The European eel may tolerate multiple infections at a low biological cost

ELVIRA MAYO-HERNÁNDEZ[1,2]*, EMMANUEL SERRANO[3,4], JOSE PEÑALVER[2,5], ALFONSA GARCÍA-AYALA[2,6], ROCÍO RUIZ DE YBÁÑEZ[1,2] *and* PILAR MUÑOZ[1,2]

[1] *Departamento de Sanidad Animal, Universidad de Murcia, 30100 Murcia, España*
[2] *Campus de Excelencia Internacional Regional 'Campus Mare Nostrum', Universidad de Murcia, Espinardo, 30100 Murcia, España*
[3] *CESAM, Departamento de Biologia, Universidade de Aveiro, Campus Universitario de Santiago, 3810-193 Aveiro, Portugal*
[4] *Servei d'Ecopatologia de Fauna Salvatge, Departament de Medicina i Cirurgia Animal, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, España*
[5] *Servicio de Pesca y Acuicultura, D.G. de Ganadería y Pesca, Consejería de Agricultura y Agua de la Región de Murcia, JUAN XXIII, 30071 Murcia, España*
[6] *Departamento de Biología Celular e Histología, Universidad de Murcia, 30100 Murcia, Spain*

(*Received 30 August 2014; revised 13 January 2015; accepted 19 January 2014; first published online 25 February 2015*)

SUMMARY

Most animals are concurrently infected with multiple parasites, and interactions among them may influence both disease dynamics and host fitness. However, the sublethal costs of parasite infections are difficult to measure and the effects of concomitant infections with multiple parasite species on individual physiology and fitness are poorly described for wild hosts. To understand the costs of co-infection, we investigated the relationships among 189 European eel (*Anguilla anguilla*) from Mar Menor, parasites (richness and intensity) and eel's 'health status' (fluctuant asymmetry, splenic somatic index and the scaled mass index) by partial least squares regression. We found a positive relationship with 44% of the health status variance explained by parasites. *Contracaecum* sp. (Nematoda: Anisakidae) was the strongest predictor variable (44·72%) followed by *Bucephalus anguillae* (Platyhelminthes: Bucephalidae), (29·26%), considered the two most relevant parasites in the analysis. Subsequently, 15·67 and 12·01% of the response variables block were explained by parasite richness and *Deropristis inflata* (Platyhelminthes: Deropristiidae), respectively. Thus, the presence of multiple parasitic exposures with little effect on condition, strongly suggests that eels from Mar Menor tolerate multiparasitism.

Key words: co-infection, multiparasitism, fluctuant asymmetry, body scaled mass, splenic somatic index, eel, teleost.

INTRODUCTION

Parasitological studies consistently demonstrate that, in natural conditions, an individual host is typically infected by multiple parasites (Petney and Andrews, 1998; Cox, 2001). The health impact of multiple infections has been known for some time, with most information from human studies (Haswell-Elkins *et al*. 1987; Ferreira *et al*. 1994; Needham *et al*. 1998; Brooker *et al*. 2000; Pullan and Brooker, 2008). However, few studies have investigated the impact on wild vertebrate host health (for a review see Bordes and Morand, 2011). There is a clear need to consider full pathogen communities rather than single relevant pathogen species when assessing the impact of infections (Serrano and Millán, 2014).

In fish, especially in predator species, both infection intensity and pathogen richness typically increase with the size and age of hosts. The rationale is rather simple since for trophically transmitted parasites infection rates rely on the ingestion of infective intermediate hosts, and hence older individuals have had more opportunities to accumulate parasites than younger ones (Dogiel 1958; Poulin, 2000; Luque and Poulin, 2004; Kamiya *et al*. 2014). Due to this bioaccumulation, fish are likely to suffer multiple infections in their lifetime, and the impact of disease severity in the host fitness may depend on interactions among co-infecting pathogens (Pedersen and Fenton, 2007) and the co-evolution between parasite and host (Lymbery *et al*. 2014). In general, hosts limit the harm caused by a given parasite burden without any direct negative effects on the parasite (tolerance), or by direct attack on parasites by activation of the immune system (resistance, according to Råberg *et al*. 2009). Tolerance and resistance are different but complementary host traits that require reallocation of host resources, and therefore tend to carry physiological costs (Råberg *et al*. 2009; Medzhitov *et al*. 2012; Schneider and Ayres, 2008). Both defensive strategies have also been described in fishes (e.g, see Boots and Bowers, 1999; Blanchet *et al*. 2010). The equilibrium between the two strategies, disease severity and host fitness, are specific to each host–parasite system (Roy and Kirchner, 2000).

