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An "omic" approach to investigate the interface between the host-parasite system: The case of *Anisakis* and their natural and accidental hosts

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1. Introduction

The advent and integration of high-throughput "omics" technologies (i.e. genomics, transcriptomics, proteomics) are becoming instrumental to assist fundamental explorations of the organism biology systems. In particular, those technologies provide unique opportunities for further investigations in parasite molecular biology (Marzano et al. 2017). For instance, studies of the transcriptome (all transcripts in an organism) of different parasitic species and/or its developmental stages provide insights into aspects of differential gene expression, regulation and function. This represents a major step to understand the biology of the parasitic life style, and adaptation in their hosts.

The present thesis aimed to investigate about the molecules (and their possible roles) involved in the host-parasite interaction formed by the species of the *Anisakis simplex* (s. l.) complex and their naturally (fish) and accidentally (humans) infected hosts. In order to reach the main and specific objectives of the research, an "omic" approach has been applied.

The ecological interaction formed by anisakids and their hosts provides fascinating examples of evolutionary adaptation (Mattiucci and Nascetti 2008). In the course of evolution, anisakids nematodes have developed numerous strategies for adapting to their hosts, survive, and increase their fitness. However, the molecular basis of molecules that regulates the mechanisms involved in the differential host responses to *A. simplex* (s. s.) and *A. pegreffii*, at a cellular and tissue level, in both natural and accidental (humans) hosts, remain largely unknown.

State of the Art

The species of the *A. simplex* (s. l.) complex (i.e. *A. pegreffii*, *A. simplex* (s. s.) *and A. berlandi*) are heteroxenous parasites, involving various marine organisms (crustacean, fish, squids, cetaceans) in their life-cycle; humans are accidental hosts. Among the *Anisakis* spp. so far genetically detected (for a review, Mattiucci et al. 2018), *A. pegreffii* and *A. simplex* (s. s) are so far recognised agents of the fish-borne parasitic zoonosis, anisakiasis. Humans became infected by consumption of parasitized raw or lightly cooked fishery products (fish and squids are intermediate/paratenic hosts in the life cycle) (for a review, Mattiucci et al. 2018; Guardone et al. 2018). Anisakiasis is of worldwide concern. Around 20,000 total anisakiasis cases are reported worldwide (EFSA 2010), most from Japan, Korea, Taiwan, China and European countries. Anisakiasis can have gastric, intestinal and gastroallergic (GAA) clinical forms. In GAA an acute parasite infection with live *Anisakis* larvae can provoke gastric symptoms together with an IgE-mediated immune response, that results in the occurrence of allergic symptoms. From an immunological point of view, *Anisakis* spp. could determine the activation of lymphokines belonging to the Th2 pattern, triggering allergic symptomatology, or to the Th1-type, with eosinophilic granulomas at the gastric and intestinal level. In Italy, the human cases are so far reported due to *A. pegreffii*. Allergy to *Anisakis* proteins has been also described in sensitized patients **(**Mattiucci et al. 2017). This implies that some patients can have allergic symptoms even when the fish

meal was properly cooked or previously frozen, although this interpretation remains controversial. Anisakis-IgE-hypersensitivity in Italian patients, determined by comparing values of iCAP and immunoblotting (WB), indicated that the only use of iCAP method could overestimate the disease (Mattiucci et al. 2017). Cross-reactivity between *Anisakis* and other antigens such as tropomyosin, might lead to misdiagnosis of *Anisakis* allergy. To date, antigens/allergens from *A. simplex* (s. l.), recognised by the human response, are 14 (Allergen Nomenclature Sub-Committee, www.allergen.org); they comprise both somatic and ESPs (Daschner et al. 2012; González-Fernández et al. 2015; Kobayashi et al. 2015). Among the excretory/secretory products (ESPs), *Ani s 1*-like *Ani s 7*-like and *Ani s 13*-like, are the major antigens of *A. pegreffii* responsible for human IgE-hypersensitivity (Mattiucci et al. 2017). Those antigens have no amino acid sequence similarity with any other allergens able to sensitize humans, thus useful for diagnosis in human infection (Moneo et al. 2000; Rodríguez et al. 2008; Daschner et al. 2012; Mattiucci et al. 2017).

Anisakids nematodes generally produce and release a variety of proteins/peptides, ESPs which have been retained to be key players in the parasite-host adaptation and interaction. It has been also suggested that ESPs production varies, both quantitatively and qualitatively, depending, for instance, by the type of host (intermediate, definitive and accidental), also as a consequence of the different structural and physiological conditions in those host groups (Mehrdana and Buchmann, 2017). On the other hand, it has been suggested that parasites ESPs have several functions during infection, e.g. penetration of host tissues and evasion of host immune responses, but also able to elicit immune responses both in fish and mammals (Kuhn et al. 2013; Skrzypczak et al. 2014; Mehrdana and Buchmann, 2017). In nematode ESPs have immunomodulatory effects interfering with host immune responses, thus, they play an important role in clinical manifestation of the disease in humans. Probably, the L3 of *Anisakis* spp. use mechanical disruption of tissue combined with the release of proteolytic enzymes (peptidases) to penetrating the gastrointestinal mucosa and for migrating into the tissue of the fish and humans (Bahlool et al. 2013). Proteases, nucleotidases, esterases, glycases, dismutases could be linked to infectivity, immune evasion and pathogenicity. Mehrdana and Buchmann, (2017) and Lee et al. (2017) have demonstrated that some metallopeptidases produced by *Anisakis* are possibly involved in the mechanical gastrointestinal penetration of experimentally infected mice. In some patients suffering from anisakiasis, the existence of the multiple, well defined, erosive and/or hemorrhagic lesions usually detected near the main lesion in the gastric mucosa could be explained by the invasive activity of the larvae and with the presence of anticoagulant substances in the ESPs.

Additionally, previous studies have shown the possible role of the temperature in the production of ESPs (Bahool et al. 2013), and in the gene expression transcripts of some proteins in *Anisakis* sp. (Chen et al. 2012). Those proteins, showing an optimum activity to host temperature of 37 °C, support the hypothesis that those enzymes are activated during the larval invasion in the homeothermic hosts

(Matthews 1992). Furthermore, the temperature plays an important role in the *A. pegreffii* larvae migration in the *post-mortem* fish tissue (Cipriani et al. 2016).

Thus, it cannot be excluded that ESPs are expressed to different degrees in intermediate/paratenic, and accidental hosts, being the temperature an important parameter for the natural infection dynamic. Therefore, the differential gene expression of those ESPs in relation to the site of infection in both natural and accidental (human) hosts has never been investigated.

Aims of the thesis

ESPs of the species of the *A. simplex* (s. l.) complex have been the target molecules of this research work, in terms of their gene expression analysis and gene sequencing approaches.

Main objectives of the research work have been the following:

1) to investigate the gene expression of those *Anisakis* species, in relation to different conditions, such as hosts temperature (homeothermic and heterothermic), site of infection and larval migration capacity, which can provide new insights into the parasite adaptation as well as to the host immune response with the two parasite species. Thus, the gene expression analysis was performed on some target genes of the parasite species *A. pegreffii* and *A. simplex* (s. s), under different thermal conditions and in different fish-host tissues/organs. (Objectives of **Papers no.1 and no. 2);**

2) One of the strategies exploited by parasites to evade the host immune response is to alter DCs differentiation and functions; those aspects have never been investigated in *A. pegreffii* and *A. simplex* (s. s.). Thus, results will greatly improve the knowledge in that aspect. Preliminary results achieved indicate that the exposure to *A. pegreffii* allergens/antigens modulate the differentiation of DC, favouring a Th2 response. Thus, transcriptomic (RNA seq) analysis of *A. pegreffii* in presence/absence of DCs was carried out **This is the objective of the paper no.3;**

3) Amongst the functional genes of the *A. simplex* (s. l.) complex, some of them have the potential to provide insights also into the existence of genetic variability (polymorphism) allowing to detect fixed alternative nucleotide positions between those parasites' species, to be used for their genetic recognition. Thus, the genetic variation at some functional genes, such as the metallopeptidases, was investigated, allowing the discovery of novel nuclear marker, to be used in a future multi-genotyping approach for their recognition. Further, the development and validation of a rapid and low cost method such as the ARMS-PCR of the target nuclear gene was performed to be used also for the identification of zoonotic parasites in human cases of anisakiasis. **This is objective of the Paper no.4.**

These objectives are included in the research Project granted by:

Istituto Pasteur - Fondazione Cenci-Bolognetti (2018-2020). Title: What shapes the parasite Anisakis-human host interaction? Integrating genetic, molecular, and immunological approaches to investigate the zoonotic disease, anisakiasis.

1.1. Current taxonomy of nematodes of genus *Anisakis*

The power of resolution of molecular/genetic methodologies in detecting anisakid species belonging to the genus *Anisakis* has revolutionized their taxonomy during the last 30 years (Mattiucci and Nascetti, 2008; Mattiucci et al. 2018). Actually, the following genetic/molecular methodologies are available for the identification of species of anisakid: Multilocus allozyme electrophoresis (MAE), sequence analysis of the mitochondrial cytochromeoxidase 2 (mtDNA *cox 2*) (Valentini et al. 2006; Mattiucci and Nascetti, 2006; Mattiucci et al. 2014) and the small subunit of rRNA (*rrnS*) genes (Nadler et al. 2005; Mattiucci et al. 2014); direct sequencing of nuclear DNA, such as the elongation factor - 1 alpha 1 of nuclear DNA (EF1 α−1) direct sequencing (Mattiucci et al. 2016); the ITS region of rDNA (Nadler et al. 2005) and the DNA microsatellite loci (Mattiucci et al. 2019).

So far, nine species belonging to the genus *Anisakis* have been identified worldwide (Mattiucci *et al.* 2014, 2018). All species have been characterized by distinct diagnostic genetic markers, possess distinct gene pools, and they are reproductively isolated. The concatenated phylogenetic analysis of the species of genus *Anisakis* obtained by nuclear and mitochondrial genes showed the existence of different clades. The first clade includes the cryptic species of the *A. simplex complex* [i. e. *A. simplex* (s. s.), *A. pegreffii* (see Nascetti et al. 1986), *A. berlandi* (Mattiucci et al. 2014); a second clade includes the two species *A. ziphidarum* (Paggi et al. 1998) and *A. nascettii* (Mattiucci et al. 2009); the third clade includes the "sibling" species: *A. physeteris* (Baylis 1920), *A. brevispiculata* (Dollfus 1966) and *A. paggiae* (Mattiucci et al. 2005). Finally, the fourth clade includes *A. typica* and *Anisakis* sp. 1 that represent a distinct phylogenetic lineage.

1.2. Basic biology of *Anisakis* spp. nematodes

Anisakid nematodes of the genus *Anisakis* Dujardin, 1845 are ascaridoid nematodes colonising the digestive system of marine vertebrates. Those parasites have an indirect life-cycle which involves various hosts at different levels of the marine food chains. Marine mammals (mainly cetaceans) serve, as definitive hosts, fish and squids are intermediate or paratenic hosts, while planktonic or semi-planktonic crustaceans act as first intermediate hosts. Eggs are released with the definitive hosts' feces, and lead eventually to free swimming unsheathed larvae in the marine environment. The larvae undergo one or two moults before being ingested by invertebrates, mostly small crustaceans (krill). Those first intermediate hosts are eaten by a wide variety of cephalopods and fishes that act as a second intermediate or paratenic hosts At this stage, many different life-cycle patterns may be observed, mostly depending on the species of *Anisakis*. The fish may be directly eaten by the definitive host when the third stage larva (L3) moults to L4 and then matures to the adult form of the parasite. Alternatively, the fish may be eaten by another fish: the larvae re-encapsulate in this new host. This phenomenon may occur several times, inducing a potential accumulation of *Anisakis* spp. larvae, in predatory fish, along the trophic chain (Zuo et al. 2016; Münster et al. 2015). Moreover, the behavior

of the larvae may be different depending on several factors, some still unknown: in some cases, most parasites will remain in the visceral cavity of the fish or within the visceral organs, whereas, in other cases the parasites can migrate to the musculature of the fish (Roepstorff et al. 1993; Karl et al. 2011; Cipriani et al. 2016).

Humans may act as accidental host acquiring infection through consumptions of raw, smoked, marinated salted, or undercooked fish and squids, infected by larval stages. Worldwide, there is an increasing recognition that the fish-borne zoonosis, known as anisakiasis, is an important emerging human disease (Chai et al. 2005; Buchman et al. 2016) (Figure 1).

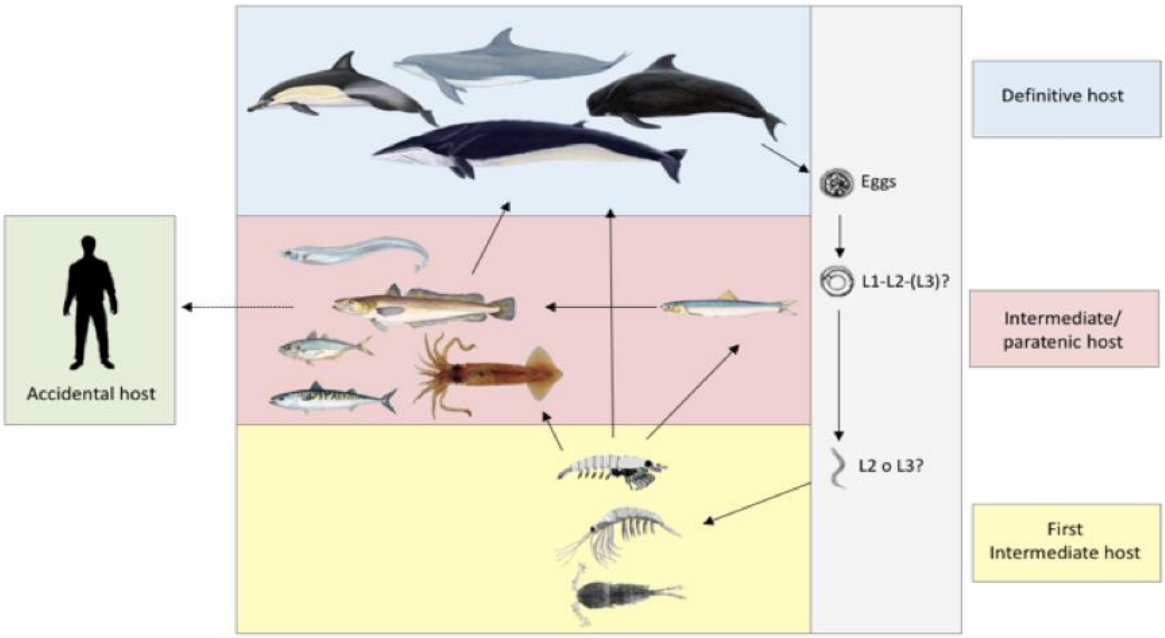

Figure 1. Schematic representation of life cycle of the *Anisakis simplex* (s. l.) complex.

1.3. Site of infection of *Anisakis* spp. larvae in fish and larval migration capacity

The different *Anisakis* species seem to have preferential localization in their fish hosts, which appear dependent not only on the *Anisakis* species, but also on the fish host species.

Anisakis larvae, especially when present at high infection intensities, tend to locate in defined parts of the fish host, mostly in the body cavity, encysted on visceral organs. These are often encapsulated and coiled up in tight spirals on the organs' surface. In some fish species the larvae tend to embed in the liver, over the gonads, in the mesentery, or attached to the intestinal wall.

Some more recent studies consider also the distribution of larvae in different parts of the fish musculature, dividing it in two (ventral or hypaxial. dorsal or epaxial) (Levsen and Lunestad 2010; Cipriani et al. 2015, 2017b), or in four parts (anterior ventral. anterior dorsal. posterior ventral. posterior dorsal) (Karl et al. 2011; Levsen and Karl 2014).

In a study of differential migration rates of *A. pegreffii* and *A. simplex* (s. s.) in flesh of the European hake, Cipriani et al. (2016) reported that the most larvae of both species penetrating the fish flesh were located in the ventral fillet (also called "belly flap"), which represents the musculature surrounding the visceral organs.

In some geographical areas, co-infection by *Anisakis* species with a differential localization in the same fish host can occur. Larval *Anisakis* belonging to different species show different relative frequencies of localization, even if overall parasite burden results similar. For instance, Suzuki et al. (2010) observed significant differences in infection patterns between two *Anisakis* species recovered in *Scomber japonicus*, sampled along the Japanese coast. Despite the parasite nematodes species occurred with similar infection levels in all fish examined, the number of *A. simplex* (s. s.) larvae present in the musculature of the fish *S. japonicus* was 12 times higher than *A. pegreffii*, which represented only the 1.7% of the larvae in the muscle.

Similar results were obtained by Cipriani et al. (2015) in the European hake, *M. merluccius*: *A. simplex* (s. s.) outnumbers *A. pegreffii* in the musculature (flesh) of the same fish host. Despite this marked difference in the relative proportions of the two species in the fish muscle, the two parasites showed the same proportion of the infection in the viscera of the same fish species, thus, highlighting that at similar infection rate, *A. simplex* (s. s.) displays a better capacity for migrating into the muscular tissue of the fish host (Cipriani et al. 2015). This phenomenon was observed also in other fish species, such as blue whiting *Micromesistius poutassou* (Gomez-Mateos et al. 2016), where *A. simplex* (s. s.) doubling the infection rate of *A. pegreffii* in the musculature.

Several hypotheses could be made to explain the differential localization of *A. simplex* (s. s.) and *A. pegreffii* in their fish hosts. When the two species are in syntopic conditions, these mechanisms could be the result of a differential use of host resources, as a result of interspecific competition between them for the same trophic resource, theoretically represented by the visceral cavity and/or organs (especially the liver). In addition, concerning to the higher migration capacity exhibited by *A. simplex* (s. s.), this finding could be the result of a differential response to the host immune system, the immune response of the fish host being lower in its musculature (Cipriani et al. 2016). Some experimental studies support the hypothesis that *A. pegreffii* has a lower capacity to invade both fish tissues and laboratory animals (Arizono et al. 2012; Quiazon et al. 2008; Romero et al. 2013).

So far, the only *Anisakis* species capable of migrating into the fish flesh are *A. simplex* (s. s.) and *A. pegreffii*. *A. pegreffii* and *A. simplex* (s. s.) were found in syntopy in European hakes, whereas, *A. physeteris* was never found in the flesh of the fish, showing a preference for the liver of its host, with the 41% of larvae detected (59% was found in the fish visceral cavity) (Cipriani et al. 2017b).

1.4. *Anisakis* spp. and their accidental host (humans)

According to the present knowledge larval stages of *Anisakis* spp. are the causative agents of human anisakiasis (Ishikura et al. 1993; Daschner et al. 2012; Nieuwenhuizen, 2016). Among the nine species of *Anisakis* described, only *A. simplex* (s. s.) and *A. pegreffii* are reported as causative agents of human anisakiasis (D'Amelio et al. 1999; Moschella et al. 2004; Umehara et al. 2007; Fumarola *et al.* 2009; Mattiucci et al. 2011, 2013; 2017d; Lim et al. 2015; Mladineo et al. 2016) (Table 1).

Anisakiasis has become increasingly more relevant as an human health risk, in particular in countries or regions where the consumption of raw, undercooked and lightly processed fish and squid is frequent and in those areas where the consumption is becoming popular. Consequently, medical cases of anisakiasis are increasingly reported in some countries of Asia (mainly Japan, Korea, China, Taiwan), representing more than 50% of the total reported worldwide. The remaining cases come from European countries, especially Italy, Spain, France and Croatia, whereas, fewer cases are from Germany, Netherlands, UK and Norway despite comparatively high *per capita* fish consumption rates in those countries (Mattiucci et al. 2018; Guardone et al. 2018). *A. simplex* (s. s.) and *A. pegreffii*, are the species most frequently found in the edible parts of commercially important fish and squid species; as a consequence, they are also the species with the highest risk to infect humans. Indeed, they are the two main species associated with human anisakiasis.

The species *A. pegreffii* was reported to cause gastric, intestinal and gastro-allergic anisakiasis (GAA). Indeed, larval stages have been identified in cases of "invasive" anisakiasis, after observing viable larvae during endoscopy (D'Amelio et al. 1999; Umehara et al. 2007; Mattiucci et al. 2013; 2017d; Arai et al. 2014; Lim et al. 2015), colonoscopy (Mattiucci et al. 2017), or in surgically removed eosinophilic granulomas (Mattiucci et al. 2011, 2017*;* Mladineo et al. 2016). The cases, molecularly identified as caused by *A. pegreffii*, were mostly from Italy (D'Amelio et al. 1999; Mattiucci et al. 2011, 2013, 2017), Croatia (Mladineo et al. 2016), from Korea (Lim et al. 2015) and, more rarely, from Japan (Umehara et al. 2007). Those findings are in concordance with the range of the parasite's geographic distribution and its spatial distribution in the infected fish (Figure 3).

Anisakis simplex (s. s.) has been identified (by molecular methods) to cause "invasive" gastric and intestinal anisakiasis in Japan (Umehara et al. 2007; Auer et al. 2007; Arai et al. 2014). Nevertheless, despite widely reported cases of "allergic anisakiasis", in some European countries, such as Spain, the etiological agent has not been identified at species level by molecular tools (Audicana and Kennedy, 2008 and ref. therein) (Daschner et al. 2012).

Table 1. Published reports of human anisakiasis worldwide, with focus on those described with the larval detection. The (*) indicates those reports in which the etiological agent was identified, to the species level by molecular methodologies.

Origin of the patient		Number of patients	Clinical features	Method used for larval detection	Etiological agent	Reference
European countries	Italy	1	Gastric	-	*Anisakis* sp.	Stallone et al. 1996
	Italy	1	Extra-gastrointestinal	Laparotomy	*Anisakis* sp.	Cancrini et al. 1997
	Italy	1	Intestinal	Endoscopy	*Anisakis* sp.	Ioli et al. 1998
	Italy	1	Gastric	Endoscopy	*A. pegreffii**	D'Amelio et al. 1999
	Italy	3	Gastric	-	*A. simplex*	Maggi et al. 2000
	Italy	1	Gastric (2), intestinal (8), spleen (1)	Endoscopy, laparotomy	*Anisakis* sp.	Pampiglione et al. 2002
	Italy	1	Intestinal	Histologic examination	*Anisakis* sp.	Caramello et al. 2003
	Italy	1	Gastric, splenic	Laparotomy	*Anisakis* sp.	Testini et al. 2003
	Italy	1	Intestinal	Histologic examination	*Anisakis* sp.	De Nicola et., 2005
	Italy	1	Intestinal	Laparotomy	*Anisakis* sp.	Moschella et al. 2005
	Italy	1	Intestinal	Laparotomy	*A. simplex* (s. l.)	Pellegrini et al. 2005
	Italy	3	Gastric	Endoscopy	*Anisakis* sp.	Fazii et al. 2006
	Italy	1	Extra-gastrointestinal	Endoscopy	*Anisakis sp.*	Avellino et al. 2007
	Italy	3	Gastric	Gastroscopy	*Anisakis* sp.	Ugenti et al. 2007
	Italy	2	Gastric	Endoscopy	*A. pegreffii**	Fumarola et al. 2009
	Italy	1	Intestinal	Histologic examination	*A. simplex* (s. s.)	Marzocca et al. 2009
	Italy	1	Intestinal	Endoscopy, histologic examination	*A. simplex* (s. l.)	Aloia et al. 2011
	Italy	1	Intestinal	Histologic examination	*A. pegreffii**	Mattiucci et al. 2011
	Italy	2	Gastric	Endoscopy	*A. simplex* (s. l.)	Pontone et al. 2012
	Italy	8	Gastric (6), (GAA) (2)	Endoscopy	*A. pegreffii**	Mattiucci et al. 2013
	Italy	1	Intestinal	Colonoscopy	*A. simplex* (s. l.)	Mumoli and Merlo, 2013
	Italy	1	Intestinal	Endoscopy, histologic examination	*A. simplex* (s. l.)	Andrisani et al. 2014
	Italy	1	Gastric	Gastroscopy	*Anisakis* sp.	Mariano et al. 2015
	Italy	3	Gastric (1), intestinal (2)	Endoscopy, histologic examination	*A. pegreffii**	Mattiucci et al. 2017d
	Croatia	1	Intestinal	Histologic examination	*A. pegreffii**	Mladineo et al. 2016
	France	-	Intestinal granuloma	-	*Anisakis* sp.	Doby et al. 1975
	France	5	Intestinal	Microscopic analyses, gastroscopy	*Anisakis* sp.	Mudry et al. 1986
	France	4	Gastric	Gastroscopy	*Anisakis* sp.	Bouree et al. 1995
	Spain	2	-	-	*Anisakis* sp.	Acebes et al. 1996
	Spain	1	Gastric	Endoscopy	*A. simplex* (s. l.)	Romeo Ramirez et al. 1997
	Spain	13	Gastric	Gastroscopy	*A. simplex* (s. l.)	Daschner et al. 1998
	Spain	2	Intestinal	Histologic examination	*A. simplex* (s. l.)	del Olmo Escribano et al.,1998
	Spain	5	GAA	Gastroscopy	*A. simplex* (s. l.)	Alonso et al. 1999

	Spain	1	Gastric	-	*Anisakis* sp.	Barriga et al. 1999
	Spain	20	GAA	Gastroscopy	*A. simplex* (s. l.)	Daschner et al. 2000
	Spain	22	GAA	Gastroscopy	*A. simplex* (s. l.)	López-Serrano et al. 2000
	Spain	2	Gastric	Endoscopy	*Anisakis* sp.	del Olmo Martínez et al.,2000
	Spain	1	Intestinal	Histologic examination	*A. simplex* (s. l.)	López-Peñas et al. 2000
	Spain	9	Gastric (5), intestinal (3)	Endoscopy or intestinal resection	*A. simplex* (s. l.)	Repiso Ortega et al. 2003
	Spain	24	Gastric, intestinal	Gastroscopy	*A. simplex* (s. l.)	Alonso-Gómez et al. 2004
	Spain	1	Gastric	Endoscopy	*A. simplex* (s. l.)	Carrascosa et al. 2015
	Spain	1	Intestinal	Colonoscopy	*A. simplex* (s. l.)	Riu Pons et al. 2015
	The Netherlands	1	Gastric	-	*Anisakis* sp.	Van Thiel 1960
	Belgium	-	Intestinal	-	*Anisakis* sp.	Fain et al. 1969
	Norway	-	Gastric	-	*Anisakis* sp.	Jacobsen and Berland 1969
	Denmark	-	-	-	*Anisakis* sp.	Andreassen, 1970
	Belgium	6	Intestinal	Histologic examination	*Anisakis* sp.	Verhamme and Ramboer, 1988
	Belgium	1	Gastric	Endoscopy	*Anisakis* sp.	Vercammen et al. 1997
	Germany	1	Gastric	-	*Anisakis* sp.	Plath et al. 2001
	Germany	1	Intestinal	Laparotomy	*Anisakis* sp.	Lock et al. 2008
	Austria	2	Intestinal	Laparotomy	*A. simplex* (s. s.)*	Auer et al. 2007
	Russia	-	Intestinal	-	*Anisakis* sp.	Karmanova et al. 2002
	Boston	1	Intestinal	Histologic examination	*Anisakis* sp.	Roselyn et al. 1973
	Hawaii	1	Gastric	-	*A. simplex* (s. l.)	Deardorff et al. 1986
	Hawaii	1	-	X-ray	*Anisakis* sp.	Hiramoto et al. 1991
	Quebec	1	Intestinal	Laparotomy	*Anisakis* sp.	Couture et al. 2003
	USA	-	Intestinal	Histologic examination	*Anisakis* sp.	Pinkus et al. 1975
	USA	-	Intestinal	Laparotomy	*Anisakis* sp.	Appleby et al. 1982
	Japan	2	Gastric granuloma	Histologic examination	*Anisakis* sp.	Asami et al. 1965
	Japan	11	Intestinal	-	*Anisakis* sp.	Otsuru et al. 1965
	Japan	-	Intestinal	-	*Anisakis* sp.	Ishikura et al. 1967
	Japan	-	Pharynx mucosa	-	*Anisakis* sp.	Tanaka, et al. 1968
	Japan	1	Gastric	Endoscopic, histologic examination	*Anisakis* sp.	Hsiu et al. 1986
	Japan	2	Intestinal	Colonoscopy	*Anisakis* sp.	Minamoto et al. 1991
	Japan	1	Gastric	Gastroscopy	*A. simplex* (s. l.)	Kagei and Isogaki, 1992
	Japan	4	Intestinal	Colonoscopy	*Anisakis* sp.	Matsumoto et al. 1992
	Japan	1	Esophageal	Endoscopy	*Anisakis* sp.	Urita et al. 1997
	Japan	1	Intestinal	Laparotomy	*Anisakis* sp.	Sasaki et al. 2003
	Japan	1	Intestinal	Laparotomy	*Anisakis* sp	Ishida et al. 2007
	Japan	1	Gastric	Laparotomy	*Anisakis* sp.	Ito et al. 2007
	Japan	1	Intestinal	Laparotomy	*A. simplex* (s. l.)	Takei and Powell, 2007
	Japan	85	Gastric	Endoscopy	*A. pegreffii* (1)* *A. simplex* (s. s.)(84)*	Umehara et al. 2007

