fed during the dark phase of the day as sole is a nocturnal feeder (Lagardère, 1987). Fish were fed restrictively to 0.8 %BW/day (on dry matter basis) with one of two different feed types: a commercial dry pelleted feed or live ragworms.

Dry commercial pelleted feed, (Weanex-3000 3mm sinking pellet; BioMar, Nersac, France) contained on dry matter basis, 68% protein, 16% fat and 22 kJ/g energy. Feed was supplied using an automatic feeding belt continuously for 12h per day from 19.00h to 07.00h.

Live ragworms (*Nereis virens*) were given to the tanks once a day by hand. The individual weight of ragworms ranged from 0.5g -2.0g. Ragworms contained (on dry matter basis) 67% protein, 9% fat and 21 kJ/g energy and were supplied by a commercial ragworm producer (Topsy Baits, Wilhelminadorp, The Netherlands). Ragworms were stored in a flow through system for no longer than 3 days till consumption.

Dry matter and ash free dry matter content of both diets were determined bi-weekly according to ISO standard 6496 and ISO standard 5984 respectively. Samples of ragworms and pelleted diet were taken daily as the percentage of total amount of feed given in the experiment and stored at -20°C, pooled after termination of the experiment and subsequently analyzed for proximate composition. Diets where analysed for crude protein (Kjeldahl method), ash (gravimetrically 550°C incineration), crude fat (gravimetrically after extraction with petroleum ether). Gross energy was calculated based on analyses of the macronutrient contents, using energy contents of carbohydrate, protein, and lipid, respectively.

Behavioural test

To assess if variation in individual behavioural responses was related to variation in growth and if that relation was affected by rearing environment, a novel environment test was performed to individual fish at the beginning (before exposure to experimental rearing environment) and at the end of the experiment (after exposure to experimental rearing environment). Individual tests were done on 8 fish per tank, so in total 32 fish per treatment were tested. The novel environment consisted of a white plastic circular tank (Ø=63 cm and 37cm height) with a barren bottom and connected to a flow through system. The swimming activity of fish in the novel environment was assessed as the total time spent swimming (in seconds) throughout the first 10 min after introduction into this tank by direct observations.

Data analysis

Overall growth performance was analysed using tank as the experimental unit, whereas the individual behavioural response to the novel environment test and its relationship to individual growth was analysed using fish as the experimental unit.

Growth rate (GR) was expressed per metabolic body weight in units of g BW/kg$^{0.8}$ /d, as:

$$GR = \frac{(W2 - W1)}{T} \bigg/ \left(\frac{(W1/1000)^{0.8} + (W2/1000)^{0.8}}{2} \right)$$

, where W1 is the weight (g) at the

beginning of each growth period, W2 the weight (g) at the end of each growth period and T is the duration in days of the growth period. Coefficient of variation (CV, %) in growth was calculated as $CV=\left(\frac{\sigma}{\mu}\right)*100$, where σ is the standard deviation of all fish within tank and μ the observation mean in each tank.

Statistical calculations were made using SAS system (SAS Inst. Inc., Cary, NC). Behavioural data were transformed by a square root transformation to comply with ANOVA assumptions (homogeneity of variances and normality of residuals). Data were analysed using a three-way analysis of variance (ANOVA) followed by the Tukey's HSD post hoc test. Since this study was an incomplete factorial design, contrasts were used to test for main effects and interaction effects: the effects of feed type, environment and their interaction were tested within sole kept in tanks with sand; the effects of substrate, environment and their interaction were tested within sole fed the pelleted diet. Spearman's correlations were used to relate individual behavioural responses to a novel environment with individual growth.

Results

Effects of rearing environment at tank level on growth

Average growth of sole was influenced by environmental conditions (i.e., light, photoperiod and temperature). Fish reared in the outdoor system showed a lower growth compared to fish reared in the indoor system (3.7 ± 0.1 vs. 5.2 ± 0.1g/kg$^{0.8}$/d, respectively, $P<0.001$, Fig. 2 panel A and C). Moreover, the effect of feed type differed between systems: in indoor systems, fish fed with live ragworms tended to grow faster compared to fish fed with dry feed; however, in outdoor systems, no differences in growth were found between dry and live feed (FEED × ENV, $P<0.05$, Fig. 2 panel C). On the other hand, the presence of sand had no effect on mean growth rate per tank ($P>0.05$, Fig. 2 Panel A). The within CV in growth between the different treatments, ranged from 30 to 34%. Despite the various effects on mean growth, the within-tank variation in growth of sole was unaffected by the imposed treatments ($P>0.1$, Fig. 2 panel B and D).

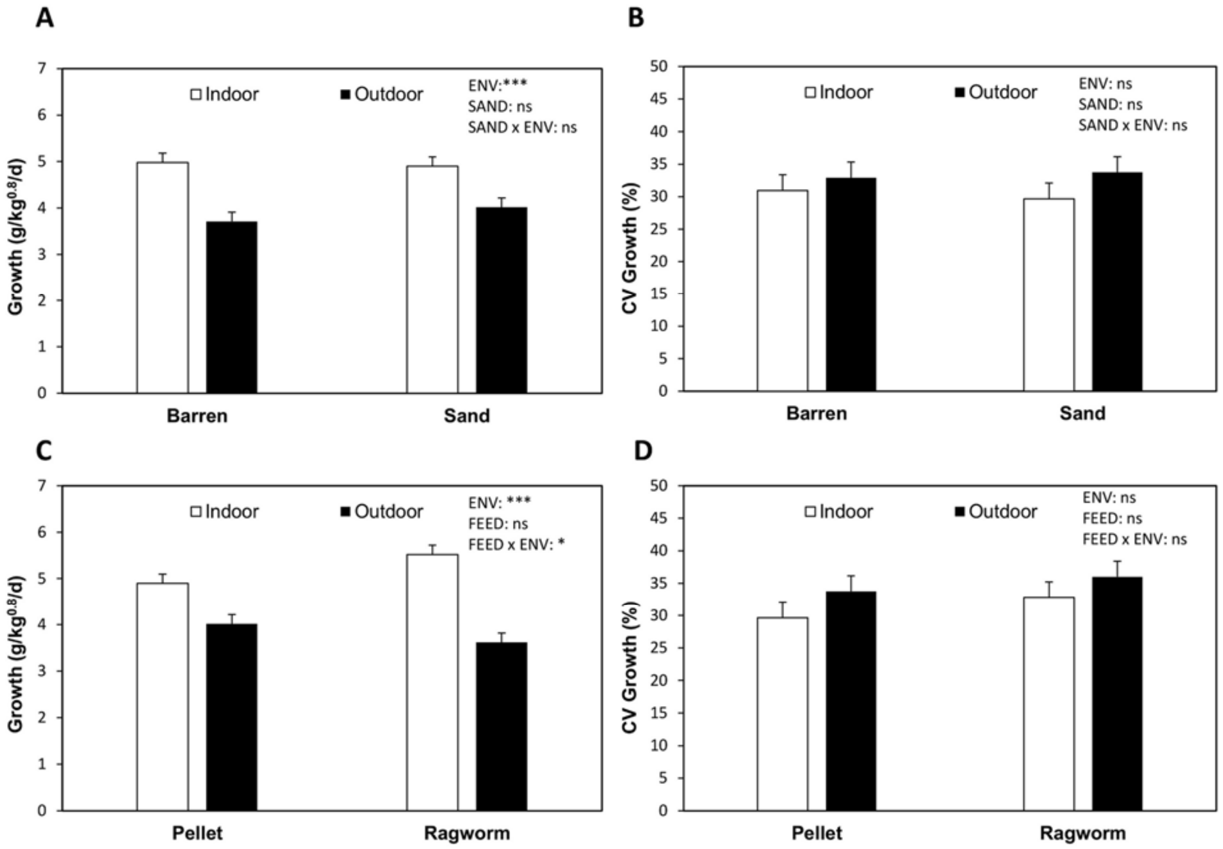

Fig. 2. Effects of rearing environment on mean growth and on the coefficient of variation in growth of sole. The effect of environment (ENV; indoor vs. outdoor) and substrate (SAND; barren vs. sand) on mean growth (A) and on variation in growth (B) being tested in sole fed pellet; The effect of environment (ENV; indoor vs. outdoor) and type of diet (FEED; pellet vs. live ragworms) on mean growth (C) and on variation in growth (D) being tested in sole kept in tanks with sand. Data are presented as averages ± S.E.M. ****P*<0.001; ***P*<0.01; **P*<0.05; ns not significant.

Effects of rearing environment on individual behavioural responses to a novel environment test

As expected, individual behavioural responses to a novel environment before exposure to the experimental rearing environments were not different between treatments (Fig. 3, Panel A and C). However, swimming activity in a novel environment of fish tested at the end of the experiment was influenced by the experienced rearing environment (Fig. 3, Panel B and D). Overall fish reared in the indoor system were more active compared to fish reared in the outdoor system (*P*<0.01, Fig. 3 panel B and D). Moreover, fish reared in tanks with sand displayed more swimming activity during the novel environment test compared to fish reared in tanks with a barren bottom (*P*<0.001, Fig. 3 panel B). We found a significant interaction effect between substrate and environment (SAND × ENV; *P*<0.05, Fig. 3 panel B). Swimming activity during the novel environment test was higher for fish reared on sandy bottom tanks in the indoor system compared to fish on sandy bottom tanks in the outdoor system.

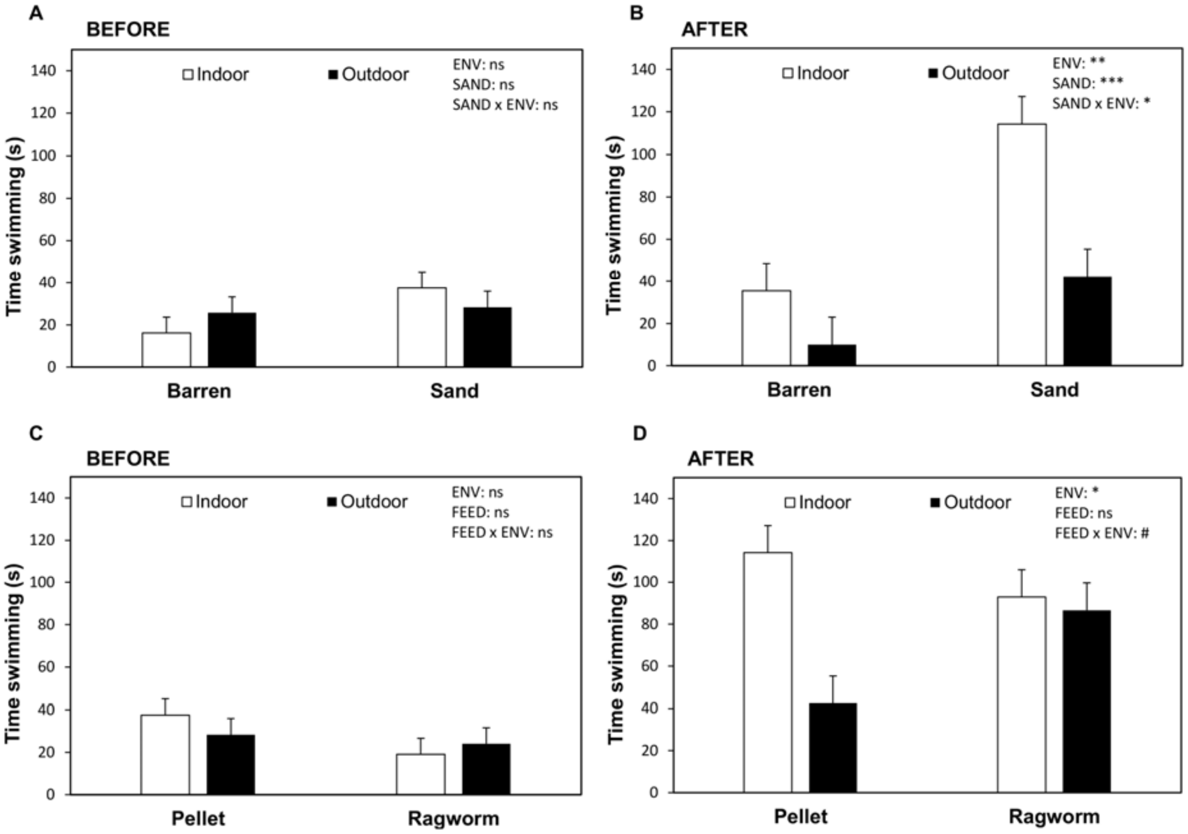

Fig. 3. Effects of rearing environment on the swimming activity during a novel environment test before and after exposure to different rearing environments. The effect of environment (ENV; indoor vs. outdoor) and substrate (SAND; barren vs. sand) on the swimming activity during a novel environment test done BEFORE (A) and AFTER (B) experimental rearing in sole fed pellet; The effect of environment (ENV; indoor vs. outdoor) and type of diet (FEED; pellet vs. live ragworms) on the swimming activity during a novel environment test done BEFORE (C) and AFTER (D) experimental rearing in sole kept in tanks with sand. Data are presented as averages ± S.E.M. ***$P<0.001$; **$P<0.01$; *$P<0.05$; #$P<0.10$; ns not significant.