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* Corresponding author: Departamento de Sanidad Animal, Universidad de Murcia, Espinardo. CP: 30100 Murcia, Spain E-mail: elviramayohdz@gmail.com

Parasitology (2015), **142**, 968–977.
doi:10.1017/S0031182015000098

However, little is known about the defensive strategies in the case of co-infection with multiple pathogens (Råberg *et al*. 2009).

The biological cost resulting from parasites in wild fishes has been evaluated by measuring the body condition (e.g. Gérard *et al*. 2013; Lefebvre *et al*. 2013), the immune response (e.g. Seppänen *et al*. 2009; Rohlenová *et al*. 2011) and stress during infection (e.g. Iwama *et al*. 2011). The rationale for using such indicators of the cost of infection is the following: body condition is defined as the tissues necessary for a functionally normal life and those accumulated in anticipation of periods of shortage. Body condition has previously been used in different fish studies to evaluate the impact of parasites on host health (e.g. Lemly and Esch, 1984; Tierney *et al*. 1996; Arnott *et al*. 2000; Maan *et al*. 2008; Lefebvre *et al*. 2013). However, we use the scaled mass index (SMI), as a proxy of body condition, because it is considered as an excellent measure of the energy capital accumulated in the body as a result of feeding and has previously been validated with data on body components such as fat and protein (Peig and Green, 2009). To the authors' knowledge, this novel condition index has only been recently used in fish (Maceda-Veiga *et al*. 2014). Due to the costs of parasitism, as in body condition, it is expected that the eel's SMI decreases as parasite intensity and richness increase.

The immune response is an evolved strategy to defend hosts efficiently against the effects of parasites on host fitness (Møller and Saino, 2004). The spleen, a secondary lymphatic organ, plays a highly important role in haematopoiesis and immune reactivity of teleost fish producing antibodies and participating in clearance of pathogens and foreign particles from the blood stream (Dalmo *et al*. 1997; Lamková *et al*. 2007). The splenic somatic index (SSI), is the simplest method for estimating the absolute or relative abundance of immunologically active cells (Owens and Wilson, 1999), and is widely used as a simple measure in immune response against parasites in fish (Manning, 1994; Kortet *et al*. 2003; Lefebvre *et al*. 2004; Ottová *et al*. 2005, 2007). If parasites cause an immune reaction, one would expect the spleen to increase in size due to its important haematopoietic function for leucocyte synthesis.

Another way to evaluate the costs of parasites in wild fishes is by measuring the stress that parasite infection produces on the host. Stress may be considered as a change in biological condition beyond the normal resting state that challenges homeostasis and, thus, presents a threat to the fish's health (Barton and Iwama, 1991). Fluctuating asymmetry (FA), a direct measure defined as a non-directional deviation from symmetry in bilateral traits, is related to stress (reviewed in Leung and Forbes, 1996). Therefore, FA could be analysed as a measure of parasite virulence (Allenbach, 2011) since parasites can cause direct stress on the host through metabolic costs (Møller, 1992; Polak, 1993). Thus, with an increase in parasite intensity and richness, FA would increase.

The present study will evaluate whether wild eel health, using FA, SSI and SMI as proxy, is affected by polyparasitism by means of partial least square regression models (PLSR). At the same time, since co-infections in European eels are common (Martínez-Carrasco *et al*. 2011), in this work we are interested in understanding whether the effects of parasitation on eel health were due to a particular species or, on the contrary, relied on the additive effects of several parasites. This analysis was used to understand the defence strategy (tolerance or resistance) used by eels to cope with co-infection.

MATERIALS AND METHODS

Area of study

Mar Menor lagoon is located in Southeastern Iberian Peninsula (37°38′N, 0°42′W), Spain (Fig. 1). It is the largest saltwater lake in Europe with an area of 180 Km^2 and 73 Km of coastline. The maximum depth is 7 m. It is isolated from the sea by a 24 Km-long sandbar known as La Manga. The water exchange and, therefore, the passage of fish species between the Mar Menor and the Mediterranean Sea occurs naturally through natural openings or passages, and there are no artificial barriers that limit the passage of eels. Its salinity levels (43–46·5 g/L) are greater than the adjacent Mediterranean Sea due to low precipitation (around 300 mm per year) and high evaporation rates (mean annual temperature 18 °C).

Eel biometry and indexes

A total of 189 wild European eels were collected by local fishermen during 2010 and frozen until processed. Eels were measured to the nearest 0·5 mm using a nylon tape (mean length: 528·46 ± 72·05 mm) and weighed to the nearest 0·01 g on a digital scale (mean weight: 261·90 ± 115·48 g).