Japan	1	Intestinal	Laparotomy	*Anisakis* sp.	Sugita et al. 2008
Japan	1	Gastric, intestinal	Gastroscopy, colonoscopy	*Anisakis* sp.	Kim et al. 2013
Japan	1	Intestinal	Colonoscopy	*Anisakis* sp.	Yorimitsu et al. 2013
Japan	44	Gastric (42), intestinal (2)	Endoscopy	*A. simplex* (s. s.)*	Arai et al. 2014
Japan	1	Gastric	Endoscopy	*Anisakis* sp.	Sonoda et al. 2015
Japan	1	Extra-gastrointestinal	Laparotomy	*A. simplex* (s. l.)	Takamizawa and Kobayashi, 2015
Japan	1	Gastric	Endoscopy	*Anisakis* sp.	Hamada et al. 2016
Japan	1	Intestinal	Endoscopy	*Anisakis* sp.	Hashimoto et al. 2016
Japan	1	Gastric	Esophagogastroduodenoscopy	*Anisakis* sp.	Hashimoto and Chonan, 2016
Japan	1	Intestinal	-	*Anisakis* sp.	Ikuta et al. 2016
Japan	1	Gastric	Endoscopy	*Anisakis* sp.	Shimamura et al. 2016
Japan	1	Intestinal	Endoscopy	*Anisakis* sp.	Tsukui et al. 2016
Thailand	1	Intestinal	Histologic examination	*Anisakis* sp.	Hemsrichart, 1993
Korea	-	Palatin tonsil	Endoscopy	*Anisakis* sp.	Kim et al. 1971
Korea	1	Intestinal	Laparotomy	*Anisakis* sp.	Kim et al. 1991
Korea	1	Gastric	Gastroscopy	*A. simplex* (s. l.)	Noh et al. 2003
Korea	1	Intestinal	Laparotomy	*Anisakis* sp.	Kang et al. 2010
Korea	1	Gastric	Laparotomy	*Anisakis* sp.	Kang et al. 2014
Korea	16	Gastric (4), intestinal (12)	Gastroduodenoscopy	*A. pegreffii* (15)* *A. simplex* (s. s.)(1)*	Lim et al. 2015
Korea	15	Gastric	Endoscopy	*Anisakis* sp.	Sohn et al. 2015
Taiwan	1	Gastric	Endoscopy	*A. simplex* (s. l.)	Li et al. 2015

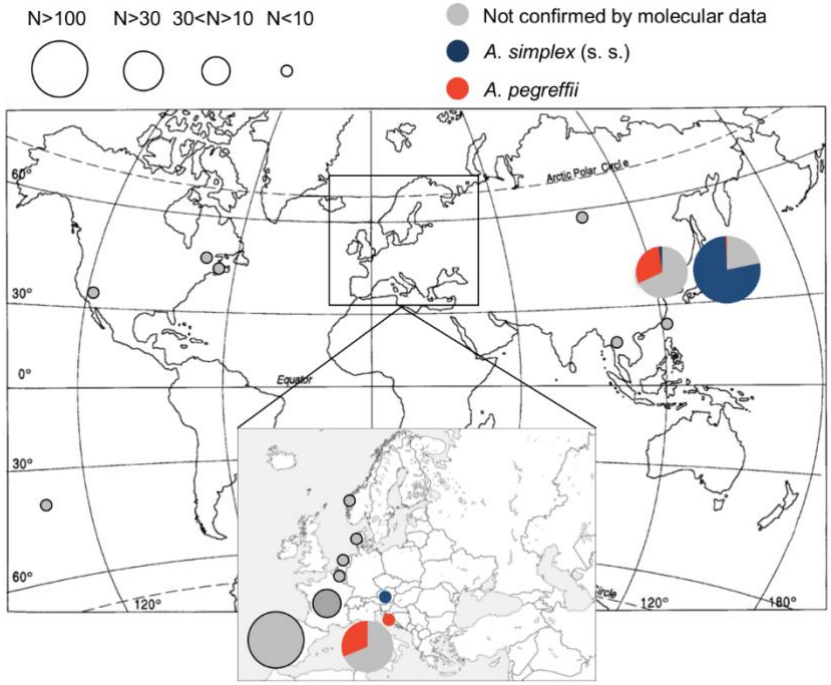

Figure 3. World mapping of human anisakiasis, based on the 1960-2018 published literature: distribution of human cases reporting the larval detection. Cases of IgE-*Anisakis* hypersensitization are not reported. *N*= number of cases. The pie graphs show relative proportion of cases due to the two zoonotic species (i.e. *A. pegreffii* and *A. simplex* (s. s.), since 1999 up to now (modified from Mattiucci et al. 2018).

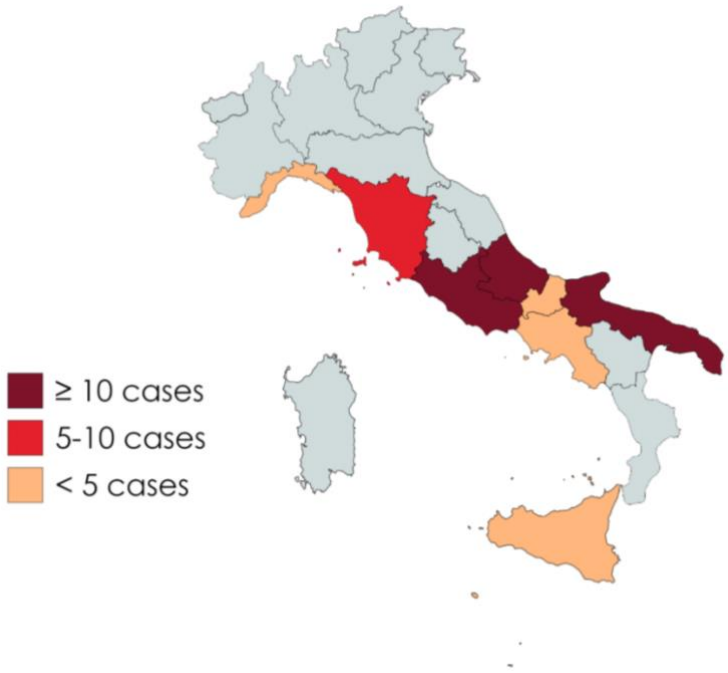

Figure 4. Map of the distribution of the Italian cases of anisakiasis, divided per region. (from Guardone et al. 2018)

1.5. Clinical manifestation of human anisakiasis

Anisakis spp. larvae do not mature in humans, but are able to provoke the fish-borne zoonosis, commonly known as "anisakiasis". Based on the site of infection reached by the living larvae in the gastro-intestinal tract based on the site of infection reached by the *Anisakis* larva in the gastrointestinal tract the disease is often subdivided into: gastric anisakiasis (GA), intestinal anisakiasis (IA), ectopic anisakiasis (EA), and gastro-allergic anisakiasis (GAA).

Clinical symptoms of gastric anisakiasis (GA) provoked by *A. simplex* (s. s.) and *A. pegreffii* are similar: dyspepsia, epigastric pain, nausea, vomiting, abdominal distension, anorexia and chest pain. As for the localization in the gastric mucosa, the majority of larvae are found in the greater curvature, followed by the posterior wall. Shibata et al. (1989) described endoscopic findings such as edematous hypertrophic gastric folds, increase in gastric secretion and mucosal lesions, including edema, redness, coagulation, haemorrhage and ulceration. Cases of GA due to *A. pegreffii* described recently from Italy (Mattiucci et al. 2013) were characterised, clinically, by epigastric pain just after 2 h following the ingestion of raw seafood, vomiting and other digestive symptoms. Arai et al. (2014) have found that in gastric anisakiasis due to *A. simplex* (s. s.), the parasite may prefer to invade and penetrate the normal intact gastric mucosa, instead of an atrophic mucosa.

Cases of IA due to *A. pegreffii* and *A. simplex* (s. s.) include a ‘mild form’ and a ‘fulminant form’. The ‘mild form’ is characterised by eosinophilic granulomas setting ‘tumour-like’ formations in the intestinal wall, whereas, ‘fulminant form’ has the symptoms of acute ileus, acute appendicitis, acute abdomen or regional ileitis. Cases of IA reported in Japan (Otsuru et al. 1965; Ishikura et al. 1967; Minamoto et al. 1991; Matsumoto et al. 1992; Sasaki et al. 2003; Ishida et al. 2007; Takei and Powell, 2007; Sugita et al. 2008; Kim et al. 2013; Yorimitsu et al. 2013; Arai et al. 2014; Hashimoto et al. 2016; Ikuta et al. 2016; Tsukui et al. 2016) ,Korea (Kim et al. 1991; Kang et al. 2010; Lim et al. 2015) and Italy (Moschella et al. 2004; Mattiucci et al. 2011, 2017) were characterised by a clinical picture of acute abdominal pain, acute appendicitis or acute abdomen.

A. pegreffii and *A. simplex* (s. s.) show similar histopathological features. The first stage is the 'phlegmon formation', and the second is the ‘abscess-granuloma formation'. The last is rather frequent in gastric anisakiasis and is characterised by abundant necrotic tissue around the larvae and by a large number of eosinophils, mast cells and macrophages. The second stage is the ‘abscess–granuloma formation’, which corresponds to the natural evolution of the disease from one to several months after ingestion of the larva. At this stage, only few debris of the larvae are found establishing a chronic inflammatory reaction at the infected tissue, the histologic features are composed by degenerated connective tissue and inflammatory infiltrate primarily consisting of macrophages forming epithelioid or giant cells, histiocytes, eosinophils, lymphocytes and other inflammatory cells - multinuclear giant cells and central necrosis may be present in some cases (Kikuchi et al. 1990).

The term Gastro-allergic anisakiasis (GAA) was described by Daschner et al. (2000) to indicate an acute allergic reaction in the context of an acute gastric response against the presence of an *Anisakis* spp. larva, when the live parasite attempts to invade the submucosal layer of the gastric wall. GAA is defined by a type I hypersensitivity which is characterized by an acute IgE-mediated reaction triggering urticaria, angioedema, itching, abdominal pain, wheezing and /or anaphylaxis, common symptoms of an acute food allergy. In this type of anisakiasis, the allergic reactions take place starting from 2 or 3 h to 2 or 3 days after the ingestion of infected fish (Daschner et al. 2011; Mattiucci et al. 2013).

1.5.1. Aspects of pathogenicity by *Anisakis* sp. in humans

1.5.1.1. Human immune response to the infection

Upon a primary exposure to a nematode, various degrees of inflammatory responses are triggered, characterized by the activation of neutrophils, tissue macrophages, monocytes, dendritic cells, basophils, and eosinophils (Buzoni-Gatel et al. 2006) and changes in blood vessel permeability and blood flow (Galioto et al. 2006; Meeusen and Balic 2000).

Epithelial cells are often the first responders to immune and inflammatory stimuli in the recruitment and activation of inflammatory cells in response to enteric parasites (Shea-Donohue and Urban 2004).

Basophils can be activated to release cytokines through specific and/or nonspecific mechanisms. For example, various lectins have been demonstrated to activate basophils by cross-linking of specific carbo*hyd*rate side chains or direct cross-linking of their high-affinity IgE receptor (FcεRI). Apart from their role in innate immunity, basophils are an important cellular source for early IL-4 production, which is strongly influential in a Th2 response (Audicana and Kennedy 2008).

The larvae usually die within a few days in humans (Sakanari 1989) and shattered in about eight weeks. During this period are surrounded by edema, necrosis and cellular inflammation composed mostly of eosinophils but also neutrophils, lymphocytes and macrophages (Ishikura et al. 1993).

Toll-like receptors (TLRs) form a major family of phylogenetically conserved transmembrane receptors involved in non-self recognition (Audicana and Kennedy 2008). TLRs have also been identified in immune system cells such as macrophages and dendritic cells. The involvement of TLR signalling pathways in parasitic infections is emerging such as with *Entamoeba histolytica* (Maldonado-Bernal et al. 2005) and other protozoa (*Leishmania* spp., *Trypanosoma cruzi*, *Trypanosoma brucei*, *Plasmodium* spp., and *Toxoplasma gondii*) (Gazzinelli and Denkers 2006). Regarding the helminth parasites, despite the lack of studies with *A. simplex* , the role of TLRs in infection by *Schistosoma* blood flukes and filarial nematodes is beginning to be investigated.

The extent of tissue damage and inflammation resulting from infection with the small (1–3 cm long) larvae points to an interaction between the host immune system and substances secreted by or contained within the larvae as the cause of the pathology (Sakanari 1989; Jeon and Kim 2015).

The main role of granulomas is to protect the host by walling off pathogens or persistent irritants. Dead *Anisakis* spp. larvae are not easily degradable; therefore, they are typical of stimuli that incite a granulomatous response (Motta et al. 2008) In some patients, the inflammation takes longer to resolve, resulting in symptoms of chronic anisakiasis (Nieuwenhuizen et al. 2016).

The granulomatous lesions observed for the chronic gastrointestinal and ectopic forms of anisakiasis are typical of type IV hypersensitivity reactions (Audicana and Kennedy 2008).

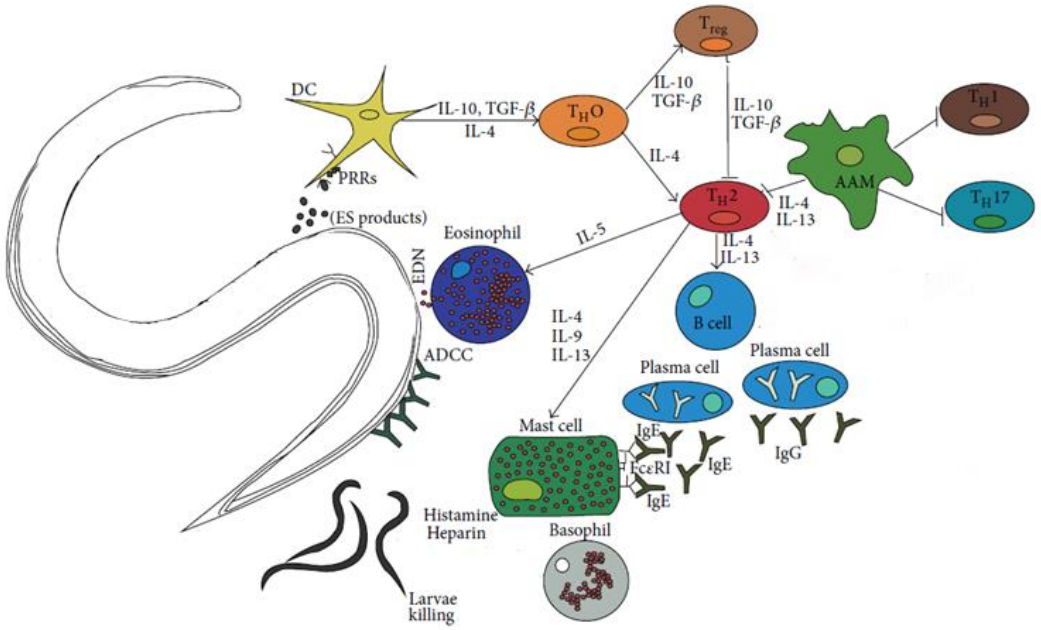

Figure 4. The helminth-induced TH2 response starts with the recognition of parasitic pathogen associated molecular patterns (PAMPs) by certain pattern recognition receptors (PRRs) that are expressed on the DCs of the host. Through contact with the antigen, the DCs become activated, allowing them to act as antigen-presenting cells (APC) after the migration to the adjacent lymph nodes, with the ability of processing and presenting the antigen to T cells to initiate an immune response. The helminth-induced host immune response is focused on the protection of the host organism and is mediated by TH2 cells. This response includes IL-4, IL-5, IL-13, and IL-10 secretion and production of IgG4 and IgE by B cells, as well as the activation of effector cells such as mast cells, eosinophils, and basophils. Affected by IL-4 and IL-13 occurs the differentiation of alternatively activated macrophages (AAMs) which can inhibit the proliferation of other cells like TH1, TH2, and TH17 cells. Thus, these cells have strong anti-inflammatory properties, which are manifested by the secretion of IL-10 and TGF-β as well as the expression of additional genes. Furthermore, IL-4 and IL-13 lead to an increased contractility of smooth muscle cells and a hypersecretion of mucus for expulsion of intestinal helminths. Immune complexes of IgE bind to high affinity IgE receptors (FcεRI) on mast cells and basophils; this leads to an activation of these cells and a secretion of inflammatory mediators like histamine, heparin (from Ditgen et al. 2014).

1.5.1.2. Adaptive immune response

Infection with anisakids larvae induces strong Th2 responses in mice and evidence of Th2 responses is also seen in humans (del Pozo et al. 1999; Gonzalez-Munoz et al. 2010; Nieuwenhuizen et al. 2006).

The Th2 cytokines driving these responses are the same as those that drive allergic diseases and are responsible for the symptoms of gastroallergic anisakiasis, such as diarrhoea, vomiting, itching, angioedema, urticaria and anaphylaxis, as well as *Anisakis* allergy, which can have symptoms such as

asthma, rhinoconjunctivitis, urticaria and atopic dermatitis, primarily driven by mediators released from mast cells (Nieuwenhuizen and Lopata 2013).

Allergic symptoms are not frequent in helminth infections, they occur more commonly in zoonotic infections such as anisakiasis (Daschner and Cuellar 2010). Allergic reactions due to infection with *Anisakis* spp. and other zoonoses could be due to the relatively low numbers of larvae to which most patients are exposed and the fact that no mutual adaptation has evolved (Daschner et al. 2000).

Several experimental rodent models have been used to investigate the immune mechanisms controlling allergic responses to *A. simplex*. A murine model of anaphylaxis in mice sensitized by intravenous or oral challenge confirmed a mixed Th1/Th2 pattern of cytokines in both cases and anaphylaxis only in those mice intravenously challenged. It has been demonstrated experimentally that rats infected by a single parasite develop higher IgE levels than do rats infected with a high number (Baeza et al. 2005).

Differences have been described between infection and antigen exposure to *A. simplex* in the sense that reinfection induces a strong Th2 response, whereas antigens alone do so to a lesser degree or not at all (Cho et al. 2006). In contrast, it has been shown recently that both live parasites and crude extracts of L3 cause allergic reaction in sensitized mice challenged orally with *A. simplex* proteins, resulting in a striking allergic reaction within as little as 1 hour (Sanchez-Monsalvez et al. 2003). This is relevant to the debate about whether antigens from dead parasites can cause allergy (Audicana and Kennedy, 2008; Nieuwenhuizen et al. 2006).

Anti-*A. simplex* antibodies in infected patients reflect a broad immunologic stimulation including both Th1 and Th2 responses (Daschner et al. 2002). Infection causes a strong immune response, and a typical progression from IgM antibody to other isotypes (IgG, IgA, and IgE, etc.) has been observed during the first month of initial infection, as analyzed from serial blood sampling and immunoblotting (Daschner et al. 1999; Audicana et al. 2003; 2008) in which the highest antigen-specific IgE, IgG, and IgA levels are seen to occur after 1 month of infection; specific IgM shows the highest levels within 24 h and a progressive decay over the following 6 months (Daschner et al. 2002).

Several research groups have studied the kinetics of specific antibody production in mice and rabbits (Iglesias et al. 1993 and 1999; Kennedy et al. 1998), but to date there are no data concerning humoral responses in humans.

1.5.1.3. Composition and role of excretory and secretory products (ESPs)

During all stages of the life cycle, nematodes produce and release a series of excretory and secretory products (ESPs), which may be key players in parasite-host interactions (Kim et al. 2005). It has also been suggested that differences among life cycles of different parasite species and even sibling species (Kuhn et al. 2013; Skrzypczak et al. 2014) may be attributed to the relative abundance and function of these bioactive molecules influencing host specificity (Hewitson et al. 2009).

At all stages, various enzyme activities have been associated with the released materials. Enzymes serving a basic metabolic role in the parasite, acid and alkaline phosphatases are found and together with enzymes connected to infectivity, immune evasion and pathogenicity (proteases, nucleotidases, esterases, glycases, dismutases) may serve roles at all life cycle stages (Mehrdana and Buchmann, 2017).

However, no studies have as yet been presented showing the action of ES products in vertebrate and invertebrate hosts and it cannot be excluded that different isotypes are expressed to different degrees in intermediate and final hosts (Mehrdana and Buchmann 2017). Probably the L3 of anisakids parasites use mechanical disruption of tissue combined with the release of potent proteolytic enzymes (Kennedy 2000; Matthews 1982; McKerrow 1989; Sajid and McKerrow 2002; Sakanari and McKerrow 1990) to penetrate and migrate in fish tissues and into the gastrointestinal mucosa of the human (Bahlool et al. 2013).

The invasive capacity of the larvae, together with the presence of anticoagulant substances in the ES products, explains the existence of the multiple, well defined, erosive, and/or hemorrhagic lesions usually detected near the main lesion within the gastric mucosa of patients suffering from anisakidosis (Namiki and Yazaki 1989; Perteguer 1996; Petithory 1991).

Some anticoagulant activities are recorded from larval *A. simplex* ES products causing prolongation of partial thromboplastin time (PTT), which may have a key role in human anisakiasis regarding larval penetration into the gastrointestinal mucosa (Perteguer et al. 1996).

Moreover, several ES compounds from *A. simplex* larvae ranging from 66 to 95 kDa may have a cytostatic effect on lymphocyte blastogenesis (Raybourne et al. 1986). Acetylcholinesterase (AChE) released by some gastrointestinal nematodes may play an important role in altering the permeability of host intestinal cells to assure parasite feeding and, therefore, its survival. This enzyme may also adversely affect coagulation and glycogenesis in the host (Lee 1996).

In general, nematode secretions have immunomodulatory effects interfering with host immune responses. AChE, glutathione-S-transferase (GST), and superoxide dismutase (SOD) secreted by the hookworm *Necator americanus* are known to suppress host inflammatory responses (Pritchard 1995).

The mechanisms by which parasites of the species *Anisakis* spp. modulate host immune responses remain largely unknown. Thus, apart from a range of enzymes and antioxidants, functional effector molecules including protease inhibitors, lectins, heat shock proteins, mucins and cytokine regulators may be detected.

1.5.1.4. Immunogenicity and allergenicity of Excretory/Secretory products (ESPs)

Many of the *A. simplex* (s. l.) ES products are highly immunogenic and can provoke antibody production both in fish (Priebe et al. 1991; Coscia and Oreste, 1998; Riggins et al. 2004) and mammals (Audicana and Kennedy 2008). Allergens in *A. simplex* (s. l.) comprise both somatic antigens (SA)

and ES products; several antigens are resistant to various freeze-, heat- and digestive processes (Daschner et al. 2012).

The allergenic potential of other anisakids, e.g. *Pseudoterranova decipiens*, molecules has not been studied to the same extent *as A. simplex* (s. l.). Several somatic antigens in *Contracaecum osculatum* (s. l.) larvae have been isolated with the molecular weight of 47, 63, and mainly 91 kDa (Coscia and Oreste 1998), but a recent study using experimental infection of mice with live *Contracaecum* sp. larvae did not show IgG or IgE antibody responses specific to SA or ES antigens (Vericimo et al. 2015).

1.5.1.5. *Anisakis* spp. allergens

To date, 14 antigens/allergens from *A. simplex* (s. l.), which includes a complex of sibling species, i.e. *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi* (Mattiucci et al. 2014) have been described (Allergen Nomenclature Sub-Committee, www.allergen.org) (Table 2).

In general. *A. simplex* (s. l.) ES allergens are known to be more potent which could be a result of their higher affinity to specific IgE compared to the somatic antigens (Baeza et al. 2004). IgE raised in patients against SA and ESP antigens of *A. simplex* may cross-react with homologous antigens of other ascarid nematodes (e.g. *Ascaris suum Ascaris lumbricoides*, *Toxocara canis*, *Hysterothylacium aduncum*), or arthropods (German cockroach, chironomids) (Pascual et al. 1997; Fernández-Caldas et al. 1998; Lozano et al. 2004). However, somatic proteins are more likely to cross-react, while ESP antigens are more specific.

Among the antigens/allergens, *Ani s 1* (24 kDa), *Ani s 7* (139 kDa) are considered the most important ESP allergens (Daschner et al. 2012). It has been reported that *Ani s 1* and *Ani s 7* have no amino acid sequence similarity with any other allergen able to sensitize humans; they are also useful for diagnosing infections with *Anisakis* spp. (Moneo et al. 2000; Rodríguez et al. 2008).

Fernández-Caldas 2015 described a globin in *A. simplex* (s. l.), as a new major allergen (*Ani s 13*) because it was recognized in a large number (≈ 64.3%) of sensitized patients and up to 80.9% of patients with gastro-allergic anisakiasis.

Finally, *Ani s 14* has recently been described; this was recognized in 54% of sera from *Anisakis*-allergic patients (Kobayashi et al. 2015).

Other allergens, such as *Ani s 4* (cystatin, 9 kDa), *Ani s 6* (serine protease inhibitor, 7 kDa) and three SXP/RAL-2 family proteins, *Ani s 5* (15 kDa), *Ani s 8* (15 kDa) and *Ani s 9* (14 kDa), are considered as minor ESP allergens, which are recognized in less than 50% of *Anisakis*-allergic patients (Anadòn et al. 2009; Daschner et al. 2012). Whereas, the allergens *Ani s 2* (paramyosin; 97 kDa) and *Ani s 3* (tropomyosin; 41 kDa), somatic antigens, are considered as panallergens of *Anisakis* (Audicana and Kennedy, 2008) and are thought to be primarily responsible for cross-reactivity between *Anisakis* and

other invertebrates, such as prawns and house dust mite (Asturias et al. 2000; Pérez-Pérez et al. 2000; Lin et al. 2014)

Recently, 36 and 29 putative allergens and some molecules possibly involved in pathogenicity were identified respectively in *A. simplex* (s. s.) and *A. pegreffii* by mean of transcriptomic analysis (Baird et al. 2016; Cavallero et al. 2018).

Table 2. Characterised antigens/allergens of *Anisakis* sp. larvae. ESP: excretory/secretory products; ? : indicates unkown function, source, or molecular weight.

Allergen	Localization	Function	Major allerger	Panallergen	References
Ani s 1	ESP	Kunitz-type tryps inhibitor	Yes	-	Moneo et al. 2000
Ani s 2	Somatic	Paramyosin	Yes	Yes	Pérez-Pérez et al. 2000
Ani s 3	Somatic	Tropomyosin	-	Yes	Asturias et al. 2000
Ani s 4	ESP	Cystatin	-	-	Rodriguez-Mahillo et al. 2007
Ani s 5	ESP	SXP/RAL protein	-	-	Kobayashi et al. 2007
Ani s 6	ESP	Serpin	-	-	Kobayashi et al. 2007
Ani s 7	ESP	Glycoprotein	Yes	-	Anadón et al. 2009
Ani s 8	ESP	SXP/RAL protein	-	-	Kobayashi et al. 2007
Ani s 9	ESP	SXP/RAL protein	-	-	Rodriguez-Perez et al. 2008
Ani s 10	Somatic?	?	-	-	Caballero et al. 2011
Ani s 11	Somatic?	?	-	-	Kobayashi et al. 2011
Ani s 11-like	Somatic?	?	-	-	Kobayashi et al. 2011
Ani s 12	?	?	Yes	-	Kobayashi et al. 2011
Ani s 13	ESP	Hemoglobin	Yes	-	González-Fernández et al. 2015
Ani s 14	?	?	Yes	-	Kobayashi et al. 2015

1.6. Parasitic nematodes adaptation to their hosts

In the course of evolution, parasitic nematodes have developed numerous strategies to adapt to their hosts and increase their fitness. Different factors such as temperature, pH, CO2, oxygen defines the preferential microhabitat of nematodes (Nieuwenhuzen and Lopata 2013).