Effects of rearing environment on the relation between individual behavioural responses and growth

Associations between individual behavioural responses to a novel environment and individual growth were influenced by rearing environment. Significant correlations between swimming activity in a novel environment and growth were only present for fish reared under similar conditions as prior to experiment, i.e., an indoor system with barren tanks and pellet feed (BP indoor, in Table 1). Spearman's correlations for BP indoor tanks were r= 0.46 (*P*<0.01) before and r= 0.35 (*P*<0.05), after the experimental period.

Table 1. Spearman's correlation between time swimming (sec) during a novel environment test and growth (GR, g/kg$^{0.8}$/d) during the experimental period. Sole were tested before and after exposure to different rearing environments (indoor vs. outdoor conditions; in barren vs. sand tanks; and with pellet vs. live ragworms diet).

	Indoor			**Outdoor**		
Spearman's correlations with GR	Barren Pellet	Sand Pellet	Sand Ragworm	Barren Pellet	Sand Pellet	Sand Ragworm
BEFORE time swim (sec)	**0.46****	-0.19ns	0.01ns	0.07ns	0.16ns	-0.06ns
AFTER time swim (sec)	**0.35***	0.03ns	0.20ns	0.08ns	0.20ns	-0.07ns

****P*<0.001; ***P*<0.01; **P*<0.05;ns not significant

Discussion

Variability in environmental conditions, such as temperature fluctuations, natural photoperiod and light intensity, which was present in the outdoor tanks in contrast to the indoor tanks, influenced final body weight and growth of sole. This effect was more pronounced when fish were fed with live ragworms, indicated by the interaction effect between feed type and variability in environmental conditions. This might suggest a reduction in the foraging behaviour of sole when searching for live prey, probably due to the lower temperatures during the night and high light intensities experienced during the day in the outdoor system. The presence of sand in tanks did not have any effect on mean growth within-tanks. A previous study showed that the absence of sand seems to increase the resting metabolic rate of sole (Howell and Canario, 1987). Moreover it seems that the presence of sand on the tank bottom can supress the increasing effect of high fish-fish interactions on within tank growth variation of sole induced by high stocking compared to tanks with barren bottom (Mas-Muñoz et al., submitted).

None of the studied environmental factors influenced within-tank variation in growth (CV, %) of sole, which ranged between 30 and 34 % for all environments. Like most fish species, sole display a pronounced variability in growth (Gjedrem, 2000). Growth variation in this study lies within values previously reported from 20% to 50% for the species *Solea solea* (Blonk et al., 2010a; Mas-Muñoz et al., 2011) and *Solea senegalensis* (Salas-Leiton et al., 2011; Sánchez et al., 2010). These results imply that individual differences in feeding behaviour and thereby of growth are maintained throughout the experimental period. The presence of novel environmental cues, such as substrate and live feed, to fish with no prior experience, does not seem to alter foraging ability or affect variation in growth of sole. Ellis et al. (1997) found that the motivation to bury in barren bottom tanks is the same for both reared and wild sole; although burying efficiency of hatchery reared sole was lower than that of wild sole at start, it increased to that of wild sole after a short period of 12 days of maintenance on sand (Ellis et al., 1997). Therefore, it seems that searching for live prey as well as burying behaviour are innate behaviours in sole, which are expressed naturally even with no prior experiences in enriched environmental conditions typically present in extensive culture conditions (i.e. ponds) or natural environments. A study on a nocturnal and benthic species burbot (*Lota lota* L.) showed that shelter availability did not affect growth nor on variation in growth, but reduced swimming activity and feeding behaviour of fish (Wocher et al.,.2011). Similarly, in the current study we found that the swimming activity of sole in the presence of substrate was reduced (data not shown).

In this study, stocking densities were kept equal in all rearing environments, and were relatively low compared to previously experienced farming conditions. Several studies in *Solea solea* (Howell, 1998; Schram et al., 2006), reported an increase in variation in growth when manipulating the social environment by increasing stocking densities. Therefore, for *Solea solea*, the social environment rather than the physical environment seem to be more important factors explaining growth variation.

Results from this study revealed interactions between environmental experiences and behavioural responses to novelty. Activity in a novel environment was influenced by rearing environment, which suggests that behavioural development in fish is influenced by prior experience. Sole reared in environments that most closely resembled to previous farming conditions in captivity (barren tanks, predictable feed and homogeneous conditions), responded consistently with low activity levels to a novel environment test before and after exposure to this environment. In contrast, fish which were kept outdoors with variable environmental conditions (light, photoperiod and temperature), or indoors but with enrichment (substrate and live feed) responded differently to a novel environment test before and after exposure to the environment. Overall, fish were more active in a novel environment when reared in an enriched environment containing sand and/or in variable environmental conditions. In line with this, a study on juvenile cod, which experienced environmental variability in food and in space (enrichment with rocks and pebbles), also showed lower latency to explore a novel environment, faster recovery of opercula rate after simulated predator attack and lower latency to eat live prey (Braithwaite and Salvanes, 2005). Salmonids seem to adjust aggression and activity over time, responding to the resources and risks they experience in their habitat (Frost et al., 2007). A study on zebrafish also showed differences in the exploratory activity in a novel environment after a short period of rearing in dark or light environments (Rosemberg et al., 2011). In brown trout, activity in a novel environment increased after a 2 months period in the wild, where new behavioural correlations appeared between activity, aggressiveness and exploration of a novel object (Adriaenssens and Johnsson, 2013). In line with this, other studies also reported the effects of environmental variability and experiences on individual behavioural responses (Coleman and Wilson, 1998; Dingemanse et al., 2010; Magnhagen and Borcherding, 2008; Stamps and Groothuis, 2010; van Oers et al., 2005; Varty et al., 2000). Furthermore, previous work also demonstrated that environmental conditions experienced by individuals can shape their development and affect the stability of genetic associations between traits (Huntingford, 2004; Mignon-Grasteau et al., 2005; Price, 1999; Price and Langen, 1992; Robinson et al., 2009; Ruiz-Gomez et al., 2008).

Associations between individual behavioural responses and growth appeared to be context dependent. Activity in a novel environment was correlated to growth of sole reared under stable intensive conditions (i.e., barren tanks, pellet feed, homogeneous environmental conditions). However, when individuals experienced enriched environments (i.e., sand, natural prey) or variable environmental conditions (i.e., light, photoperiod, temperature) this association was not present. In a previous study we also found positive associations between activity and boldness towards novelty and growth of sole reared in captivity (Mas-Muñoz et al., 2011).

Behavioural traits are a strong mediating force determining growth-mortality trade-offs. Fast growth has been associated with higher foraging activity, boldness and a greater predation risk (Biro and Stamps, 2008; Sih et al., 2004b; Stamps, 2007; Wolf et al., 2007). Associations between behavioural traits and production (growth/ reproduction) have been mostly documented for animals kept in captivity (Huntingford and Adams, 2005; Huntingford et al., 1990; Johnsson et al., 1996; Sih et al., 2003; Sundström et al., 2004). In line with our results, recent studies also showed that the relationship between behavioural traits (i.e., boldness) and growth might be more variable and weaker when animals experience different or heterogeneous environmental conditions, like commonly present in nature (Adriaenssens and Johnsson, 2009, 2013; Hojesjo et al., 2011; Magnhagen and Borcherding, 2008; Meekan et al., 2010; Smith and Blumstein, 2008). Therefore behavioural traits and fitness associations seem to be context dependent. This might explain why in nature the maintenance of individual differences in behavioural traits has been explained as adaptive behavioural strategies to specific contexts or situations, which are considered to be crucial for the survival of a species in a continuous changing environment (Wilson, 1998; Wilson et al., 1994b).

In conclusion, this study suggests that physical environmental factors, such as substrate, feed type or variable outdoor conditions, influence mean growth but not variation in growth of sole. Individual behavioural responses towards novelty are influenced by environmental experiences. In consequence, correlations between activity in a novel environment are present under stable intensive conditions whereas correlations are absent in high variable or “enriched” environments.

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CHAPTER 6

Genotype by environment interaction for growth of sole (*Solea solea*) reared in an intensive aquaculture system and in a semi-natural environment

Julia Mas-Muñoz, Robbert Blonk, Johan W. Schrama, Johan van Arendonk, Hans Komen

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Abstract

The objective of the current study was to assess the extent of genotype by environment interaction for growth of sole (*Solea solea*) reared in an intensive recirculation aquaculture system (RAS) and in a semi-natural outdoor pond (POND). The RAS environment consisted of four indoor shallow raceways without sand, where fish were stocked at a density of 40 fish/m^2 and fed with dry pelleted feed. The POND environment consisted of one 100 m^2 outdoor pond, where fish were stocked at densities of 1.4 fish/m^2, and fish could prey on live ragworms present in the sediment. *Solea solea* (*n*=2800) offspring, produced by natural mating of wild broodstock, and with initial body weight of 64 ± 20 g, were randomly divided over the two environments. After a growing period of 165 days, all fish were harvested and harvest weight (HW, g), specific growth rate (SGR, %BW/d) and sex of all fish was assessed; molecular relatedness between animals was estimated using 9 microsatellite markers. In POND 980 fish and in RAS 774 fish were successfully genotyped and used in the analysis. SGR was higher for sole reared in POND compared to RAS (0.60 ± 0.01 vs. 0.57 ± 0.01). Pearson correlation of initial body weight with SGR was negative, and more so in POND compared to RAS (-0.30 vs. -0.16, respectively). Genetic variance and heritability estimates for SGR were higher in POND (h^2 0.20 ± 0.05) than in RAS (h^2 0.04 ± 0.02). Genetic correlations for HW and SGR of sole reared in RAS and in POND were 0.56 ± 0.34 and 0.27 ± 0.3 respectively. The differences in heritable variation and the low genetic correlations of growth of sole between environments suggest strong genotype by environment interaction. These results are important in developing breeding programs for sole because the accuracy of selection and genetic gain for growth of sole may differ between environments. Low genetic correlations for growth between environments imply that the best genotypes in an intensive aquaculture environment are not to be necessarily the best genotypes in more natural environments such as ponds.

Keywords: Solea solea, genotype by environment interaction, genetic correlation, heritability, pond, aquaculture

Introduction

Common sole (*Solea solea*) is considered to have a high potential for marine aquaculture in Europe because of its consumer's popularity and high market value (Dinis et al., 1999; Howell, 1997; Imsland et al., 2003). However, its slow and variable growth is a major economic constraint for the commercial culture of sole. Slow growth is thought to be a consequence of its undomesticated status (Exadactylos et al., 1999; Howell et al., 2009; Imsland et al., 2003). Individual variation in growth results from both genetic and environmental sources of variation, as well as the interaction between the two (Falconer, 1990).

Past and current research initiatives on sole have focused on improving growth and feed intake by improving nutritional conditions (i.e. applying attractants to pellets, feeding methods, etc.) and environmental conditions (i.e. temperature, photoperiod, stocking densities, etc.) (Howell et al., 2011; Imsland et al., 2003). More recently, efforts have been directed towards selective breeding to improve economic viability of farms (Blonk et al., 2010a, b).

In the Netherlands, production of sole is carried out in two different systems: (1) intensive land-based indoor culture using recirculation, with high stocking densities in terms of fish weight per square metre and total dependence on artificial feeds; (2) experimental semi-natural outdoor ponds, which employ low stocking densities of fish reared on substrate and with total reliance on natural food preys (i.e. live ragworms).

The expression of growth derived from a single genotype can vary across different environmental conditions. This environmental sensitivity or reaction norm among genotypes may vary and lead to considerable genotype by environment (G×E) interaction. As a result, differences among genotypes are not consistent from one environment to the next (Falconer, 1990). The only existing breeding program for sole is being executed in an intensive recirculation aquaculture system (RAS). Therefore, when G×E interaction exists, selection under conditions of RAS may lead to different genetic gains than expected in other production environments such as outdoor ponds.

There are two main types of G×E interaction: scaling and re-ranking. Scaling means that the amount of genetic variation (V_a) in two environments differs ($V_{a,RAS} \neq V_{a,POND}$). If the environmental variance (V_e) is not changing along proportionally or stays the same this may result in heterogeneous heritability (h^2) estimates. Consequently the accuracy of selection in different environments will not be equal and the genetic gain for growth of sole will differ.

Re-ranking means that the order of genotypes changes across different environments (Lynch and Walsh, 1998). Re-ranking across environments can be estimated using the genetic correlation (r_g) between measurements on the same trait in two environments (Falconer, 1952) and is commonly considered biologically significant when r_g is lower than 0.8 (Robertson, 1959). Re-ranking of genotypes in particular is a challenge for selective breeding because it implies that the best genotypes in one environment are not the best in other environments. It is hypothesized that environmental factors, which differ between the rearing systems (i.e., food type, feeding method, stocking density, presence of substrate, light intensity, etc.), will influence feeding behaviour of sole, resulting in G×E interaction regarding growth. Therefore the objective of the current experiment was to assess the extent of G×E interaction for growth of common sole reared in an intensive recirculating aquaculture system (RAS) and in a semi-natural environment (POND).

Materials and methods

Ethics

This study was performed from April until October of 2011. All procedures involving animals were conducted in accordance with the Dutch law on experimental animals, which complies with the ETS123 (council of Europe 1985) and the 86/609/EEC directive. The Ethical Committee for Animal Experiments (DEC) of Lelystad in the Netherlands approved the experiment (2011020.a).