The maximum pectoral fin length (LFR, LFL for right and left measures, respectively) and the distance between the operculum and labial commissure on each side (O–MR, O–ML for right and left measures, respectively) (Fig. 2) were measured with a digital calliper. Maximum otolith length (MOLr; MOLl, for right and left measures, respectively) and maximum otolith width (MOWr; MOWl, for right and left measures, respectively, Fig. 3) were measured using a stereoscope ZEISS model Stemi 2000-C connected to a computer-enhanced video image-analysis (SPOT version 4.6). All measurements were carried out twice by the same observer according to the recommendations of Palmer and Strobeck (1986).

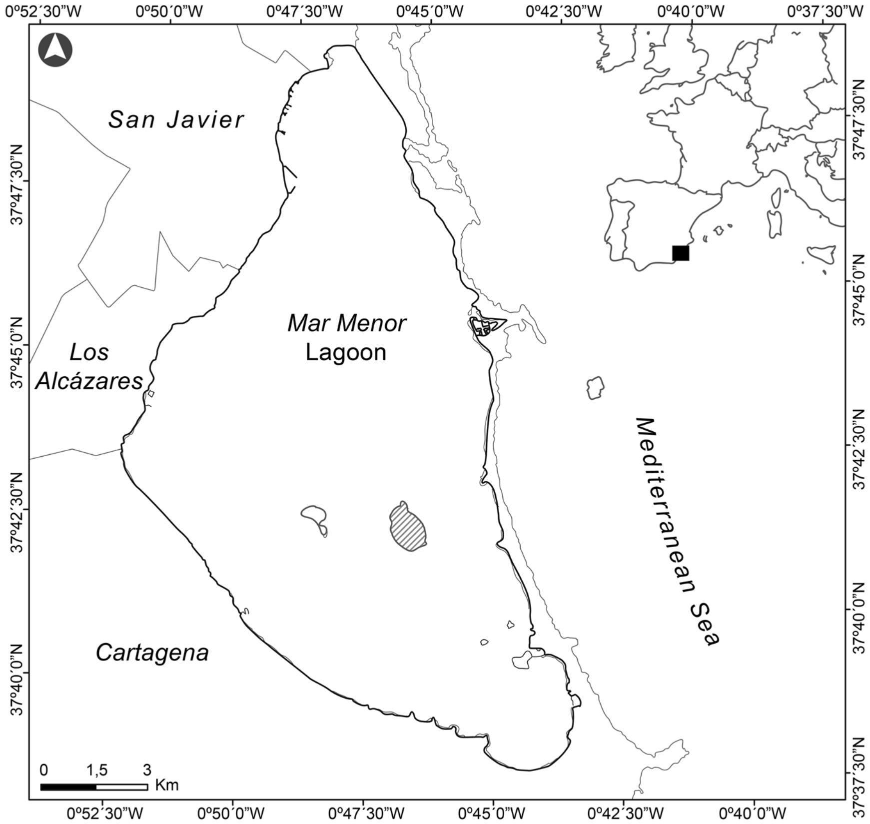

Fig. 1. Map showing the location of Mar Menor lagoon.

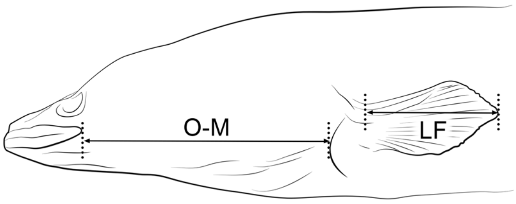

Fig. 2. Anterior part of the eel body. Arrows indicate the distances in mm between operculum and dorsal fold mouth of each side (O–M) and maximum length of pectoral fins (LF).

SMI was calculated according to Peig and Green (2009) as $M_{ii} = M_i (L_o/L_i)^{b}_{SMA}$ where M_i and L_i are the body mass and linear body measurement of an individual$_i$ respectively; $^{b}_{SMA}$ is the scaling exponent estimated by the standardized major axis (SMA) regression of ln$M$ on ln$L$; L_0 is an arbitrary value of L (e.g., the arithmetic mean value for the study population); and *Mii* is the predicted body mass for individual *i* when the linear body measure is standardized to $L0$. SSI was calculated as the residuals of the ratio of the spleen weight to total body weight, and used to correct the fish condition effect.

Age determination

In brief, sagittal otoliths were removed from each eel by slicing the head open longitudinally from the dorsal surface with a sharp, heavy-bladed knife. Thus, they could easily be removed from the otic capsules with forceps, cleaned and stored dried. For better reading, due to their small size and thinness, whole otoliths were cleared in glycerine (ICES, 2009), and examined under a stereoscopic microscope (40X magnification), with reflected light, against a dark background and immersed in glycerine to allow increased light penetration.

By convention, the age reference date was set as the 1st of January, eels were age 0 in their year of arrival to continental water, and age was estimated based on counts of otolith winter rings. As is recommended, age was estimated from each left otolith by two independent readers, EMH and PM (ICES, 2009).