The pH is an important parameter for the nematode's fitness in the host. These organisms are able to tolerate a wide pH range (1.6-11.0) (Neher and Powers 2005); for example, *Anisakis simplex* larvae (s. s.), survive at a pH 1.8 (Arizono et al. 2012). The temperature is one of the parameters that regulate the abundance and distribution of anisakid nematodes in all the aquatic environments of the world (Basànez et al. 2012).

Parasites have developed the ability to synthesize proteins that are released under unfavorable environmental conditions (for example low or high temperature conditions). HSP90 (Heat Shock Protein 90) is involved in a variety of cellular processes from development, to immune response of the host) and regulates the physiological response to thermal, chemical (exposure to heavy metals), and biological stress in almost all living organisms, from bacteria to humans, including anisakid nematodes parasites (Chen et al al. 2014).

Several studies showed that when *A. simplex* larva is exposed to a 50 ° C for 5 minutes, there is a 50% decrease in motility, with the deterioration of the cuticle (Tejada et al. 2006; Vidacek et al. 2010; 2011). Another study showed that at low temperatures (4-5 °C) in saline water (0.85%), larvae are able to survive much longer, even though with reduced motility (Chen et al. 2014).

Because parasitic nematodes are found in an anoxic environment, they have evolved one myoglobin particular with the role of scavenger capable of binding 25,000 times better than human haemoglobin, oxygen molecules, but also oxide nitrogen (NO) and *hyd*rogen peroxide (H2O2) released by the host (Dixon et al. 1992; Hardison et al. 1996; Goldberg 1999; Sherman et al. 1992; Minning et al. 1999).

The role of CO_2 in the survival of *Anisakis* spp. larvae does not seem to be fully clear yet. Davila et al. (2006) showed the potential effect of CO_2 on the development and survival of larvae. Like other parasites, above all helminths, anisakid nematodes possess the ability to fix carbon dioxide and high CO2 levels may be related to the need to incorporate carbon atoms into the energy metabolism of the parasite. Since CO2 is a catabulary of many hosts, its use represents an adaptive advantage by the parasite (Gutteridge and Coombs 1977; Kilgour 1980).

2. I - Gene expression profiles of antigenic proteins of third stage larvae of the zoonotic nematode *Anisakis pegreffii* in response to temperature conditions Parasite 26, 52 (2019)

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Abstract - *Anisakis pegreffii* is a parasite of homeothermic hosts at the adult stage, while it infects ectothermic hosts as third stage larvae. Currently, *A. pegreffii* has been recognised as etiological agent of human anisakiasis. Among distinct factors, the temperature appears to be crucial in affecting parasite hatching, moulting and to modulate parasite-host interaction. In the present study, we investigated the gene transcripts of proteins having antigenic role among the Excretory Secretory Products (ESPs) (i.e. a Kunitz-type trypsin inhibitor, *A.peg-1*; a glycoprotein, *A.peg-7* and the myoglobin, *A.peg-13*) after 24h, in *A. pegreffii* larvae maintained *in vitro,* under controlled temperature conditions (i.e. 37°C, 20°C resembling respectively homeothermic and ectothermic hosts condition, and 7°C, cold stress condition *post mortem* of the fish host). Primers of genes coding for those ESPs to be used in Quantitative Real-Time PCR were newly designed, and qRT-PCR conditions developed. Expression profiles of the genes *A.peg-1* and *A.peg-13* were found to be significantly up-regulated at 20°C and 37°C, with respect to the control (larvae kept at 2°C for 24h). Conversely, transcript profiles of *A.peg-7* did not significantly change among the chosen temperature conditions. In accordance with the observed transcript profiles, the SDS-PAGE revealed the presence of the three target ESPs at 37°C, while only *A.peg-13* was observed at 7°C. The results obtained seem to indicate that chosen temperature conditions do regulate gene expression profile of *A.peg-1* and *A.peg-13* in *A. pegreffii* larvae. While, the regulation of the glycoprotein, *A.peg-7* could be most related to other factors such as host's immune response.

Key words: *Anisakis pegreffii*, gene expression, immune-related genes, qRT-PCR, temperature conditions

2.1. Introduction

Anisakis pegreffii is a parasite of homeothermic hosts (mainly cetaceans) at the adult stage, while it infects ectothermic hosts (teleost fish and squids) as third stage larvae. *Anisakis pegreffii* is the dominant species of *Anisakis* in the Mediterranean Sea; indeed, it is the most common nematode in several pelagic and demersal fish of the Mediterranean Sea (Mattiucci et al. 2018). In the Atlantic waters, the northerly limit of geographical range of *A. pegreffii* is represented by the Iberian coast. It is also widely distributed in temperate sea waters of the Austral region, between 30°S and 60°S (Aibinu et al. 2019; Mattiucci et al. 2014; Mattiucci et al. 2018; Shamsi et al. 2012,).

Anisakis pegreffii has been recognised as etiological agent of human anisakiasis. Humans can acquire an accidental infection with *A. pegreffii* mainly through consumptions of raw, marinated, or undercooked fish fillets (mainly anchovies in Mediterranean countries) infected by the third stage larvae of this parasite (reviewed in (Guardone et al. 2018; Mattiucci et al. 2018)). Several cases of anisakiasis, due to the species *A. pegreffii*, have been reported in Italy (D'Amelio et al. 1999; Fumarola et al. 2009; Guardone et al. 2018; Mattiucci et al. 2011; Mattiucci et al. 2013; Mattiucci et al. 2017a; Mattiucci et al. 2018), Croatia (Mladineo et al. 2015), Japan (Umehara et al. 2007) and South Korea (Lim et al. 2015). Those geographical areas, where human anisakiasis due to *A. pegreffii* have been described, correspond to where a high rate of parasite infection has been reported in commercial fish species (Mattiucci et al. 2018). Clinical signs associated to the appearance of the pathology due to *A. pegreffii* are: gastric anisakiasis (GA), intestinal anisakiasis (IA), ectopic anisakiasis (EA) and gastro-allergic anisakiasis (GAA) (reviewed in Guardone et al. 2018; Mattiucci et al. 2018)).

So far, several molecular components of the L3 larvae have been identified in *A. simplex* (s. l.) as targets of the human humoral IgE mediated antigenic response (Allergen Nomenclature Sub-Committee, www.allergen.org). The antigens from *A. simplex* (s. l.) comprise both somatic antigens and excretory and secretory products (ESPs) (Daschner et al. 2012; Gonzales-Fernandez et al. 2015). Recently, the antigens *Ani s 1 like* (24 kDa, kunitz-type trypsin inhibitor), *Ani s 7 like* (139 kDa, a glycoprotein) and *Ani s 13 like* (37 kDa, myoglobin) were recognised in human cases of anisakiasis, as well as in IgE-hypersensitised patients due to the species *A. pegreffii* (Mattiucci et al. 2017a). These antigens do not display amino acid sequence similarity with any other allergen able to sensitize humans. Indeed, the cross-reaction with other antigens/allergens has not been documented. Such antigens are also useful for diagnosing of the *Anisakis* spp. infection (Gonzales-Fernandez et al. 2015; Kobayashi et al. 2015; Mattiucci et al. 2017a; Moneo et al. 2000; Rodríguez et al. 2008). Other putative allergens/molecules involved in the pathogenicity have been recently suggested by a RNAseq transcriptomic analysis (Baird et al. 2016; Cavallero et al. 2018; Kim et al. 2018). A characterization of transcriptomic by RNAseq of affected tissues of rats infected with *A. pegreffii* L3 have recently indicated a regulated expression modulation of hosts' immune-related genes in response to the larval infection (Bušelić et al. 2018; Hrabar et al. 2019; Nieuwenhuizen and Lopata 2016). However, the

expression profiles of immune-related genes in those *Anisakis* larvae in that homeothermic host-infected tissue was not investigated.

Temperature has been generally accepted as among the drivers that influences the rate of biological functions and adaptation of marine parasites (Widmann 2013). Larval development, and time of acclimate to an altered temperature, are generally to be 5-12X slower in parasite species of ectothermic hosts living near 0-10°C, compared with those from temperate ectothermic host species (Clarke 2004). Infective larval stage of nematodes generally requires specific stimuli for hatching and moulting, such as temperature, pH, and pepsin, which are known to be fundamental in the larval development to the adult stage, in *in vitro* experiments (Iglesias et al. 2001; Möller 1978). For many parasitic nematodes transmitted between ectothermic and homeothermic hosts - such as in the case of *Anisakis* spp. life-cycle - the stress resulting from the different body temperature of the hosts would be a signal for a further development in that host, or to maintain a homeostasis (Kim et al. 2018). It is generally known that mRNA transcripts to be a measure of the signal to make proteins synthesis by on organism in relation to the temperature (Peck 2016). Thus, those genes involved in adaptation to hosts' temperature, parasite development and host immune response can be differentially expressed in distinct temperature conditions. Temperature appears to modulate survival of *Anisakis* spp. (Vidacek et al. 2010, 2011), as well as metabolic processes as the production of ESPs (Bahlool et al. 2013). Furthermore, temperature and storage time may play important role in the *post-mortem* motility of *A. pegreffii* larvae, indicating that storage temperature higher than 7°C increases larva motility and therefore migration to the fish muscle (Cipriani et al. 2016).

Recently, a comparative transcriptomic analysis of the L3 and L4 *A. simplex* (s. l.) showed that several protein metabolism-related genes, likely involved in the host invading tissues, were highly expressed in L3 larvae of *A. simplex* (s. l.) (Kim et al. 2018). In particular, the mRNA expression levels of some selected genes (i.e. *Nas-13, EF-TsMt, SFX2, dhs*) were found to be significantly higher in *A. simplex* (s. l.) third stage larvae, compared to the fourth stage larvae (Kim et al. 2018). Furthermore, the expression patterns of Heat shock proteins (i.e. *Hsp70* and *Hsp90*) appeared to be modulated in L3 *vs* L4 stage larvae of *A. pegreffii*, with higher expression profiles in L4 larvae (Chen et al. 2015). However, the expression profiles of those genes coding immuno-related proteins in L3 larvae of *Anisakis* in response to the temperature was never investigated.

Aim of this study was to investigate the impact of temperature changes on the gene expression profiles of *A. pegreffii* proteins previously found to be target of the human IgE mediated immune response to L3 larvae, i.e. the Kunitz-type trypsin inhibitor, *A.peg-1*; the glycoprotein *A.peg-7*; the myoglobin, *A.peg-13*. A qRT-PCR method to detect the gene transcript levels was developed and the gene expression profiles were analysed in *A. pegreffii* L3, cultured *in vitro*, in response to different temperature conditions (i.e. 20°C, 37°C, and 7 °C), speculated by a third stage larva of the parasite species in ectothermic, homeothermic hosts, and under a cold stressed condition, respectively.

2.2. Materials and Methods

2.2.1. Sampling and experimental design

Anisakis spp. alive larvae were collected from anchovies *Engraulis encrasicolus*, caught in the Adriatic Sea (off S. Benedetto del Tronto coast), from where high rate of infection with the target parasite species, i.e. *A. pegreffii*, was previously recorded (Cipriani et al. 2018). After the capture, the fish were maintained at 2°C, using a data logger, until the parasitological examination. The gentle removal of *Anisakis* spp. larvae, and the checking for their integrity - the last procedure under a dissecting microscope - was performed in a refrigerate room (2°C). Live and not disrupted larvae of *Anisakis* spp. were washed in saline solution (0.9%), several times, and then treated for 1 min, with 4% acetic acid to inhibit bacterial contamination.

In order to analyse gene expression profiles and excretory secretory products released, following temperature exposure, the larvae were cultured *in vitro* at chosen thermal conditions, i.e. 7°C, temperature at which a significant increase of *A. pegreffii* motility *post mortem* of the fish host was previously observed (Cipriani et al. 2016); 20°C, average temperature of an ectothermic fish host of the Mediterranean Sea infected by *A. pegreffii*; and 37°C, temperature of the definitive homeothermic cetacean host, as well as of accidental human host. The gene expression and the excretory secretory products released were analysed after 24 h incubation. For each experimental condition, 50 larvae were seeded in 3.5 cm Petri Dishes in 3.5 mL of PBS 1X containing penicillin (1%) and streptomycin (1%) and incubated at the distinct temperature, 7°C, 20°C and 37°C in 5% CO_2. As control condition, larvae (50) were also kept at 2°C. A total of 200 *Anisakis* spp. larvae were used. The experiments were prepared in triplicate and repeated at the three temperatures.

At 24h post-incubation, the excretory secretory products were stored at -80°C for the proteins characterization by SDS-PAGE and the survived *Anisakis* spp. larvae were collected, and stored in RNA later at -80°C for RNA and DNA extraction, to study the gene expression by qRT-PCR, and for larval identification to the species level by DNA sequences analysis. A larva was considered dead if it was not motile. At 2°C temperature, the larval motility of *A. pegreffii* was observed almost absent *in vivo* (Cipriani et al. 2016), as well as *in vitro* experiments (Mattiucci unpublished data).

2.2.2. DNA and RNA extraction

Both DNA and RNA were extracted from each individual *Anisakis* larva used in the experiments, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, each larva was homogenized using a motorised pestle and homogenised tissue in 1 mL of TRIzol, with the addition of 0.2 mL chloroform. The mixture was vortexed and centrifuged (12,000 x *g*, 15 min, 4°C) resulting in an aqueous (containing RNA) and organic phase (containing DNA and proteins). The aqueous layer was then transferred into a separate tube; the RNA was precipitated with 500 μL of isopropanol at room temperature for 10 min and pelleted by centrifugation (12,000 x *g*, 15

min, 4°C), then washed in 1 mL of 75% ethanol, centrifuged again (7,500 x *g*, 5 min a 4°C), and dried for 10-30 min. The pellet was re-suspended in 40 μL of nuclease and RNase-free water at 60°C for 10 min.

DNA was precipitated from the remaining interphase/organic layer with 300 μL absolute ethanol, and mixed by inverting the tube several times. The mixture was centrifuged (2,000 × *g*, 5 min, 4°C), and the upper aqueous layer (containing proteins) was removed. The pellet was washed in 1 mL of 0.1 M sodium citrate in 10% ethanol pH 8.5, mixed, occasionally, by gentle inversion. The DNA was stored in sodium citrate/ethanol for 2 hours, and the mixture was then centrifuged (8,000 × *g*, 5 min, 4°C). The supernatant was discarded with a micropipette and the pellet was re-suspended in 0.3–0.6 mL of 8 mM NaOH. The mixture was centrifuged (12,000 × *g*, 10 min, 4°C) and the supernatant was transferred the to a fresh tube. RNA and DNA concentration and quality were measured by a NanoDrop®TC1-E20 spectrophotometer (BioTek Synergy HT). DNA was stored at −20°C, while RNA was stored at −80°C, until use.

2.2.3 Molecular identification of *Anisakis* spp. larvae

Each *Anisakis* spp. larvae used in the gene expression experiments, were identified to species level by means of genetic/molecular markers. For this purpose, a multi-locus approach based on sequences analysis of mitochondrial (mtDNA *cox-2*) (629 bp) and nuclear (elongation factor EF1 α-1 of nDNA) (409 bp) genes was applied.

The mitochondrial cytochrome c oxidase subunit II (*cox-2*) gene was amplified using the primers 211F (5'-TTTTCTAGTTATATAGATTGRTTYAT-3') and 210R (5'-CACCAACTCTTAAAATTATC-3'). Polymerase chain reaction (PCR) was carried out according to the procedures as previously described (39). The sequences obtained at the mtDNA *cox-2* for those larval specimens analysed in the present study were compared with those already obtained for the same gene in the species *A. pegreffii* and with respect to those from other *Anisakis* spp. previously sequenced (Mattiucci et al. 2014).

The elongation factor (EF1 α−1 nDNA) nuclear gene was amplified using the primers EF-F (5'-TCCTCAAGCGTTGTTATCTGTT-3′) and EF-R (5′-AGTTTTGCCACTAGCGGTTCC-3′) and employing experimental conditions as previously described (Mattiucci et al. 2016). The sequences obtained at the EF1 α−1 nDNA for the larval specimens analysed in the present study were compared with those already obtained for the same gene in the species *A. pegreffii* and *A. simplex* (s. s.), under the accession numbers KT825684 and KT825685, respectively. The genomic DNA obtained from those samples is stored at the Department of Public Health and Infectious Diseases (Section of Parasitology) - 'Sapienza University of Rome'.

2.2.4. RT-PCR, amplification of *A.peg-1, A.peg-7* and *A.peg-13* cDNA

Total RNA from each individual larva was treated with DNase (DNase I, Invitrogen): 1μL DNase I (1 U/μl, Invitrogen) was added to 1 μg of RNA sample. RNA concentration and quality were measured by a NanoDrop®TC1-E20 spectrophotometer (BioTek Synergy HT).

High-Capacity cDNA Reverse Transcription Kits (ThermoFisher) was used for cDNA synthesis. The reverse transcription was performed in a final volume of 20 μL, in the presence of dNTP mix, (0,5 mM each), RNAse inhibitor (10 U) and with MultiScribeTM Reverse Transcriptase (4 U). Oligo(dT) and random hexamers (0.5 μM) were used as primers. The reaction was carried out in a thermal cycler at 37 °C for 2 hours.

To obtain cDNA sequences of *A.peg-1, A.peg-7* and *A.peg-13* genes, species-specific primers for *A. pegreffii* larvae were first designed (Table 1), by using Primer3 web software (version 4.1.0), starting from those sequences data at the same gene coding loci, previously deposited in GenBank as *A. simplex* (s. l.) larvae (Anadón et al. 2009; Gonzales-Fernandez et al. 2015; Moneo et al. 2000); they were synthesized by Eurofins Genomics (Ebersberg, Germany). The amplification was performed by PCR in a total volume of 25 μL containing 1X PCR buffer, 25 mM $MgSO_4$, 2 mM dNTPs (each), 10 mM primer (each) (Table 1), 50 ng cDNA, 0,12U Taq polymerase in bi-distilled water. PCR was performed as follows: 95°C for 5 min followed by 35 cycles of 1min at 95°C, 1 min at different temperature of annealing for each gene (see Table 1), 1 min at 72°C with a final extension at 72°C for 15 min.

The PCR products were separated using agarose gel electrophoresis and, subsequently, the PCR products with expected molecular weight were purified, and sequenced with the same primers used for PCR (Macrogen, Europe). Nucleotide sequences of the Kunitz-type trypsin inhibitor, the glycoprotein and the myoglobin were aligned using ClustalW v2.0 (Larkin et al. 2007).

Table 1. Primer sequences of target genes coding for antigenic proteins, used in this study. *GAPDH*: gene housekeeping.

	PCR end-point			Real Time PCR		
Gene locus	Sequences (5' to 3')	Ta/°C	Size (bp)	Sequences (5' to 3')	Ta/°C	Size (bp)
A. peg-1	F: ATCCTCTTCACATTCGCTTT R: GGATAATAATGGTCGGGCAA	57°C	639	F: CATGTGCCGATAAATGCGGG R: CCCTGTGAGCATGCATCCTT	57°C	130
A. peg-7	F: ACACCTCCATCTGAACAAA R: CCTAACATGCAGGCGATTA	57°C	899	F: TATCGGAATGCGTGACTGCA R: AGGCAGTTTCCATGGTGTATG	57°C	130
A. peg-13	F: CATGAAATCACTCGAACACG R: TGTTCCTCCTTGTGCTCT	55°C	931	F: AAACATTCGACGCCTACACC R: CATCGTGGTCTTCTCTGCGA	60°C	108
GAPDH	-	-	-	F: CCCCTTCATCAACATCGACT R: TCAGCTCCCCATTTGATTTC	60°C	152

2.2.5. Quantitative Real-Time PCR

The gene-specific primers of those target genes, to be used in qRT-PCR were first designed, by using Primer Expression 3.0 software (Applied Biosystem, USA) from the sequences of the selected *A. simplex* (s. l.) genes available in GenBank. Primer sequences of target genes used in this study in both PCR end point, and qRT-PCR are reported in Table 1. Relative gene expression profiles of L3 stage of *A. pegreffii* under different thermal conditions, were evaluated by fluorescent real-time PCR. The cDNA synthesised was used as a template. The housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was evaluated as a reference gene; GAPDH specific primers were employed (Chen et al. 2014).

The quantitative Real time PCR (qRT-PCR) was carried out in 50 µL of a reaction mixture with 25 µL of 2X SYBR™ Green PCR Master Mix (Applied Biosystems™) containing SYBR Green I dye, AmpliTaq Gold DNA polymerase, dUTP and buffer, 1 µL of each primer (10Mm), 21 µL of bi-distilled water, and 2 µL of cDNA. PCR amplification was carried out using a gradient cycler StepOnePlus™ Real-Time PCR Detection System (Applied Biosystems™). The conditions used were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C, according to the primers used, for 1s. Melting curve data were collected at 55–95°C. All reactions were performed three times, in triplicate. Amplification data were gathered and analysed. Relative expression was calculated using the ΔΔCt method (Livak and Schmittgen 2001), with all expression normalised to GAPDH levels in initial control samples. Relative levels of GAPDH were confirmed to be approximately equal across all treatments. The qRT-PCR experiment was designed and performed according to the MIQE guide recommendation (Bustin et al. 2009).

2.2.6. SDS-PAGE analysis

SDS-PAGE analysis was carried out on the excretory secretory products (ESPs) obtained from the supernatant of cultured *A. pegreffii* larvae, collected at 7°C, 20°C and 37°C, after 24h of incubation. ESPs were concentrated as previously described (Mattiucci et al. 2017a). Briefly, culture supernatant was mixed with 90% acetone (1:1), vortexed for 1 min, and kept in ice for 15 min. Following centrifugation at 1,100 *g* for 10 min, the supernatant was discarded and the residual acetone was removed by evaporation at room temperature; the pellet was dissolved in PBS 1X. Protein concentration was determined using the Quick Start Bradford Protein Assay (Bio Rad), with Bovine Serum Albumin as a standard control. For the protein preparation, samples from ESPs were individually diluted 1:1 with Laemmli buffer, and then heated at 95ºC for 5 min. Protein electrophoresis was carried out in Mini-PROTEAN 3 Cell (Bio-Rad, Hercules, California, U.S.A.) (12% polyacrylamide separating gel with 5% stacking gel), following the manufacturer's instructions.

2.2.7. Statistical analysis

Statistical analyses were carried out using Prism8 Statistical Software from GraphPad Prism 6.0 (GraphPad Software, Inc. La Jolla, CA). Shapiro-Wilk test was performed to check the normality of the data. The one-way test was employed to compare three groups of data, while the Student's *t*-test was used to assess the occurrence of significant differences between two set of data. All data were expressed as mean ± standard deviation (SD). Significance was set at $p< 0.05$.

2.3. Results

2.3.1. Molecular identification of *A. pegreffii* larvae

On the basis of the sequence analysis of the mitochondrial (mtDNA *cox-2*) and nuclear (EF1 α−1 nDNA) gene loci, the *Anisakis* L3 larvae employed in the present study were assigned to the species *A. pegreffii*. The obtained mtDNA *cox-2* sequences showed 99% or 100% similarity with sequences previously deposited in GenBank for *A. pegreffii*. Further, the same larval *Anisakis* specimens were confirmed to belong to the species *A. pegreffii*, as defined by the presence of the specific nucleotide residues present in the 409 bp length EF1 α−1 nDNA sequences - i.e. a T and C nucleotides in position 186 and 286, respectively that have shown to be diagnostic molecular features of *A. pegreffii* (Mattiucci et al. 2016).

2.3.2. Sequencing of antigenic ESPs in L3 of *A. pegreffii*

The comparison of the sequences of *Ani s 1*, *Ani s 7* and *Ani s 13* genes, obtained in the present study for the species *A. pegreffii*, hereby indicated as *A.peg-1*, *A.peg-7* and *A.peg-13*, with previously described in literature (Abe and Teramoto 2017; Anadón et al. 2009; Gonzalez-Fernandez et al. 2015; Moneo et al. 2000; Park et al. 2012; Rodríguez et al. 2008), revealed sequences of 534bp, 816bp and 931bp, respectively (Figures 1-3). Nucleotide sequences of *A.peg-1*, *A.peg-7* and *A.peg-13* showed some nucleotide differences with respect to those previously deposited as *A. simplex* (s. l.) in GenBank. *A.peg-1* gene locus showed ten variable bases at positions 69, 87, 130, 135, 265, 268, 282, 336, 450 and 479, in comparison with those of *A. simplex* (s. l.) previously deposited in GenBank (Accession No. JN241677) (Figure 1a). Four bases positions 44, 89, 90 and 160 were found in the amino acid sequence alignment of *A.peg-1* in *A. pegreffii* compared to *A. simplex* (s. l.) sequence (Figure 1b). The *A.peg-7* gene obtained in the present study showed variable nucleotides at position 86, 113, 114, 249, 500, 686 in *A. pegreffii* when compared with those previously deposited in GenBank as *A. simplex* (s. l.) (Figure 2a) (Accession No. EF158010). Two variable bases at positions 29 and 229 were found in the amino acid sequence alignment of *A.peg-7* in *A. pegreffii* with respect to *A. simplex* (s. l.) (Figure 2b). Finally, *A.peg-13* gene locus obtained in the present study showed several variable bp sites, with respect to that previously deposited in GenBank as *A. pegreffii* (Accession No.LC209224). Six variable bases at positions 82, 117, 118, 120, 121, 249 were found in the amino

acid sequence alignment of the protein, with respect to that available sequence (Figure 3a-b). Sequences obtained at those gene loci for the species *A. pegreffii* were deposited in the GenBank with the following accession numbers: MG962417 MG962418 MG962419 MG962420 (*A.peg-1*); MG962411, MG962412, MG962413 (*A.peg-7*) and MG962414, MG962415, MG962416 (*A.peg-13*).

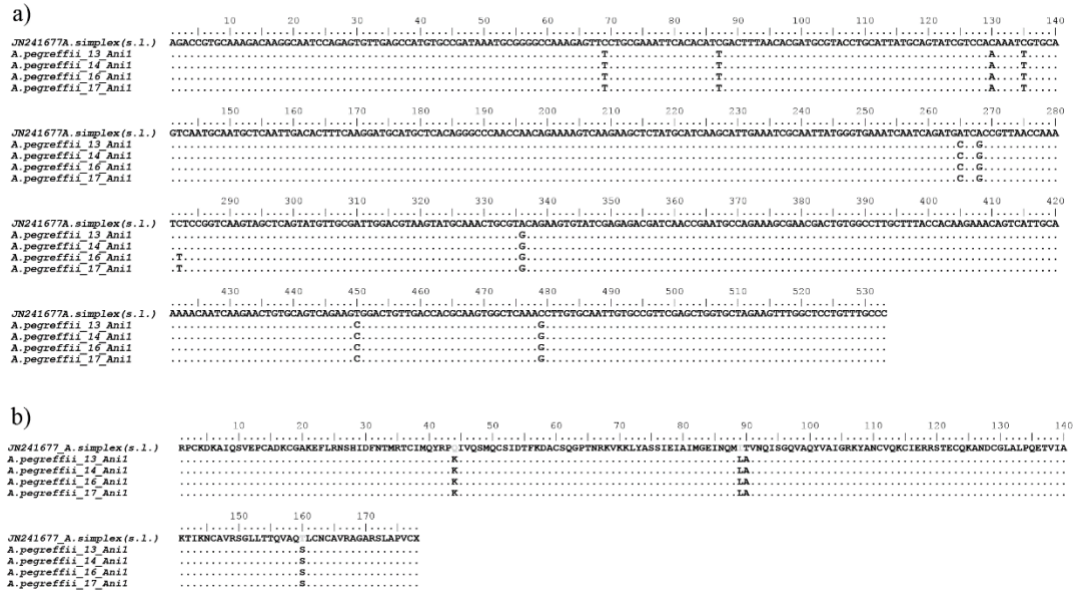

Figure 1. Nucleotide and amino acid sequences alignment of the *A.peg-1* cDNA. a) Nucleotide sequence alignment obtained from *A. pegreffii* larvae from the present study in comparison with those previously deposited in GenBank as *A. simplex* (s. l.) (Accession No. JN241677). Dots indicate identity with the consensus sequence. b) Amino acid sequence alignment of the deduced amino acid sequences of the *A.peg-1* allergen showing the variable sites between *A. pegreffii*, and previously reported *A. simplex* (s. l.). Dots represent residues identical to the reference *Ani s 1* sequence.

