Gymnorhynchus gigas **in** *Lepidopus caudatus* (Actinopterygii: Perciformes: Trichiuridae)**: Prevalence and Related Effects on Fish Quality**

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Abstract

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We evaluated the effects of *Gymnorhynchus gigas* on the freshness and hygienic quality of *Lepidopus caudatus*. Total Volatile Basic Nitrogen (TVB-N), Trimethylamine Nitrogen (TMA-N), as well as Specific Spoilage Organisms (SSOs) are the most important freshness indicators in fish. Our study was carried-out on 65 specimens of *L. caudatus* kept in ice and stored at 2°C for different period of time. The microbiological charge of SSOs recovered on a portion of parasitised muscles (MP) was compared with those recovered on portions of parasite-free muscles (M). The contents of TVB-N and TMA-N on MP, M, and *G. gigas* larva/ae were measured using the Conway microdiffusion method. High prevalence (72.31%) of *G. gigas* in the specimens of *L. caudatus* from the Mediterranean sea was observed. No statistically significant differences ($P < 0.05$) between M and MP were found during storage. However, massive infestation of *G. gigas* on the muscle of the silver scabbardfish could negatively influence TVB-N values, without compromising the sensorial characteristic of fish.

Keywords: Trypanorhyncha; TVB-N; TMA-N; muscle parasite; specific spoilage organisms

The silver scabbardfish, *Lepidopus caudatus* (Euphrasen 1788) (Actinopterygii: Perciformes: Trichiuridae), is a mesopelagic species, widely distributed in the Atlantic, Pacific, and Indian Oceans and in the Mediterranean Sea (Demestre *et al.* 1993). The fish presents a laterally compressed, blade-like structure with a highly silvered and scaleless body. *L. caudatus* is well known to be often parasitised by nematodes of genus *Anisakis* and Trypanorhyncha plerocercoid larvae.

The presence of anisakid nematodes was extensively reported in specimens of *L. caudatus* with a high prevalence and intensity of infestation (Klimpel *et al.* 2006; Mattiucci & Nascetti 2007; Cavallero *et al.* 2012; Sola 2012).

Among Trypanorhyncha plerocercoid larvae, *Gymnorhynchus gigas* has been commonly described in the muscle of silver scabbardfish (Panebianco 1953, 1994; Pelayo *et al.* 2009; Panebianco *et al.* 2011). Trypanorhyncha are common parasites of marine elasmobranchs, in which they mature in the stomach or spiral valve. The typical life cycle of Trypanorhyncha includes a copepod as the first host, euphausiid or schooling fish as the second host, and an elasmobranchi as the final hosts (Klimpel *et al.* 2006). The juvenile stages (larva and plerocercoid) are located in the musculature of several commonly consumed teleost in the human diet such as swordfish (*Xiphias gladius,* Linnaeus, 1758) (Manfredi *et al.* 1993; Muscolino *et al.* 2012), ray's bream (*Brama raji,* Schneider & Bloch 1801) (Panebianco 1952; Seyda 1976; Vázquez-López *et al.* 2001b) and silver scabbardfish.

The massive infestation by larvae of the genus *Gymnorhynchus* in *B. raji* can cause changes in the

sensorial characteristics during storage (Panebianco 1952, 1994; Seyda 1976). In *L. caudatus,* the parasites do not seem to cause organoleptic modifications (Panebianco 1953), however, it is reported that they may interfere with the index of freshness (TVB-N and TMA-N contents) (Panebianco *et al.* 2011). The aim of the present study was to improve the knowledge on the prevalencein the silver scrabbardfish of *G. gigas* and its effects on the freshness and hygienic quality during storage.