Parasitological analysis

Briefly, eels were examined for ectoparasite detection. They were then dissected and the body cavity and mesenteries were inspected. Internal organs were cut and observed under a stereomicroscope. Digestive content was also examined under a stereomicroscope. Swim bladders were inspected for the presence of adult and larval stages (L3 and L4) of *Anguillicoloides crassus*. Muscle tissue was artificially digested for detection of anisakid larvae. Parasites were stored in 70% ethanol until their identification. Prevalence, intensity and abundance of infection for each parasite species were defined according to Bush

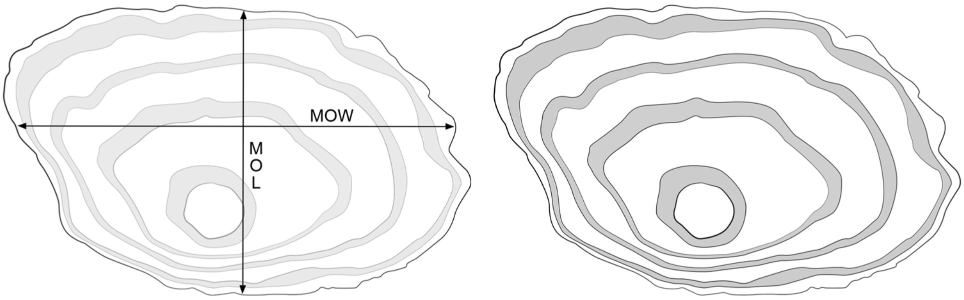

Fig. 3. Eel otolith and an overlook of the otolith rings. MOL, maximum otolith length and MOW maximum otolith width.

et al. (1997). Specifically, the prevalence is the number of hosts infected with a particular parasite species (or taxonomic group) divided by the number of hosts examined for that parasite species, whose value is measured as percentage. The intensity is the ratio among number of individuals of a particular parasite species in a single infected host and the number of infected individuals of the host species in the sample, whose value is measured in specimens per infected animal. And finally, the abundance is number of individuals of a particular parasite species per host examined, whose unit is measured as specimens per analysed animal.

Statistical analysis

Two analyses were performed to examine FA with multiple traits. For the first analysis, a FA-index that reflects absolute differences between right and left side separately for each trait for each individual was calculated following the recommendations of Palmer and Strobeck (2003) ($FA_1 = |(\text{mean R}) - (\text{mean L})|$). The Composite Fluctuating Asymmetry index 2 (CFA_2) was then estimated (Leung *et al*. 2000) as the summation of standardized absolute FA_2 values (FA_1 values of a given trait divided by the average absolute FA for that trait). Every FA index of a single trait depends heavily upon statistical inference, and consequently a number of conditions should be evaluated beforehand to obtain reliable CFAs.

Linear regression, Pearson correlation and a Kruskal–Wallis test were used to analyse the relationship of eel age (treated as continuous or categorical variable) and size (length) to richness, total intensity of infection and individual parasite intensity. To assess whether older individuals had accumulated more parasites than the younger ones, *post hoc* tests were also performed between age classes.

Moreover, the influence of parasite community traits (i.e. parasite composition and intensity of infection due to specific parasite species) on the selected eel health-related parameters was assessed using a PLSR approach. This statistical tool is an extension of multiple regression analysis where associations are established with factors; that is to say, combinations of dependent variables extracted from predictor variables that maximize the explained variance in the dependent variables. The relative contribution of each variable to the derived factors is calculated by means of square predictor weights. PLSR is a distribution-free technique and does not require specific distribution data. In our case, the response variables (SMI, the residuals of SSI regression and CFA_2) make up the Y-component, which is defined as health status, whereas the explanatory variables (called ‘parasites block’) were parasitic richness (i.e. the number of different parasite species found in an eel) and specific parasite intensity (i.e. number of individuals from a parasite species found in an individual eel). We also obtained the variable importance for projection (VIP) and modified weights and scores of the variables. VIP explains the power of a given predictor X_j on the block of responses Y. Moreover, VIP provides another way to classify the predictors in terms of their explanatory power of Y. Those predictors with a $VIP > 1$ are considered the most relevant to the construction of the Y-component. The modified weights are the values used for calculating the components with the original block of predictors. The X-scores, which contain the extracted PLS components, and the Y-scores are the components associated with the response variable. PLSR was implemented in the ‘plspm’ library (Sanchez and Trinchera, 2012) of the statistical package *R* version 3.0.3 (R Development Core Team, 2013).

RESULTS

Eel biometry and age

Morphological and otolithometrical characteristics are shown in Table 1, while Table 2 summarizes all FA values. FA values were significant in two traits analysed (O–M and MOW) while no significant FA were observed in LF or in MOL (see Appendix).