Animals

Juvenile common sole (*n*=2800) with an initial body weight 64 ± 20 g (mean ±SD) coming from a commercial farm (Solea BV, IJmuiden, the Netherlands) were used in this study. At the farm of origin (prior to the experiment), fish were reared under intensive recirculating aquaculture conditions (RAS) in barren shallow raceways, at a high stocking density (8 kg/m^2), constant temperatures (18-23°C) and fed with a commercial diet (Weanex-3000; 3mm dry pellets). These animals were offspring of wild broodstock parents (*n*=90), produced by natural mating. At the start of the experiment, the pedigree of the experimental animals was unknown. The total number of fish required for evaluating G×E interaction (1000 fish/environment) was estimated assuming a heritability for growth of 0.2-0.3 and the presence of 20-30 full sib families with a skewed distribution in family size due to unequal contributions of parents to total offspring (Blonk et al., 2009; Sae-Lim et al., 2010).

Experimental procedure

At the beginning of the experiment, fish were individually tagged (Trovan ID100A: 2.12x11.5mm, DORSET GP, Aalten, The Netherlands), weighed and blood sampled for DNA analysis and pedigree reconstruction while anaesthetized with 2-phenoxyethanol (1ml/l). Next, fish were randomly assigned to two different rearing environments. After 165 days (Period: 21st of April until 3rd of October 2011), fish were harvested and harvest body weight (HW, g) recorded. The sex of each animal was examined using ultrasound (System: Esaote PieMedical MyLab30Vet; Transducer: Esaote LA435 6–18 MHz).

Rearing environments

The rearing conditions in the two experimental environments are summarized in Table 1.

The intensive recirculating aquaculture system (RAS) at the experimental facilities of IMARES Wageningen UR (Yerseke, The Netherlands) consisted of a water treatment unit including mechanical filtration (sandfilter), biological filtration (trickling filter) and bacterial disinfection (UV unit). The fish were held in 4 barren shallow raceways of 5x1.75x0.2 (L x W x H, in m). Stocking density was 40 fish/m^2 (350 fish /raceway). Fish were kept indoor under constant photoperiod 12D:12L and fed with commercial pelleted feed (Weanex-3000, 3 mm sinking pellet, BioMar, Nersac, France) supplied continuously with automatic feeding belts for 12h/day, during daytime.

The semi-natural environment (POND) at the Zeeuwse Tong project (Colijnsplaat, The Netherlands) consisted of an outdoor pond of 100x10m in surface and 0.8 m depth, which was lined with a sheet of EPDM-rubber and a layer of 20 cm of sandy sediment. Stocking density was of 1.4 fish/m^2. Fish were kept under natural photoperiod and light intensity and ambient temperature. Feeding was on natural prey consisting mainly of live ragworms (*Nereis virens*) present in the sediment of the pond.

Table 1. Environmental parameters of the two rearing environments, POND and RAS.

Variable	RAS	POND[a]
Temperature range (°C)	18.2-24.0	12.5-22.6
Daily mean temperature (°C)	21.3 ± 0.1	18.1 ± 0.1
Temp. fluctuations day/night (°C)	0-0.1	0.7-4.5
Oxygen range (mg/l)	6.2-9.4	4.5-11.6
Light/photoperiod	Artificial/ 12L:12D	Natural/ variable
Substrate (cm)	0	20
Stocking density	40 fish/m^2	1.4 fish/m^2
Feed type	Dry pellets	Live prey
Feeding method	Automatic feeding belts	Natural prey search

[a] GPS coordinates: 51° 35' 37.734" N, 3° 52' 7.6944" E

Data analysis of traits

For every fish we analysed two traits at the end of the experimental trial: harvest body weight (HW, g) and specific growth rate (SGR, %BW/d). SGR was used to correct for unequal variances of initial and harvest body weight and was calculated using fish wet weight (g) at the beginning (W_0) and at the end (W_1) of the experimental growth period (T=165 days), as:

$$SGR = 100 * \left(\frac{Ln(W_1) - Ln(W_0)}{T} \right).$$

Coefficient of variation (CV, %) for each trait, and in each environment, was calculated as CV=$\left(\frac{\sigma}{\mu} \right) * 100$, with σ the standard deviation and μ the mean growth rate of all fish in each environment.

Statistical analyses on phenotypic data were performed using SAS (SAS, 2002). A multivariate analysis of variance (MANOVA) was done for initial body weight, HW and SGR with sex, tank and environment as fixed effects followed by the Tukey's HSD post-hoc test. The error terms were tested for homogeneity of variances and normality with the Shapiro-Wilk test. Pearson's correlations of initial body weight with HW and SGR were calculated to analyse the effect of rearing environment on the relationship between initial weight and HW and SGR of fish.

DNA analysis

Blood samples from all fish and available parents were taken with heparinized syringes at the beginning of the experiment and stored at −20 °C for further analysis. For DNA isolation of blood, nucleospin blood columns (NucleoSpin®96 Blood, Machery–Nagel) were used. To test if DNA was extracted successfully, DNA concentrations were measured from several samples in all plates using a spectrophotometer (Nanodrop technologies ND-1000). DNA concentrations were diluted to 5-10 ng/µl for further analysis.

The following 9 microsatellite markers identified in common sole were used for DNA analysis:

AF173855, AF173854, AF173852, AF173849 (Iyengar et al., 2000), AY950593, AY950592, AY950591, AY950588, AY950587 (Garoia et al., 2006). The number of alleles per marker in this population ranged from 7 to 26, with 152 alleles in total present.

PCR amplification involved 5 min of denaturation at 95 °C followed by 36 cycles of consecutively 30 s denaturation at 95 °C, 45 s annealing at 55 °C and 90 s elongation at 72 °C. After 36 cycles, a final elongation step of 10 min at 72 °C was applied. After PCR amplification, marker samples were pooled per individual and analyzed on an ABI 3730 automatic sequencer. Fragment sizes were set relatively to Genescan LIZ 500 size standard (Applied Biosystems). Output data were analyzed using Genemapper software (Applied Biosystems) in order to determine allele profiles at each locus.

Molecular relatedness

Genetic relationships between animals were estimated using a relatedness estimator as parental information was incomplete which made pedigree reconstruction of all the offspring not possible. To estimate molecular relatedness, we used the method described by (Toro et al., 2002; Blonk et al., 2010b). Coancestry (f) is calculated from similarity (S) of alleles between two individuals x and y. Here $S_{xy,l}$= ¼ [I_{ac} + I_{ad} + I_{bc} +I_{bd}], where at locus l, a and b are alleles of individual x and c and d are alleles of individual y (Li and Horvitz, 1953). When at I_{ac} allele a is identical to allele c, I_{ac} equals one, and zero otherwise, etc. Molecular coancestry (f_{xy}) is then calculated as $f_{xy} = \frac{1}{L}\sum_{l=1}^{L}\frac{S_{xy,l} - s_l}{1 - s_l}$,

where L is the number of markers (markers) and S_l is mean similarity (sum of squared allele frequencies, $\sum P^2$) at locus l in the base population (Lynch and Walsh, 1998).

Estimated relatedness r, in diploid species between two animals, is calculated as $r = 2f$. When ignoring alleles that are alike in state (AIS), molecular relatedness between two individuals is calculated from coancestry as: $r_{xy} = \frac{2}{L}\sum_{l=1}^{L} S_{xy,l}$.

Consequently, values of molecular relatedness are continuous and may range between 0 (no alleles are similar) and 2 (all alleles are similar).

Genetic analysis

To estimate heritabilities (h^2) and genetic correlations (r_g) for growth in the two environments, restricted maximum-likelihood estimation (REML) was used with ASReml software (Gilmour et al. 2006). Analyses were performed using a bivariate animal model, in which one trait in two environments is treated as two different traits:

$$GR_{ijk,\,RAS},\ GR_{ijk,\,POND} = \mu + T_{i,\,RAS} + G_j + a_k + e_{ijk}$$

where GR is the response variable for growth (HW, SGR) in one of the two environments (RAS or POND) for individual k, μ is the overall mean of the trait, T_i, is the effect of tank i (i=1,2,3,4) within RAS environment, G_j is the fixed effect of gender (j=male, female), a_k is the random additive genetic effect of animal k, and e_{ijk} is the random error term. Residual covariance was fixed to zero, as each individual inhabited only one environment (either RAS or POND).

A model for harvest weight with initial weight at stocking as co-variable gave similar solutions as the model using SGR as the response variable, and is therefore not included.

Heritabilities (h^2) were calculated from the additive genetic variance (V_a) and environmental variance (V_e), obtained from ASREML, as $h^2 = \frac{V_a}{(V_a + V_e)}$. Genetic correlations ($r_g$) between environments were calculated using the genetic covariance between measurements of the trait in the two environments, as $r_g = \frac{\operatorname{cov}_A(GR_{RAS}, GR_{POND})}{\sqrt{V_{a,RAS} * V_{a,POND}}}$.

Results

Phenotypic growth traits

At the end of the experimental period, 2492 animals (1304 in POND and 1188 in RAS) were harvested. From these, 1754 fish were successfully genotyped and used in the analysis: 980 fish in POND and 774 fish in RAS. As expected from earlier work (Blanco-Vives et al., 2011; Howell et al., 2011; Mas-Muñoz et al., unpublished data), sex ratio was 7:1 (males:females) where females grew (SGR, %) more than males (0.65 ± 0.01 vs. 0.52 ± 0.00; $P<0.001$). Mean HW and SGR were significantly higher for sole reared in POND compared to RAS (Table 2). Moreover, there was a trend for a tank effect on HW and SGR of fish reared in the RAS, with mean SGR (±SE) of 0.55 (±0.01) for tank 1 and 2, and of 0.58 (±0.01) for tank 3 and 4 ($P<0.10$, Table 2).

Table 2. Effects of environment (ENV), tank, and sex on initial weight, harvest weight (HW) and SGR of sole reared in an intensive recirculating aquaculture system (RAS) and in a semi-natural environment (POND).

	RAS		POND		Effects		
	Mean ± SE	CV	Mean ± SE	CV	ENV	Tank	Sex
Initial BW (g)	64.7 ± 0.9	32	64.6 ± 0.8	36	0.88	0.36	<0.0001
HW (g)	168.7 ± 2.0	37	175.1 ± 1.9	35	0.005	0.06	<0.0001
SGR (%BW/d)	0.57 ± 0.01	32	0.60 ± 0.01	28	<0.0001	0.10	<0.0001

Pearson's correlation between initial body weight and HW was positive in both environments, 0.76 and 0.73 in POND and in RAS, respectively (Table 3). However the correlation between initial body weight and SGR was negative and significantly more in POND compared to RAS (-0.30 vs. -0.16, $P<0.001$; Table 3). Moreover, there was a significant interaction effect between initial body weight and rearing environment on SGR of sole (IBW×ENV, $P<0.05$; Table 3). Together, these results show that fish with a low initial weight grew more in POND compared to RAS .

Table 3. Pearson's correlations and effects of initial body weight on harvest weight (HW) and SGR of sole reared in an intensive recirculating aquaculture system (RAS, n=774) and in a semi-natural environment (POND, n=980).

	RAS	POND	Effects[a]	
	Initial BW (g)	Initial BW (g)	IBW	IBW x ENV
HW (g)	0.73***	0.76***	***	#
SGR (%BW/d)	-0.16***	-0.30***	***	*

[a] Linear regression model includes fixed effects for sex, environment (ENV), and tank with initial body weight (IBW) as covariate and the interaction effect of initial body weight with environment (IBW x ENV). ****P*<0.001, ***P*<0.01, **P*<0.05, #*P*<0.1

Molecular relatedness

Out of the 2800 DNA samples analyzed, 1754 animals were genotyped successfully with at least eight markers. The distribution of estimated molecular relatedness values was similar for both environments. Molecular relatedness values were continuous and ranged between 0 and approximately 1.75 with an average (±SD) of 0.43 (±0.18) and 0.42 (±0.19), for RAS and POND respectively (Table 4).

Table 4. Molecular relatedness estimates [mean, standard deviation (SD), minimum (MIN) and maximum (MAX)] between pairs of common sole offspring reared in an intensive recirculating aquaculture system (RAS, *n*=774) and in a semi-natural environment (POND, *n*=980).

	RAS	POND
Mean	0.43	0.42
Median	0.43	0.39
SD	0.18	0.19
CV (%)	42.51	44.23
MAX	1.67	1.75
MIN	0	0

Heritability and genetic correlations for growth

Estimated variance components and heritabilities from bivariate analysis using molecular relatedness are shown in Table 5.

Genetic variances (V_a) for SGR were significantly lower in RAS compared to POND: $9.9x10^{-4}$ ($\pm 6.2x10^{-4}$) vs. $4.3x10^{-3}$ ($\pm 1.2x10^{-3}$) respectively. However the opposite was observed for HW: 96.6 (± 52.2) vs. 78.2 ± (40.9) in RAS and POND, respectively. Phenotypic variances (V_p) for HW and for SGR were similar in RAS and POND. Heritability estimated (h^2) for SGR was lower in RAS (0.04 ± 0.02) compared to POND (0.20 ± 0.05). However, low heritability values for HW were found in both rearing environments (0.04 ± 0.02).