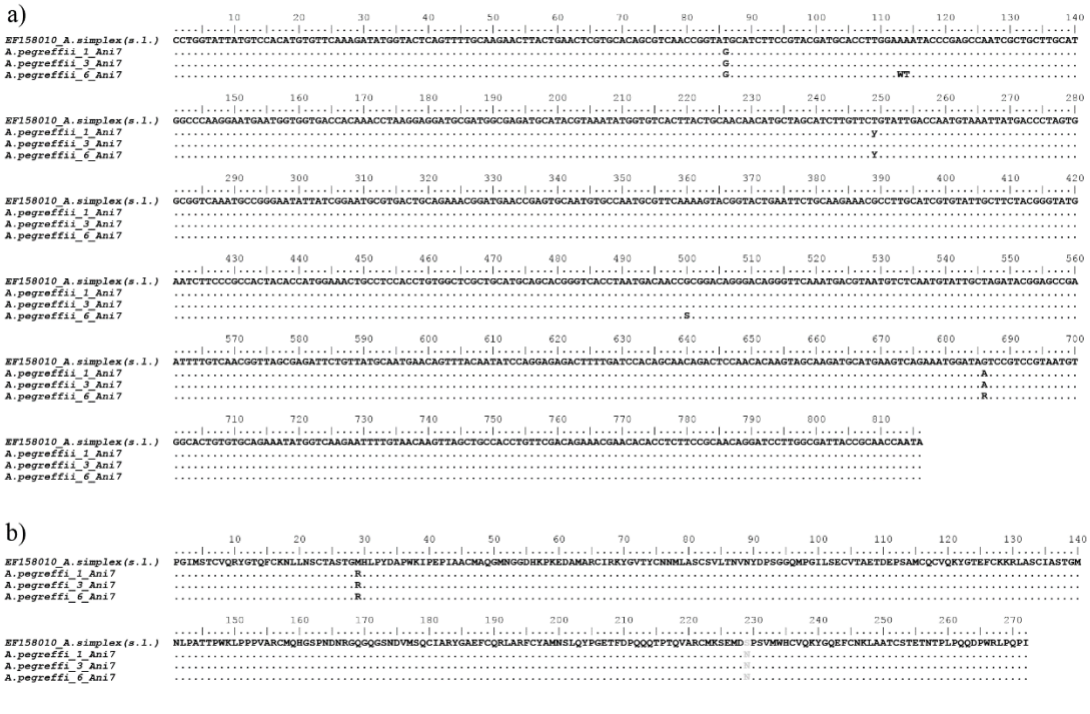

Figure 2. Nucleotide and amino acid sequences alignment of the *A.peg-7* cDNA. a) Nucleotide sequence alignment obtained from *A. pegreffii* larvae from the present study with respect to the internal *Ani s 7* cDNA (nucleotide (nt) 1203–2139) fragment (a potential antigenic region) of the previously deposited in GenBank as *A. simplex* (s. l.) (Accession No.: EF158010). Dots indicate identity with the consensus sequence. b) Amino acid sequence alignment of the deduced amino acid sequences of the *A.peg-7* allergen showing the variable sites between *A. pegreffii*, and previously reported *A. simplex* (s. l.). Dots represent residues identical to the reference *Ani s 7* sequence.

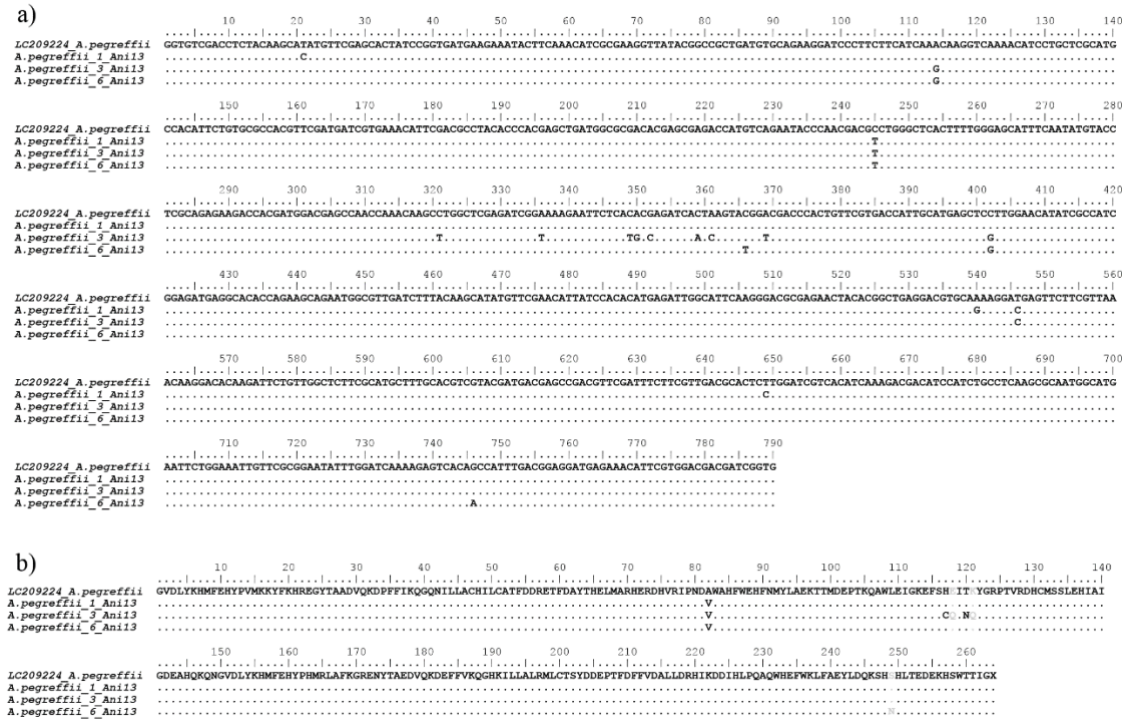

Figure 3. Nucleotide and amino acid sequences alignment of the *A.peg-13* cDNA. a) Nucleotide sequence alignment obtained from *A. pegreffii* larvae from the present study in comparison with those previously deposited in GenBank for *A. simplex* (s. l.) (Accession No.: LC209224). Dots indicate identity with the consensus sequence. b) Amino acid sequence alignment of the deduced amino acid sequences of the *A.peg-13* allergen showing the variable sites between *A. pegreffii*, and previously reported *A. simplex* (s. l.). Dots represent residues identical to the reference *Ani s 13* sequence.

2.3.3. Gene expression analysis

Quantitative Real-time PCR analysis (qRT-PCR) revealed a differential sensitivity of the three target genes in response to the exposure to the chosen temperature conditions in third larval stage of *A. pegreffii.* The Shapiro-Wilk tests supported a normal distribution of the data obtained from the qRT-PCR assay tests (all the tests $p<0.05$).

Figure 4 summarises the profile expression patterns of the *A.peg-1, A.peg-7* and *A.peg-13* genes as detected by qRT-PCR. Change in expression levels of *A.peg-1* appeared to be highly significant ($p<0.0001$) among the three distinct temperature exposure conditions chosen by ANOVA test (Table 2). When the comparison was performed between pair samples employing Student's *t* test, the gene expression of *A.peg-1* after 24h, was not significantly down-regulated by exposure at the cold temperature (7°C), as compared to the control (larvae maintained at 2°C). However, increase of the

temperature to 20°C and 37°C appeared to trigger significantly the expression of the *A.peg-1*, with respect to the control (p= 0.026, and p<0.0001, respectively). Furthermore, a significantly increase was observed between *A.peg-1* at 37°C *versus* 20°C (p<0.001).

Regarding the *A.peg-13* transcripts, significantly higher gene expression was found for each temperature condition in comparison with the control. Already at 7°C, an increase of the gene expression occurred (p= 0.0092) and it was maintained throughout the increasing temperature, with a slight decrease at 20°C (p= 0.0292) and with a maximum expression at 37°C, when compared to the control (2°C) (p= 0.0004) (Figure 4). However, the relative gene expression of the myoglobin, did not show a significant ANOVA value (p= 0.1795), in the comparison with the relative expression profiles reached at different temperature conditions (Table 2).

On the contrary, the transcript levels of *A.peg-7* were not significantly modulated by distinct temperature conditions (ANOVA p= 0.7111) (Table 2). A general trend of down-regulation of *A.peg-7* was observed at each temperature condition as compared to the control one, with a decreased, although not significant (p=0.0970) (Figure 4).

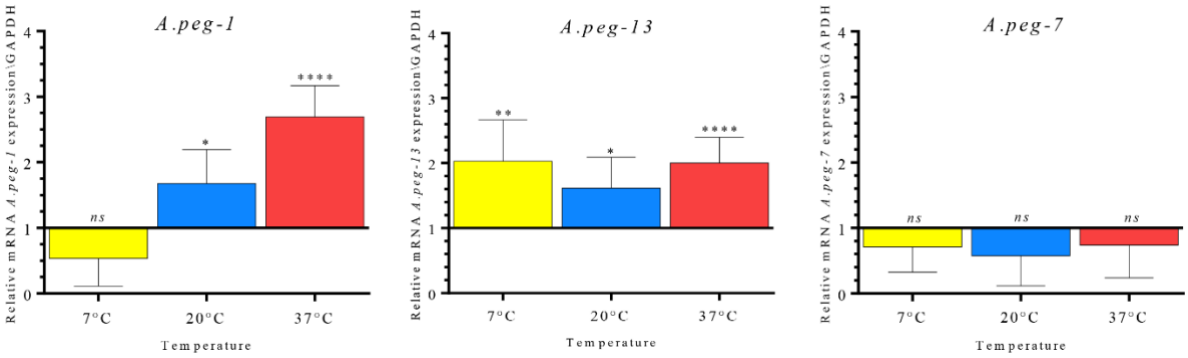

Figure 4. Relative expression profiles of the gene coding for the ESPs proteins, *A.peg-1, A.peg-7, A.peg-13* (shown vertically on the right side of the graph), normalized to the geometric mean of GAPDH (*gpd2*), in *A. pegreffii* larvae, in response to temperature conditions (7°C, 20°C and 37°C, after 24h). The control at 2°C is shown as normalized to a value of 1. Each value represents the mean ± s.d. of 3 biological replicates. Significant differential expression was assessed by Student' *t* test. Significance was fixed at $P < 0.05$ (*p<0.05, **p<0.01, ***p<0.001, p<0.0001).

Table 2. One-way ANOVA analysis on the relative gene expression profiles of *A.peg-1*, *A.peg-7* and *A.peg-13*, in response to temperature conditions. Significance was fixed at p< 0.05 (**** p< 0.0001); ns= not significant. d*f*: degrees of freedon; MS: mean square; F: F ratio.

Gene	d*f*	MS	F	p
A.peg-1	2	10.49	47.09	****
A.peg-7	2	0.070	0.345	ns
A.peg-13	2	0.485	1.846	ns

2.3.4. Protein characterization

The observed gene expression patterns resulted in accordance with the analysis of the relative protein levels of the parasite at the three distinct temperature conditions in SDS-PAGE. While no bands were

detected in the supernatants of *A. pegreffii* larvae maintained at 2°C for 24h, a band likely to be corresponding to the *A.peg-13*, 37 kDa protein, was detected in the supernatants of larvae at the three different temperature conditions (7°C, 20°C and 37°C). A 24 kDa molecule, likely corresponding to *A.peg-1*, was observed only in the culture media of *A. pegreffii* larvae maintained at 20°C and 37°C. Finally, another band of molecular weight around 139 kDa likely corresponding to *A.peg-7* was revealed at 37°C, while, at less extent, at 7°C and 20°C (Figure 5).

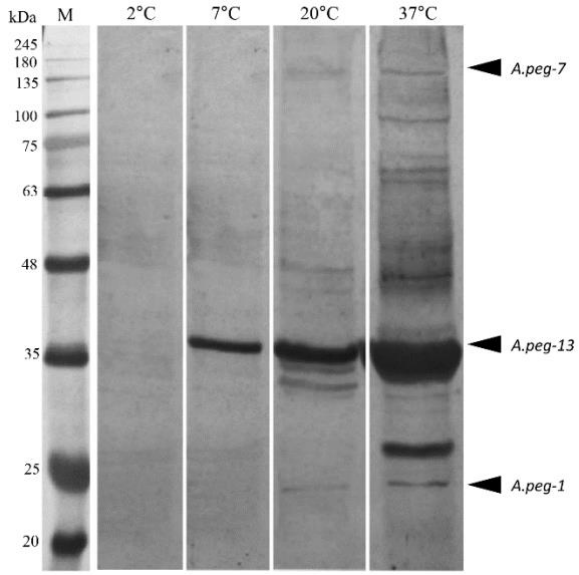

Figure 5. Excretory secretory proteins from advanced third stage larvae of *A. pegreffii* (*A.peg-1*, *A.peg-7*, *A.peg-13) in vitro* cultured in response to temperature (7°C, 20°C and 37°C, after 24h). These proteins were examined by SDS-PAGE. Supernatant of *A. pegreffii* maintained at 2°C, used as control. M: marker.

2.4. Discussion

Anisakis spp. larvae produce and release Excretory Secretory Products (ESPs) which may serve roles during the development stages of their life cycle. In addition, the relative abundance and function of ESPs are retained to be molecular determinants in defining host specificity of different *Anisakis* species (Mehrdana et al. 2017). It has also been suggested that ESPs in the third stage larvae of *Anisakis* varies quantitatively, depending on the type of the physiological conditions of natural host groups (fish and mammals), as well as of the accidental ones (human host). The changing of thermal conditions experimented by *A. pegreffii* in the microhabitat of ectothermic and homeothermic hosts, involved in its life cycle, would require, among the others, molecular and biochemical responses to temperature, also as a result of ecological adaptation of the parasite species to those hosts. Indeed, for instance, temperature was found a sensitive parameter to trigger the development of *A. pegreffii* L3 *in vitro* cultured to L4, up to the adult stage (Mattiucci pers. observ.). A temperature-dependent relative concentration of ES proteins produced by *A. simplex* larvae, after incubation at different temperatures was previously shown (Bahlool et al. 2013).

The present study represents the first analysis, by using an experimental approach, on the expression patterns of genes coding for ES antigenic proteins, which were found to elicit antibody response (IgE) in humans. Indeed, target ES products of this study have been already described as the most important antigens responsible for IgE sensitization in humans by *A. pegreffii* (Mattiucci et al. 2017a). In *A. simplex* (s. l.), the antigen *Ani s 1* contains a Kunitz-domain (Kennedy 1991; Matthews 1982, 1984; Quiazon et al. 2013), with trypsin inhibitor activity and aspartic proteases (Moneo et al. 2000). Proteins with Kunitz domains are degrading enzymes that act as protease inhibitors, with a functionally conserved role in cuticle formation in a diverse range of nematodes, and possibly involved in the inhibition of thrombin and coagulation factors in the homeothermic accidental human host (Mehrdana and Buchmann 2017). Proteins with Kunitz domains, showing homology with *Anis 1*, has been found in expressed transcript products obtained from pharyngeal tissues of *A. pegreffii* and *A. simplex* (s. s.) larvae (Cavallero et al. 2018). These proteins have been also previously identified in studies examining the proteolytic enzymatic activity of *Anisakis* spp. (Matthews 1982, 1984), showing an optimum of activity at the host body temperature of 36–37°C, thus supporting the hypothesis that these enzymes are activated during the infection in homeothermic hosts of *Anisakis* spp.. In accordance with these findings, we have shown that transcripts of the gene coding for *A.peg-1* from *A. pegreffii* are up-regulated in response to thermal conditions of both 20°C, and, at higher and significant extent, at 37°C. While, the larvae exposed to colder thermal condition (7°C), were still alive, but the transcript levels of *A.peg-1* were observed to be down-regulated - even if not at significant rate - with respect to the control larval samples maintained at 2°C temperature. The up-regulation of *A.peg-1* at 20°C and 37°C in comparison with the control, and its release in the supernatant of *in vitro* culture, as detected by SDS-PAGE, suggests that this molecule may be related to the temperature conditions, as those found in their intermediate/paratenic fish hosts (on average, 20°C), and in definitive, as well as in accidental human hosts (37°C) (Figures 4, 5). In particular, the finding of transcripts of *A.peg-1* antigen, that has been significantly released at 37°C after 24 hours, suggests a possible role of *A.peg-1* also in tissue invasion in the accidental (humans) hosts. Indeed, *A.peg-1* has been recognised by the IgE immune response in human serum as described in a case of Gastro-Allergic-Anisakiasis (GAA) due to *A. pegreffii* (Mattiucci et al. 2013; Mattiucci et al. 2017a). Further, in an *in vivo* animal model of *Anisakis* infection, it was found that C5BL/6 mice infected with *Anisakis* spp. larvae significantly increased the production of Th17-related cytokines IL6 and IL17A, after the treatment with recombinant *Ani s 1* (Cho et al. 2014). In another model, Wistar rats exposed orally to fresh and frozen *Anisakis* larvae, had shown that crude larval extracts did not induce significant IL17 by the experimentally infected host (Bušelić et al. 2018). This might suggest that live larvae *A. pegreffii* and ES production of *A.peg-1* protein could trigger different immune recognition and activation pathways in accidental human host.

This *in vitro* study seems also indicate that other molecules are up-regulated by the temperature change. The gene coding for the myoglobin *A.peg-13* is highly expressed at 37°C and 7°C, with respect

to the control; at less extent, a significant relative expression was found in the larvae exposed to 20°C (Figure 4). In ascaridoid nematodes, the function of *Ascaris* haemoglobin (and other nematodes myoglobin) is largely unknown. It has been retained that myoglobin is able to bind oxygen too tightly to be involved in its delivery, and to sequester oxygen in order to maintain an anaerobic environment (Goldberg 1999; Minning et al. 1999). Furthermore, nematode myoglobin can bind and break down the nitric oxide (NO) and *hyd*rogen peroxide (H_2O_2), suggesting that it may provide protection against host oxidative defences (Barrett and Brophy 2000; Goldberg 1999; Minning et al 1999). *Anisakis* spp. myoglobin was previously found in L3 larval stages, and associated with the excretory secretory products (Nieuwenhuizen and Lopata 2016). Also, *A.peg-13* is recognised, at high percentage, by the IgE-immune response in human cases of anisakiasis (Gonzalez-Fernandez et al. 2015; Mattiucci et al. 2017a). The lower relative transcripts of *A.peg-13* observed at 20°C, prompts for future investigation aimed to estimate its gene expression in *A. pegreffii* larvae infecting tissues of intermediate/paratenic hosts.

Differently from the other two genes, in our experimental study, expression profiles of the gene coding for the glycoprotein *A.peg-7* did not change significantly at the temperatures and time interval considered (Figure 4, Table 2). *A.peg-7* is a glycoprotein which has been considered as one of the major excretory secretory (ES) antigens, being detected by the 85% and 100% of infected patients with *A. simplex* (s. l.) (Daschner et al. 2012; Mattiucci et al. 2017a). Previous studies have suggested that *Anisakis* spp. larvae secrete ES products whose glycosylated components regulate the fundamental processes of antigen recognition, processing and presentation (Bahlool et al. 2013; White and Artavanis-Tsakonas 2012). On the other hand, ES glycoprotein of the parasites, can down-modulate the host immune response. For instance, in the case of *Schistosoma mansoni* eggs, an ES omega-1 glycoprotein was found to promote Th-2 skewing of dendritic cells (DCs) and T cells during infection (Coakley et al. 2015). DCs are the antigen presenting cells (APCs) performing the essential role in the regulation and coordination of innate and adaptive immune responses. DCs are equipped with a wide range of receptors (PRRs-pathogen recognition receptors) for the recognition of parasites. Among the PRRs, the C-type lectins recognise carbohydrate structures on self and non-self glycoproteins and glycolipids (Van Vliet et al. 2008). In particular, Macrophage C-type lectin (MGL) has been identified as selective binder of the carbohydrate residue GalNAc-O-S/T (Tn) carried by several parasites as well as expressed by cancer cells (Napoletano et al. 2007, 2012; Zizzari et al. 2015). Further, it has been found that the interaction between the MGL lectin expressed by Dendritic Cells (DCs) and the parasite *A. pegreffii* likely induces a Th-2 switch, involved in the IgE mediated responses against this parasite (Napoletano et al. 2018). Thus, since the transcript levels of *A.peg-7* in *A. pegreffii* seems to be maintained in the selected temperature-conditions, investigated in the present study, its gene expression would be rather modulated by the host (both of intermediate and definitive/accidental human hosts) immune response. *Ani s 7* has been suggested to be involved in the regulation of the IgM

response by the intermediate/paratenic host (fish) (Haarder et al. 2013). Teleost fishes are capable of producing specific immunoglobulins towards parasitic antigens, as an integrated part of the adaptive immune response (Buchmann 1993; Coscia and Oreste 1998; Coscia et al. 2000; Haarder et al. 2013; Priebe et al. 1991). This would allow the larval worms to establish persistent infections in the fish host.

In our experiments, the expression profiles of this gene resulted to be down-regulated (even if not at significant rate, with respect to the control sample), at the temperature of 20°C. On the other hand, significant differential transcripts of the gene *A.peg-7* in *A. pegreffii* larvae, from different tissues of infected fish host with the parasites, have been observed (Palomba pers. observ.), thus suggesting a possible role of the fish host' immune system in the up-regulation of this gene, when infected by *A. pegreffii* larvae. Merdhana (Mehrdana et al. 2017) demonstrated that ES products from *C. osculatum* activate most immune genes in a dose-dependent manner. It is noteworthy that a high concentration of *C. osculatum* ES proteins also induced higher expression of the fish host gene indicating activation of T regulatory cells (Tregs) which, in turn, produces inhibitory and anti-inflammatory cytokines in the host.

It has been also suggested that using the glycoprotein *A.peg-7*, the parasite *Anisakis* sp. would be able to induce and regulate the Th-2 polarizing response associated to *Anisakis* infection in the accidental host (humans) (White and Artavanis-Tsakonas 2012). On the other hand, the capacity of *A. pegreffii* larvae to impair human DC cells biology and functions, has been also experimentally shown (Napoletano et al. 2018), suggesting a possible up-regulation of this gene under effective cellular immune response of homeothermic human host.

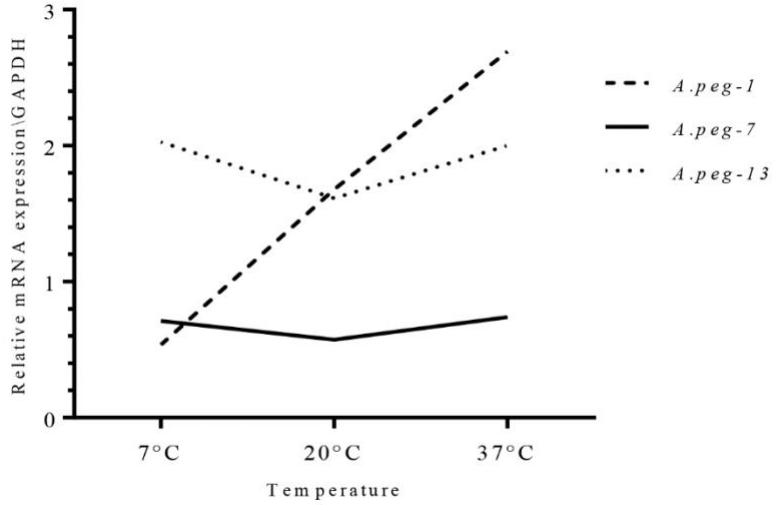

Figure 6. Trend of the relative expression of three selected genes coding for the ESPs proteins, *A.peg-1*, *A.peg-7*, *A.peg-13* in response to temperature conditions, using the normalization reference gene, *gpd2*.

2.5. Conclusions and perspectives

This study presents novel results on the expression of the *A.peg-1*, *A.peg-7* and *A.peg-13* genes coding for proteins among the ES products in the species *A. pegreffii*, having a major immuno-

modulatory role in the host response, under the effect of temperature conditions. Particularly, a significant positive correlation between the expression levels of *A.peg-1* and *A.peg-13* were found with the temperature increase at 37°C. This phenomenon might be capable to enhance larval parasite tolerance and adaptive response to the homeothermic host microhabitat, and host immune response. On the contrary, other genes, such as the glycoprotein, *A.peg-7,* resulted to be down-regulated at the chosen temperature conditions (Figure 6).

These results seem suggest that it may be that the high temperature may have an effect on the increase of both mRNA productions of those proteins having an adaptive role, such as *A.peg-13* and *A.peg-1* (Figure 6). Further investigation should be performed to elucidate the role of other variables, as the influence of immunological factors of both intermediate/paratenic and accidental human hosts, as players in triggering the mRNA gene transcripts and relative proteins production, such as *A.peg-7*, as well as from other target gene-related proteins, among those ESPs produced by the zoonotic parasite, *A. pegreffii*.

All the three genes resulted to be not significantly expressed and released, except in the case of *A.peg-13* which is an adaptive protein, when the larvae were reared at 7°C. The temperature range between 2°C and 7°C seems not to represent an optimum thermal condition for the parasite species *A. pegreffii*. Indeed, as we have demonstrated in our previous studies (Cipriani et al. 2016), larval migration of *A. pegreffii* from the viscera to the musculature of the fish host (anchovies, *Engraulis encrasicolus*) does not occur when the fish is maintained at the temperature range comprised between 2°C and 7°C. Accordingly, in our experiments, the expression levels of those target genes did not significantly increase at those temperature conditions. This aspect would acquire importance in fish storage, potentially infected with *A. pegreffii* larvae, before its human consumption. The storage of fish at temperature below 7°C would impede not only the larval motility from the viscera to the musculature, but it would also enhance a lower level of transcripts of genes coding for parasite-derived ES products having an immunogenic role in humans, as those considered in the present study.

Finally, quantitative qRT-PCR assay of target genes would be used, in future studies, for the detection of parasite-derived gene transcripts of those antigenic proteins, in the supernatants of *in vitro* larvae maintained at 37°C. This, in turn, would allow, in future investigations, to detect those parasite-derived secreted RNA, as possible "biomarkers" of the infection in human anisakiasis.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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3. II - Exploring the gene expression profiles of seven target proteins in the third-stage larvae of *Anisakis simplex* (s. s.) (Nematoda: Anisakidae), infecting blue whiting (*Micromesistius poutassou*). Submitted to *Genes* (special issue on Anisakid nematodes)

Marialetizia Palomba, Paolo Cipriani, Lucilla Giulietti, Arne Levsen, Giuseppe Nascetti, Simonetta Mattiucci

Abstract – The third-stage larvae of the parasitic nematode genus *Anisakis* tend to encapsulate in different organs incl. the musculature, of fish. Host tissue penetration and degradation involve both mechanic processes as well as the production of proteins encoded by an array of genes. Investigating larval gene profiles during the fish infection has relevance in understanding biological traits in the parasite's adaptive ability to cope with the fish hosts' defence responses. The present study aimed to investigate the gene expression levels of some proteins in L3 of *A. simplex* (s. s.) infecting different tissues of blue whiting, *Micromesistius poutassou*, a common fish host of the parasite in the NE Atlantic. The following genes encoding for *Anisakis* spp. proteins were studied: a kunitz-type trypsin inhibitor (*TI*), a glycoprotein (*GP*), the haemoglobin (*hb*), the sideroflexin 2 (*sfxn 2*), an ubiquitin-protein ligase (*hyd*), an trehalase (*treh*), and the zinc metallopeptidase 13 (*nas 13*). Upon comparison with *A. simplex* (s. s.) larvae recovered from the stomach and used for control, significant differences in gene transcripts of the selected proteins were observed in larvae located in various other organs of the fish host. Thus, ANOVA analysis showed that relative gene expression levels of the seven target genes in the larvae are linked to infection site in the fish host. The genes encoding for some of the present adaptive proteins seem to have protective properties, facilitating host tissue migration and, consequently, survival of the parasite in the basically hostile target tissues of the fish host.