Relationship among parasite status and age and eel size

The composition of the parasite community (prevalences, abundances and intensities) in this eel

Table 1. Morphological and otolithometrical characteristics of the 189 eels sampled from Mar Menor lagoon

Mean age ± s.d. (range) in years	4·73 ± 1·49 (0–8)
Mean weight ± s.d. (range) in g	261·9 ± 115·487 (61–916·2)
Mean length ± s.d. (range) in mm	528·5 ± 72·06 (355–760)
Mean LF ± s.d. (range) in cm	**R** 2·48 ± 0·45 (1·30–4·2) **L** 2·45 ± 0·45 (1·20–4·3)
Mean O–M ± s.d. (range) in cm	**R** 4·86 ± 0·67 (3·1–7) **L** 4·85 ± 0·65 (3·2–7)
Mean MOL ± s.d. (range) in mm	**R** 2·05 ± 0·21 (1·35–2·65) **L** 2·05 ± 0·22 (1·39–3)
Mean MOW ± s.d. (range) in mm	**R** 3·22 ± 0·37 (2·41–4·13) **L** 3·26 ± 0·38 (2·23–4·34)

LF, length of the pectoral fins; O–M, distance between the operculum and labial commissure; MOL, máximum length of the otoliths; MOW, máximum width of the otoliths; s.d., standard deviation, L, left; R, right.

Table 2. Fluctuating asymmetry values of four bilateral characters

FA		
Mean ± s.d. (range)	LF	0·02 ± 0·10 ((−0·30)–0·35)
	O–M	(0·017 ± 0·25) ((−0·75)–0·8)
	MOL	−0·027 ± 0·13 ((−0·75)–0·45)
	MOW	−0·058 ± 0·14 ((−0·77)–0·34)

FA, means raw values of fluctuating asymmetry for each measured, LF, length of the pectoral fins; O–M, distance between the operculum and labial commissure; MOL, maximum length of the otoliths; MOW, maximum width of the otoliths; s.d., standard deviation.

population was described in Mayo-Hernández *et al.* (2014) (see Table A1 in the Appendix). The nematode *A. crassus* and Proteocephalidae larvae were removed from further analysis due to their low prevalences (<5%).

Age, analysed as a quantitative variable, was positively and significantly correlated to the log-transformed whole parasite intensity (R^2 = 4%, $F_{1,154}$ = 6·55, *P*-value = 0·01) while no significant correlations to specific parasite species were observed. When age was pooled in classes (e.g. 1 year, 2 years, etc), *Contracaecum* intensity of infection was significantly different between age classess (Kruskal– Wallis chi-squared = 14·83, d.f. = 6, *P*-value = 0·022), and had a positive and significant linear correlation to log-transformed *Bucephalus anguillae* intensity (R^2 = 8·6%, $F_{6,145}$ = 2·27, *P*-value = 0·04). Nevertheless the post-hoc comparisons indicated no significant effect of age on *Contracaecum* sp. and *B. Anguillae* intensity of infection. No significant correlation was found to richness or to *Deropristis inflata* intensity.

Regarding size (length), we detected a positive significant relationship with: richness (R^2 = 7·4%, $F_{4,182}$ = 3·64, *P*-value = 0·01) and with parasite intensity (R^2 = 7·9%, $F_{1,184}$ = 15·8, *P*-value < 0·01); also with log-transformed intensities of *D. inflata* (*r* = 0·15, *P*-value = 0·05), *B. anguillae* (R^2 = 8·2, $F_{1,184}$ = 16·64, *P*-value = $6{\cdot}718 \times 10^{-5}$); and *Contracaecum* sp. intensities of infection (R^2 = 12%, $F_{1,185}$ = 25·26, *P*-value = $1{\cdot}179 \times 10^{-6}$). It should be noted that the residuals of the last linear correlations did not show a normal distribution.

Impact of co-infections on host health

The results of the PLSR analysis are represented graphically (Fig. 4), and predictor and explanatory variable weights and scores are shown in Table 3. The graphic shows a linear and positive correlation between individual parasitic intensity and richness (*X*-component) and SMI, FA and SSI (*Y*-component). Moreover, the analysis revealed that the parasites block (*X*-component) is responsible for 44% of the variance of the eel's health status (*Y*-component). Moreover, the nematode *Contracaecum* sp. was the strongest predictor variable, explaining 44·72% of the response variables block. The 29·26% was explained by *B. anguillae*, which along with the *Contracaecum* sp., was considered the most relevant to the construction of response variables Y, since they had a VIP = 1·0 and a VIP = 1·5, respectively. Subsequently, 15·67 and 12·01% of the response variables block were explained by richness and *D. inflata*, respectively.

