Caligus elongatus and Photobacterium damselae subsp. piscicida
Concomitant Infections Affecting Broodstock European Seabass, 
Dicentrarchus labrax, with Special Reference to Histopathological Responses

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Abstract
Caligus elongatus and Photobacterium damselae subsp. piscicida are pathogens of serious infections in European seabass, Dicentrarchus labrax. In this study, both agents were concomitantly isolated from moribund broodstock European seabass cultured within the hatchery unit at El-Max Research Station (NIOF), Alexandria governorate, Egypt. Externally, fish were heavily infested with Caligus elongatus ectoparasitic copepods. The overall prevalence, mean intensity and mean abundance of C. elongatus on examined fish were 92.3%, 23.3 and 21.5: respectively. Majority of samples noticed severe haemorrhages on the external body surface and fins. Internally, moribund fish showed characteristic whitish nodules and extensive adhesions of visceral organs. 88.46% of investigated fish were concurrently found to be infected with P. damselae subsp. piscicida. No other bacterial species were detected. P. damselae subsp. piscicida was also isolated from C. elongatus infesting clinically diseased fish. All P. damselae subsp. piscicida isolates were confirmed by sequencing of the 16S rRNA gene. Microscopically, multiple granulomas were regularly observed in haemopoietic organs. Our results as a whole indicate that C. elongatus may serve as a potential vector for P. damselae subsp. piscicida and possibly enhance photobacteriosis dissemination among co-habiting fish, thus suggesting the desirability of redesigning the protocols presently used for microbial recognition during fish epidemiological studies to improve fish health.

Keywords: Photobacterium damselae subsp. piscicida; Caligus elongatus; Vector; European seabass; Mortalities

Introduction
European seabass, Dicentrarchus labrax, is one of the most valued marine fish species used in fish farming worldwide [1]. Several pathogens can put the life of cultured seabass in jeopardy with consequent detrimental impacts on growth, fecundity and productivity [2]. Photobacteriosis caused by the halophilic bacterium Photobacterium damselae subsp. piscicida, has long been considered among the dominant limiting factors in mariculture all over the world [3]. The disease has caused substantial mortalities in seabass and many other marine fishes with colossal economic losses [3-8].

The pathogenesis of P. damselae subsp. piscicida is not completely elucidated. This pathogen possesses many virulence mechanisms that significantly contribute to the capacity of this bacterium to devastate and overcome fish immune defense mechanisms including a capsule and an iron uptake system [9,10]. Moreover, this pathogen produces variety of destructive extracellular products with phospholipase, cytotoxic, and hemolytic activities that contribute significantly to the development of the disease [8].

The ability of P. damselae subsp. piscicida to invade and replicate intracellularly is a critical issue in the pathogenesis of photobacteriosis enabling this pathogen to evade host defenses as well as decrease the need for adherence to fish surfaces to establish infection [11].

P. damselae subsp. piscicida infections are enhanced with fish ectoparasites infestations as they could facilitate the invasion and settlement of bacteria in fish blood stream [12]. Skin injuries, induced by fish ectoparasites, including sea lice, are effective portals of entry for diversity of opportunistical bacterial infections [13-15].

P. damselae subsp. piscicida is considered an obligate pathogen and its survival is short lived outside the host [16,17]. The transmission of this fish pathogen is not fully understood. A symptomatic carrier and/or reservoir of infection may coexist [18].

Sea lice, Caligus elongatus, feed on host mucus, tissues and blood thereby they could be potential vectors as well as transmitters for numerous pathogens among fish [19,20]. From this perspective, copepods, Caligus elongatus may be involved in dissemination of photobacteriosis infections.

Bacterial pathogens vectored by copepods pose serious threats to aquaculture and human health. Therefore, this study aimed to investigate the link between copepods infestation and P. damselae subsp. piscicida infections in European seabass broodstock with large scale mortalities, and to provide information about the histopathological alterations induced by host pathogens interactions. Additionally with the aim to improve its diagnosis, full phenotypic and molecular analyses were employed to identify P. damselae subsp. piscicida.