Key words: *Anisakis simplex* (s. s.), gene expression, infected tissues, fish host, *Micromesistius poutassou*

3.1 Introduction

Third-stage nematode larvae (L3) of the species *Anisakis simplex* (s. s.) infect several marine species of the Arctic-Boreal Region (Mattiucci et al. 2018). Planktonic or semi-planktonic crustaceans are intermediate hosts, fish and squids act as paratenic/intermediate hosts, while cetaceans are definitive hosts in the life cycle of these parasites (Mattiucci & Nascetti 2008, Mattiucci et al. 2018). Fishes acquire *Anisakis* spp. larvae through the diet, preying upon infected crustaceans and/or other fish species. Once infected prey is digested, the larvae are able to penetrate the stomach wall of the fish (Levsen & Berland 2012) and migrate into the visceral cavity. After crossing the stomach wall, *Anisakis* spp. larvae usually settle on the external surface of internal organs, such as liver, gonads and mesentery, eventually followed by host-generated encapsulation. However, some larvae may migrate intra-vitam or post-mortem into the body musculature of the fish host (Smith, 1984; Smith & Wootten, 1975; Karl et al. 2002; Quiazon et al., 2011; Cipriani et al. 2016, 2017).

The L3 stage of *Anisakis* spp. are considered parasites of generally low pathogenicity and virulence in fish (Levsen and Berland, 2012). However, during tissue migration, the larvae can modify the structure and function of the host tissues, thus causing haemorrhages and focal immune reactions (Bahool et al. 2012). The severity of the pathological effects differs widely, depending on the intensity of infection, and the parasitized tissue (Abollo et al. 2001, Dezfuli et al. 2008, Bahool et al. 2012, 2013). Moreover, various fish host species may show differential susceptibility to the infection (Bahlool et al. 2012). For example, considerable differences seem to exist between fish species with respect to their ability to respond, immunologically, against the larvae (Levsen and Berland, 2012; Bahlool et al. 2012). *Micromesistius poutassou* is apparently not associated with any significant tissue damage, unlike the Atlantic mackerel that appears to be capable of reducing the *A. simplex* (s. l.) infection by immunological means (Levsen & Berland, 2012). Additionally, infection-site selection of the L3 would differ with both fish species and species of *Anisakis* (Bahool et al. 2012; Mattiucci et al. 2018).

In an experimental study, Bahool et al. 2013 elucidated the influence of larval excretory/secretory (ES) products on the fish immune system, by measuring the immune gene expression in spleen and liver of rainbow trout (Oncorhynchus mykiss), in response to an intraperitoneal injection of excretory/secretory (ES) products isolated from L3 of *A. simplex* (s. l.). The overall gene expression profile of the injected hosts showed a down-regulation of certain immune genes, suggesting that ES products from the nematode larva can dampen the immune reactions of fish (Bahlool et al. 2013; Mehrdana et al., 2017). Recently, also Marnis et al., 2019 showed a worm-induced immune suppression (i.e. downregulation of genes encoding cytokines) locally in the liver infected of the Baltic cod (Gadus morhua) with C. osculatum. However, some ES products seem also to be able to elicit immune responses and their immunogenicity can provoke antibody production (Priebe et al 1991, Kim et al. 2005, Kuhn et al. 2013, Skrzypczak et al. 2014, Mehrdana & Buchmann 2017).

The analysis of transcribed mRNAs of *Anisakis* spp. larvae can provide useful information on the genes associated with the parasite-host interactions (Kim et al. 2018). In particular, gene expression profiles of adaptive molecules may provide clues related to their role(s) in the biological pathways and pathogenesis of L3 *Anisakis* spp. in naturally and accidentally infected hosts (human), and it remains an aspect of the host-parasite interaction to be further investigated (Cavallero et al. 2017, Palomba et al. 2019, Lopienska-Biernat et al. 2019). According to Palomba et al. (2019), water temperature can play an active role in modulating the gene expression profiles of immunogenic and adaptive proteins (i.e. the kunitz-type trypsin inhibitor and the haemoglobin) in L3 of *A. pegreffii*. Likewise, a down-regulation of the glycoprotein (*GP*) gene expression was found under different temperature conditions, suggesting a possible role of other factors, such as host immune responses, as well (Palomba et al. 2019).

In this respect, one may assume that during the penetration process as well as after having settled in or on a target organ, the L3-larva transcribes mRNAs related to different biological processes (e.g. invasion, migration and survival). Thus, the present study aimed to evaluate the gene expression profiles of some proteins of the parasite species *A. simplex* (s. s.) in different organs of a naturally infected fish host, the blue whiting (*Micromesistius poutassou*). Target genes were selected among those coding for proteins which have previously found by other authors to be highly expressed in L3 of *Anisakis* sp. (Kim et al. 2018), as well as genes that are either up- or down-regulated under the effect of various abiotic conditions (Palomba et al. 2019). In addition, the herein investigated genes are also known to be involved in facilitating parasite survival and beneficial adaptations to the host on the one side, but on the other side, also for triggering host immune responses (Kim et al. 2018; Baird et al. 2016).

3.2 Materials and Methods

3.2.1 *Anisakis* spp. larvae sampling

A total of N= 60 blue whiting (total mean length 280 ± 35 mm) were sampled in April 2018 off St. Kilda (N58°04' W09°40'), in the NE Atlantic Ocean (FAO 27 area, Division VI a, Northwest Coast of Scotland and North Ireland or West of Scotland) on-board the commercial fishery and research vessel MS Kings Bay (Institute of Marine Research cruise no. "Kings Bay" 2018843). Fish were freshly analysed on board, and *Anisakis* spp. L3 were collected within one hour post catch. Larval abundances per infection site were recorded in each fish. A total of N= 27 larvae, with 9 each from the viscera (mesenteries), liver and muscle, were rinsed several times in sterile saline solution (0.9% NaCl) and stored in RNA laterTM (24h at 4°C, then stored at -20°C) for further molecular identification and gene expression analysis. A set of *Anisakis* spp. larvae (N= 9) recovered from the fish stomach were used as control in the qPCR assay. Larvae collected from the stomach were considered as control because they were considered as just acquired by the fish during predation upon other infected

intermediate/paratenic hosts and mainly from amphipods, appendicularians and euphausiids (Prokopchuk and Sentyabov 2006) (i.e. not migrated).

3.2.2. Total DNA/RNA isolation and cDNA synthesis (RT)

DNA and RNA were simultaneously extracted from each *Anisakis* larva using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with some modifications, as described in Palomba et al. 2019. DNA was stored at −20°C for the larval identification to species level, based on the direct sequence analysis of mitochondrial (mtDNA *cox 2*) and nuclear (elongation factor EF1 α-1 of nDNA) gene loci.

The total RNA was subsequently treated with DNase (DNase I, Invitrogen) to remove any genomic DNA contamination, according to the manufacturer's protocol. RNA concentration and purity were determined by spectrometry using a NanoDrop®TC1-E20 spectrophotometer (BioTek Synergy HT) and by 1.5% agarose gel electrophoresis. RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription (RT) kits (Applied BiosystemsTM) from a RNA pool of three larvae of *Anisakis simplex* (s. s.) previously identified, to increase the efficiency of normalization and maximize the number for which quantitative data were obtained.

The RT reaction was carried out at 37° C for 2 hours. A final volume of 20 µl of cDNA was stored at –20°C for further qPCR analysis.

3.2.3 DNA sequencing for larval identification

The *Anisakis* spp. larvae sampled (N= 36) were identified to species level by using the mtDNA *cox 2* and EF1 *α*-1 nDNA genes sequences analysis.

For sequencing the mitochondrial cytochrome C oxidase subunit II (*cox 2*) gene, PCR amplification was performed using the primers 211F (5'-TTTTCTAGTTATATAGATTGRTTTYAT-3') and 210R (5'-CACCAACTCTTAAAATTATC-3') (Mattiucci et al. 2014). Polymerase chain reaction (PCR) was carried out according to the procedures provided by Mattiucci et al. (2014). The mtDNA *cox 2* sequences obtained were compared with those already deposited for the same gene in GenBank for other *Anisakis* species. All the *Anisakis* spp. larvae, identified by mtDNA cox 2 gene, were sequenced at the elongation factor (EF1 α−1 nDNA) nuclear gene. The EF1 *α*−1 nDNA was amplified using the primers EF-F (5'-TCCTCAAGCGTTGTTATCTGTT-3′) and EF-R (5′-AGTTTTGCCACTAGCGGTTCC-3′) (Mattiucci et al. 2016). The PCR conditions and procedures followed those previously reported (Mattiucci et al. 2016). The sequences obtained for the EF1 α−1 nDNA gene for the larval specimens were compared with those previously deposited in GenBank, at the diagnostic positions (i. e. 186 and 286) as previously detailed (Mattiucci et al. 2016).

3.2.4 Gene expression by quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) was carried out using the StepOnePlusTM system (Applied BiosystemsTM) to detect and quantify the transcript amounts of *TI* (kunitz-type trypsin inhibitor) *GP* (glycoprotein), *hb* (haemoglobin), *sfxn 2* (sideroflexin 2), *hyd* (ubiquitin-protein ligase), *treh* (trehalase) and *nas 13* (zinc metallopeptidase 13) in *A. simplex* (s. s.) larvae recovered from different sites of infection of the blue withing.

Prior to the analysis, absence of primer-dimer formation, as well as the identification of the appropriate cDNA working dilution for each assay, were determined by running a dilution curve. Reactions were performed in triplicates, and the protocol used was optimized for a total reaction volume of 20 µl: 0.5 ul forward and reverse primers (10 uM each) (Table 1), 10 µl of SYBRTM Green PCR Master Mix (Applied BiosystemsTM) containing SYBR Green I dye, AmpliTaq Gold DNA polymerase, dUTP and buffer, 1 µl of 10-fold diluted cDNA (Table 1).

Primers used for the *sfxn 2*, *hyd*, treh and *nas 13* genes were previously designed by Kim et al. (2018); whereas the primers for *TI*, *GP* and hb were designed by Palomba et al. (2019). Gene expression results were normalized to the geometric mean of gpd (Glyceraldehyde-3-phosphate dehydrogenase), used as reference gene (Chen et al. 2014) (Table 1). The qPCR assays conditions were the following: one cycle at 95°C for 10 min, 40 cycles of a denaturation step at 95°C for 15 s, and annealing step at 57-60°C for 1s (Table 1). Melting curve data were collected at 55–95°C.

Gene expression data were analyzed according to the ΔΔCt method (Livak et al. 2001). Change in threshold cycle (ΔCt) was calculated as the difference between the target gene and the reference gene for each sample. ΔΔCt was calculated as the difference between the ΔCt of the control group and the ΔCt of the experimental groups.

Table 1. Primers sets of target genes used in real time PCR analysis.

Gene locus	Sequence (5' to 3')	Ta/°C	Product size (bp)	Reference
TI	CATGTGCCGATAAATGCGGG CCCTGTGAGCATGCATCCTT	57°C	130	Palomba et al. 2019
GP	TATCGGAATGCGTGACTGCA AGGCAGTTTCCATGGTGTATG	57°C	130	Palomba et al. 2019
hb	AAACATTCGACGCCTACACC CATCGTGGTCTTCTCTGCGA	60°C	108	Palomba et al. 2019
sfxn 2	TTAGAATGGCGTTGAAGCAGTAGTAG AGTATCGGTTCTGACCAGTTTTTTG	60°C	130	Kim et al. 2018
hyd	CCATCCAGTGAAGAAGGATTCC GAATAGAGCGGTAAGTAGAGCCTTGA	60°C	116	Kim et al. 2018
treh	TCAGCAAGCATTTGAGTGAAGAGT TAACATGATTGAAAACTTCGCAACA	60°C	117	Kim et al. 2018
nas 13	AGCAATAGCAGCACGATGA CTGCGGTAGCCAATGCTTTT	60°C	131	Kim et al. 2018
gpd	CCCCTTCATCAACATCGACT TCAGCTCCCCATTTGATTTC	60°C	152	Kim et al. 2018

3.2.5 Statistical analyses

Statistical analyses were performed by using GraphPad Prism version 6.0 (GraphPad Softwere, La Jolla, California USA, www.graphpad.com). Normality was analyzed using the Shapiro–Wilk test. Statistical differences between the gene expression levels of *Anisakis* spp. larvae by fish host tissues were assessed by applying one-way ANOVA followed by Dunnett's multiple comparison test to compare the mean of each organ group with the mean of the control group, and between the organ groups.

Significant differences were considered at $p < 0.05$ for all statistical tests performed. Data are presented as mean (M) ± standard deviation (SD). Significant differences are indicated with different asterisks.

3.3 Results

3.3.1 Identification of *Anisakis* spp.

According to the sequences of 629 bp in length of the mtDNA *cox 2* gene locus (Mattiucci et al. 2014) all the *Anisakis* spp. larvae used in this study were assigned to the species *A. simplex* (s. s.). The sequences obtained in the present study matched 99 – 100%, with the sequences previously deposited in GenBank for the species. In addition, the identity of the specimens recognized by mtDNA cox 2 seq. analysis as belonging to *A. simplex* (s. s.), was confirmed by analysis of the two diagnostic nucleotide positions observed at the locus EF1 α-1 nDNA (Mattiucci et al. 2016).

3.3.2 qPCR analysis of gene coding for seven target proteins

The analysis of gene expression profiles of seven adaptive proteins of the L3 of *A. simplex* (s. s.) recovered from different sites of the parasite infection (liver, mesentery, muscle), in *M. poutassou* are shown in Figure 1.

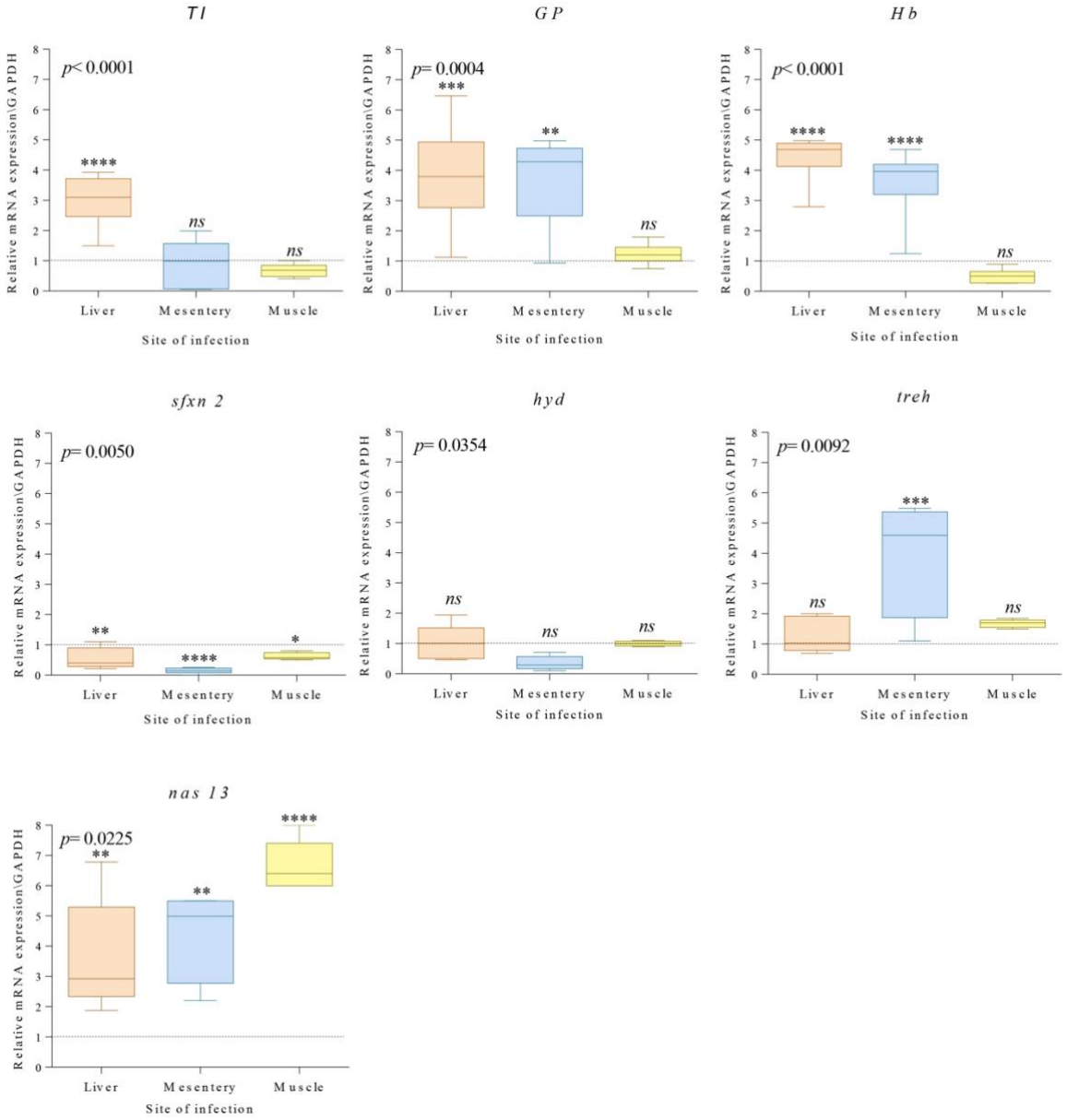

Figure 1. Box-plot representation of relative expression profiles of 7 genes coding for adaptive proteins, i.e. *TI, GP, hb, sfxn 2, hyd, treh, nas 13*, normalized to the geometric mean of *gpd* in *A. simplex* (s. s.) larvae infecting different host-fish tissues (liver, mesentery, muscle). The control, represented by larvae recovered in the stomach lumen of the fish host, is given as normalized to a value of 1 (dashed line). Each box shows the minimum, first quartile, median, third quartile, and maximum gene expression values of three biological replicates. Asterisks indicate the significant level of the differences between each group of *A. simplex* (s. s.) larvae from different sites of infection and the control group. Significance was fixed at p <0.05 [*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001]. ns= no significant differences. One-way ANOVA analysis on the relative gene expression is shown in the top left of each box.

Quantitative real-time PCR (qPCR) analysis revealed differential regulation of the target genes in response to the infection site of the *A. simplex* (s. s.) larvae. Shapiro-Wilk test supported a normal distribution of the data obtained from the qRT-PCR assay tests (all tests $p < 0.05$). Comparison with the control, differences in the expression levels of the genes coding for Kunitz-type trypsin inhibitor, glycoprotein, haemoglobin, sideroflexin 2, ubiquitin-protein ligase, trehalase and zinc metallopeptidase 13, were observed in some groups of *A. simplex* (s. s.) larvae located in the different organs of the fish host (Figure 1).

The gene coding for the trypsin inhibitor in the *A. simplex* (s. s.) larvae located in the liver showed statistically significant higher levels of expression ($p < 0.0001$) (Figure 1) compared to larvae of the control (stomach lumen). No significant differences were recovered at the same gene in *A. simplex* (s. s.) larvae located in the mesentery and muscle ($p = 0.571$, $p = 0.301$, respectively) (Figure 1).

The *GP* gene was found to be significantly up-regulated in *A. simplex* (s. s.) larvae collected from the liver ($p < 0.0001$) and mesentery ($p = 0.0015$) while the expression values of this gene in larvae from the muscle were at a similar level as the control ($p = 0.760$).

A statistically significant up-regulation of the hb gene, coding for the haemoglobin, was observed in A simplex (s. s.) larvae recovered from liver ($p < 0.0001$) and mesentery ($p < 0.0001$) (Figure 1). Standard levels of the transcripts at the gene hb ($p = 0.208$) were measured in the larvae located in the muscle, with respect to the control.

The transcripts levels of the *sfxn 2* gene coding for the sideroflexin 2 were significantly down-regulated ($p < 0.05$) in *A. simplex* (s. s.) larvae located in all fish organs.

A low gene expression level was recorded also for the *hyd* gene coding for the ubiquitin-protein ligase, studied in the larvae from liver, mesenteries (i.e. viscera) and muscle of the fish host; nevertheless, non-significant differences ($p > 0.05$) were recorded in comparison with the larvae of the control.

No significant difference was recorded in the expression levels of the treh gene coding for the trehalase, between *Anisakis* larvae collected from the fish liver ($p = 0.596$) and muscle ($p = 0.230$), and the control. Conversely, significant ($p = 0.0002$) were the differences in the transcripts at this gene estimated in the parasites recovered from mesenteries.

Finally, the *nas 13* gene of *A. simplex* (s. s.) showed a generally significant ($p < 0.05$) up-regulation of the larvae located in all of infection sites, compared to the larvae of the control group (Figure 1).

Dunnett's multiple comparison test for differences in expression level per target gene between larvae from different fish host organs, are shown in Table 2. The ANOVA analysis showed that the overall transcripts values of the seven selected genes in *A. simplex* (s. s.) were significantly ($p<0.05$) affected by the infection site of the parasite species in the fish host (Figure 1).

Table 2. Dunnett's multiple comparison test for differences in gene expression level per target gene between larvae from different fish host organs/tissues. Significance was fixed at p <0.05 [*p <0.05, **p <0.01, ***p <0.001, ****p<0.0001]. ns= not significant.

Gene locus	Liver *vs* mesentery	Liver *vs* muscle	Mesentery *vs* muscle
TI	****	****	*ns*
GP	*ns*	***	**
hb	*ns*	****	****
sfxn 2	*	*ns*	****
hyd	*	*ns*	*ns*
treh	*	*ns*	*
nas 13	*ns*	*	*ns*

3.4 Discussion

In the present study, target gene transcripts of L3 of *A. simplex* (s. s.), correlated with their tissue localization in a commonly infected fish host, have been investigated for the first time.

The selected fish host species, blue whiting, is a paratenic host in the life cycle of *A. simplex* (s. s.) which acquires the parasite through predation on other infected hosts (i.e. crustaceans, small fish and cephalopods) (Prokopchuk and Sentyabov 2006; Fernandez et al. 2018). The fish host populations caught in the NE Atlantic Ocean from off St. Kilda island (FAO VIa), are known to have high infection levels both in the liver, mesenteries and the flesh (Gomez-Mateos et al. 2016; Levsen et al. 2018; Mattiucci et al. 2018). Moreover, blue whiting from the present fishing area are usually not infected with other anisakid nematode species (Højgaard 1998, Levsen et al. 2018, MacKenzie 1979) which, in this respect, makes this fish host a suitable model since interference from other closely related anisakid species is not likely to occur.

Anisakis simplex (s. s.) larvae usually penetrate the gastric wall of the fish host to reach the visceral cavity. There, some L3 settle on the surface of the visceral organs (i.e. mesenteries, liver, gonads), followed by host-induced encapsulation, where the capsule thickness seems largely to depend on infection site, i.e. the host target organ (Berland, 2006; Levsen and Berland 2012). However, some larvae may migrate into the musculature of fish or squid (Smith, 1984; Karl 2008; Cipriani et al. 2016, 2019; Mattiucci et al. 2018; Levsen et al. 2018). But so far, only L3 of *A. simplex* (s. s.) and *A. pegreffii* have been recorded in the muscular tissue of various fish and squid hosts, with *A. simplex* (s. s.) having apparently a higher capability to invade the flesh of fish than its sibling species *A. pegreffii* (Suzuki et al. 2010; Quiazon et al., 2011; Cipriani et al. 2014; Gomez-Mateos et al., 2016). Once established and encapsulated, the larvae go through a period of latency where they need to survive and remain infective till the next host, either another transport host, or a definitive marine mammalian host (see review by Mattiucci and Nascetti 2008). To date, few studies have investigated the interactions between *Anisakis* spp. and their intermediate/paratenic hosts. Levsen and Berland (personal observation, 2012) observed

that different fish host might show different degrees of immunological reactions to larvae. In some fish host, including the target species of this study, M. poutassou, evident melanomacrophage aggregates around larval infections sites on the liver have been observed (Levsen and Berland, 2012). The role of these aggregates is not yet completely understood but Agius and Roberts (2003) suggested that the melanomacrophage aggregates might develop in association with late-stage chronic inflammations induced by parasites. Recently, a cellular immune response in the liver of blue whiting infected by *A. simplex* (s. s.) larvae was found. Interestingly, however, no host reaction was apparently present around larvae that were lodging in the musculature of fish from the same sample as the former (Dezfuli et al. submitted).

Larsen et al. (2002) assumed that the parasite-secreted molecules could be the cause of fish host cellular reactions. In other studies, the gene expression of target proteins of L3 *A. simplex* (s. l.) were compared with L4 stage larvae (Kim et al. 2018), while also the *A. pegreffii* L3 gene expression at different temperature conditions was tested and compared (Chen et al. 2014; Palomba et al. 2019). However, no study concerning the differential gene expression related to the *Anisakis* spp.-fish host interaction has so far been investigated.

Amongst the selected target genes in this study, a significant differential gene expression of the TI gene coding for a Kunitz-type trypsin inhibitor protein, was detected in *A. simplex* (s. s.) larvae removed from the fish liver (Figure 2). The Kunitz-type trypsin inhibitor protein seems to have important functions in the parasites development (Rhoads et al. 2000; Stepek et al. 2010) and in the host proteases' inhibition (Moneo et al. 2000; Mehrdana & Buchman 2017). Indeed, a wide range of diverse nematode species has been found to express proteins with trypsin inhibitor-like domains, including *Ascaris suum* (Bernard and Peanasky, 1992), Ancylostoma caninum (Stanssens et al. 1996) and *Trichuris suis* (Rhoads et al. 2000). Many of these nematode proteins have been shown to inhibit serine proteases, e.g. chymotrypsin, trypsin, elastase and, in the case of the hookworms, host' thrombin and coagulation factor (Matthews et al. 1982; 1984; Kennedy et al. 1991; Quiazon et al. 2013). Hawley and Peanasky (1992) assumed that, in order to parasitize a host, a nematode requires those protease inhibitors that interact strongly with those host proteases located in different sites of infection. The kunitz-type trypsin inhibitor protein would aid *A. simplex* (s. s.) to counteract the host proteolytic enzyme and coagulation factors. Indeed, proteins with Kunitz domains have previously been detected in expressed transcripts sets obtained from pharyngeal tissues of *A. pegreffii* and *A. simplex* (s. s.) larvae (Cavallero et al. 2018). In this study, an up-regulation of transcripts of *TI* in *A. simplex* (s. s.) located in the liver could be correlated to the fish host's proteolytic enzymes, i.e. serine proteases. Fish organs are a source of those enzymes, especially digestive proteases, such as aspartic protease pepsin and serine proteases (i.e. trypsin, chymotrypsin and elastase) (Khangembam et al. 2012; Klomklao and Benjakul 2018). In addition, some serine proteases such as the tryptases, are produced and stored in the mast cell granules located in different host immune tissues (McNeil et al. 2007). Those host serine

proteases would be implicated in the response to an invading parasite larva which again could react with increasing production of inhibitor proteins, e.g. the kunitz-type trypsin.

Nematode haemoglobins are molecules with an unusual high oxygen affinity. Several studies indicate that they may represent an important component of parasite adaptation to the co-existence with the host (Abe & Teramoto 2017; Gonzalez-Fernandez et al. 2015, 2017; Palomba et al. 2019). Thus haemoglobin, sequestering oxygen, has been suggested to aid the parasite in maintaining a locally anaerobic environment, while their ability to break down nitric oxide (NO) and *hyd*rogen peroxide produced by innate immune cells would also aid the parasite survival. Myoglobin´s catalase and nitric oxide dioxygenase activities are important in protection against host immune responses to infection (Nieuwenhuizen et al. 2013). Results obtained in this study seems to support that possible function of the parasite's haemoglobin in increasing its protection against the host immune response. Indeed, an up-regulation of the gene is reported, in the present study, for the larvae located in both host viscera (i.e. mesentery) and liver (Figure 2).

In previous investigations (Palomba et al., 2019) we found that since the transcript levels of the GP in *A. pegreffii* remained stable under certain temperature conditions, its gene expression level would be modulated by the host immune response. Indeed, we observed in the present study that the transcripts of a GP gene were significantly up-regulated in *A. simplex* (s. s.) located in the liver and mesentery, where the immune reactivity of the host seems to be stronger (Secombes and Wang 2012). Conversely, a standard GP expression similar to the control was detected in larvae recovered in the host skeletal muscle, where the host's immune reactivity seems to be weaker (Dezfuli et al submitted) (Figure 2). Indeed, it is generally recognized that the parasite glycoproteins which occur abundantly on worms' surface and in excretory/secretory (ES) products (Prasanphanich et al. 2013), are able to down-modulate the host immune response (White 2012; Prasanpanich et al. 2013). For instance, in the case of Schistosoma mansoni eggs, an omega-1 glycoprotein was found to promote Th-2 skewing of dendritic cells (DCs) and T cells during infection (Coakley et al. 2015). Previous studies have suggested that *Anisakis* spp. larvae secrete ES products, whose glycosylated components regulate the fundamental processes of antigen recognition, processing, and presentation (Bahlool et al. 2013). The *Anisakis* glycoprotein would be able to interact with both innate and adaptive branches of the immune system in both intermediate and definitive hosts. Indeed, these molecules are involved in the regulation of the IgM response of the intermediate/paratenic fish host (Hrabar et al. 2019).