DISCUSSION

Relationship among parasite status and age and eel size

Our results from wild eels are in line with previous studies (Dogiel 1958; Bell and Burt, 1991; Poulin, 2000; Luque and Poulin, 2004; Kamiya *et al.* 2014), which have shown that both parasite intensity and diversity of infection increase with age or size of fish hosts due to parasites accumulated over time.

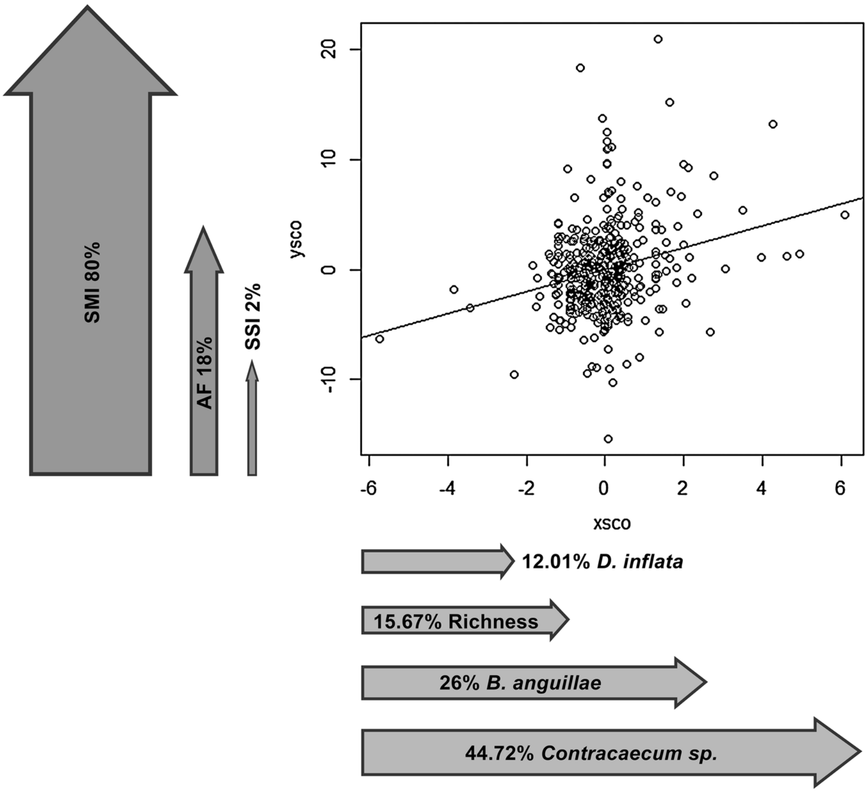

Fig. 4. Relationship on a PLSR model among co-infecting helminths intensities and parasites richness (X-component) and the body condition, the SSI and the FA (Y-component) on 189 eels from Mar Menor. This plot represents the best PLSR model shown in table 3. Arrow direction indicates either an increase or a decrease of the component value, and arrow thickness directly indicates the weight of the component. The nematode *Anguillicoloides crassus* and Proteocephalidae larvae were excluded from the analysis due to their low prevalences (< 5%).

Table 3. Predictor weights of the partial least squares regression (PLSR) model explaining the effects of co-infection by community parasites on health of 189 eels from Mar Menor lagoon

Predictor variables	Weights	% Variance explained
Contracaecum sp	0·78	44·72
Bucephalus anguillae	0·51	29·26
Richness	0·27	15·67
Deropristis inflata	0·21	12·01
Explanatory variables	Weights	% Variance explained
SMI	0·95	80
CAF_2	0·45	18
Residuals-SSI	0·16	2

Predictor weights (parasitic richness and specific parasite intensity) were transformed in percentage to explain the contribution of each to the PLSR's Y axis, represented by SMI, fluctuating asymmetry and SSI. Explanatory weights (correlation between the Y-components and the Y) of the partial least squares regression (PLSR) model explaining the percentage of the Y-component explained by the explanatory variables.

Impact of co-infections on host health

The analysis revealed that the parasites block (X-components) is responsible for 44% of the variance in eel health status. The present study showed that although parasite intensity and richness increase, causing stress and immune system activation, eels continue to develop. The parasites block included two digenea (*D. inflata* and *B. anguillae*), the nematode *Contracaecum* sp. and parasite richness. Both Digenea, native and eel-specific, the most prevalent parasitic group in Mar Menor eels, can destroy the mucosal epithelium covering the villi, and cause its necrosis and degeneration (Dezfuli *et al.* 1997), showing strict specificity for their niche (intestine). Therefore, *D. inflata* and *B. anguillae* compete for the same sources and may interact directly (Pedersen and Fenton, 2007). On the other hand, eels serve as intermediate hosts for *Contracaecum* spp., a generalist parasite that matures in piscivorous birds and mammals (Anderson, 1992). The L3 larvae of this nematode can be found in musculature and viscera, with its migration and feeding leading to lesions (Rohde, 1984; Williams and Jones, 1994). However, the host can induce L3 larvae encystment in the tunica propia and along the stomach and intestinal wall, as previously reported in eels by Dezfuli *et al.* (2009), which may contribute to their evasion of the host's immune response (Sitjà-Bobadilla, 2008).