When a genetic analysis was performed using only the best performing tanks in terms of growth rate (tanks 3 and 4) in RAS, heritability was higher 0.11 ± 0.05 (*n*=366). The effect of tank became even more pronounced when performing genetic analysis on only the best performing tank (tank 3). Here, heritability was 0.21 ± 0.10 (*n*=168), although with a high standard error.

The estimated genetic correlations of sole reared in RAS and in POND were 0.56 ± 0.34 and 0.27 ± 0.3, for HW and for SGR respectively.

Table 5. Phenotypic variance (V_p), genetic variance (V_a) and heritability (h^2) estimates (± standard error) for harvest weight (HW) and SGR of sole reared in an intensive recirculating aquaculture system (RAS, *n*=774) and in a semi-natural environment (POND, *n*=980).

	$V_{p,\ RAS}$	$V_{p,\ POND}$	$V_{a,\ RAS}$	$V_{a,\ POND}$	h^2, $_{RAS}$	h^2, $_{POND}$
Initial BW (g)	425.1 ± 14.09		25.76 ± 8.08		0.06 ± 0.02	
HW (g)	2283 ± 117.6	2012 ± 91.4	96.6 ± 52.2	78.2 ± 40.9	0.04 ± 0.02	0.04 ± 0.02
SGR (% BW/d)	0.025 ± 0.0013	0.022 ± 0.0014	0.00099 ±0.00062	0.0043± 0.0012	0.04 ± 0.02	0.20 ± 0.05

Discussion

Growth

Sole is a relatively slow growing fish species which in a commercial RAS farm currently takes 2-3 years to reach a commercial harvest weight of 250 g. Harvest weight represents the growth realized over the entire period, from hatch to harvest. Growth rate on the other hand is specific for the period in which it is measured. In this study, we analysed both specific growth rate and harvest weight, since our fish had been reared for approximately 1.3 years in a common RAS before being tested in the two environments for 6 months. In this relatively short growth period, harvest weight is strongly determined by initial body weight, especially when growth rate is low. This was demonstrated by the relatively high phenotypic correlations between initial body weight and harvest weight of 0.73 and 0.76 in RAS and POND. This might also explain why h^2 estimates on HW (0.04 ± 0.02) were similar to h^2 estimates on initial body weight (0.06 ± 0.02). In contrast, phenotypic correlations between initial body weight and SGR were negative and low: -0.16 for RAS and -0.30 for POND. Specific growth rate reflects only the performance of the fish during the experimental period of 165 days and thus more accurately reflects the effect of the test environment on growth.

Heritability estimates

Heritability estimates using molecular relatedness for HW were equally low in both RAS and POND, 0.04 ± 0.02. However, heritability for SGR was higher in POND, 0.2 ± 0.05, compared to RAS, 0.04 ± 0.02. Heritability estimates can be low because they are biased downwards. Using molecular relatedness can lead to underestimation of genetic variance by assigning erroneous relationships between offspring due to the use of few markers and thus an increased variance of relatedness and sampling error (Thomas et al., 2002; Thomas, 2005). However previous work on common sole by Blonk et al., (2010b), showed that molecular relatedness can perform equally well in estimating breeding values compared to pedigree reconstruction, especially in cases where there are missing parents and a skewed contribution of parents to total offspring, as in our study. Furthermore, mean and variance of relatedness was similar for both environments (0.43±18 in RAS and 0.42±19 in POND), while there was a considerable difference in additive genetic variance estimated for SGR in POND and RAS. The low h^2 estimates for HW (and for SGR in RAS) are therefore not a consequence of using molecular relatedness but more likely a consequence of the environmental conditions to which fish were exposed.

To compare our estimates to those obtained with pedigree reconstruction, we also estimated heritability on a small number of fish for which we were able to reconstruct the pedigree (n=270 and n=360, for RAS and POND respectively). Heritability estimates for HW were 0.06 ± 0.09 in RAS and 0.13 ± 0.09 in POND; for SGR estimates were 0.10 ± 0.10 in RAS and 0.46 ± 0.19 in POND. Although these estimates are higher, they also have much higher standard errors and are probably upward biased due to the low numbers of offspring used (Sae-Lim et al., 2010).

Heritability for SGR estimated in this study for POND is within the range 0.2-0.3 for growth in other fish species (Gjedrem, 2000) and higher compared to values estimated for HW of common sole in a previous study (Blonk et al., 2010b). Blonk et al. (2010b) estimated heritability for HW of common sole using molecular relatedness in a commercial RAS system at 0.11- 0.13. Our estimates of heritability for HW of sole in RAS are considerably lower compared to those estimated by Blonk et al. (2010b). However, in the latter study, sole were reared in an intensive commercial RAS farm for 3 years, while in our study the age of the fish at harvest was approximately 1.9 yrs. (1.3 yrs. + 6 months). It is possible that due to the relatively low growth rates commonly achieved in sole, differences in growth can only be accurately assessed after a prolonged testing period when the effect of measurement errors is reduced. In that case, heritability estimates for RAS will likely be closer to the values reported by Blonk et al. (2010b).

Heritability for SGR in RAS in this study was lower than for POND. This lower estimate was due to a lower ratio of estimated genetic variance over residual variance in RAS. It is possible that under less favourable conditions the genetic potential of organisms is not fully expressed. Conditions in POND were more similar to natural (wild) conditions of sole in term of temperature, photoperiod, substrate and food type, than conditions in RAS. It is possible that offspring from wild parents better express their genetic potential for growth in a more natural environment. However, heritability estimations could also decrease due to persistent measurement errors (Hoffmann and Merilä, 1999). Within RAS, tanks seem to have had an effect on the estimation of genetic components for growth. Heritability estimated for SGR in RAS increased when only tanks with the best growth performance where included in the analysis (0.11 ± 0.05 for analysis with only tank 3 and 4; results not shown). As molecular relatedness was very similar for the four tanks, the most likely explanation is that in some tanks the genetic potential for growth was either more suppressed or estimated with higher error.

Genotype by environment interaction between intensive and semi-natural environments

G×E interaction is commonly considered biologically significant when genetic correlation (r_g) is lower than 0.8 (Robertson, 1959). In this study the genetic correlation estimated for HW and SGR of sole reared in two different environments was low: 0.56 (±0.34) and 0.27 (±0.30), respectively. The estimates came with large standard error, which is probably due to low heritability estimates of these traits and relatively low number of families and unequal family sizes of sole produced by natural mating (Blonk et al., 2009; Sae-Lim et al., 2010).

For aquaculture species, significant G×E, i.e. low genetic correlations for growth in different environments, has been well documented. For example, in rainbow trout (*Oncorhynchus mykiss*) genetic correlations ranging from 0.86 to 0.58 were obtained for harvest weight at three different farms which differed mainly in feeding regime, water quality and salinity (Sylvén et al., 1991). Genetic correlations ranging 0.54-0.17 were reported also for rainbow trout when reared at different temperatures (McKay et al., 1984). More recently, Sae-Lim et al., (unpublished data) estimated genetic correlations for harvest weight in trout, reared in four different production systems on three different continents at 0.19-0.48. In European sea bass (*Dicentrarchus labrax* L.) genetic correlations for growth rate between four different sites and production systems ranged from 0.21 to 0.78 (Dupont-Nivet et al., 2010) and from 0.4-0.5 for body weight of fish reared at different temperatures and stocking density conditions (Saillant et al., 2006). For sea bream (*Sparus aurata* L.), genetic correlations for body weight between cage and tank rearing systems were 0.70 ± 0.10 (Navarro et al., 2009). In contrast, most studies in Nile tilapia (*Oreochromis niloticus*) found no apparent family re-ranking, and high genetic correlations for harvest weight, i.e. 0.74 to 0.84 between high input and low input farms (Khaw et al., 2009) or 0.89-0.90 for tilapia grown in cages and low-input ponds (Trinh et al., 2013). In rainbow trout high genetic correlations of 0.86 ± 0.026 were also found for fish reared at different temperatures (Fishback et al., 2002) and of 0.80-0.74 for Chinook salmon (*Oncorhynchus tshawytscha*) weighted at two different ages (Winkelman and Peterson, 1994). However, in these latter two studies only one specific environmental factor (temperature or age) was studied, and not the whole production systems.

The biological basis for G×E interactions is complex and G×E can arise from multiple environmental factors. In our study rearing environments differed in an extreme way and many factors such as water temperature, light (intensity and photoperiod), fish density, feed type and composition, the presence of sandy substrate, water quality, associated pathogens, etc. were different. All these factors may have contributed to G×E interactions.

We found a significant interaction effect between initial body weight and rearing environment on SGR. The phenotypic correlations between initial body weight and SGR were negative and stronger in POND compared to RAS (-0.30 vs. -0.16, respectively), showing that fish with relatively low initial body weight after a common rearing environment in RAS grew relatively faster in POND. Unfortunately, bivariate analyses between initial body weight and HW in either POND or RAS did not converge and genetic correlations could not be estimated. However, the phenotypic correlations suggest that re-ranking mainly concerned small fish which were not able to display their full genetic potential for growth in RAS.

Cultured fish are usually selectively bred for desirable production traits, such as fast growth. However, the striking differences between the environment experienced in intensive aquaculture systems and their natural environment brings with them unplanned inherited behavioural differences arising from differential experience (Ruzzante, 1994; Price, 1999; Huntingford, 2004). Fish with a “wild” genetic background (wild broodstock offspring) were reared in an artificial or a natural environment, therefore in the presence or absence of stimuli that are specific to these conditions. This might generate (non)-feeding behavioural differences which then result in different growth. Slow growing sole in RAS might reflect less adaptive capacity to intensive rearing conditions, which among others, entails social stress caused by high stocking densities and the absence of hiding substrate.

Conclusions

To our knowledge, this is the first study that describes genetic correlations for growth of “genetically wild” fish in a commercial and in a semi-natural environment. Genetic variability for specific growth rate of sole was present as a direct response to different environmental conditions in intensive RAS compared to semi-natural POND conditions. Genetic variance (V_a) and heritability (h^2) estimates for SGR using molecular relatedness were lower in RAS compared to POND. The differences in heritable variation and the low genetic correlation for growth of sole between environments suggest strong genotype by environment interaction effects. Heterogeneity of variance between environments can have implications for developing breeding programs in sole as the accuracy of selection and the predicted genetic gain for growth may differ between environments. Moreover the low genetic correlations for growth between environments implies that the best genotypes selected in an intensive aquaculture environment will not necessarily be the best ones in terms of growth in other, more natural environments. Therefore, selection of parents should be done in environments that are similar to commercial production.

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CHAPTER 7

General Discussion

Conceptual framework

Feeding behaviour might be the result of a trade-off between hiding motivation and explorative behaviour. This phenomenon has been explained by Sih et al. (2004b) as the covariance in these behaviours across situations or personality. The rationale followed by most life-history studies (Réale et al., 2007; Sih et al., 2004a; Sih et al., 2004c; Stamps, 2007), is that genetic (co)variation in (non-) feeding behaviour influences the phenotypic expression of fitness traits (i.e., growth, reproduction and/or survival under predation risk). In this respect, consistent individual differences in (non-) feeding behaviour are explained in the context of growth-mortality trade-offs. Individuals that choose to spend more time foraging and less time hiding grow faster but are at a higher risk of predation. On the other hand, individuals that spend more time hiding display less foraging behaviour and grow slower but are at a lower risk of predation. In nature, both strategies within a population can result in equal fitness, and thereby maintaining behavioural variation (Biro and Stamps, 2008; Stamps, 2007) (Fig. 1).

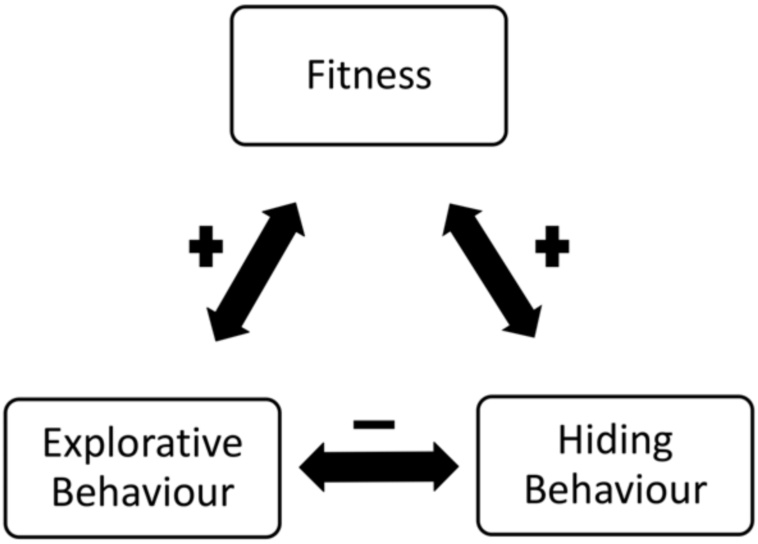

Fig. 1. Conceptual framework for behaviour (co)variation and its relation to fitness traits (Sih et al., 2004b).