Lopienska-Biernat et al. (2019) have recently described a pathways of genes coding proteins of trehalose and glycogen metabolism in *A. simplex* (s. l.). They suggest an important role of the trehalose in the energy storage, circulatory sugar, glucose uptake in absence of food and nematode protection in adverse environmental conditions (Elbein et al. 2003; Tang et al. 2018; Lopienska-Biernet et al. 2020). Indeed, in parasitic nematodes, e.g., *A. simplex* (s. s.), the trehalose is essential for their survival (Lopienska-Biernat et al. 2015, 2019b).

L3 larvae of *A. simplex* does not use external sources of glucose (Smith and Wootten 1978; Lopienska et al. 2019b). Indeed, even if the alimentary system is developed, there is no intake and assimilation of food, so the trehalose is necessary to the glucose storage and to survive without directly feeding (Grabda 1976; Iglesis et al. 2001; Lopienska et al. 2006). In this study, no significantly higher levels of treh gene in *A. simplex* (s. s.) larvae recovered from the liver and muscle compared with larvae from the stomach, were observed. However, significantly higher treh gene expression levels were detected in larvae recovered from the mesenteries (Figure 2). These results could reflect the necessity of the parasite L3 to transcribe treh gene since trehalose appears to be indispensable to accumulate endogenous saccharide reserve compounds (Lopienska et al. 2006). The *Anisakis* spp. larvae are typically surrounded by fibrous connective tissues generated by the fish host (Levsen and Berland 2012); the capsule's thickness formed around the larva seems to be related to the abundance of connective tissue in different infection sites in the host. Thus, it would occur that where the connective tissue layers become more compact (i.e. mesenteries), the host's immune system produces a capsule around the parasite and the larva, which remains 'imprisoned' (Levsen et al. 2008) would need of more energy to contrast the host immune response, with a consequent increase of the treh transcripts.

In parasitic nematodes, peptidase proteins represent a conspicuous percentage of expressed genes (Sajid and McKerrow 2002); they are involved in host-parasite interactions incl. parasite immune evasion, transmission and pathogenesis (Malagon et al. 2013). In many cases, parasite peptidases constitute the main component of antigens, triggering immune responses of the host (Hotez and Pritchard 1995; Todorova and Stoyanov 2000). For example, the metallopeptidases of the nematodes, with their proteolytic activity, are a group of peptidases that facilitate the invasion of the host tissue by degrading the extracellular matrix. In some cases, it has been suggested that the metallopeptidases conserve their proteolytic activity, even after the cellular, antibody-based responses of the host during the infection to the parasite (Malagon et al. 2011; Bruschi and Pinto 2017). Kim et al. (2018) found that some metallopeptidases genes are differentially regulated in the *A. simplex* (s. l.) larvae transcriptome. In particular, the authors observed that the *nas 13* was up-regulated in L3 larvae in comparison with L4 stage of the parasites species. These enzymes were considered to be involved in host tissue penetration in *Anisakis* spp. larvae (Kim et al. 2018). In this study, a generalized up-regulation of *nas 13* in *A. simplex* (s. s.) larvae located in different host organs compared to the larvae from the stomach, suggests a role in the host tissues invasion and migration, as in other ascaridoids.

The role of ubiquitin-protein in *A. simplex* (s. l.) was not yet fully elucidated. Anyhow, Adhikari and Adams (2011) hypothesised that the ubiquitin-proteasome system could be involved in the protein degradation and apoptosis in parasitic nematodes, as well as in the withstanding to oxidative stress stress-induced genes. A Ubiquitin pathway is also known to be involved in the survival of parasitic nematode Trichinella spiralis and in the development of the parasitic trematode Schistosoma mansoni (Shang et al. 1997). Nematodes rely on proteomic plasticity to remodel themselves during periods of

developmental change, to endure varying environmental conditions and to respond to biotic and abiotic stressors. Considering the different tissues of *M. poutassou* examined here, the gene *hyd* of *A. simplex* (s. s.) was apparently not differentially regulated compared to the control (Figure 2). This could indicate that the larvae are in a normal physiological environment and the parasites do not need to regulate the internal state, usually mediated by the stress genes expressed as the ubiquitin (Adhikari and Adams 2011).

Since Kim et al. (2018) observed a significantly higher *sfxn 2* expression in *A. simplex* (s. s.) L3 larvae compared to L4, here we investigated the gene expression of the sideroflexin 2 in different sites of fish infection. To date, it is known that the sideroflexins are mitochondrial multiple transmembrane proteins, which are associated with the iron accumulation in the mitochondria (Miotto et al. 2007). In the present study, a down-regulation of *sfxn 2* was recorded in *A. simplex* (s. s.) larvae removed from different fish organs. However, because no information on the function of sideroflexin in parasitic nematodes is so far available, further studies are needed to elucidate their possible roles, out of those here attempted, of the sideroflexin 2 in *A. simplex* (s. s.) larvae.

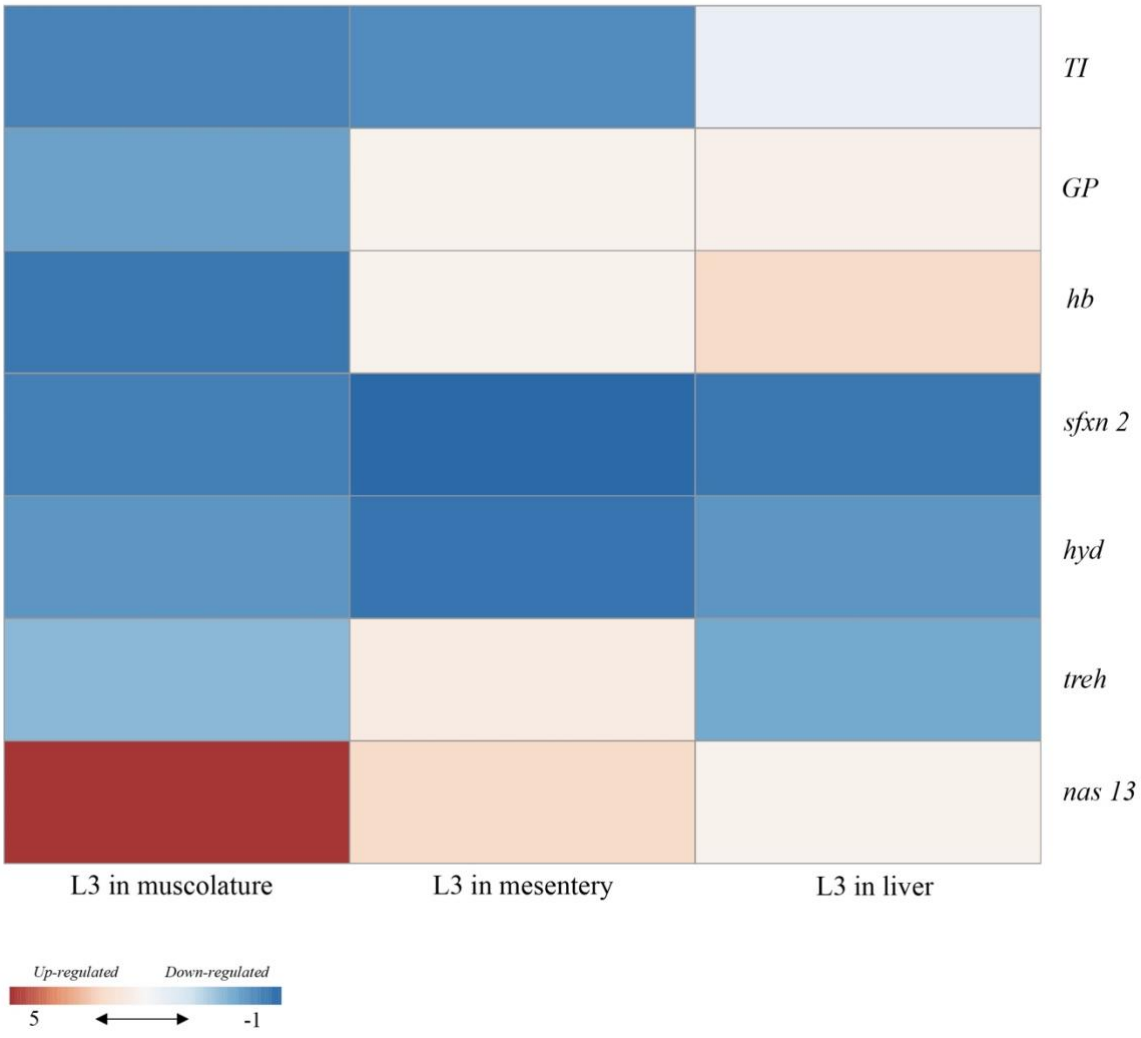

Figure 2. Heat maps presenting the changes in the gene expression of *Anisakis simplex* (s. s.) larvae collected from different fish organs. Gene expression was first calculated relative to the geometric mean of gpd and then was standardized following a standard score normalization against the corresponding control (larvae recovered in the internal lumen of the stomach) samples. Shades of red and blue, respectively, indicate the highest and lowest expression levels, as specified in the scale bar at the bottom of the figure. This heatmap was produced with ClustVis web tool (Metsalu and Vilo 2015).

3.5. Conclusions

Anisakis is an endoparasite exposed to changing life-conditions, considering the hosts varying from the poikilothermic crustaceans, heterothermic fish, to homeothermic marine mammals. Survival in an array of different habitats must require costly molecular and biochemical adaptations. Natural selection should remove the behaviours there are not compensating benefits (Read and Sharping 1995). For example, during migration through the tissues, the larvae can became lost or kill by the fish host; migration is not necessary for development. Probably migration in different site of infections of fish is an advantageous life-history strategy; however, the evolutionary factors responsible for the larval migration in the fish are poorly understood. The findings acquired during this study show that the relative gene expression levels of the seven target genes in the *A. simplex* (s. s.) larvae are linked to infection site in the fish host, blue whiting. Indeed, different sites of infection influence the gene expression levels of *A. simplex* (s. s.) larvae. The genes encoding for some of the present adaptive proteins seem to have protective properties, facilitating host tissue migration (i.e. *nas 13*) and, consequently, survival (i.e. *TI, GL, hb, treh*) of the parasite in the basically hostile target tissues of the fish host. To conclude, while the L4 stage and adults of *A. simplex* (s. l.) parasitizing a more stable environment such as the stomach of the cetacean definitive hosts and the levels of transcripts are more stable with respect to L3, the third stage larvae invade different tissues/organs of the same fish species and also part of genes encode various proteins which ensure the survival of the parasite transcribe differentially mRNAs depending on different host tissues environments.

Conflict of interest

The authors declare that they have no conflicts of interest.

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4. III - Human dendritic cells influence the transcriptome of *Anisakis pegreffii* larvae (Nematoda: Anisakidae). (*In preparation)*

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Abstract - Anisakiasis is a zoonotic human disease provoked by the accidental ingestion of larvae of anisakid nematodes, so far reported as belonging to the two zoonotic species *Anisakis pegreffii and A. simplex* (s. s.). In humans, the larvae-induced a T-cell-mediated inflammatory response through TH2 differentiation, starting with the recognition of parasitic pathogen by pattern recognition receptors (PRRs) that are expressed on the dendritic cells. They are capable of inhibiting *in vitro* generated dendritic cells (DCs). The effects of the DCs on transcriptional responses in *Anisakis pegreffii* larvae were investigated by high-throughput RNA-sequencing (RNA-seq) technologies. In total, 1203 differential expressed genes were identified: 573 and 628 genes as significantly up- or down-regulated, respectively. Functional analysis based on gene ontology (GO) classification system and the Kyoto encyclopaedia of genes and genomes (KEGG) database revealed a repository of *Anisakis pegreffii* genes encoding a variety of glycosylated molecules, acting on glycosyl bonds and *hyd*rolyzing O-glycosyl compounds. Understanding the roles of glycoconjugates in host–parasite interactions may provide targets in further intervention of the *Anisakis* spp. in the diagnosis of the infection and in therapy treatment.

Key words: *Anisakis pegreffii*, dendritic cells, transcriptome, glycosylated molecules

4.1. Introduction

Anisakiasis is a zoonotic human disease provoked by the accidental ingestion of larvae of anisakid nematodes belonging to the genus *Anisakis*, infecting edible parts of fish or squids, which are consumed as raw and/or undercooked dish preparations (Mattiucci et al. 2018). The two species so far mostly associated with human anisakiasis are *Anisakis simplex* (s. s.) and *A. pegreffii*. The symptoms can occur 1-12 hours after the ingestion of alive third stage larvae with the sudden onset of severe stomach pain, nausea and vomiting (Audicana and Kennedy, 2008). After 2- 4 weeks from ingestion of the infected fish, the larva dies and its debris provoke a chronic inflammation causing ulceration in the gastrointestinal mucosa or eosinophilic granulomas (Audicana and Kennedy 2008).

The pathological outcome within the gastrointestinal tract during an infection by *Anisakis* spp. larvae are the combined result of the direct invasive capacity of the larva, the secreted antigens during its invasion and the interaction with the host's immune response.

Generally, the immune response to helminths, including the genus *Anisakis*, begins by the direct damage on the epithelial surface in gastric or intestinal mucosa, causing the secretion of alarmins (TSLP, IL-33 and IL-25), subsequently, the activation of antigen-presenting cells (APCs) such as dendritic cells (DCs), innate lymphoid cells 2 (ILC2) and macrophages (Bruschi and Dupouy-Camet 2014; Entwistle et al. 2017). The activation of APCs trigger a very strong Th2 response characterized by production of high levels of IL-4, IgE antibodies, and large number of eosinophilic and mast cells. Thus, the activation and modulation of the human immune response are strictly dependent on the functions of APCs engaged by helminth pathogens (Entwistle et al. 2017; Cortes et al. 2017).

Dendritic cells are key elements for the development of immunity against parasites: they control the responses required to eliminate these pathogens, while maintaining host homeostasis (Motran et al. 2017). They play a crucial role in the coordination of both natural and adaptative immunity driving the polarization of naive T cells towards Th subsets (Ueno et al. 2007; Pulendran et al. 2015).

When matured in the presence of alive *A. pegreffii* the DCs show remarkable phonotypic changes (Napoletano et al. 2018). These modifications frequently elicited a polarization towards Th2 cells and regulatory T cells, thus, contributing to immunological tolerance against those pathogens. Indeed, it was found that *A. pegreffii* larvae impact DC viability, hampered DC maturation by reducing the expression of molecules involved in antigen presentation and migration (i.e. HLA- DR, CD86, CD83 and CCR7), increased the phagosomal radical oxygen species (ROS) levels and modulated the phosphorylation of ERK1,2 pathway (Napoletano et al. 2018).

The aim of the present study was to investigate if the transcriptome of the zoonotic parasite *Anisakis pegreffii* - the common causative agent of human anisakiasis in Italy, is influenced by human dendritic cells (DC).

4.2. Materials and Methods

4.2.1. *Anisakis* spp. larvae sampling

Live *Anisakis* sp. larvae were removed from the viscera and body cavity of the fish host (anchovy, *Engraulis encrasicolus;* and European hake, *Merluccius merluccius*) caught from the Adriatic Sea (off San Benedetto del Tronto coast). After their removal, the live *Anisakis* spp. larvae were first washed in 4% acetic acid (Carlo Erba, Cornaredo, Italy) and then in phosphate- buffered saline (PBS, Sigma, St Louis, MO).

4.2.2. Dendritic cells generation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient (1,077 g/mL; Pharmacia LKB, Upsala, Sweden) from buffy coats of healthy donors obtained by the Transfusion Center, Policlinico Umberto I "Sapienza" University of Rome (Ethics Committee - Protocol nr.4212). Monocytes (CD14+ cells) were purified from PBMCs by CD14 MicroBeads (Miltenyi Biotech, Paris, France) and cultured in RPMI 1640 (HyClone, South Logan, Utah) supplemented with 2 mM L-glutamine (Sigma Chemical Company, St. Louis, MO, USA), penicillin 100 U/mL (Sigma), streptomycin 100 µg/mL (Sigma) with 5% heat-inactivated Fetal Calf Serum (Hyclone). A total of 50 ng/mL rH Granulocyte-Macrophage colony-stimulating factor (RandD System, Minneapolis, MN) and 1000 U/mL rhIL-4 (RandD System) were added at day 0 and day 2 for DCs' differentiation. Immature DCs (iDCs) were collected at day 5.

4.2.3. Dendritic cell and *A. pegreffii* co-culture

Seven alive third stage larvae of *Anisakis* spp. were co-cultured with iDCs (1 × 106 cells/mL) for 24 hours in a trans well system at 37°C and 5% CO_2. Larvae were plated in the upper chambers, while iDCs in the lower chambers. In parallel, live larvae L3 were seeded in culture medium in the absence of DCs (7 larvae/mL). At 24h post-incubation the survived *Anisakis* spp. larvae were collected, and stored in RNA later at -80°C for RNA and DNA extraction.

4.2.4. DNA extraction and molecular identification

Total DNA was extracted from a tissue portion (≈2 mg) of each *Anisakis* larva. The *Quick*-gDNA™ Miniprep Kit (ZYMO RESEARCH) was used as extraction method. DNA obtained was quantified by using a NanoDrop®TC1-E20 spectrophotometer (BioTek Synergy HT).

For sequencing the mtDNA *cox 2* gene locus, PCR amplification was performed using the primers 211F (5'- TTT TCT AGT TAT ATA GAT TGR TTT YAT - 3') and 210R (5' - CAC CAA CTC TTA AAA TTA TC - 3') (Valentini et al. 2006; Mattiucci et al. 2014). PCR conditions were established as previously described (Mattiucci et al. 2014).

The amplification of EF1 α-1 nDNA was performed using the primers EF-F (5'- TCC TCA AGC GTT GTT ATC TGT T -3') and EF-R (5'- AGT TTT GCC ACT AGC GGT TCC -3') under the conditions previously described (Mattiucci et al. 2016).

4.2.5. RNA extraction and quality check

Total RNA (~ 2,5 µg) was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), as described by Palomba et al. 2018. RNA was subsequently treated with DNase (DNase I, Invitrogen) to remove any genomic DNA contamination. Three independent biological replicates were analyzed, each one consisting of total RNA extracted from three *Anisakis* spp. larvae co-cultured with DCs and three larvae in absence of DCs. RNA concentration, purity and integrity were verified and measured on agarose gel (2%) and by a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany), respectively.

4.2.6. cDNA preparation and Illumina next-generation sequencing

Following the manufacturer's protocol, the cDNA library was prepared using TruSeq Stranded mRNA kit (Illumina, San Diego, USA). In brief, polyadenylated (PolyA+) RNA was purified from 10 mg of total RNA of *Anisakis pegreffii* larvae using Sera-Mag oligo (dT) beads, fragmented to a length of 100-500 nucleotides and reverse transcribed to cDNA using random hexamers. The size-fractionated cDNA was end-repaired and adaptor-ligated according to the manufacturer's protocol (Illumina). Ligated products of 200 bp were excised from agarose gels and PCR amplified. Products were cleaned using a MinElute PCR purification kit (Qiagen Hilden, Germany) and paired-end sequenced on an Illumina HiSeq 2000, according to the manufacturer's protocol.

4.2.7. Bioinformatic data preparation

Raw reads were trimmed and filtered for adaptor sequences, sequences with suboptimal read quality (i.e. PHRED score of 20) and sequences shorter than 50 bp using Trim Galore 0.4.3 and cutadapt 1.18. The high-quality single-end reads were mapped to the available *Anisakis pegreffii* reference transcriptome (http://anisakis.mncn.csic.es/public/) using the *pseudo-aligner* kallisto-v0.43 (options: --rf-stranded --single -b 30 -l 280 -s 80). Resulting alignments were extracted and, information linked to genome regions to which reads were mapped, (e.g. intron-exon boundaries) were obtained from the GTS annotation file available from *Anisakis* DB (http://anisakis.mncn.csic.es/public/). Bioconductor tximport package, ver. 1.6.0 was used to perform read counting. Bioinformatic data analysis was carried out by Biofab Research s.r.l.

4.2.8. Differentially expressed genes

Differential expression Genes (DEG) was determined using EdgeR package ver. 3.28.0 (Robinson et al. 2010) for R software (R Core Team). We considered DEG to be significant at a false discovery rate (FDR) <0.05 (Shao et al. 2014). The trimmed mean of M-value normalization method (TMM) was used for cross sample normalization, and the normalized expression of each transcript was presented as transcripts per million (TPM). The low counts were filtered by row sum of transcripts with < 80 and eliminated from subsequent analyses. Volcano Plot and heatmap representations of the gene expression patterns of differentially expressed genes were plotted using ggplot2 package (Wickham et al. 2016).

Using Blast2Go (Conesa et al. 2005), Gene Ontology (GO) terms (or KEGG pathways) statistically over-represented were compiled to help interpret the biological implications of the expression patterns (Young et al. 2010). Selected Gene Ontology (GO) terms were divided into subcategories.

4.3. Results

4.3.1. *Anisakis* sp. larvae identification

On the basis of the sequence analysis obtained from the *Anisakis* L3 larvae used in the present study, at the mitochondrial (mtDNA *cox 2*) and nuclear (EF1 α−1 nDNA) gene loci, were assigned to the species *A. pegreffii*. Indeed, mtDNA *cox 2* sequences, here obtained from those sequenced specimens, showed 99% or 100% similarity with sequences previously deposited in GenBank for *A. pegreffii* (Mattiucci et al. 2014). Further, according to the diagnostic positions studied, in the 409 bp length EF1 α−cording to the diagnostic positions studied, *A. pegreffii*, and the position 286, showing a C in *A. pegreffii* (Mattiucci et al. 2016), the same larval *Anisakis* spp. specimens, were confirmed to belong to the species *A. pegreffii*.

4.3.2. Comparison of transcripts expression in *A. pegreffii* larvae co-cultured in presence and absence of DCs

In *A. pegreffii* co-cultured with DCs, a total of 68,071,234 bp reads were generated for *A. pegreffii*. Combined and filtered data (Table 1) revealed 1203 differentially expressed genes with *p* values of ≤0.01 and FDR≤0.05. Of these, 530 were over-expressed in *A. pegreffii* larvae with DCs. The distribution of the differentially expressed genes is presented in the heatmap and in the volcano plot in Fig. 1.

Table 1. Summary of RNA-seq bioinformatics analyses.

	Number of reads	Sequence length	%GC
Sample 1	27880321	35-76	46
Sample 2	22795434	35-76	47
Sample 3	24071703	35-76	47
Control 1	27798561	35-76	44
Control 2	24772741	35-76	44
Control 3	26728447	35-76	44

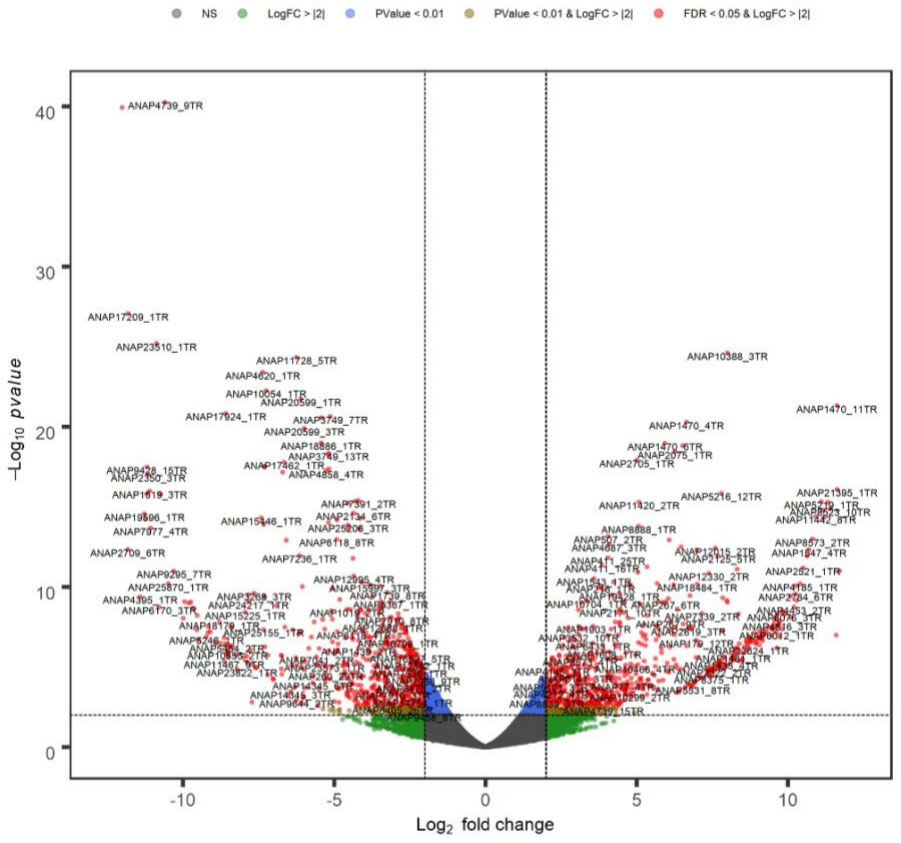

Figure 2. Volcano plot shows the differential levels of transcripts up and down regulated in *Anisakis pegreffii* larvae *in vitro* co-cultured of DCs. On the left the statistically significant expressed genes up-regulated in *A. pegreffii* L3 larvae co-cultured in presence of DCs are shown; while, on the right the statistically significant expressed genes down-regulated in presence of DCs are given.

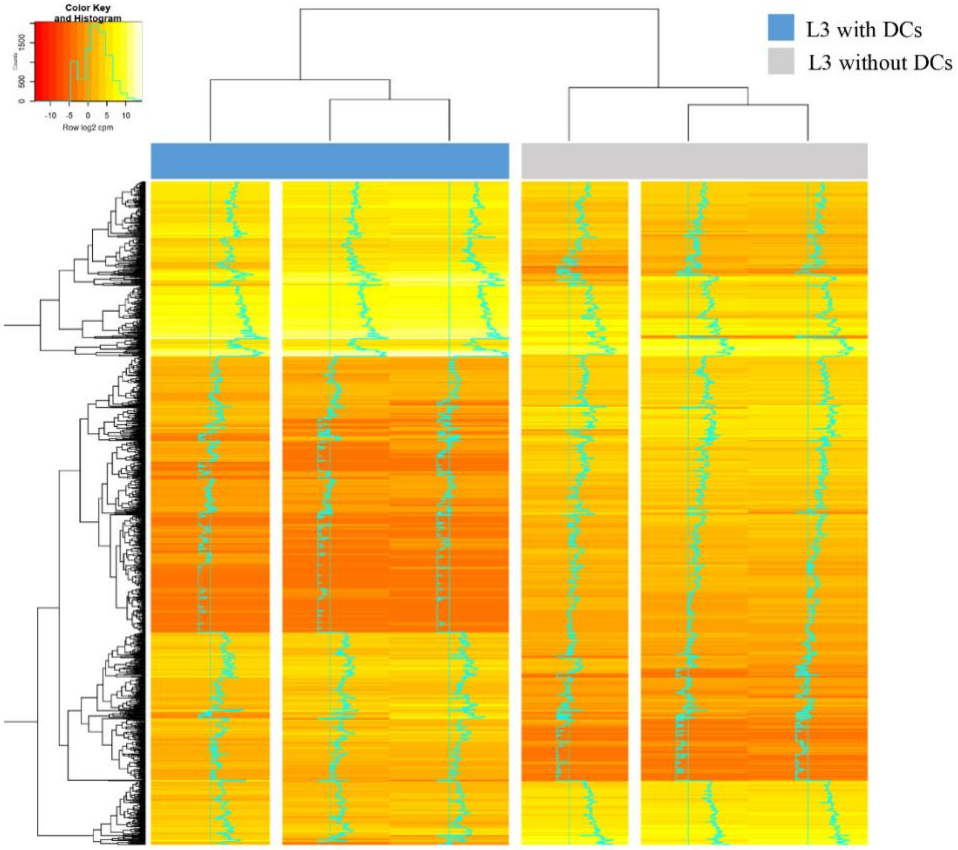

Figure 3. Heatmap of 1203 differentially expressed genes. Hierarchically clustered genes (rows) and *A. pegreffii* larvae (columns) with dendrograms and flat cluster (colored bars). Yellow colour in the heatmap denotes upregulation, while orange colour denotes down-regulation.