The eels' health status block (*Y*-component) was defined by the response block (SMI, SSI and FA). Eighty percent of the health status was represented by SMI variation. Negative parasite effects on host condition are widely known (e.g. Maan *et al.* 2008; Gérard *et al.* 2013*) and expected,* but there is no a priori reason to assume that this relationship would always be negative as there are also reports of better relative condition among parasitized fish (Arnott *et al.* 2000; Costa-Dias *et al.* 2010; Guidelli *et al.* 2011; Lefebvre *et al.* 2013). The present study showed a positive relationship between SMI and parasite intensity and richness, which corroborates the finding that the relationship between fitness loss and infection intensity is not necessarily linear (intensity-dependent virulence) (Rollinson and Hay, 2011). Some studies concluded that changes in body condition depend on the presence of determinate parasites and interactions among them (e.g. Arnott *et al.* 2000; Khokhlova *et al.* 2002; Hoffnagle *et al.* 2006). Seppänen *et al.* (2009), Khan (2012) and Santoro *et al.* (2013) reported that effects on body condition can be influenced by the level of infection, the size of the parasites and the host tissue or organ affected. In Mar Menor eels, the parasite community composition, the low mean intensity of parasitization (102 ± 200·87) and parasite localization (explained in the parasites block) may be responsible for this positive relationship.

The association between spleen size and parasitism by metazoan parasites has been tested in many intraspecific studies (e.g. Taskinen and Kortet, 2002; Ottová *et al.* 2005; Vainikka *et al.* 2009). Positive correlations between spleen enlargement and nematodes (Arnott *et al.* 2000; Morand and Poulin, 2000; Lefebvre *et al.* 2004) or digenean (Seppänen *et al.* 2009) parasites have been reported. However, in our study, the parasite community showed a very low variance in the SSI residuals (2%) reflecting a weak spleen immune response to those species. Similar results have been described by Vainikka *et al.* (2009) and may be due to the long-term coevolution between the parasites in the PLSR model, all of them native, and the eel populations (Peeler *et al.* 2011).

Eighteen percent of the *Y*-component (health status) was due to stress, measured by FA, indicating a slight influence of parasite intensities and richness on FA. A positive relationship between parasite infection level and developmental instability in fish has been reported previously (Møller, 2006; Reimchen, 1997; Sasal and Pampoulie, 2000; Reimchen and Nosil, 2001; Bergstrom and Reimchen, 2002), while no consistent relationship was found in other studies (Escos *et al.* 1995; Berg *et al.* 1997; Campbell and Emlen, 1996). Fazio *et al.* (2005) found no asymmetry in European eels due to *A. crassus*, considered highly pathogenic in eels (Kennedy, 2007); however, similar to our results, they did find a positive relationship between asymmetry and digenean parasites in yellow eels. They suggested that digeneans may be responsible for part of the asymmetry by disturbing, either directly or indirectly, the metabolism of some nutrients. It is also important to note that some asymmetrical fishes are free of parasites (and the reverse is also true), indicating that it is impossible to determine whether the presence of parasites is the cause or the consequence of the phenotypic variation in the selected traits (Sasal and Pampoulie, 2000). Therefore, according to Allenbach (2011), both FA and parasitism can be used as biological indicators of stress. For this reason, we recommend further studies to test other possible stressors as contamination.

Tolerance vs resistance

Our results show a positive link between parasitism and eel health status. Undergoing multiple parasitic exposures while maintaining good condition, strongly suggests that Mar Menor eels tolerate multiparasitism. Tolerance ameliorates the damage that parasites cause, allowing infected hosts to live longer, and thus, increases the infectious period and increases rather than decreases parasite prevalence, which in Mar Menor eels was 95%, leading to a positive feedback loop (Roy and Kirchner, 2000). The low prevalence (3%) of the invader species *A. crassus* in Mar Menor eels (Mayo *et al.* 2014) should be emphasized. The severe adverse effects produced by this nematode at the population level could be due to a lack of immunity in new hosts (Peeler *et al.* 2011), since there has been no co-evolution between host and parasite. In fact, the eventual higher prevalence of this invasive parasite may be responsible for poorer health status in eels.