Approach in this thesis

In cultured sole, high growth variation is a main concern for the economic viability of the farming industry of the species. Individual differences in feeding behaviour (i.e. feeding consistency, feeding motivation or meal size) and of non-feeding behaviour (i.e. willingness to take risks, activity or hiding motivation) provide a possible explanation for the high variation in feed intake and thereby in growth of common sole (*Solea solea*). Behavioural responses of an individual are influenced by the physical environment (i.e., temperature, light, substrate, feed type, etc.) and the social context (i.e., frequency and density of fish-fish interactions). The relation between individual differences in behaviour and growth can be determined by an animal's genotype, the environment and the interaction of its genotype with the environment. In this context, the aim of this thesis was to investigate: 1) the importance of (non-) feeding behaviour of sole in relation to growth; 2) the effect of (social and physical) environmental factors on behaviour, growth and the relation between them; 3) the existence of G×E interaction on growth (Fig. 2).

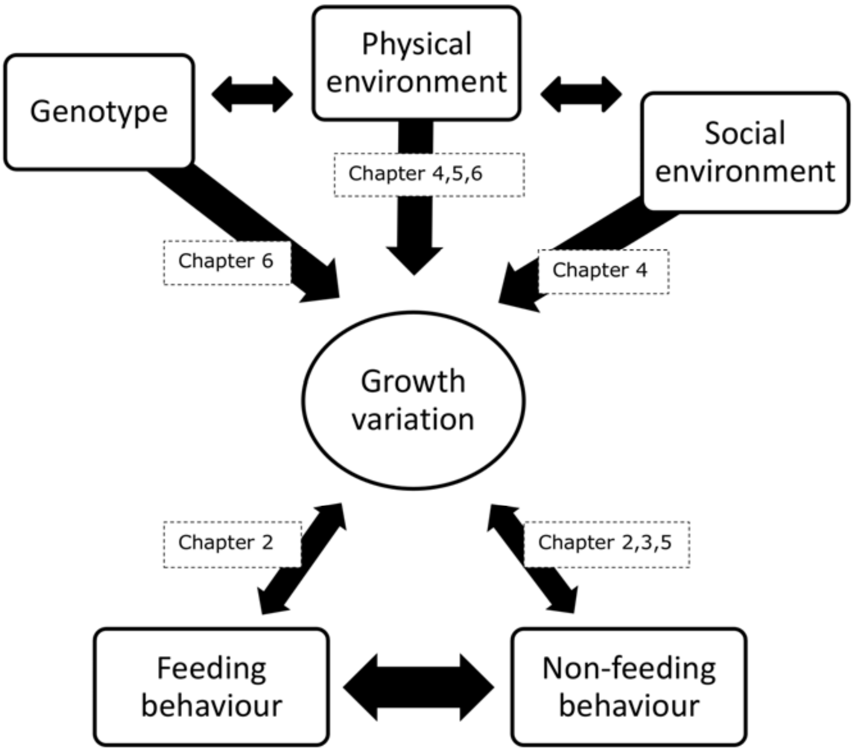

Fig. 2. Overview of factors (studied in this thesis) explaining behaviour and growth variation in common sole.

For each objective results will be discussed within a broader context. Moreover, the existence of personalities in sole and the behavioural changes occuring during domestication are critically reviewed. Finally, practical implications and concluding remarks are given.

Role of (non-) feeding behaviour in growth variation

Growth variation in sole

Variation in growth between individuals is common among cultured animals even when reared under optimal conditions (reviewed by Stamps, 2007). Coefficients of variation (CV) for growth in livestock animals range from 7 to 10 %. However, fish show even a more pronounced variability in growth, ranging from 20 to 40% for most fish species (Gjedrem, 1997, 2000). In spite of this, the majority of studies in fish only focus on mean data regarding growth and do not consider individual variations within an experimental group. The latter is treated as “statistical noise or random variation”. In this way, the importance of biological mechanisms involved in individual differences in growth, such as the role of (non-)feeding behavioural traits, are underestimated. To some extend this may explain the occurrence of contradictory results between studies.

The relation of (non-)feeding behavioural differences with variation in feed intake/efficiency and growth, and potential factors influencing this relationship are shown in Fig. 3.

This thesis showed large inter-individual variability for both feed intake (CV of 23 %, Chapter 2) and growth (CV of 25-35%, Chapter 2, 4, 5 and 6) of common sole. Variation in growth rate between individuals is generally coupled with consistent growth rates within individuals in sole (Pearson’s correlations between consecutive periods of 0.6-0.7, Chapter 3). Consistent individual differences in growth of sole were even observed when animals were reared individually in the absence of social factors (Chapter 2). Differences in growth can be caused by feed intake and/or feed efficiency (Fig. 2). In this thesis it was shown that 79% of the individual variation in growth of individually housed sole is explained by the variation in feed intake. The remaining 21% of variation in growth is the sum of variation caused by variation in feed efficiency (residual feed intake) and random error (Chapter 2).

Large individual variations in feeding behaviour (i.e., consistency of feed intake between and within days) of 27-55 % (Chapter 2) and in non-feeding behaviour (i.e.,swimming activity, burying behaviour, escape behaviour, hiding motivation, etc.) of 30-170% (Chapter 2, 3 and 5) were also measured.

Feeding behaviour and growth of sole

Individual differences in the consistency of feeding behaviour (between and within days) were related to variations in feed intake (regression model with R^2=0.43 and R^2=0.60) and growth (Pearson's correlations of r= -0.52 and r= -0.60) of individually housed sole (Chapter 2). High "voracity" of individuals showing consistent feeding rates within the day and between days, explains variation in feed intake and growth of sole.

However, individual differences in (non-) feeding behaviour and its relationship to feed intake/efficiency were only measured for a limited number (n=16) of individually housed fish in Chapter 2. Nevertheless, strong correlations between feed intake and growth and weak correlations between behavioural traits and feed efficiency, indicate that measurements on individual growth (Chapter 3, 4, 5 and 6) will give a good approximation for inter-individual variations in feed intake. On the other hand, the relationship between (non-) feeding traits and feed efficiency might become stronger when considering social interactions, since stress or activity levels are expected to increase energy requirements for maintenance.

Monitoring individual feed intake in groups of fish requires relatively complex non-invasive techniques such as radiography or invasive methods, such as looking at stomach contents of fish after feeding (Jobling et al., 2001). Therefore, the relations between feeding behaviour or feed intake and growth using individual fish as the unit of analysis, is only possible with individual housing or with the use of X-radiography. Since, the use of X-rays does not allow to measure day-to-day variations in feed intake (Jobling et al., 2001), in a non-schooling species like sole we prefer the use of individual housing to study individual variations in feed intake. The alternative of doing behavioural observations on groups of fish or using self-feeding systems, does not allow to identify the amount of food consumed by each individual in the group and thereby to assess a relationship between individual feed intake and growth. On the other hand, working with individually housed fish might compromise the validity of extrapolation to group housed individuals.

To overcome this problem and be able to measure individual feeding behaviour in group housed sole and its relation to growth, the feeding behaviour of sole fed in a restricted area which was connected to two pit-antennas (ANT SQR300, DORSET GP, Aalten, The Netherlands) was investigated (unpublished work). The pit-antennas were allocated parallel to each other in the entrance and recorded all fish entering or leaving the feeding area, thus individual information on feeding frequency and total feeding time were measured. The results from this work showed high consistency of individual feeding behaviour (i.e., frequency to visit feeding area, time in feeding area, and latency to enter the feeding area) over time (repeatability values between days of 40 to 80 %, unpublished data). Moreover, individual differences in feeding behaviour showed relative correlations with growth of the group housed sole (Spearman's correlations, r_s ranging from 0.2 to 0.5; n=200; P<0.05; unpublished data). The relations between individual feeding behaviour and growth of group housed sole seems to be more variable compared to individually housed sole, which can indicate either a lower precision in the measurements or the effect of the social environment.

Non-feeding behaviour and growth of sole

Individual differences in non-feeding behavioural traits (activity, exploration in a novel environment and burying behaviour) were measured during rearing (Chapter 2) and by conducting individual behavioural tests (Chapter 2, 3 and 5). Parallel to data in a wide variety of species (Biro and Stamps, 2008; Dingemanse and Réale, 2005; Sih et al., 2004a; Stamps, 2007), sole individuals that are more exploratory in a novel environment, and display less hiding behaviour, grow faster (chapter 2, 3 and 5). Behavioural factors derived from a principal component analysis, with all behavioural traits measured during 4 different behavioural tests and sex, explained 23 % of the variation in growth of group housed sole. Correlations between individual behavioural responses and growth were also present when considering only single behavioural traits for individually housed sole (r= 0.55 to 0.77, Chapter 2) and for group housed sole (r= 0.35 to 0.46, Chapter 5).

Individual behavioural responses are often plastic and thus influenced by environmental conditions (Braithwaite and Salvanes, 2005; Dingemanse et al., 2010; Frost et al., 2007). Maybe, this explains why repeatability measures for behavioural traits are generally relatively low compared to production traits (Bell et al., 2009). In our study, the individual behavioural traits measured in sole during different tests, show low to moderate consistency over time (Spearman 's correlations ranging 0.27-0.56, Chapter 3). Moreover, no strong correlations were found between behavioural traits measured across different tests (Chapter 3).

In summary, there is strong evidence for the influence of (non-) feeding behaviour on growth variation in sole. However, the consistency and strength of this relation is low to moderate, which suggests that the relation between individual behaviour and growth of sole is highly reactive to its environment.

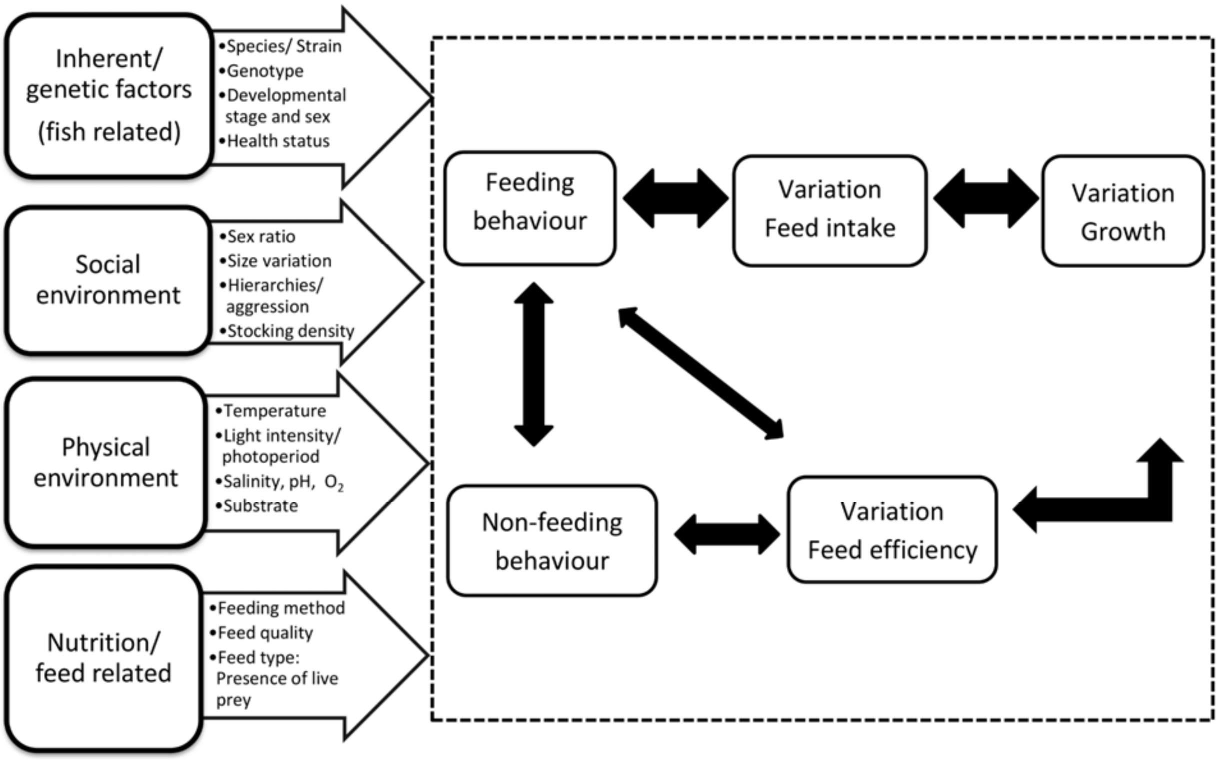

Fig. 3. Potential factors influencing (non-)feeding behaviour and its relation to feed intake/efficiency and growth variation.

Environmental influences on behaviour and growth variation

Potential environmental factors influencing (non-)feeding behaviour and growth variation are presented in Fig.3. In this thesis, the effects of social (Chapter 4) and physical (Chapter 5) environmental factors on behaviour and growth of common sole were investigated.

Social environmental factors

Environmental factors, like social interactions, are regarded as a major cause for growth variation in fish (Jobling and Wandsvik, 1983; Koebele, 1985). In this thesis, we showed that the social environment is a very important factor influencing behaviour and growth variation of common sole.

Sole is a non-schooling species which in nature is known to be predominantly a solitary fish, rarely in contact with other sole since it spends most of the time buried in sand (Kruuk, 1963). We observed that growth variation in sole is largely influenced by fish-fish interactions when reared in barren bottom tanks. Growth variation of sole increases when fish are grouped housed compared to when sole are kept in isolation (CV, 70 % vs. 25 %, Chapter 2).