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Materials and Methods

Fish sampling

On June 2014, twenty-six moribund broodstock European seabass *D. labrax* were obtained from El-Max Research Station, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. This farm noticed mass mortalities approaching 70% among broodstock seabass reared within concrete ponds. Fish were fed on pelleted diet 45% protein. Samples were preserved in isothermal boxes within ice, to be transferred with the minimum time of delay to Fish diseases Lab. Cairo University. The average body weight of examined fish ranged from 1100 to 1300 gm. All fish were visually inspected for any lesions before the examination is adopted. The average recorded values for salinity, water temperature, pH, dissolved oxygen and ionized ammonia in the investigated farm were 32‰; 25°C, 8.8, 3.6 mg/L and 0.9 mg/L respectively.

Parasitic investigation

Detection of external parasites relied firstly upon visual inspection by naked eye. Furthermore specimens, including the gills, fins, body cavity and internal organs of fish, were also examined in petri-dishes under the dissecting microscope. Identification of copepods was based mainly on characteristic morphological features according to [21]. The prevalence, mean intensity and mean abundance of copepods infestations were calculated using quantitative Parasitology web version 3 [22].

Bacterial isolation

Loopfuls from lesions in liver, spleen and kidney of moribund fish were streaked onto marine agar (Difco), thioulate citrate bile salt sucrose agar (TCBS, Oxoid) and blood agar containing 2% NaCl. Cultures were incubated at 25°C for 48-72 h. Representative inocula of single colonies collected from the plates were re- streaked onto tryptic soy agar supplemented with 1.5% NaCl (v/v) (TSA, Difco) for purity and identification.

The ectoparasitic copepods were removed from infested fish by sterilized forceps then washed three times with saline (0.85% NaCl). Five randomly selected copepods from each fish were processed as one group and homogenized using a sterile plastic rod. The homogenates were serially 10-fold diluted with saline and inoculated onto marine agar and TCBS as described by [13] with minor modification. Identification of retrieved bacterial isolates from both moribund seabass and copepods was mainly performed by using API 20E systems (BioMerieux). Furthermore, sensitivity to 150 mg vibrio static agent (O/129) and motility on soft agar were also investigated [23].

Sequencing of *P. damsel* subsp *piscicida* 16s rRNA gene

Genomic DNA was extracted from cultivated *P. damsel* subsp *piscicida* strains using prepMan Ultra reagent (Applied biosystems, USA) according to protocol supplied. To amplify a 267-bp fragment of the target 16s rRNA gene; the specific primer pair of Car 1:5'-GCTTGGAAGAGATTCCAGT-3', and Car 2:5'-CACCTCGCGGTCTTGCTG-3' was used [24]. The PCR product was directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with 310 Automated DNA Sequencer (Applied Biosystems, USA) using the same primers for annealing. The nucleotide sequences of the 16s rRNA genes of *P. damsel* subsp *piscicida* isolates retrieved from both *Caligus elongatus* and seabass were submitted to the DNA Data Bank of Japan (DDBJ) nucleotide sequence database.

Histopathological examination

Specimens from gills, liver, spleen and kidney of infected fishes were taken for histopathological studies. The trimmed samples were fixed in 10% phosphate buffered formalin for 24 hours, dehydrated by a series of upgraded ethanol solution and embedded in paraffin. Finally, sections were stained with Hematoxylin and Eosin (H & E) to be examined under light microscope [25].

Results

Clinical examination

The prominent clinical findings of moribund broodstock European seabass were lethargy and widespread haemorrhages on the external body surface and fins (Figure 1a). Majority of samples showed haemorrhagic and bleeding vent (Figure 1b). Internally, whitish nodules and extensive adhesions of visceral organs were characteristic.

Parasitological examination

Moribund seabass were heavily infested with *Caligus elongatus* copepods appeared as extensive foci brown spotted lesions on the skin, fins, head region and buccal cavity. No other parasitic infestations were recorded.