Although, we did not investigate the immune system in its entirety but the low percentage of SSI variance explained by the parasite community would indicate little investment in immunity. Although further studies are needed to identify other factors affecting European eel fitness, such as contaminant accumulation or other immunity indexes, as oxidative stress. Based on parasite community damage, we propose that eels from the hypersaline Mar Menor lagoon are probably able to migrate and reproduce, contributing to the recruitment and gene pool of the *A. anguilla* population, but more definitive studies using swim tunnels will be required to assess whether health effects caused by parasites impair migration of eels from this ecosystem to the spawning area.

ACKNOWLEDGEMENTS

The authors are thankful to Jose Miguel Mayo H. for his collaboration with the illustrations and João Carvalho (Unidade de Vida Selvagem, Universidade de Aveiro) for the map.

FINANCIAL SUPPORT

This work was supported by La Fundación Séneca, Coordination Centre for Research (grant 04538/GERM/06). E. Serrano was supported by the postdoctoral program (SFRH/BPD/96637/2013) of the Fundação para a Ciência ea Tecnologia, Portugal.

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APPENDIX

Table A1. The composition and levels of infection of the helminth community in 189 eels from the Mar Menor lagoon; mean values ± standard deviation

Helminth species	Site of infection	Prevalence (%)	Mean intensity (range)	Mean abundance
Digenetic trematodes				
Deroprisitis inflata	Digestive tract	67	101 ± 162·85 (0–1240)	68 ± 173·55
Bucephalus anguillae	Digestive tract	60	39 ± 47·30 (0–400)	23 ± 51·19
Nematodes				
Contracaecum sp.	Digestive tract	46	8 ± 9·98 (0–158)	4 ± 8·15
	Muscle	12	1 ± 0·39 (0–4)	1± 0·45
Anguillicoloides crassus[a]	Swim bladder	3	3 ± 0·44 (0–10)	1 ± 0·75
Cestodes				
Proteocephalidae larvae	Digestive tract	2	0·01 ± 0·10 (0–1)	1 ± 0·12

[a] includes adult and larval stages.

Fluctuating asymmetry analysis for O–M; LF; MOL and MOW was carried out according to Palmer and Strobeck (2003).

1) First, data were inspected for poor raw measurements or aberrant individuals. The box plots of replicate measurements of right (R) and left (L) and (R–L) differences of all traits (O–M; LF; MOL; MOW) did not reveal any outliers.
2) Differences due to measurement error between-sides by a Mixed-ANOVA of sides (fixed factor) * individuals (random factor) were tested, and the results revealed that the variation between-sides was significantly greater than that expected due to measurement error in traits for O–M (sides * individuals $F_{184,369} = 8·99$, P-value $< 0·0001$), LF (sides * individuals, $F_{185,371} = 2·5$, P-value $< 0·0001$) and MOW (sides * individuals, $F_{42,42} = 3·09$, P-value $= 0·0002$). However for MOL, measurement error was higher than the variation between-sides (sides * individuals $F_{42,42} = 0·567$, P-value $= 0·9652$), meaning that the measurements were not made correctly, and these measurements are thus rejected from the analysis.
3) The independence between FA (|R−L|) and trait size [(R+L)/2] was then checked through a Pearson correlation test, and independence was found in LF measurements ($r = -8·603568$ e-05, $t = -0·0012$, P-value $= 0·9991$) and MOW measurements ($r = 0·103295$, $t = 1·2761$, P-value $= 0·2039$). However, correlation in the size parameter in O–M ($r = 0·1820509$, $t = 2·5046$, P-value $= 0·01313$) was found, and for this reason, O–M FA corrected by size (FAs-O–M) was selected to calculate the CAF2.
4) Because the advantage of combining FA indices (Leung *et al*. 2000) from different traits decreases as the degree of correlation between traits increases, we previously checked for the correlation between FA indices through a Pearson correlation test. Values for all analyses were uncorrelated (LF~O–M: $r = 0·0120833$, $t = 0·163$, P-value $= 0·8707$; LF~MOW: $r = 0·09862025$, $t = 1·2056$, P-value $= 0·2299$; O–M~MOW: $r = -0·07307574$, $t = -0·8884$, P-value $= 0·3758$).
5) We also checked for departures for directional asymmetry. The R–L of MOW (Mixed-ANOVA step 2, sides $= F_{1,42} = 1·19$, P-value $= 0·2810$) and O–M (Mixed-ANOVA step 2, sides $= F_{1,369} = 8·20$, P-value $= 0·0044$) did not exhibit directional asymmetry. However, LF did exhibit directional asymmetry (Mixed-ANOVA step 2, sides $= F_{1,371} = 18·0$ P-value $<$ $0·0001$). For this reason, LF was not included in the CAF_2 calculations.
6) The last step was to calculate the CAF_2 as the summation of standardized absolute FA values of MOW and M–O, which fulfilled all requirements.

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