The presence of social hierarchies is a major factor causing an increase in growth variation in group housed fish (Jobling and Baardvik, 1994), Fig.3. In this study, “slow growers” when taken from a large population and held in individually (Chapter 2) or reared separately from “fast growers” (Chapter 4), show a marked growth recovery. The opposite occurs when fast growers are reared together (Chapter 4). Similarly, this effect was reflected in literature when sorting *Solea senegalensis* (Salas-Leiton et al., 2011) and *Solea solea* (Overton et al., 2010), did not result in overall improvements of growth. However, in our study the effect of sorting was tested between consecutive growth periods, and thus the effect of time and sorting could be confounded. Next to this, the absence of active aggression between individuals to establish their social status makes it difficult to test the presence of hierarchies in sole.

Sexual dimorphism for growth in sole can also be an important source of variation influencing feeding behaviour and thereby growth (Fig. 3). A recent study by Sánchez et al. (2010), assigned a prevalent role to sex instead of size in variation of growth in juveniles of *Solea senegalensis*. Females were considered the dominant individuals, especially at high stocking densities. We also found indications for dominance of females over males (similar in size) during group housing of sole. Rearing male sole in mixed sex groups increases the coefficient of variation in growth compared to male sole reared in all-male groups (CV of 90 % vs. 32 %, data not shown). This increase in growth variation is mainly caused by the decrease in growth of males when reared in mixed sex groups. On the other hand, females show consistent growth figures in both mixed sex and all-female groups. Therefore, it seems female sole establishes a dominant relationship over males, which influences growth variation of group housed sole.

Another important social factor influencing fish-fish interactions is stocking density (Fig. 3). In Chapter 4 we showed that an increase in stocking density in tanks without substrate, increases swimming activity, reduces feed efficiency and results in an increased variation in growth of common sole, *Solea solea* (FCR of 1.04 vs. 1.12; CV in growth of 28.4% vs. 49.6%, Chapter 4). However, contradictory results are found in literature on the effect of stocking density on *Solea senegalensis* (Salas-Leiton et al., 2008; Sánchez et al., 2010). Moreover, the negative effect of stocking density can disappear if sand is provided since sole then spend most time buried (Chapter 4). Therefore, it seems that stress conditions caused by intensive fish-fish interactions at high stocking densities can be alleviated by providing sand to the environment. Similarly, providing some enrichment to the environment in rodents and pigs reduces reactivity of animals to stressful situations and can stimulate feed intake (Chapillon et al., 1999; Moncek et al., 2004; Oostindjer et al., 2011).

The effects of social environment (individual vs. group housing) on the associations between individual behaviour and growth variation in sole are presented in Fig. 4.These results show that sole, which were active in a novel environment test, have lower growth rates under group housing, whereas when housed individually the activity in the novel environment test did not relate to growth.

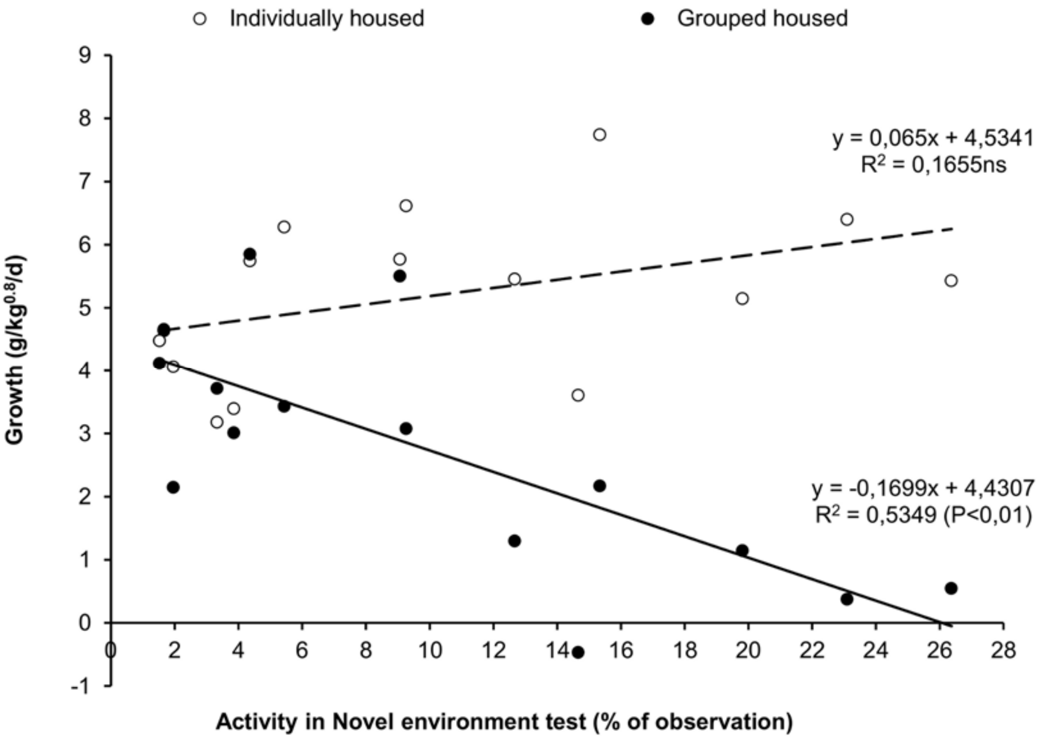

Fig. 4. Relationship between swimming activity in a novel environment and growth of sole group and individually housed (data originates from experiment presented in Chapter 2).

Physical environmental factors

Contrary to what was expected, physical aspects of rearing conditions, such as feed type, presence of sand and variability of environmental conditions (i.e., light/photoperiod and temperature), did not influence variation in growth (CV, %) of sole. The coefficient of variation in growth was the same in all rearing environments studied (30 to 34 %, Chapter 5).

Unfortunately, in this study the effects of variability of environmental conditions were confounded with the effects of lower mean temperatures, which influenced mean feed intake and thereby growth. For a better comparison, mean temperatures should have remained the same.

The presence of substrate reduces the resting metabolic rate of sole (Howell and Canario, 1987) and, as mentioned earlier, is able to alleviate the negative effects of increasing stocking density on growth variation in sole reared in barren environments (Chapter 4). Next to this, sole reared with sand, reduces activity and surface swimming under culture conditions (Chapter 4) and has no effects in neither variation nor mean growth (Chapter 5). In literature, providing substrate has also been suggested for the prevention of diseases in cultured sole (McVicar and White, 1979 cited by Howell, 1997). Therefore, the strong drive of sole for burying even under barren environments, and the positive effects shown on stereotypic/stress related behaviour and performance, point it out as an importance factor when considering welfare of fish in captivity.

The presence of substrate and variability in the environmental conditions (i.e., light/photoperiod and temperature) influences the activity of sole in a novel environment and its relationship to growth (Chapter 5). Activity in a novel environment was correlated to growth of sole reared under stable intensive conditions (i.e., barren tanks, pellet feed, homogeneous environmental conditions). However, when individuals experienced enriched environments (i.e., sand, natural prey) or variable environmental conditions (i.e., light, photoperiod, temperature) this association was not present. Therefore behavioural traits and fitness associations seem to be context dependent.

To conclude, social environmental factors are more important than physical environmental conditions for growth variation in sole. The presence of substrate can alleviate stress in sole induced by intensive farming conditions. Associations between growth and behavioural activity measured in novel environment tests are only present under intensive aquaculture conditions, which suggests a relation between reactivity to stress situations and the ability of sole to cope with high fish-fish interactions in barren bottom tanks.

Genotype by environment interaction regarding growth

The role of behavioural traits in growth variation in different environments suggests potential genotype by environment (G×E) effects. Genetic variation for growth in fish has been determined with heritability estimates ranging from 0.2 to 0.4 (Gjedrem, 2000). Heritability estimates for harvest body weight in common sole reared in intensive aquaculture systems range between 0.2-0.3 (Blonk et al., 2010). This thesis showed heterogeneity of heritable variation for growth of sole reared in an intensive and in a semi-natural environment, of 0.04 and 0.2, respectively (Chapter 6). Moreover, we found low genetic correlations for harvest body weight (0.56 ± 0.34) and growth (0.27 ± 0.30) of sole. These differences in heritable variation (scaling effects) and the low genetic correlations (re-ranking) regarding growth of sole between environments indicate strong genotype by environment interaction effects.

In this regard, it has to be noted that in literature results are not consistent and thus it is not possible to draw a general conclusion about the importance of G×E for aquatic species (reviewed by Gjedrem et al., 2012). However, G×E seem to be important when environments are very variable, especially when interactions between the behaviour and its environment can take place. This is the case in sole, where environmental conditions, such as feed type, substrate, light, temperature and photoperiod are known to affect the feeding behaviour of the species (De Groot, 1969; De Groot, 1971; Imsland et al., 2003).

We did not measure G×E interactions in behavioural traits of sole due the labour intensity and time consuming measuring individual behaviour for a genetic study. In addition, no golden standard behavioural test has been developed for sole, thus interpretation of results is difficult when reared in different environments. However, low repeatability estimates for most behavioural traits over time and across situations (Chapter 3), high sensitivity of individual behavioural responses to the environment (Chapter 5), and context dependant association with growth (Chapter 5), indicates that strong G×E interactions regarding growth reflect the flexibility of different types of behaviour to environmental conditions.

In conclusion, low genetic correlations for growth between intensive and semi-natural culture systems means that breeding selection of parents for sole should be done in environments that are similar to its commercial production.

Do personalities exist in sole?

It is well known, that individuals within a population differ in their behavioural responses. Consistent individual differences in feeding behaviour (feeding consistency, feed motivation, foraging under predation risk, etc.) and non-feeding behaviour (boldness, aggressiveness, activity levels, coping styles, etc.) have been documented in many animals (Bell, 2007; Gosling, 2001; Sih et al., 2004a; Sih et al., 2004b). These behavioural differences have been termed as personality (Dingemanse and Réale, 2005; Gosling, 2001), temperament traits (Reale et al., 2007), coping styles/strategies (Koolhaas et al., 1999) or boldness (Wilson et al., 1994).

The personality traits are commonly presumed to be consistent over time and/or across situations and thus reflect underlying processes that can affect an animal’s response to a wide range of stimuli and situations (Sih et al., 2004a; Sih et al., 2004b). However, the development of certain flexibility in personality traits can be important from an ecological and evolutionary point of view since it allows animals to adapt to environmental changes (Dingemanse et al., 2010).

Four properties of individual variation are important to clarify if copying styles or personalities exist: (1) consistency of behavioural responses to the same situation over time; (2) consistency in behavioural reactions across situations; (3) bimodal distribution of individual behavioural responses; (4) genetic base for behavioural variation (Jensen, 1995).

For sole, measuring variation in behavioural traits is not an easy task: 1) feeding behaviour of individual fish is complicated and very laborious because of long feeding times and lack of feeding motivation after handling; 2) non-feeding behavioural traits in sole are mainly based on basically two behavioural traits, activity and burying, since the species does not show signs of active aggression; 3) under stress conditions, sole reacts mostly with immobility being difficult to measure variation in behavioural responses.

Moreover, behavioural responses measured under laboratory conditions are largely influenced by environmental conditions and prior experiences. For instance, activity levels of sole are affected by the level of fish-fish interactions at tank level, by increasing stocking density and the presence of substrate (Chapter 4). The effect of substrate and variability of environmental conditions (i.e., light/photoperiod and temperature) also influenced individual responses of sole to a novel environment (Chapter 5). Next to this, individual behavioural traits measured during tests, showed low to intermediate consistency over time and lack of correlations across situations in sole (Chapter 3).

The fact that different people differ in their boldness to take risks is an obvious feature of human personality. However, making sense of observations of analogous behaviour in animals is not always easy. In common sole, low consistency of behavioural measures and high reactivity of behavioural traits to its environment with indications for potential G×E interactions make it difficult to study.

Therefore, although behavioural differences in sole are present and seem to be important explaining variation in growth in captivity, the existence of personalities in sole cannot be confirmed based on data in this thesis. Further research should be done in this area in combination with measurements in physiological traits.

Behavioural development and domestication

Behavioural traits are thought to be among the first traits affected by domestication (Ruzzante, 1994). It is known, that behaviour in animals undergoing domestication has evolved and differentiated from those of their wild ancestors. This seems reasonable since the environment experienced by cultured animals, including fishes, is very different from that experienced by their wild counterparts (Price, 1999). Domestication is defined as the process of adaptation of animals to captive conditions, which involves both genetic and developmental changes (Price, 1999). Huntingford (2004) proposed three mechanisms in which behavioural development may take place: (1) Differential experience: for example, the physical environment in which species are cultured is much simpler and less challenging than the one of their wild conspecifics, since they live in the absence of predators and protected from diseases. However, space is restricted and generally animals are stocked at high densities and without shelter, which can promote competition for feed; (2) Differential survival of behavioural traits within a single generation: for example wild animals cohabit areas with variable abundance of food and in the presence of predators. This may lead to different behavioural strategies between cultured and wild animals; 3) Differential selection (natural or artificial) of behavioural traits over generations: for example, many cultured animals have been selected over generations for desirable production traits, such as fast growth (Gjedrem, 2000). Such selection may be correlated with differences in behavioural traits (deliberate or unintended) between wild and cultured populations, such as increased appetite, boldness or aggression (Huntingford, 2004; Ruzzante, 1994).