4.3.3. Functional classification based on GO and KEGG assignments

A total of 1201 differentially expressed genes were detected, of which 573 were up-regulated and 628 were down-regulated. GO has three ontologies, namely, molecular function, cellular component and biological process. Genes related to terms “catalytic activity” and “binding” were dominant in *A. pegreffii* larvae in presence of iDCs. The “metabolic” and “cellular” process are dominant in the biological process (Figure 1).

KEGG analysis revealed that the highest number of genes were identified for 5 metabolic pathways: biosynthesis of antibiotics, purine metabolism, thiamin metabolism, drug metabolism and starch and sucrose metabolism.

Complete list of the differentially expressed genes is presented in Supplementary file 1.

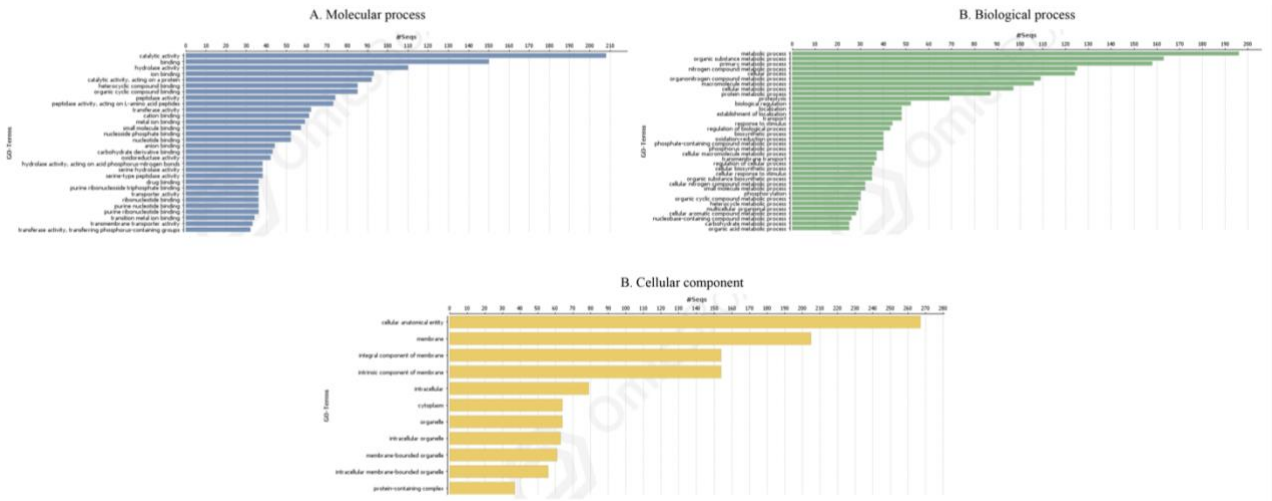

Figure 1. Prediction of differentially over-expressed genes in *A. pegreffii* larvae co-cultured with DCs according to Gene Ontology categories. A) Molecular function, B) Biological process, C) Cellular component.

Examining the function of transcripts with different expression pattern using GO terms, the results revealed a high percentage of genes coding for glycoconjugates included in the metabolic process. Those transcripts exhibited a gene up-regulation (Table 2).

Table 2. Differentially expression of transcripts coding glycosylated proteins. FC: fold change; FDR: False Discovery Rate.

ID	Description	logFC	FDR
ANAP10413_15TR	Cbr-Cht-1	2,09	0,00061
ANAP12860_1TR	C50b6.7	2,16	0,0107
ANAP10413_1TR	Cbr-Cht-1	2,73	2,85E-06
ANAP14781_2TR	Cbr-Tre-5	2,78	0,045
ANAP5646_7TR	Trehalase	2,91	0,008
ANAP15611_1TR	Beta-Glucuronidase	3,41	0,031
ANAP12330_4TR	Maltase	7,02	0,001
ANAP2032_2TR	C-Type Lectin	4,22	0,004
ANAP2032_1TR	C-Type Lectin	3,39	6,0123E-05
ANAP667_34TR	UA3-Recognized Allergen (*A. peg-7*)	3,29	0,08
ANAP17606_1TR	Cytosolic Endo-Beta-N-Acetylglucosaminidase	3,19	0,11
ANAP18150_1TR	Trehalase	2,98	0,09
ANAP10413_11TR	Chitinase And Chia	2,91	0,09
ANAP22913_1TR	UDP-Glucoronosyl And UDP-Glucosyl Transferase	2,67	0,07
ANAP17627_1TR	Acyltransferase	2,65	0,002

ANAP19312_1TR	Udp-Glucuronosyltransferase 1-6	2,59	0,003
ANAP2867_2TR	Fructose-Bisphosphatase Isozyme 2	2,56	2,9339E-05
ANAP4606_10TR	Prolyl Carboxypeptidase 1	2,53	0,0056
ANAP10413_13TR	Chitinase And Chia	2,49	4,1098E-05
ANAP9961_1TR	UDP-Glucoronosyl/UDP-Glucosyl Transferase	2,49	3,6086E-05
ANAP10413_6TR	Putative Endochitinase	2,45	0,0001
ANAP10413_3TR	Putative Endochitinase	2,40	0,0008
ANAP14781_4TR	Cbr-Tre-5	2,35	0,0311
ANAP1182_5TR	32 Kda Beta-Galactoside-Binding Lectin 3 (Galectin)	2,32	0,0001
ANAP10170_1TR	Cytosolic Beta-Glucosidase	2,32	0,0002
ANAP1637_1TR	Carboxylesterase 1e	2,29	0,0747
ANAP10684_1TR	Udp-Glucuronosyltransferase 1-6	2,29	0,0010

4.4. Discussion

Each human helminth has a complex, multi-stage lifecycle, which depends on particular intermediate and definitive hosts and an ecological niche (Prasanphanich et al. 2013). The species *A. pegreffii* involves various hosts at different levels across the marine food webs (Mattiucci and Nascetti 2008). Humans represents an accidental host in its life cycle (Audicana and Kennedy 2008; Mattiucci et al. 2018). The interface between *Anisakis* with human host immunity is complicated. The science has yet to understand of protective immunological mechanisms existent. Therefore, *Anisakis* larvae can influence and regulate the dendritic cells functions in order to promote a more permissive environment for their survival (Napoletano et al. 2017; Motran et al. 2017). In this study we investigated for the first time the influence of the dendritic cells on the transcriptome of *A. pegreffii* L3 and discuss how this interaction is crucial in shaping the host response. The analyses of transcribed mRNAs can provide useful information for the characterization of the genes associated with these processes. Amongst these differentially expressed molecules, we investigate the potential role of the glycosylated molecules. It is widely understood that it is these carbohydrate moieties, or glycans, that are most important for the interface with host DCs (Thomas and Harn 2004; Rhiannon et al. 2012). Indeed, the helminths carbohydrates complex presents an exciting opportunity to fill the existent gap between the anisakids larvae and human host immunity system (Prasanphanich et al. 2013, Lopienska et al. 2019).

The Committee on Assessing the Importance and Impact of Glycomics and Glycosciences reports the host-pathogen interactions as a typical example of the key roles played by carbohydrates in living organisms. Parasitic helminths utilize a wide variety of glycoconjugates to successfully infect their vertebrate and/or invertebrate hosts (Prasanphanich et al. 2013). Many glycans within glycoproteins and glycolipids are unique to helminths or to a particular worm species, they are abundant on worm

surfaces and secretions, and humans vigorously target these glycans in the natural immune response. Helminth glycans also have potent immunomodulatory effects. Indeed, carbohydrates and glycoconjugates exert pro-inflammatory effects on DCs and elicit pathogen-induced innate immunity and antigen processing (Mascanfroni et al. 2011).

Lorenzo et al. (2000) suggested the possible role of a glycoprotein (O-glycans) as a diagnostic protein, in the human anisakiasis. This glycoprotein *Ani s 7* is considered one of the major antigens, detected in 85-100% of patients infected with *A. simplex* (s. l.) (Daschner et al. 2012; Mattiucci et al. 2017; Palomba et al. 2019). However, the role of the glycoprotein is still unknown. Palomba et al. (2019) hypothesized a differentially gene expression of *A. peg-7,* correlated to host immunity system. Interestingly, in the present study, we have observed a differentially gene expression of the UA3-Recognized Allergen (*A. peg-7*) in *A pegreffii* larvae co-cultured with iDCs.

Recognition of pathogens' glycans is known to be mediated by at least two classes of special-receptors (TLRs) and C-type lectin receptors (CLRs), which are instrumental in regulation of adaptive immunity (Geijtenbeek et al. 2004; Banchereau et al. 1998). There are over a dozen different C-type lectins expressed in DC and Langerhans cells, and many other glycan-binding proteins, that have potential to interact with parasite-derived glycans (Mascanfoni et al. 2011; van den Berg et al. 2012). Several evidences indicated a key role of the pathogen's glycan carbohydrates in the modulation of dendritic cells (Mascanfoni et al. 2011; Prasanphanich et al. 2013). Pathogens have evolved several strategies to shape adaptive immunity through modulation of DC function (Vallejo et al. 2009); they may evade immune responses by shaping the DC compartment and selectively altering adaptive immunity through glycan-mediated control of intracellular signaling pathways (Vallejo et al. 2009). *S. mansoni* soluble egg antigens express fucosylated-LDN sequences and $Lewis_x$ or pseudo-$Lewis_y$ antigens in the cercaria of the parasite, suppressing the dendritic cell activation (van Liempt et al. 2007). Sialylated glycans present on the protozoan *T. cruzi* interact with DCs to trigger inhibitory signals (Erdmann et al. 2009).

Romaris et al. (1996) have shown that a group of enzymes involved in carboxylation reactions in pathways such as gluconeogenesis and fatty acid synthesis are widely distributed in *Anisakis* and other nematodes. Recently Lopienska et al. (2019) described a valuable repository of genes encoding proteins of carbohydrates metabolism. However, the gene expression analysis of glycan carbohydrates of the L3 of *A. pegreffii* under the effect of dendritic cells have been here investigated for the first time. C-type lectins were found to be differentially expressed in *A. pegreffii* larvae co-cultured with dendritic cells. Indeed, helminth C-TLs sharing sequence and structural similarity with mammalian immune cell lectins have recently been identified from nematodes parasites, suggesting clear roles for these proteins at the host-parasite interface, notably in immune evasion (Loukas and Maizels 2000). In particular, the galectin-3, have shown a differential gene expression in *A. pegreffii* L3 larvae in presence of dendritic cells. Shi et al. (2018) reviewed some recent studies on the roles of the galectins produced by parasitic protozoa, nematodes, and trematodes and their hosts. For examples, gal-3, an important modulator of

biological processes and an emerging player in the pathogenesis of various immune/inflammatory diseases (Dumic et al. 2006), reduces the frequency and function of $CD4^+CD25^+Foxp3^+$Treg cells during *L. major* infection via modulation of the Notch signaling pathway (Fermino et al. 2013). Several galectins have been also reported to play a role in infections of gastrointestinal nematodes of domestic animals, including *Cooperia oncophora*, *H. contortus*, *Ostertagia ostertagi*, *Teladorsagia circumcincta*, and *Trichuris muris*.

4.5. Conclusions

The study of the expression genes coding for immunomodulatory glycans of L3 stage of the zoonotic species *A. pegreffii* represents a field of research still not deeply investigated. Thus, the knowledge on this aspect is likely contributing to our overall understanding of innate immunity, particularly in defining how Th2 PAMPs activate native DCs and drive maturation to DC2s. The parasites' glycans have many other as yet un-described immunomodulatory properties and that taken together, will provide much insight into understanding the evolution and maintenance of polarized Th2 responses (Thomas and Harn 2004). Understanding the roles of glycoconjugates in host–parasite interactions could also provide targets for diagnosis and possible therapies of the parasitic infections.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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5. IV - A novel nuclear diagnostic marker and development of ARMS-PCR assay at the metallopeptidase 10 (*nas 10* nDNA), for genotyping of the sibling species of the *Anisakis simplex* (s. l.) complex (Nematoda: Anisakidae). Under review in *Parasite*

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Abstract - The genus *Anisakis* represents one of the most widespread groups of ascaridoid nematodes in the marine ecosystem. Three closely related taxa are recognized in the *A. simplex* (s. l.) complex: *A. pegreffii*, *A. simplex* (s. s.) and *A. berlandi*. They are widely distributed in populations of their intermediate/paratenic (fish and squids) and definitive (cetaceans) hosts. A novel nuclear gene locus at the metallopeptidase 10 (*nas 10*) (451 bp) was sequenced and validated on a total of N= 219 specimens of the three species of *Anisakis*, collected in fish and cetacean hosts from allopatric areas included in their ranges of distribution. The specimens of *Anisakis* were first identified by allozymes and sequence analysis of the mtDNA *cox 2* and EF1 α-1 nDNA. The novel nuclear marker has shown fixed alternative nucleotide positions in the three species, i.e. resulted to be diagnostic at 100%, permitting the species determination of a large number of specimens analyzed in the present study. In addition, primers to be used for amplification-refractory mutation system (ARMS) PCR of the same gene locus were designed at those nucleotide positions. Thus, direct genotyping determination, by double ARMS, was developed and validated on N= 219 specimens belonging to the three distinct species. Complete concordance was observed between the tetra-primer ARMS PCR assays and direct sequencing results obtained at the *nas 10* gene locus. The novel nuclear diagnostic marker will be useful in future works of a multi-locus genotyping approach also to study possible hybridization and/or introgression events occurring between the three species in sympatric areas.

Key words: *Anisakis simplex* (s. s.), *A. pegreffii*, *A. berlandi*, diagnostic markers, metallopeptidase *nas 10* nDNA, ARMS-PCR

5.1. Introduction

The *Anisakis simplex* (s. l.) species complex includes heteroxenous nematodes belonging to three distinct species, namely *A. pegreffii, A. simplex* (s. s.) and *A. berlandi* (see Mattiucci et al. 2018). The three taxa, first genetically detected by allozymes (Mattiucci et al. 1997), were later morphologically described (Mattiucci et al. 2014), and recognized as distinct biological species based on different nuclear (Pontes et al. 2005) and mitochondrial gene loci (Valentini et al. 2006). The geographical range of the three species *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi* is quite distinct; however, some oceanographic basin waters are reported as overlapping areas and contact zones between the three species (for a review, Mattiucci et al. 2018). In allopatric and sympatric areas, they are known to infect several definitive and intermediate/paratenic hosts; the three species are often sharing the same host-species range (Mattiucci et al. 2018). In addition, *A. pegreffii* and *A. simplex* (s. s.) are recognized as zoonotic parasites that cause human gastro- (GA) intestinal- (IA) anisakiasis and a gastro-allergic (GAA) anisakiasis (Mattiucci et al. 2018). Gastro-intestinal anisakiasis is currently widespread in Europe, mostly occurring in Italy, Spain, France, Portugal and Croatia (reviewed in Mattiucci et al. 2018; Guardone et al. 2018); cases have also been widely reported in Japan and South Korea (Lim et al. 2015). Humans are accidental hosts who become infected following the ingestion of raw fish and squids containing, in their edible parts, the third-stage larvae (L3) of *A. pegreffii* and *A. simplex* (s. s.). No data are so far available about the zoonotic role of the species *A. berlandi*. On the other hand, while it was widely demonstrated that *A. simplex* (s. s.) and *A. pegreffii* (Levsen et al. 2018a, 2018b; Cipriani et al. 2016) are infecting the musculature of the fish hosts, there is not knowledge about the capacity of *A. berlandi* to invade the fish host musculature.

In the recent years, the multi-locus genotyping approach has been suggested to be applied in the identification of the anisakid nematodes of the genus *Anisakis* and in the investigation about the micro-evolutionary aspects, such as the hybridization and introgression phenomena (Mattiucci et al. 2018). In this aim, several attempts to provide new nuclear markers have been recently performed. Among them, there are: 1) the sequences analysis of the nuclear locus EF1 α-1 nDNA showing the presence of fixed alternative nucleotide positions between the two species *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci et al. 2016), and 2) the development of DNA microsatellite loci (Mladineo et al. 2017); according to the results given by those authors, however, no diagnostic markers were discovered between the two taxa. Whereas, opposite results were obtained by a further recent development of polymorphic SSRs DNA markers (Mattiucci et al. 2019). Indeed, diagnostic SSRs DNA loci between the species *A. pegreffii* and *A. simplex* (s. s.) were detected (Mattiucci et al. 2019), also with respect to the species *A. berlandi* (Bello et al. in press), allowing the reliable identification of specimens of the two species. It was also found that among the diagnostic loci detected at the SSRs loci between the three *Anisakis* species (Mattiucci et al. 2019; Bello et al. in press), sex-linked loci were existing. The last finding, however, highlighted the problem that, being male worms hemizygotes at those diagnostic

SSRs loci, their genotypes cannot be considered in a large genotyping analysis in the investigation of hybridization and introgression phenomena (Mattiucci et al. 2019). On the other hand, it has been also underlined that a multi-locus nuclear genotyping approach is generally mandatory and highly recommended to investigate the occurrence of mixed ancestry just in the hosts, and in the geographic areas where parasite species are overlapping their range of distribution (Anderson 2001; Anderson and Thompson 2002; Criscione et al. 2007; Mattiucci et al. 2018). Therefore, the discovery of further nuclear diagnostic markers to be used for disentangling hybridization and/or introgression events between those sibling species of *Anisakis*, are desirable.

The amplification-refractory mutation system (ARMS) can distinguish a single base sequence difference using one-step PCR (Patel et al. 1991). This method is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. Following an ARMS reaction, the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele. ARMS-PCR assay was recently developed on the ITS rDNA for the species identification of *A. pegreffii*, *A. simplex* (s. s.) and *A. typica* occurring in fish from Korean waters (Kim et al. 2019).

The scope of this work was to: 1) provide new nuclear diagnostic marker among the three *Anisakis* species (i.e. *A. pegreffii, A. simplex* (s. s.) and *A. berlandi*), based on the direct sequences analysis of a coding gene locus at a metallopeptidase-10 (*nas 10*), to be used in future multi-locus approach; 2) validate the novel marker on a large number of specimens of the three *Anisakis* species; 3) develop and validate an amplification-refractory mutation system (ARMS) PCR tool based on that gene locus, for the rapid genotyping of individuals of *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi* at that locus.

5.2. Materials and Methods

5.2.1. Parasite sampling and molecular identification

A total of 219 *Anisakis* spp. third-stage larvae and adults collected, respectively, from intermediate/paratenic fish and cetacean hosts, were obtained from the frozen and alcohol collection stored in the Section of Parasitology, Department of Public Health and Infectious Diseases of 'Sapienza - University in Rome'. Details concerning the sampling localities are given in Table 1.

The allozymes have proven to be diagnostic for the species of the *A. simplex* (s. l.) complex, have been used (Mattiucci et al. 2009; Mattiucci et al. 2014). Standard horizontal starch gel electrophoresis was performed at those enzyme loci; their staining procedures are those previously reported (Mattiucci et al. 2014). Total DNA was extracted from a tissue portion (≈2 mg) of each *Anisakis* larva (L3, L4) and each adult specimen (Table 1). The *Quick*-gDNA™ Miniprep Kit (ZYMO RESEARCH) was used as extraction method. DNA obtained was quantified by using a NanoDrop®TC1-E20 spectrophotometer (BioTek Synergy HT). The specimens were identified to the species level by the sequence analysis of the mtDNA *cox 2* gene locus, which allows to distinguishing the three species

A. simplex (s. s.), *A. pegreffii,* and *A. berlandi (*Mattiucci et al. 2014). Additionally, the specimens recognized at the mtDNA *cox 2* as belonging to *A. pegreffii* and *A. simplex* (s. s.) were also identified by sequence analysis of the EF1 α-1 nDNA (Mattiucci et al. 2016).

For the sequencing of the mtDNA *cox 2* gene locus, PCR amplification was performed using the primers 211F (5'- TTT TCT AGT TAT ATA GAT TGR TTT YAT - 3') and 210R (5' - CAC CAA CTC TTA AAA TTA TC - 3') (Valentini et al. 2006; Mattiucci et al. 2014). PCR conditions were established as previously described (Mattiucci et al. 2014). The amplification of EF1 α-1 nDNA was performed using the primers EF-F (5'- TCC TCA AGC GTT GTT ATC TGT T -3') and EF-R (5'- AGT TTT GCC ACT AGC GGT TCC -3') under the conditions previously described (Mattiucci et al. 2016).

Table 1. Sampling area, host species and life-history stage (A: adult; L4: 4th stage larvae; L3: 3rd stage larvae) of the specimens of *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi* analysed.

Species	Sampling area	Host species	*N*	N_A	N_{L4}	N_{L3}
A. simplex (s. s.)						
	NE Atlantic Ocean					
	Norwegian Sea	*Clupea harengus*	22	-	-	22
	(68°52'N – 3°08'E)	*Scomber scombrus*	2	-	-	2
	North Sea	*Clupea harengus*	3	-	-	3
	(59°13'N – 00°14'W)					
	Baltic Sea	*Clupea harengus*	9	-	-	9
	(58°29'N – 19°51'E)					
	English Channel	*Clupea harengus*	19	-	-	19
	(48°38'N – 4°34'W)					
	Gran Sole Bank	*Merluccius merluccius*	16	-	-	16
	(49°38'N – 10°10'W)					
	Scotland coast	*Stenella coeruleoalba*	13	13	-	-
		Lagenorhynchus albirostris	9	9	-	-
		Tot.	93	22	-	71
A. pegreffii						
	Mediterranean Sea					
	Tyrrhenian sea	*Merluccius merluccius*	1	-	-	1
	(41°7'N – 13°24'E)	*Lepidopus caudatus*	11	-	-	11
		Scomber scombrus	4	-	-	4
		Trachurus trachurus	1	-	-	1
	Western Adriatic Sea	*Stenella coeruleoalba*	22	11	1	10
	(42°18'N – 15°35'E)	*Lophius piscatorius*	1	-	-	1
		Scomber scombrus	5	-	-	5
	SW Pacific Ocean					
	New Zealand	*Globicephala melas*	31	20	11	-
	(44°30'S – 172°58'E)					
		Tot.	76	31	12	32
A. berlandi						
	SW Pacific Ocean					
	New Zealand	*Globicephala melas*	50	19	2	3
	(44°30'S – 172°58'E)					
		Tot.	50	19	2	3

5.2.2. Primers selection for the *nas 10* nDNA and PCR condition

For the direct sequencing of the metallopeptidase (*nas 10* nDNA) gene locus of *Anisakis*, oligonucleotide primers were designed based on the sequence at the metallopeptidase *asnas 10* of *Ascaris suum* deposited in the GenBank under the accession number JI170268.1, by using the software program PRIMER3 plus (http://bioinfo.ut.ee/primer3-0.4.0/). The total sample of N= 218 individuals, belonging to the three species, *A. pegreffii*, *A. simplex* (s s.) and *A. berlandi*, previously identified by allozymes, mtDNA *cox 2* and EF1 α-1 nDNA, were thus sequenced at the metallopeptidase 10 (*nas 10*) nDNA gene locus. Thus, PCR amplification was performed using the primers nas10-F (5′-GAT GTT CCT GCA AGT GAT TG-3′) and nas10-R (5′-CGC TAT TAA GAG AGG GAT CG-3′) (Table 2). PCR reactions were carried out in a 25 μL volume containing 0.6 μL of each primer 10 mM, 2μL of $MgCl_2$ 25 mM (Promega), 5 μL of 5× buffer (Promega), 0.6 μL of dNTPs 10 mM (Promega), 0.2 μL of Go-*Taq* Polymerase (5U/ μL) (Promega) and 2 μL of total DNA. PCR temperature conditions were the following: 94°C for 5 min (initial denaturation), followed by 40 cycles at 95°C for 1 min (denaturation), 53.1°C for 1 min (annealing), 72°C for 1 min (extension) and followed by post-amplification at 72°C for 15 min.

Table 2. Primer sequences of the metallopeptidase 10 (*nas 10*), product size and annealing temperature (Ta/°C). In bold is indicated a deliberate second mismatch.

Name	Primer sequences (5' to 3')	Genotyping pattern (bp)	Ta/°C
nas 10	F: GATGTTCCTGCAAGTGATTG	451 bp	53.1°C
	R: CGCTATTAAGAGAGGGATCG		
Primer set-1	Out-F1: TATGGCAAATATTATTATCGTA	373 bp (control fragment)	49°C
	Out-R1: TATTTCCGACAGCAAACAA	296 bp (T allele)	
	In-F1: GCATTGTACACTTCGTA**T**ATT	117 bp (C allele)	
	In-R1: ATTTCTYCAGCAATCGT**A**AG		
Primer set-2	Out-F2: GAAAGACAGGTTCATCTCA	321 bp (control fragment)	49°C
	Out-R1: TATTTCCGACAGCAAACAA	117 bp (G allele)	
	In-F2: AACGGATATGAATGAT**C**CC	216 bp (C allele)	
	In-R2: HAAATGAAAGTAGAAAGAATT**T**AC		

5.2.3. Sequences data analysis

Clustal X (version 2.0) software (Larkin et al. 2007) was used for the the sequences alignment, obtained at the studied gene loci (i.e. mtDNA *cox 2* , EF1 α-1 nDNA and *nas 10* nDNA). In the case of mtDNA *cox 2* and EF1 α-1 nDNA, the generated sequences were aligned with those from the same

species previously obtained (Mattiucci et al. 2014, 2016), in order to detect those fixed diagnostic nucleotide positions which permit to discriminate *A. simplex* (s. s.), *A. pegreffii* and *A. berlandi* (Mattiucci et al. 2014, 2016).

5.2.4. Development of ARMS-PCR assay of the *nas 10* nDNA

The diagnostic nucleotide positions found between the three *Anisakis* species, were used to develop ARMS-PCR assay experiments. This resulted in the use of 4 pairs of primers organized into 2 primers sets (Table 2). The two sets were indeed designed on those diagnostic nucleotide positions (i.e. 173 and 251) observed at direct sequencing of *nas 10* nDNA, in the three species of the *A. simplex* (s. l.), by using the web-based program, Primers3 plus (https://primer3plus.com/primer3web/primer3web).

Each primer set includes four primers, one pair of primers comprises the forward and reverse outer primers which serve as internal amplification control; the other pair of primers comprises the forward and reverse inner primers (Table 2). Importantly, in tetra-primer ARMS-PCR, there is not only a 3' terminus mismatch, but also a second deliberate mismatch at position-2 from the 3' terminal of the inner primers to increase the specificity of the reaction. The two primers sets (Table 2) were used in two PCR reactions planned to work simultaneously, to identify *A. pegreffii*, *A. simplex* (s. s.) and *A. berlandi*. Each PCR was performed in a total volume of 25 μL: 3.5 μL of 10X solution buffer, 1.5 μL containing 10 μM of four mixed dNTPs, 1.5 μL of 50 Mm $MgCl_2$, 1.2 μL of each primer (10 Mm) (Table 2), 0.25 μL of Taq DNA polymerase (5U/ μL) (Promega) and 3 μL of total DNA. PCR amplification conditions were the following: 95°C for 5 min (initial denaturation). Followed by 35 cycles at 95 °C for 30 s (denaturation), 48 °C for 35 s (annealing), 72 °C for 30 s (extension) and a final elongation step at 72 °C for 7 min. The PCR products generated from the outer primers were used as positive controls in the ARMS-PCR system to ensure the normality of PCR reaction (373 bp for primers set-1, 321 bp for primers set-2). The PCR products, obtained from each analyzed *Anisakis* specimen, by use of two sets of primers (Table 2), were separated by electrophoresis using agarose gel (1.5%) stained with GelRed; 3μL of the amplification products were visualized. The distinct banding patterns were detected by the use of ultraviolet transillumination.

5.3 Results

5.3.1. Identification of *Anisakis simplex* (s. l.) specimens

Allozyme analysis of *Anisakis* (N= 93 specimens) (Table 1) corresponded to *A simplex* (s. s.), N= 50 to *A. berlandi*, and finally N= 75 to *A. pegreffii*, according to alleles found at the diagnostic loci between the three sibling species (i.e. *Adk-2*, *Pep C-1*, *Pep C-2*, and *Mdh-1*) (Mattiucci et al. 2014). Similarly, according to the sequences of 629 bp in length of the mtDNA *cox 2* gene locus (Mattiucci

et al. 2014), 75 specimens were assigned to *A. pegreffii*, 93 to the species *A. simplex* (s. s.) and, finally, 50 individuals corresponded to *A. berlandi*. The sequences obtained in the present study matched, at 100% or 99%, with the sequences previously deposited by us in GenBank for those species. In addition, the specimens recognized at the mtDNA *cox 2* as belonging to *A. pegreffii* and *A. simplex* (s. s.), according to the diagnostic positions of the locus EF1 α-1 nDNA (Mattiucci et al. 2017), were also identified as *A. pegreffii* and *A. simplex* (s. s.).