MATERIAL AND METHODS

The present study was carried out on 65 specimens of *L. caudatus*. Fifty specimens were captured off the coast of the city of Milazzo (Sicily, Italy) (FAO 37.1.2) during October 2011. The remaining 15 specimens came from the Strait of Messina (FAO 37.2.2) in the period from July 2012 to November 2012. The specimens were collected and immediately transported at the refrigeration temperature to our laboratories for analysis. Then they were numbered, measured for the standard length (SL) and body weight (BW). Each *L. caudatus* was then filleted and carefully examined macroscopically in order to evaluate the presence and number of *G. gigas* in the muscle tissue. Due to the known absolute prevalence of *G. gigas* in *L. caudatus,* a representative number of larvae was identified each time on the basis of morphologic characteristics using stereo microscope (Leica M205C; Leica, Milan, Italy).

Thirty of 50 specimens (captured on the same day and condition) were also processed for microbiological and chemical analyses (TVB-N and TMA-N determination). The fish were kept in boxes with ice in cold stores at 2°C in the laboratory and processed after the periods of storage of 0, 24, 72, 120, and 168 hours. From 6 specimens, examined after each period, we proceeded to collect, under sterile conditions the following samples:

MP – pool of portions of the muscles (11.5 ± 1 g) immediately surrounding the location of parasite;

M – pool of portions of the parasite-free muscles (11.5 ± 1 g), collected at least 10 cm away from the parasitic locations;

P – larva/ae extracted from the parasitised muscle.

Due to the variable number of parasites observed, from each group of 6 samples, a significant number of MP, M, and P were collected and immediately processed for microbiological and chemical analyses. A total of 150 samples were obtained after 168 h of storage.

The microbiological investigation was carried out only on the portions (10 g) of MP and M after each period of storage. Each sample was transferred to a stomacher bag and Phosphate-Buffered Saline (PBS) pH 7.4, was added in a ratio of 1 : 10; the samples were homogenised for 120 s at 230 rpm, with a stomacher (Stomacher® 400 Circulator; International PBI s.p.a., Milan, Italy) and tenfold dilutions in PBS were prepared. One ml aliquots were plated in duplicate, in Iron Agar Levine (Oxoid, Milan, Italy) (Gram *et al.* 1987). The count of Specific Spoilage Organisms (SSOs) (black colonies producers of hydrogen sulphide) was determined after incubation at 25°C for 72 hours.

All 150 sample units (1 g) (MP, M, P) were processed for the determination of TVB-N and TMA-N, according to the method of Conway and Byrne (1933). The TVB-N and TMA-N contents were estimated by the method of microdiffusion using Conway cell, as described by Mahmud *et al.* (2007). This method is one of the methods applied in accordance with EC Regulation 2074/05. The extract was prepared by mixing 1 g of the minced/ground fish muscle with 4 ml of 4% trichloroacetic acid (TCA) (Sigma-Aldrich, Milan, Italy) in aqueous solution and was homogenised properly in sovirel. Then it was centrifuged at 3000 rpm for 10 minutes. To the edge of the outer ring of each unit sealing agent was applied (Glicerine; Carlo Erba, Milan, Italy). One ml of 1% boric acid solution containing the indicator (Carlo Erba, Milan, Italy) was pipetted into the inner ring of each unit. Into the outer ring of each unit, 1 ml of the sample extract was pipetted. The indicator solution was made the dissolving 0.01 g of Bromocresol green and 0.02 g of methyl red (both Sigma-Aldrich, Milan, Italy) in to 10 ml of ethanol absolute (Carlo Erba, Milan, Italy). One ml of 50% Potassium carbonate (K_2CO_3) (Carlo Erba, Milan, Italy) solution was carefully pipetted into the outer ring of each unit to prevent any entering into the inner ring, and immediately after the units were covered and closed with clips. The solutions in the units were then mixed gently, to prevent any solution entering from one ring into the other one. The units were placed in an incubator at 40°C for 60 minutes. After incubation, the inner ring solution was titrated with 0.01N HCl (Carlo Erba, Milan, Italy) until green coloured solution turned to pink. TMA-N was determined in the same way as TVB-N but prior to the addition of potassium carbonate (K_2CO_3), 1 ml of 10% neutralised formalin (Carlo Erba, Milan, Italy) was pipetted into the extract to react with ammonia and thus allow only the TMA-N to diffuse over the unit.