From a breeder's point of view, domestication implies the control of the animal's growth and reproduction to suit human's purposes (Lush, 1943 reviewed by Russell, 2002). In genetic terms, domestications does not seem to have reduced the genetic variance of traits in farmed species (Price, 1999). Behavioural variations are known to be inherited and selection can steer which phenotypes will prevail in a specific environment (Dingemanse et al., 2004; Mignon-Grasteau et al., 2005). Behavioural heritability estimates have been reported for vertebrates (mammals, fish, reptiles, birds) and invertebrates (insects and crustaceans) (reviewed by Stirling et al., 2002).

For most farmed animals breeding programs are already well developed. In cultured fish, genetic selection has only been recently implemented and its use is still very low (Gjedrem, 2000; Gjedrem, 2012). Species like Atlantic salmon, *Salmo salar*, Nile tilapia, *Oreochromis nicoticus*, rainbow trout, *Oncorhynchus mykiss*, Gilhead seabream, *Sparus aurata* and European sea bass, *Dicentrarchus labrax,* already have large scale breeding programs (Gjedrem et al., 2012). However, many species in aquaculture production, still rely completely or partly on spawning of wild broodstock (Gjedrem, 2000). This is the case for sole, which is still a species in an early stage of domestication, where mainly natural reproduction of wild broodstock is used for offspring production (G1). More recently, first generation individuals selected for growth have been successfully reproduced obtaining a second generation offspring (G2) (Blonk and Blom, IMARES personal communication). Consequently, animals with a "wild' genetic background are generally used for culturing sole. Therefore, behavioural differences with their wild counterparts can imply differences in behavioural development caused by environmental differences or genotype by environment interaction effects. For instance, reproduction success of G1 fish is often unpredictable, and a systematic failure of G1 males to display normal sexual courtship behaviour, has been observed (Howell et al., 2011). In addition, even after only one generation of selection, striking differences in behavioural characteristics on the feeding behaviour and activity of sole juveniles have been observed (personal observation). G2 offspring selected for fast growth are more active when feed is delivered and display more surface swimming in captivity compared to G1 fish. In line with this, literature on salmonids also found increased aggression levels during domestication. Domesticated fish (G2) are more active swimmers and more distributed in the water column compared to wild offspring (G1), that remain less active in the bottom of tanks (Ruzzante, 1994).

Therefore, selection for fast growth in fish reared in intensive aquaculture systems have generated behavioural differences between cultured fish and their wild counterparts from which they originated. Artificial selection in cultured animals, is related to higher levels of competitive interaction during feeding due to the presence of high fish-fish interactions. Consequently, foraging behaviour, boldness and aggressive behaviour seems to be favoured over generations under captive conditions (Huntingford, 2004; Huntingford and Adams, 2005; Ruzzante, 1994). As a result, shy fish, less active or non-aggressive might not prevail under intensive farming conditions.

In Chapter 2, 3, and 5 fast growth of G1 offspring of sole was related to higher feeding consistency, activity and surface swimming during behavioural tests. In nature, wild sole generally remain immobile or buried in the sand during most part of the day (Kruuk, 1963). Therefore, surface swimming has been attributed as a stereotypic behaviour in flatfish related with stress or boldness in captive conditions (Kristiansen et al., 2004). Stereotypical behavioural in barren environments have been well documented in vertebrates (Gonyou, 1994). Deviations from natural behaviour can create unnatural stress levels and other behavioural problems (i.e. aggression), thus stereotypical behaviour is believed to be an indicator of poor welfare (Williams et al., 2009).

In conclusion, these type of correlations with behavioural traits, such as aggression, activity or stereotypic behaviour, when selecting for growth can have negative implications for welfare in intensive aquaculture conditions where animals are stocked at high densities and in the absence of shelter.

Practical Implications and future perspectives

Overall, the presence of large individual variations in behaviour and growth in common sole suggests scope for improvement in aquaculture.

From a welfare perspective, the strong drive of sole for burying next to the positive effects of sand on growth variation under high stocking densities, implies that developing rearing systems/conditions with substrate is recommended. This could bring a conflict between welfare and practical/economic issues. However, stability of market prices for sole and the possibility of an added value of these type of production systems might make it reasonable to include sand for sole culture. Moreover, the heritable variation of burying behaviour should be investigated to provide information on whether domestication or breeding could select sole which copes better under intensive farming conditions.

Furthermore, the (non-)feeding behaviour of sole is highly reactive to environmental conditions. Therefore huge differences between natural and farming conditions might make it difficult to culture undomesticated fish stocks in captivity. Sole seems to be highly sensitive to fish-fish interactions when reared at high stocking densities. Therefore, extensive rearing systems (i.e., low density and sand) or breeding might provide a solution for this.

The development of breeding programs in sole is important to progress with sole farming. Moreover, the presence of G×E interaction effects regarding growth between different rearing systems, means that separate breeding programs should be developed for the environmental conditions in which production will take place. Low heritability estimates for sole reared under intensive aquaculture systems compared to semi-natural pond systems found in this study, proves that the genetic potential for growth in sole is not reached under intensive conditions. In intensive aquaculture conditions, environmental factors seem to be responsible for a large part of the variation in growth of sole. Therefore improvement, in both nutritional and husbandry conditions of current intensive production systems is needed. On the other hand, options can be investigated to increase productivity/ m^2 for extensive systems. This can either be by optimization of environmental conditions (i.e., light or temperature) or food availability. This thesis (and other research) has shown that the availability of live feed (ragworms) in combination with the effort to forage on ragworms in the sand during night (when temperatures are lower) might be negative for the maximum growth of sole in these systems. Therefore, co-feeding or feeding pelleted feeds could increase production as the feed is more easily available for the sole.

The relation between (non-)feeding behavioural traits and growth variation in sole should be taken into consideration when selecting fish that are able to cope best with potential stress conditions in commercial aquaculture and to avoid unwanted correlated behavioural responses with growth. Further research should follow to find suitable behavioural tests measuring stress reactivity of sole which could be implemented in practice.

Concluding remarks

- The role of (non-)feeding on growth variation should be taken into account to foster progress in the farming of sole.
- Environmental effects on behaviour and growth can be used to optimize culture systems, and to adapt these to the fish's needs and requirements. In this respect, substrate seems to be an important feature which can improve welfare and performance of sole.
- Lack of consistency when measuring behaviour in animals under laboratory conditions indicates that behaviour as a selection trait is highly reactive to environment. Nevertheless, repeatable associations between behaviours and production traits in a specific context should not prevent individual differences to be targets of selection.
- The existence of correlations between behavioural traits, such as feeding activity and boldness, and growth, suggest that under intensive aquaculture conditions bold sole with higher competitive ability when stocked at high densities in the absence of sand will be selected. A selection for this type of behaviour could influence the welfare of cultured fishes.
- The results from this thesis provide further insight for future genetic selection strategies. Such strategies should focus on behavioural characteristics which are related to increased feed intake and uniform growth. In addition, the knowledge obtained from the genotype by environment interaction should be used to select sole which will be able to cope and grow best in the different rearing conditions present in commercial aquaculture.

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APPENDICES

SUMMARY

Common sole (*Solea solea*) is considered to have a high potential for commercial aquaculture because of its consumer popularity and high market values in Europe. However, a major economic constraint for the culture of sole is its slow and variable growth. Variation in growth may result from both genetic and environmental factors, as well as the interaction between the two. It was hypothesized that individual variation in feeding behaviour (i.e., feeding motivation, meal size, feeding consistency) and non-feeding behaviour (i.e., willingness to take risks, activity or hiding motivation), can determine individual variation in feed intake and thereby variation in growth. The role of feeding and non-feeding behaviour in growth of sole is thought to be influenced by environmental factors (i.e., food type, feeding method, stocking density, presence of substrate, light intensity, etc.), a potential cause for genotype by environment (G×E) interaction. Therefore the aim of this thesis was to investigate: 1) the importance of (non-) feeding behaviour of sole in relation to growth; 2) the effect of (social and physical) environmental factors on behaviour, growth and the relation between them; 3) the existence of G×E interaction regarding growth.

In **Chapter 2,** the relationship between feed intake, growth and (non-) feeding behaviour of individually housed sole was assessed. In the absence of social interactions, both variation in feed intake within and between days and activity in the tanks was recorded. Individual differences in behavioural reactions of sole towards challenge tests (a novel environment test and light avoidance test) were related to growth. Results showed that differences in feed intake account for 79% of the observed individual differences in growth. Feeding consistency, swimming activity in the tank, and boldness during challenge behavioural tests are related to variation in feed intake and growth of sole reared in captivity.

In **Chapter 3,** the consistency of individual behavioural responses to a range of tests (a novel environment, a novel object, a hiding motivation and a net restrain test), and its relationship with variation in growth for communally reared sole was assessed. Individual behavioural traits measured in sole show relative consistency over time. However consistency of behavioural traits across situations (between tests) was very low. Behavioural factors derived from a principal component analysis during the first time tests were done to the fish together with sex explained 23% of the variation in growth. The motivation to bury was negatively related to growth, whereas the motivation to explore a novel environment was positively related to growth. Female sole that are less reactive upon an artificial predator threat grow faster, whereas in males this factor had no impact.

In **Chapter 4,** the effect of social interactions, both in quality (i.e., size hierarchies) and in quantity (i.e., stocking density), on (non-) feeding behaviour and growth of sole in the presence and in the absence of sand were investigated. Individual growth variation seems to be regulated by the hierarchical social structure of the group. Growth of subordinate smaller individuals is improved when they are separated from larger conspecifics. On the other hand, larger conspecifics show lower growth when isolated from subordinates. High stocking density in tanks without substrate results in more fish-fish interactions, which increases activity, FCR and variation in growth of sole. These conditions seem to induce social stress in sole, which is alleviated when sand is provided.

The effects of rearing environmental factors on the relationship between behaviour and growth of sole were studied in **Chapter 5**. Environmental factors which differ between nature and commercial farming conditions, such as food type (live versus dry food), sand (presence versus absence of hiding substrate), and variability of environment (indoor versus outdoor) were studied. Moreover, individual behavioural responses to a novel environment test were measured before and after exposing fish to different rearing environments. Environmental conditions (i.e., light, photoperiod and temperature) present in an outdoor system compared to a controlled indoor system influenced mean growth of sole. None of the studied environmental factors influenced individual variation in growth of sole (CV,%). Swimming activity of sole in a novel environment test was influenced by previously experienced environmental conditions. Moreover consistent associations between individual behavioural responses to novelty and growth were only present for fish reared under commercial farming conditions prior and during the experiment (indoor, barren and pellet). These results suggest that consistent relationships between behaviour and growth develop when fish are reared in stable barren environments but not when fish experience more variable, enriched/natural environments.

The role of environmental factors on the relationship between (non)-feeding behaviour and growth of sole suggests a potential cause for genotype by environment (G×E) interaction. Therefore in **Chapter 6**, we assessed the extent of genotype by environment interaction for growth of sole. Common sole offspring produced by natural mating of wild broodstock were randomly assigned to one of the two following environments: (1) an intensive recirculation aquaculture system (RAS) and (2) in a semi-natural outdoor pond. Heritability (h^2) estimates for growth (SGR, %BW/d) using molecular relatedness, were lower in RAS (0.04 ± 0.02) compared to Pond (0.20 ± 0.05). We also found low genetic correlation ($r_g = 0.27 \pm 0.3$) for

growth of sole between intensive RAS and semi-natural pond environments. The differences in heritable variation and the low genetic correlations of growth of sole between environments suggest strong genotype by environment interaction. These results are important in developing breeding programs for sole because the accuracy of selection and genetic gain for growth of sole may differ between environments. Low genetic correlations for growth between environments imply that the best genotypes in an intensive aquaculture environment are not necessarily the best genotypes in more natural environments such as ponds. Therefore selection of parents should be done in environments that are similar to commercial production.

The main results from this thesis are discussed in **Chapter 7**. Moreover, the presence of personalities in sole and behavioural changes occurring during domestication are critically reviewed. Overall the following conclusions can be drawn from this thesis:

- The role of (non-)feeding on growth variation should be taken into account to foster progress in the farming of sole.
- Environmental effects on behaviour and growth can be used to optimize culture systems, and to adapt these to the fish's needs and requirements. In this respect, substrate seems to be an important feature which can improve welfare and performance of sole.
- Lack of consistency when measuring behaviour in animals under laboratory conditions indicates that behaviour as a selection trait is highly reactive to environment. Nevertheless, associations between behaviours and production traits in a specific context should not prevent individual differences to be targets of selection.
- The existence of correlations between behavioural traits, such as feeding activity and boldness, and growth, suggest that under intensive aquaculture conditions bold sole with higher competitive ability when stocked at high densities in the absence of sand will be selected. A selection for this type of behaviour could influence the welfare of cultured fishes.
- The results from this thesis provide further insight for future genetic selection strategies. Such strategies should focus on behavioural characteristics which are related to increased feed intake and uniform growth. In addition, the knowledge obtained from the genotype by environment interaction should be used to select sole which will be able to cope and grow best in the different rearing conditions present in commercial aquaculture.