5.3.2. Genotyping of *Anisakis* spp. by direct sequencing of *nas 10* nDNA

A fragment of 451 bp in length of the *nas 10* nDNA region was obtained for all of the 218 specimens here analyzed. The sequences revealed the presence of one diagnostic nucleotide site between *A. simplex* (s. s.) *versus A. pegreffii/A. berlandi*, and another one between *A. berlandi versus A. pegreffii/A. simplex* (s. s.): those nucleotide diagnostic positions were 173 and 251, respectively (Figure 1). In particular, the position 173 showed the T allele in all the *A. simplex* (s. s.) individuals (N= 93); while it showed always the C allele in the specimens of *A. pegreffii* and *A. berlandi* here considered (Figure 1). The position 251 showed the G allele in the individuals of *A. pegreffii* and *A. simplex* (s. s.), while it was always C in all the *A. berlandi* specimens (N= 50), here analyzed (Figure 1). All the specimens belonging to the three *Anisakis* species, were found to be homozygous at those nucleotide positions; no heterozygous between the three species were found in the present study at those positions. As a consequence, those nucleotide positions resulted fixed for alternative nucleotide among the three species, and were considered as having diagnostic value. Sequences of the *nas 10* nDNA region were deposited in GenBank under the following accession numbers: MN897674, MN897675, MN897676 for *A. pegreffii*, MN897671, MN897672, MN897673 for *A. simplex* (s. s.), and MN897677, MN897678, MN897679 for *A. berlandi*.

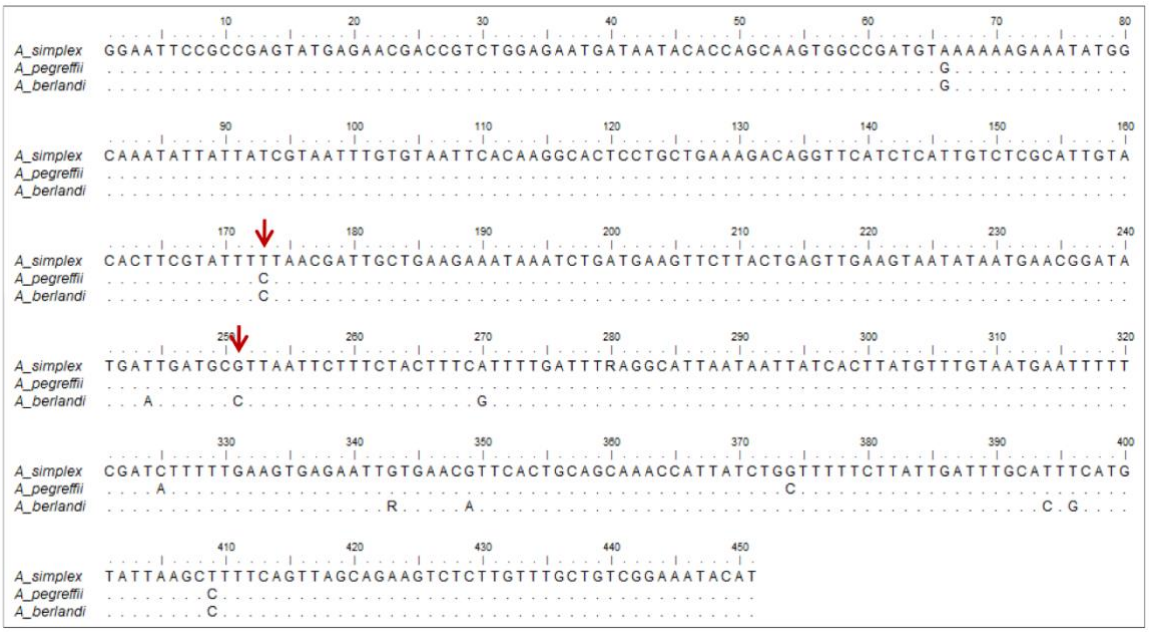

Figure 1. Sequence alignment of the nucleotide sequences of the metallopeptidase 10 (*nas 10*) of *A. simplex* (s. s.), *A. pegreffii* and A. *berlandi*. Dots indicate identity with the consensus sequence. Arrows indicate the nucleotide diagnostic position.

5.3.3. Genotyping of *Anisakis* spp. by ARMS-PCR of *nas 10* nDNA

The two point mutations diagnostic between the three *Anisakis* species and the tetra-primers ARMS-PCR analysis allowed the genotyping of the individuals belonging to the three species here studied. Indeed, all the specimens belonging to three taxa, previously identified by allozymes, mtDNA *cox 2* , EF1 α-1 nDNA and sequenced at the *nas 10* nDNA, were directly analyzed by ARMS-PCR at the last locus.

One set of designed primers (primers set-1) (Table 2) was able to detect the first point mutation (i.e. 173) allowing to distinguish *A. simplex* (s. s.) *versus A. pegreffii/A. berlandi*; while, the second set of primers detected the point mutation 251, which identified always a C/C genotype in *A. berlandi*; while it showed a G/G genotype in both *A. simplex* (s. s.) and *A. pegreffii*. In particular, in the primer set 1 (Table 2) the combined use of *nas 10*-reverse outer primer (OUT-R1) and *nas 10*-forward inner primer (INN-F1), amplifying the T-allele at the position 173, generated a specific PCR product at 296 bp in *A. simplex* (s. s.). (Figure 2; Table 3). Whereas, the combined use of *nas 10*- forward outer primer (OUT-F1) and *nas 10*-reverse inner primer (INN-R1), amplifying the C-allele at the position 173, generated a specific PCR fragment of 117 bp in *A. pegreffii* and *A. berlandi* specimens (Figure 2; Table 3). In the primers set-2 (Table 2), the combined *nas 10*- forward outer primer (OUT-F2) and the *nas 10*-reverse inner primer (INN-R2) amplified the G-allele at the diagnostic nucleotide position 251, producing a specific PCR fragment of 148 bp in *A. simplex* (s. s.) and *A. pegreffii* (Figure 2; Table 3).

While, in the same primer set, the *nas 10*-forward inner primer (INN-F2) and the *nas 10*-reverse outer primer (OUT-R1) amplified the C-allele (diagnostic position 251) and determined a PCR product of 216 bp in *A. berlandi* (Figure 2; Table 3). All the specimens resulted homozygous for those fixed alternative nucleotide positions; no heterozygous at those SNPs were found in the sample of *Anisakis* spp. here tested.

According to the gel electrophoresis patterns (Figure 3) obtained by the combined use of tetra-primers set-1 and tetra-primers set-2, 75 specimens were resulted *A. pegreffii*, 93 *A. simplex* (s. s.) and, finally, 50 individuals corresponded to *A. berlandi*. Thus, it was found a 100% concordance between the genotyping obtained by ARMS-PCR assay and direct sequences analysis of the same gene locus *nas 10* nDNA.

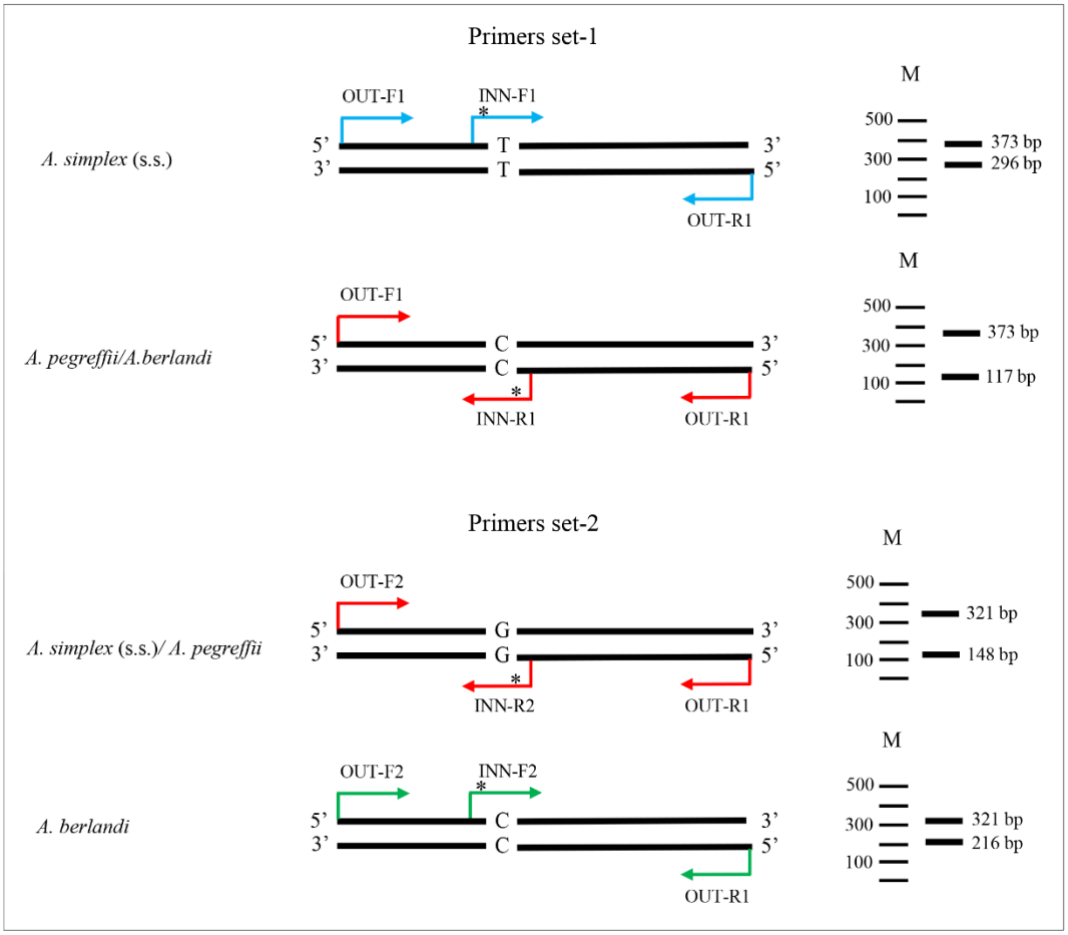

Figure 2. Schematic illustration of the two set of the tetra-primers ARMS-PCR primer design and DNA gel patterns of the different single nucleotide genotyping (on the right). Asterisk indicates the second mismatch of the inner primer.

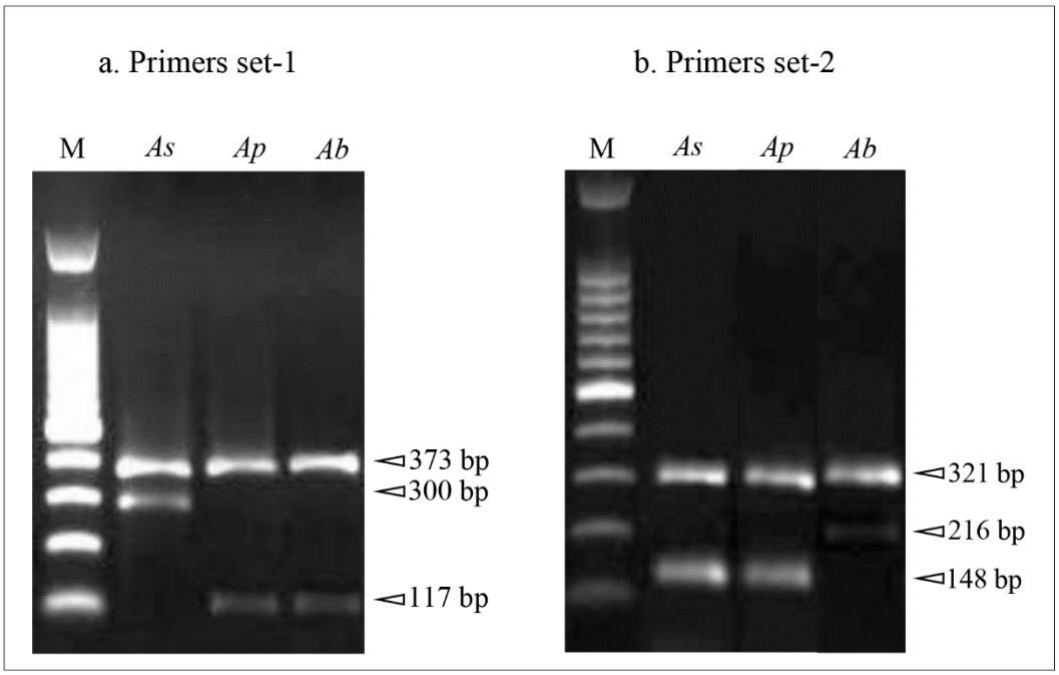

Figure 3. Products (genotypes) of tetra-primers ARMS-PCR obtained at the locus *nas 10* in the three species of the *A. simplex* (s. l.) complex.

5.4. Discussion

In parasitic nematodes, peptidase proteins represent around the 2% of gene expressed; they are involved in host-parasite interactions, parasite immune evasion, life cycle transmission and pathogenesis (Malagon et al. 2013). In many cases, peptidases are also the main source of parasite antigens, favouring the host immune response; many of them are especially immunogenic (Hotez and Pritchard 1995; Todorova and Stoyanov 2000). In particular, metallopeptidases are a group of peptidases which involve in the invasion of the host tissue by the parasite, as they are able to degrade the extracellular matrix. In some cases, it has been suggested that the metallopeptidases conserve their proteolytic activity even after the antibodies action has exhibited by the host during the infection to the parasite (Malagon et al. 2011; Benitez et al. 2013; Bruschi and Pinto, 2017).

Recent RNA seq analysis revealed a high percentage of gene coding for metallopeptidases highly expressed in *A. pegreffii* third-stage larvae maintained *in vitro* co-culture with dendritic cells, at 37°C (Palomba et al. in preparation). These enzymes were postulated to be involved in host tissue penetration by *Anisakis* spp. larvae (Kim et al. 2018). The tissues migration capacity of parasite would represent an evolutionary advantage for the larvae of *Anisakis* spp. during the intermediate/paratenic host infection. In turn, this would mean that selective pressure would have conferred an evolutionary advantage to the genes coding for the group of peptidase proteins, allowing to the parasite species to successfully invade different fish hosts. As a consequence, high level of mutation rate could have

occurred in those gene loci over an evolutionary time scale, resulting as advantageous genotypes for the parasite species, in terms of invasion and adaptation to their hosts' range. Interestingly, previous allozyme studies in anisakids have demonstrated that amongst the scored gene loci, those coding for the peptidase proteins represent a high percentage, showing fixed alternative alleles between related anisakid species belonging to the genera *Anisakis*, *Pseudoterranova* and *Contracaecum* (Paggi et al. 1991; Nascetti et al. 1993; Orecchia et al. 1994; Mattiucci et al. 2009). Furthermore, in the case of the three species of the *A. simplex* (s. l.) complex, the gene locus coding for the enzymatic proteins: Peptidase-C, Peptidase-B and Leucine-amino-peptidase (i.e. coding loci: *Pep-C-1*, *PepC-2*, *Pep B*, *Lap-1*, respectively) showed fixed alternative alleles, that are diagnostic in those species. Thus, it seems that those gene loci coding for peptidases are fast evolving loci in anisakids (Mattiucci and Nascetti 2008), suitable for discriminating between closely related taxa (Ferguson 1980).

The possibility to look directly into the gene sequences coding for those functional proteins, such as peptidases, allows to detect fixed nucleotide positions (SNPs). Single nucleotide polymorphism (SNP) refers to a polymorphic sequence generated by a single nucleotide mutation at a specific locus in the DNA sequence. This is the case of the direct sequencing of the gene coding for the metallopeptidase 10 (*nas 10* nDNA), that shows single nucleotide positions (SNPs), alternative among the three *Anisakis* species: i.e. a further diagnostic nuclear gene. These diagnostic single nucleotide positions are: the position 173 allowing to distinguishing *A. simplex* (s. s.) from the other two taxa (i.e. *A. pegreffii*, and *A. berlandi*) while, the position 251 identifies *A. berlandi* with respect to *A. pegreffii* and *A. simplex* (s. s.). A molecular key based on those nucleotide differences is presented in Table 3. No heterozygous genotypes were observed: this is another demonstration that *A. pegreffii*, *A. simplex* (s. s.) and *A. berlandi* conserve independent gene pools.

The presence of those fixed differences can be considered as Mendelian characters - being the *nas 10* nDNA a nuclear gene locus. This means that these SNPs can be also tested for Hardy–Weinberg equilibrium (HWE), and they can be included in Bayesian clustering multi-genotyping analysis (Mattiucci et al. 2016; 2019) in future multi-locus approaches.

A sufficient sample size (i.e. number of specimens) is required to ensure that those detected fixed differences are maintained. In the present study, we have validated the existence of those fixed difference in a large number (218) of larval and adult specimens of the three *Anisakis* species collected from different fish and cetacean hosts.

The present study has also shown that the combined use of two primer sets of ARMS-PCR, established on the basis of those diagnostic SNPs at the *nas 10* nDNA, is suitable for the direct and fast genotype determination of the three *Anisakis* species. In particular, the primers set-1 discriminated the species *A. simplex* (s. s.) that showed the two fragments at 373 bp and 296 bp, from the species *A.*

pegreffii/*A. berlandi*, which have the same banding pattern of 373 bp and 117 bp (Table 3, Figure 3). Then, the primers set-2 allowed the discrimination between *A. berlandi* with the fragments of 321 bp and 216 bp, *versus A. simplex* (s. s.)/*A. pegreffii* (321 bp, 148 bp). Thus, the use of both primers set is mandatory in order to discriminate between the three *Anisakis* species, (Table 3, Figure 3).

The tetra-primer ARMS-PCR could be a useful tool for genotyping, faster, less technically demanding, and cheaper than the direct DNA sequencing.
For the genetic identification of parasites such as anisakid nematodes, infecting economic important fish, fast and economical assays that can be performed with standard PCR instruments are highly desirable. Compared to other genotyping techniques, tetra-primer ARMS-PCR has been reported to be a rapid, reliable, simple and economical assay for SNP genotyping (Little 2001; Okayama et al. 2004; Ye et al. 2001). The amplification-refractory mutation system (ARMS) can distinguish a single base sequence difference using one-step PCR. This method has been recently developed on the SNPs at the ITS rDNA for the species identification of *A. pegreffii, A. simplex* (s. s.) and *A. typica* occurring in fish from Korean waters (Kim et al. 2019). However, it has been also recently suggested that polymorphic nucleotide positions at those SNPs detected at the ITS region of the rDNA may occur, especially in sympatric areas. The polymorphism, at those position, may compromise the correct identification of the nematodes belonging to the species *A. pegreffii* and *A. simplex* (s. s.) (Mattiucci et al. 2016).

Thus, in the present study, tetra-primer ARMS-PCR based methodology was developed on the basis of the novel nuclear marker, for genotyping *A. simplex* (s. l.) nematodes. The assay would be also more convenient than the traditional PCR-RFLP, since it eliminates the need for incubation with restriction enzymes. This not only avoids any consequent errors and artefacts from such procedures, but also reduces the amount of DNA required for the digestion step in PCR-RFLP. No special equipment, and only a small amount of standard PCR reagents is needed in tetra-primer ARMS-PCR. The tetra-primer ARMS-PCR protocol described in the present study is the first reported method allowing to genotype novel SNPs in *nas 10* gene locus. This method is rapid, simple, reliable, easy to perform, economical, and requires minimum level of expertise that can be used for both large- and small-scale genotyping studies.

Table 3. Molecular key based on the diagnostic positions.

Position (bp)	Genotype	Species identification	ARMS-PCR fragment pattern (bp)
(1.) 173	T/T ⟶	*A. simplex* (s. s.)	373 and 300
	C/C ⟶	*A. pegreffii*/*A. berlandi* **(2.)**	373 and 117
(2.) 251	G/G ⟶	*A. pegreffii*	321 and 148
	C/C ⟶	*A. berlandi*	321 and 216

5.5. Conclusions

The novel nuclear marker (*nas* 10 nDNA) and the development of ARMS-PCR protocol, based on the diagnostic SNPs detected between the three species of *Anisakis*, proposed in the present study, will allow to genotyping the specimens at a further locus when a multilocus-approach is needed. The markers were validated on 219 samples belonging to the three species of *Anisakis* (i.e. *A. pegreffii*, *A. simplex* (s. s.) and *A. berlandi*). A concordance was observed between the identification and genotyping obtained by tetra-primer ARMS PCR assay and the direct DNA sequencing. The protocols detailed here outline methods that can be used to analyze further genomic DNA of the three sibling species of *Anisakis* for two diagnostic mutations (SNPs).

Currently, SNP markers are one of the preferred genotyping approaches, because they are abundant in the genome, genetically stable and amenable to high throughput automated analysis of *Anisakis simplex* (s. l.) (Lopienska-Biernat et al. 2019). Diagnostic SNPs in candidate nuclear genes in anisakid nematodes may be useful in multilocus genotyping protocols required for studying their microevolutionary aspects. Indeed, the novel nuclear locus reveals to be useful to our further understanding around the debate of the hybridization and/or introgression phenomena between the species of the *A. simplex* (s. l.) complex in sympatric areas.

Conflict of interest

The authors declare that they have no conflict of interest.

6. Concluding remarks

With respect to the state of the Art, we have improved knowledge about some target proteins ESPs of the two main zoonotic species, i.e. *A. simplex* (s. s.) and *A. pegreffii*.

In particular, concerning the specific objectives of the Thesis:

Objective no. 1: Besides their role as antigens/allergens, the Excretory Secretory Products (ESPs), are thought to be key players in modulating the parasite-host interaction, also in response to temperature, pH, CO_2, oxygen, as well as in response of the host tissues (both invertebrates and vertebrates, including humans). Particularly, for instance, the temperature has been considered as a key factor in modulating both the motility and migration of *A. pegreffii* larvae in the hosts' tissues.

In this regards the gene transcripts of proteins having the antigenic role, among the ESPs, (i.e. a Kunitz-type trypsin inhibitor, *A.peg-1*; a glycoprotein, *A.peg-7* and the myoglobin, *A.peg-13*) were investigated. In particular, the gene expression of those target proteins after 24h, in *A. pegreffii* larvae maintained *in vitro*, under controlled temperature conditions (i.e. 37°C, 20°C resembling respectively homeothermic and ectothermic hosts condition, and 7°C, cold stress condition *post mortem* of the fish host) revealed differential expression of transcripts. Expression profiles of the genes *A.peg-1* and *A.peg-13* were found to be significantly up-regulated at 20°C and 37°C, with respect to the control (larvae kept at 2°C for 24h). Conversely, transcript profiles of *A.peg-7* did not significantly change among the chosen temperature conditions. In accordance with the observed transcript profiles, the SDS-PAGE revealed the presence of the three targets ESPs at 20°C and 37°C. The results obtained seem to indicate that chosen temperature conditions can regulate the gene expression profile of *A.peg-1* and *A.peg-13* in *A. pegreffii* larvae. While, because the transcripts of *A.peg-7* in *A. pegreffii* seems to be maintained in all the selected temperature-conditions (i.e. 7°C, 20°C and 37°C), it would indicate that the regulation of the glycoprotein, *A.peg-7* could be most related to other factors such as the intermediate/paratenic and accidental (human) hosts' immune response. The results achieved were the object of the **Publication no. 1.**

The differential gene expression of those ESP and other molecules in the zoonotic *A. simplex* (s. s.), involved in the larval invasiveness in naturally infected hosts' tissues, was also carried out. In the intermediate/paratenic fish hosts, *Anisakis* spp. larvae, migrate from the stomach to other fish tissues, tend to encapsulate in different organs; sometimes can reach the muscles. The processes are thought to be involved with both mechanical and biochemical process for host tissue penetration and degrading the extracellular matrix. The aim of this specific objective allowed to: 1) study for the first time, the gene expression levels of some proteins in L3 stage of *A. simplex* (s. s.) infecting different host tissues/organs of a fish species, i.e. the blue whiting *Micromesistius poutassou*. The following genes encoding for the proteins: a kunitz-type trypsin inhibitor (*TI*), a glycoprotein (*GP*), the haemoglobin

(*hb*), the sideroflexin 2 (*sfxn*), the ubiquitin-protein ligase (*hyd*), the trehalase (*treh*), and the zinc metallopeptidase 13 (*nas 13*) were studied. With respect to the control (i.e. larvae recovered in the stomach), significant differences of expression levels of *trypsin inhibitor*, *glycoprotein*, *hemoglobin*, *sideroflexin 2*, *ubiquitin-protein ligase*, *trehalase* and *zinc metallopeptidase 13,* were observed in *A. simplex* (s. s.) larvae located in the different organs of the fish host. The ANOVA analysis performed on comparative values obtained at the *q*PCR of the *TI*, *GP*, *hb*, *sfxn*, *hyd*, *treh* and *nas 13,* showed that gene expression levels were affected by their infection site in the host.

The results, so far, obtained could suggest the role played by the environment (i.e. site of infection and host immune response) in the paratenic/intermediate host on determining the different expression profiles at some target genes of the parasite *A. simplex* (s. s). The results were reported in **paper no. 2.**

Objective no. 2. The aim was to investigate the mechanisms by which molecules in *A. pegreffii* influences the human immune response through the modulation of DCs. Generally, the human dendritic cells (DCs) show remarkably phenotypic changes when matured in the presence of helminth-derived products. Those modifications frequently elicited a polarization towards Th2 cells and regulatory T cells, thus contributing to an immunological tolerance against those pathogens.

Accordingly, the transcriptomic analysis (RNAseq) was carried out for the first time in *A. pegreffii* larvae maintained *in vitro* co-cultured in the presence and absence of DC. The comparison of transcripts (RNAseq) was performed by high-throughput RNA-sequencing (RNA-seq) technologies, revealing a total of 573 and 628 genes, up- or down-regulated, respectively in presence of DCs. The functional analysis based on gene ontology (GO) classification system and the Kyoto encyclopaedia of genes and genomes (KEGG) database highlighted that genes related to terms “catalytic activity” and “binding” were dominant in *A. pegreffii* larvae in presence of iDCs. Whereas, the “metabolic” and “cellular” process are dominant in the biological process. KEGG analysis showed that the highest number of genes identified corresponding to 5 metabolic pathways: biosynthesis of antibiotics, purine metabolism, thiamin metabolism, drug metabolism and starch and sucrose metabolism. Likewise, GO and KEGG analyses revealed a repository of *Anisakis pegreffii* genes encoding a variety of glycosylated molecules, acting on glycosyl bonds and *hyd*rolyzing O-glycosyl compounds. Understanding the roles of glycoconjugates in host–parasite interactions may provide targets in further interventions in the diagnostic tools and medical treatment on *Anisakis* spp. infection. The results so far achieved were reported in the **paper (in preparation) no. 3.**

Objective no. 3. Among the genes coding for functional proteins of those zoonotic species, genetic variants fixed for alternative nucleotide positions were detected at some gene loci. A novel nuclear gene locus at the metallopeptidase 10 (*nas 10*) (451 bp) was sequenced and validated on a total of N=

218 specimens of the three species of *Anisakis*, collected in fish and cetacean hosts from allopatric areas included in the range of distribution of those parasite species. The specimens of *Anisakis* were first identified by allozymes and sequence analysis of the mtDNA *cox 2* and EF1 α-1 nDNA. The novel nuclear marker showed fixed alternative nucleotide positions in the three species, i.e. resulting to be diagnostic at 100%, permitting the identification at species level of a large number of specimens analyzed. In addition, primers to be used for amplification-refractory mutation system (ARMS) PCR of the same gene locus were designed at those nucleotide positions. Thus, direct genotyping determination, by double ARMS, was developed and validated on N= 218 specimens belonging to the three distinct species. Complete concordance was observed between the tetra-primer ARMS PCR assays and direct sequencing results obtained at the *nas 10* gene locus. The novel nuclear diagnostic marker will be useful in future works of a multi-locus genotyping approach also to study possible hybridization and/or introgression events occurring between the three species in sympatric areas.

Additionally, this new approach has the potential to be used as a rapid, and low cost molecular diagnosis of larvae removed by endoscopy, biopsy or in *Anisakis*-granulomas formed in human cases of anisakiasis (Mattiucci et al. 2017; 2018). The overall results achieved were the object of **Paper no. 4.**

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