For the statistical analysis, all specimens were grouped in quartiles according to the following groups of the parasites number observed (nP): no parasites (first quartile), 1–2 parasites (second quartile), 3 parasites (third quartile), and 4–12 parasites (fourth quartile). Simple linear regression analysis was conducted to determine the relations between the average values of nP for each group and their average standard length (SL), body weight (BW), and condition index (K).

Condition index was obtained applying the Fulton's condition index, calculated using the following equation $K = (W/L^3) \times 100$, where: W – body weight (BW); L – standard length (SL) (Stevenson & Woods 2006; Gabriel *et al.* 2010). Statistical significance between MP and M (TVB-N, TMA-N, and SSOs) was determined by Student's *t*-test ($P < 0.05$).

RESULTS AND DISCUSSION

The standard length (SL) of the fish varied from 6 cm to 125 cm. The mean SL of the fish was 94.00 ± 11.48 cm. These specimens had an average weight of 696.94 ± 292.02 g (range 208–1600 g). Of the 65 specimens observed, 47 (72.31%) showed *G. gigas* larvae in the muscle tissue. On these 47 fish, a total of 135 parasites were counted, with the average of 2.87 ± 2.16 nP for each specimen (range 1–12). No *G. gigas* larvae were observed in the remaining 19 samples (27.69%). On fifty *L. caudatus* (BW: 709.68 ± 234.38 g; SL: 94.94 ± 8.29 cm) from FAO 37.1.2, thirty four specimens (68%) were parasitised by *G. gigas* larvae with a nP of 2.7 ± 1.27. The remaining 15 fish (BW: 654.47 ± 441.66 g; SL: 90.87 ± 18.66 cm) from FAO 37.2.2 showed a prevalence of 86.67% with a nP of 3.31 ± 3.64.

G. gigas larvae appeared like a whitish ribbon structure resembling myosepta, with a variable length ranging from 3 cm to 29 cm. The smaller larvae were usually located in hypaxial muscle tissue of the tail, while longer parasites were observed in epaxial and hypaxial muscles of the entire body of fish. These larvae were often curled upon themselves. Good statistical correlation between the fish length (SL) ($R^2 = 0.8559$), weight (BW) ($R^2 = 0.9121$), K index ($R^2 = 0.9635$), and nP were noted (Figure 1).

Table 1 shows the charge of SSOs during storage in M and MP. At hour 0 the mean value observed for M was 2.27 ± 0.48 log CFU/g and reach at hour 168, the respective value was 5.38 ± 0.39 log CFU/g. The parasitised muscle (MP) showed the mean charge at hour 0 2.81 ± 0.32 log CFU/g, and 5.55 ± 0.53 log CFU/g at hour 168. The mean values were always