SAMENVATTING

Noordzeetong (*Solea solea*) is een interessante vissoort voor aquacultuur vanwege de populariteit bij de consument en de hoge marktprijs. Een van de grote problemen om de kweek van tong economisch rendabel te krijgen is de trage en variabele groei. Variatie in groei wordt mede veroorzaakt door genetische en milieufactoren, en de interactie hiertussen. De hypothese is dat individuele variatie in eetgedrag (motivatie om te foerageren, totale voeropname, het aantal keren van voeropname) en niet-eetgedrag (bereidheid tot het nemen van risico's, activiteit of schuilgedrag) variatie in groei kan bepalen. Eet- en niet-eetgedrag van tong wordt waarschijnlijk beïnvloed door milieufactoren (soort voer, manier van voeren, dichtheid, aanwezigheid van substraat, lichtintensiteit, etc.) en is mogelijk onder invloed van genotype x milieu ("Genotype by Environment", G×E) interacties. Het doel van dit proefschrift is het onderzoeken van: 1) de relatie tussen niet-eetgedrag en groei van tong; 2) het effect van milieufactoren (sociaal en fysiek) op gedrag, groei en de relatie daartussen; 3) de aanwezigheid van G×E interacties op het gebied van groei.

In **hoofdstuk 2** is de relatie tussen voeropname, groei en (niet-) eetgedrag van individueel gehuisveste tong onderzocht. Zowel de variatie in voeropname per dag als de variatie in voeropname tussen dagen, en de activiteit in de bakken is onderzocht. Individuele verschillen in gedrag na blootstelling aan een nieuwe omgeving en het vermijden van licht bleken gerelateerd aan groei. De resultaten laten zien dat 79% van de waargenomen verschillen in groei wordt verklaard door voeropname. De mate van het vertonen van eetgedrag en activiteit tijdens de testen bleek van invloed op de voeropname en groei van tong in kweeksystemen.

In **hoofdstuk 3** is de consistentie van individuele verschillen in gedragstesten (een nieuwe omgeving, een nieuw object, de wil om te verschuilen en gevangenschap in een net) in relatie tot de variatie in groei van in groepen gehuisveste tongen onderzocht. Individueel gedrag bleek per test vrij consistent over tijd , maar er bleek weinig consistentie in gedrag tussen verschillende testen. Kenmerken gerelateerd aan het ontwijken van predatoren, zoalsingraven) lijken aangeboren te zijn. Dit is aangetoond doordat deze gedragingen de meeste consistentie binnen testen en een lichte consistentie tussen verschillende testen vertonen . Gedragsfactoren verkregen via principaal component analyse van gedrag tijdens de eerste blootstelling van de vis aan een test verklaren samen met het geslacht 23% van de groei. De neiging om in te graven is negatief gecorreleerd met groei terwijl de neiging om een nieuwe omgeving te verkennen positief gecorreleerd is met groei. Vrouwelijke tongen die minder reageerden op de aanwezigheid van een gesimuleerde predator groeiden sneller, terwijl deze dreiging bij mannelijke tongen geen invloed had op de groei.

In **hoofdstuk 4** zijn de effecten van sociale interacties, in kwaliteit (door een populatie te sorteren op groeisnelheid) en in kwantiteit (door populaties in te delen in verschillende dichtheden), op niet-eetgedrag en de prestaties van de tong met of zonder zand onderzocht. Variatie in groei lijkt te worden beïnvloed door sociale structuur binnen de groep. De groei van kleinere tong binnen een populatie verbetert op het moment dat grotere exemplaren uit de populatie worden verwijderd. Daarentegen groeien grotere exemplaren minder goed op het moment dat de kleinere exemplaren worden verwijderd. Hoge dichtheden in bakken zonder zand verhogen de kans op vis-vis interacties, welke vervolgens activiteit, voederconversie en variatie in groei van de tong verhogen. Deze condities lijken de sociale stress in tong te verhogen, maar deze stress wordt verminderd wanneer er gebruik wordt gemaakt van zand.

De effecten van milieu op de relatie tussen gedrag en groei zijn onderzocht in **hoofdstuk 5.** Fysieke milieufactoren aanwezig in natuurlijke en kweeksystemen, zoals voersoort (vers versus droogvoer), zand (wel of niet aanwezig) en verschil in omgeving (indoor versus outdoor), zijn onderzocht. Het gedrag van tong is voor en na de introductie in een nieuwe omgeving gemeten. Milieufactoren zoals licht, daglengte en temperatuur, hebben invloed op de gemiddelde groei van tong. Geen enkele van de onderzochte milieufactoren heeft invloed op de individuele variatie van tong (CV, %). De zwemactiviteit van tong wordt wel beïnvloed door de aangeboden milieuomstandigheden. Een verband tussen groei en gedrag in een nieuw milieu was echter alleen aanwezig bij vissen die voor en tijdens het experiment opgekweekt werden in een indoor systeem zonder zand. Deze resultaten lijken aan te tonen dat er een stabiele relatie ontstaat tussen gedrag en groei wanneer vissen worden opgekweekt in een stabiel milieu zonder substraat, maar niet wanneer de vissen opgekweekt worden in een meer variabel natuurlijk milieu.

De invloed van milieufactoren op de relatie tussen niet-eetgedrag en groei van tong lijkt een reden te zijn voor genotype en milieu (G×E) interacties. Daarom is er in **hoofdstuk 6** gekeken in hoeverre genotype x milieu interacties de groei van tong beïnvloeden. Nakomelingen van wilde broodstock zijn willekeurig toegewezen aan de volgende twee milieus: (1) een intensief indoor recirculatie systeem (RAS) en (2) een semi-natuurlijk outdoor vijversysteem. De schattingen voor erfelijkheid van groei (SGR, %BW/dag), bepaald middels moleculair verwantschap tussen dieren, waren lager in RAS (0.04 ± 0.02) dan in vijvers (0.20 ± 0.05). Ook is er een lage genetische correlatie (r_g=0.27 ± 0.3) tussen de groei van tong in een intensief RAS en semi-natuurlijk vijvermilieu. De verschillen in erfelijke variatie en de lage genetische correlatie tussen de groei van tong tussen de twee milieus wijzen op een sterke genotype×milieu interactie. Deze resultaten zijn belangrijk voor het opzetten van fokprogramma's voor tong omdat de manier van selecteren en het resultaat hiervan kunnen verschillen per milieu. De lage genetische correlatie voor groei tussen de milieus wijzen erop dat de beste genotypen in een intensief aquacultuurmilieu niet vanzelfsprekend de beste genotypen zijn in een meer natuurlijk vijversysteem.

De belangrijkste resultaten van dit proefschrift worden in **hoofdstuk 7** besproken. De aanwezigheid van persoonlijkheid in tong en de veranderingen in gedrag die plaatsvonden, worden bediscussieerd. De belangrijkste conclusies uit dit proefschrift zijn:

- Er moet rekening gehouden worden met de invloed van niet-eetgedrag op de variatie in groei om verder te komen met de kweek van tong
- Effecten van milieu op gedrag en groei van tong kunnen worden gebruikt om kweeksystemen te optimaliseren door deze aan te passen aan de behoeften van de vis. De aanwezigheid van substraat lijkt een belangrijke parameter die welzijn en prestaties van tong bevordert.
- Gebrek aan consistentie bij het meten van gedrag van dieren onder laboratoriumcondities geeft aan dat gedrag als selectie kenmerk in sterke mate reageert op de omgeving. Desalniettemin, associaties tussen gedrag en productiekenmerken in een specifieke context moeten het gebruik van individuele verschillen voor selectie niet in de weg staan.
- Het bestaan van correlaties tussen gedrag, zoals eetgedrag en activiteit, en groei, wijst erop dat onder condities van intensieve aquacultuur zonder zand en onder hoge dichtheden met name actieve en competitieve vis zal worden geselecteerd. Selectie voor dit type gedrag kan invloed hebben op de welzijn van kweekvis.
- De resultaten van dit proefschrift geven verder inzicht in het verbeteren van selectiemethoden voor tong in de toekomst. De selectie moet gericht zijn op hogere voedselopname en uniforme groei. De kennis over interacties tussen genotype en milieu zouden moeten worden gebruikt om tong te selecteren die het best in staat is om te gedijen en te groeien in de verschillende condities van de huidige commerciële aquacultuur.

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Hartelijk bedankt jullie allen!

Julia

CURRICULUM VITAE

ABOUT THE AUTHOR

Julia Mas Muñoz, was born on April 22, 1982 in Valencia, Spain. She went to a bilingual school from 1985 until 1996 at the English School Los Olivos (Valencia, Spain). After the completion of her high school education in 2000, she undertook a 5 year degree in Agricultural Engineering, with specialization in Animal production (Zootechnics) at the Polytechnic University of Valencia, Spain (ETSIA, UPV). During the last year of this study she took part in European student exchange programme in 2006 at Wageningen University (Wageningen, the Netherlands) and completed a thesis at the Animal Production Systems group on "Ecological sustainability of biomass as a future energy source". She was invited to present the results from this research at the XVII Foro Universitario Juan Luís Vives: Biocombustibles: solución o problema in Valencia, Spain. In 2008 she obtained her MSc study at Wageningen University with honours during which she completed another thesis at the Adaptation Physiology group on "Effect of free-ranging sows and environmental enrichment on behaviour, feed intake and performance of piglets". Her MSc thesis received first NZV thesis award (Dutch Zootechnical Society, the Netherlands) in 2009.

In September 2008 she started her PhD research at the Aquaculture and Fisheries Group (in cooperation with the Animal Breeding and Genetics Group and Wageningen IMARES), which resulted in this thesis. Her PhD research proposal was awarded as the best research proposal during the Research master cluster course in 2008 and received the WIAS Science Day best poster presentation prize in 2009 and 2010. Continuing with her interest in animal production and triggered to work in applied animal nutrition, she is currently employed by De Heus Animal Nutrition B.V. (Ede, the Netherlands).

Contact: julsmas@gmail.com

LIST OF PUBLICATIONS

Referred scientific journals

Oostindjer, M., **Mas Muñoz, J.,** Brand, H. van den, Kemp, B., Bolhuis, J.E., 2011. Maternal presence and environmental enrichment affect food neophobia of piglets (Online first published 16 June 2010). Biology Letters 7(1): 19-22.

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Mas-Muñoz, J., Schneider, O., Komen, H. Schrama, J.W., 2010. Consistency of individual differences in burying and anti-predator behaviour and its relationship with body weight and growth in sole (*Solea solea*). In: Don Mackinlay (Ed.), Proceedings of the 9th International Congress on the Biology of Fish, Barcelona, Spain, 5-9 July 2010 (pp. 158).

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Mas-Muñoz, J., Komen, H., Blonk B., Verreth, J.A.J. Schrama J.W, 2012. Rearing environment affects growth and behaviour of sole, *Solea solea*. In: Proceedings of the International conference on Global Aquaculture “Securing our future” AQUA 2012, Prague, Czech Republic, 1-5 September 2012.

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European Aquaculture Society, Rhodos, Greece.

World and European Aquaculture Society (AQUA 2012), Prague, Czech Republic.

Seminars and workshops

IV Workshop on the Cultivation of Soles, Faro, Portugal

Aspects of lactation management for piglets and sow, Wageningen, The Netherlands

Mechanisms and evolution of animal locomotion, Wageningen, The Netherlands

Natural behaviour and welfare assessment, Wageningen, The Netherlands

IPOP Sea and Coastal Zones Symposium, Wageningen, The Netherlands

Sole culture in the Netherlands, past, present and future, Wageningen, The Netherlands

Learning how to eat like a pig, Wageningen, The Netherlands

Scientific Research in Animal Welfare: Do We Make a Difference? Wageningen, The Netherlands

WIAS Science Day 2009-2012, Wageningen, The Netherlands

Presentations

Oral presentation at the IPOP Sea and Coastal Zones Symposium, Wageningen, The Netherlands

Oral presentation at the 44rd congress of the International Society for Applied Ethology, Uppsala, Sweden

Oral presentation at the 9th International congress on the Biology of Fish, Barcelona, Spain

Oral presentation at the European Aquaculture Society conference 2011, Rhodes, Greece

Oral presentation at seminar "Sole culture in the Netherlands, past, present and future", Wageningen, The Netherlands

Poster presentation at the WIAS Science Day 2009 & 2010, Wageningen, The Netherlands

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“Individual variation in growth and feed intake of sole (*Solea solea*) in relation to behaviour (pilot study)”. MSc Thesis 2009, Sander Visch.

“Effect of stocking density, growth class and their interaction on growth/feed intake and behaviour of Dover Sole (*Solea solea*)". MSc Thesis 2010, Sara Leigo.

“Variation in feeding behaviour of group- housed Dover sole (*Solea solea*) and its relationship with growth”. MSc Thesis 2011, Jurre Zaal.

“The effects of altering the group composition (homogenous versus heterogeneous) regarding sex class and size class on feed intake, growth and (non-) feeding behaviour of Dover sole (*Solea solea*)”. MSc Thesis 2011, Marit Nederlof.

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COLOPHON

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