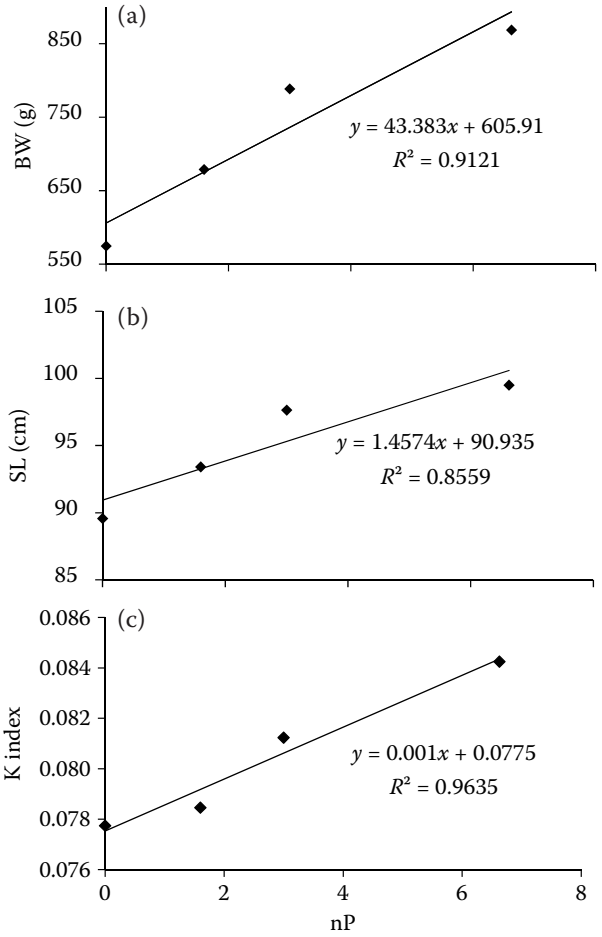

Figure 1. Simple linear regression analysis on the relation between(a) standard length (SL) and number of parasites (nP), (b) body weight (BW) and number of parasites (nP), and (c) condition index (K) and number of parasites (nP) of *L. caudatus*

slightly lower in M than MP, with statistically significant differences observed only after 72 h of storage.

The parasite-free muscle (M) had the initial TVB-N value of 16.04 ± 5.21 mg/100 g while the parasitised muscle (MP) had 19.98 ± 5.97 mg /100 g. At hour 168 of storage, the mean values of TVB-N for M and MP observed were 31.20 ± 5.88 mg/100 g and 31.44 ± 3.86 mg/100 g, respectively. No statistically significant differences between parasite-free muscle and parasitized muscle were observed during storage. The mean values of MP were always higher than those of M, becoming approximately equal at hour 168 of storage (Figure 2a).

No statistically significant differences between TMA-N contents were observed with M and MP, in fact, they showed an equal trend (Figure 2b). The M had the initial TMA-N value 1.04 ± 0.80 mg/100 g and the MP had 1.80 ± 1.38 mg/100 g reaching at hour 168 the values of 9.05 ± 3.29 mg/100 g and of 10.13 ±

Table 1. Specific Spoilage Organisms (SSOs in log CFU/g) in muscle (M) and in parasitised muscle (MP) of *L. caudatus*

	0 h	24 h	72 h	120 h	168 h
Muscle (M)	2.27[a] ± 0.48	2.30[a] ± 0.48	3.46[a] ± 0.56	4.18[a] ± 0.53	5.28[a] ± 0.39
Muscle parasitized (MP)	2.81[a] ± 0.32	2.51[a] ± 0.19	4.62[b] ± 0.38	4.81[a] ± 0.73	5.55[a] ± 0.53

[a a]no statistically significant difference ($P > 0.05$); [a b]statistically significant difference ($P < 0.05$)

3.80 mg/100 g, respectively. TVB-N and TMA-N contents were constant in larvae (P) (Figure 2).

The results of this study show the high prevalence (72.31%) of *G. gigas* in *L. caudatus* coming from the Mediterranean Sea. *G. gigas* larvae increased their number in this species with the growth of fish, as demonstrated by the correlation found between the fish size (standard length and body weight) and nP observed. The high correlation observed between the condition index (K) and nP shows that the presence of *G. gigas* does not influence the normal growth of *L. caudatus*.

This study confirms that the values of TVB-N in MP are higher than in M during the first days of storage, as reported by Panebianco *et al.* (2011). In this regard, the highest values of TVB-N observed in MP are probably linked to the regressive phenomena of the muscle tissue induced by compression, inflammation response and circulatory disturbances related to the presence of parasite. Moreover, the parasite can cause the lysis of the muscle cells by the activation of endogenous proteases, and with various types of endo- and exoproteases that showed activity against various substrates including collagen (Vázquez-López *et al.* 1999; Giuffrida *et al.* 2002).

The trend of TMA-N and SSOs contents in M and MP was almost identical during storage. TMA-N production, in fact, is related to the activity of the fish spoilage bacteria (SSOs) (Dalgaard 1995). *G. gigas* can carry several bacteria in the cuticle or within the larval body. In this regard, Panebianco *et al.* (1997) have isolated from the larvae of this parasite different Gram-negative bacteria, pathogens such as *Yersinia enterocolitica* and *Yersinia pseudotubercolosis*, and SSOs such as *Pseudomonas* spp., *Acinetobacter* spp., and *Erwinia* spp. Moreover, their multiplication could be favoured by the presence of natural inhibiting substances in inside the larvae content, more active against Gram-positive (Panebianco & Giuffrida 1996).

The SSOs growth, in combination with the normal proteolytic activity of the muscle tissue, was responsible for the overlap of TVB-N value for MP and M observed at hour 168. The TVB-N and TMA-N values, observed in plerocercoid larvae (P), were almost constant during the storage period.

Massive infection by *G. gigas* the silver scabbardfish of muscle, could negatively influence TVB-N values, without compromising organoleptic characteristics of the fish. Furthermore, this parasite does not significantly affect the lipid profile of the muscle (total lipids 3.95 g/100 g: 11.68% ω-6 and 27.62% ω-3) (Ziino *et al.* 2002) and does not constitute a zoonotic risk for the consumer. On the other hand, a potential immune-mediated toxicity of the tissues and body fluids of the parasite should be taken into account. The larvae of *G. gigas* possess, in fact, antigenic compounds potentially capable to

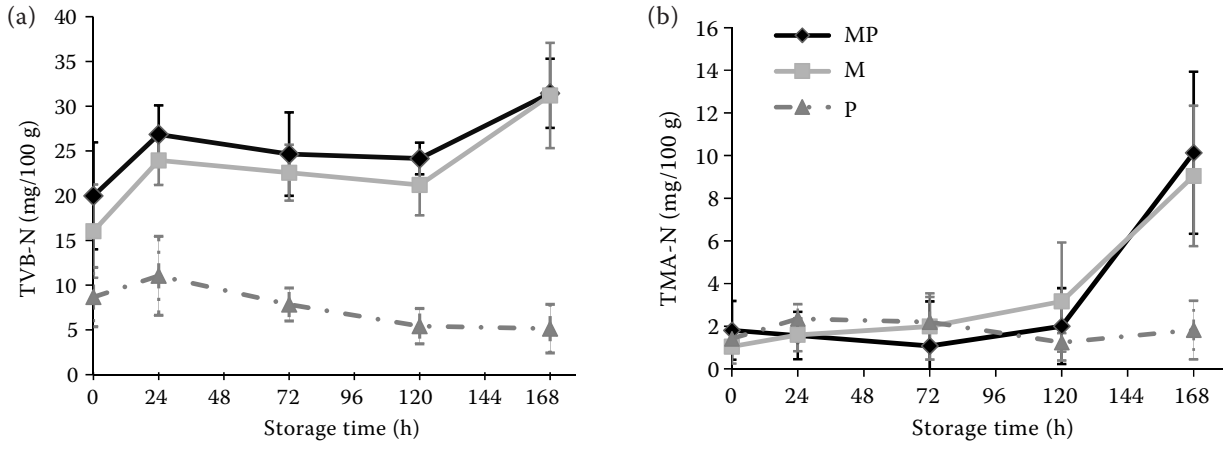

Figure 2. (a) TVB-N and (b) TMA-N value muscle (M), muscle parasitised (MP), and larvae P of *L. caudatus* during storage

produce anaphylactic reactions in laboratory animals (Rodero & Cuéllar 1999, 2000; Vázquez-López *et al.* 2001a). Moreover, more recently a seroprevalence of Anti-*Gymnorhynchus gigas* antibodies in the Spanish population was observed (Pelayo *et al.* 2009).

According to EC Regulation 2074/2005 Annex II, Section I, Chapter I, *G. gigas* can be considered as "visible parasites" (because of their dimensions and morphologic characteristics such as colour or texture which are clearly distinguishable from the fish tissues). Therefore, besides to the potential risks for consumers and considering the repugnant appearance of the parasitised tissues, according to the Regulation 853/2004 EC (Annex III, Section VIII, Chapter V, Part D), the fishery products "that are obviously contaminated with parasites are not to place on the market for human consumption". In this regard, a careful visual inspection by food business operators during filleting, according to Regulation EC 2074/2005, should reduce the sale of the products parasitised by *G. gigas*.

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