Taxonomic summary

*Caligus elongatus* Nordmann (1832)

**Family:** Caligidae Burmeister (1835)

**Host:** European seabass *Dicentrarchus labrax* Linnaeus (1758) (Perciformes: Serranidae)

**Locality:** The hatchery unit at El-Max Research Station, the National Institute of Oceanography and Fisheries (NIOF), Alexandria City, Egypt

**Site of infection:** Skin, fins, head region and buccal cavity of infected fish

Prevalence, mean intensity and mean abundance of infection:

Figure 1: (a) Moribund seabass showing extensive haemorrhages on the external body surface and fins (b) Moribund seabass showing haemorrhagic and bleeding vent.
92.3% of examined fish were infected with 23.3% as mean intensity and 21.5% as mean abundance.

**Bacteriological examination**

88.46% of examined fish were found to be infected. *P. damselae* subsp. *piscicida* was the bacterial species solely obtained from investigated samples. *P. damselae* subsp. *piscicida* was retrieved also from all *C. elongatus* homogenates. No other bacterial species were detected in investigated fish specimens or copepod homogenates. Isolates were Gram negative, non-motile, pleomorphic rod-shaped with characteristic bipolar staining as well as sensitive to O/129 vibriostatic agent (150 mg). All *P. damselae* subsp. *piscicida* isolates obtained from both moribund seabass (23 isolates) and *C. elongatus* homogenates (24 isolates) had unique API 20 E profile, 2 005 004 (Table 1). All isolates reacted positively with the 16s rRNA gene specific primers and yielded the expected size of 267-bp. The accession numbers of sequenced 16s rRNA genes are LC017838 and LC017839 in this study. All sequences results revealed 100% homogenous with that of *P. damselae* subsp. *piscicida* ATCC29690 (GenBank accession number Y18496).

**Histopathological findings**

The tissues of infected fish revealed various proliferative, degeneration and circulatory changes. The circulatory changes included severe congestion, edema, hemorrhage in gills, kidney, liver and spleen. Moreover, diffuse hyperplasia of secondary lamellae is recorded. (Figure 2a).

Different sized granulomas were noticed in between hepatocytes, spleen and renal tubules together with vacuolar degeneration of hepatocytes and renal tubules. Moreover, inflammatory cell aggregation were noticed in pancreatic tissues. (Figure 2b, c and d).

Different granulomas were also noticed in kidney and spleen, with pronounced activation of melanomacrophage centers in spleen and activation of goblet cells in gills. (Figures 2e and 2f).

**Discussion**

In the aim to increase productivity per unit spaces, fish are intensively cultured in ponds and cages consequently they become feasible target for infectious diseases. At the top of the most critical pathogens limiting mariculture expansion, *P. damselae* subsp. *piscicida* and *C. elongatus* rank firstly [5,6,13]. These pathogenic agents are responsible for substantial economic losses among farmed marine fishes worldwide [26,27].

The majority of investigated moribund seabass, 88.46%, were found to be infected with *P. damselae* subsp. *piscicida*. No other bacterial infections were detected. *P. damselae* subsp. *piscicida* isolates were Gram negative, non-motile rod-shaped with bipolar staining and sensitive to O/129 vibrio static agent (150 mg). In addition, all *P. damselae* subsp. *piscicida* strains showed a unique API 20E profile, 2 005 004. All PCR reactions yielded definite amplicons of 267-bp fragment and the sequences of 16S RNA were 100% homogenous with

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### Table 1: Phenotypic and biochemical characteristics of retrieved *P. damselae* subsp. *piscicida* isolates.

| Gram-staining | Gram-negative pleomorphic rod |
|---------------|--------------------------------|
| Bipolar staining | + |
| Motility | Non motile |
| O/129 sensitivity (150 mg) | + |
| Cytochrome oxidase (OX) | + |
| Catalase | + |
| B-Galactosidase production (OPNG) | - |
| Arginine dihydrolase production (ADH) | + |
| Lysine decarboxylase production (LDC) | - |
| Orotate decarboxylase production (ODC) | - |
| Citrate utilization (CIT) | - |
| H2S production (H2S) | - |
| Urea production (URE) | - |
| Tryptophane deaminase production (TDA) | - |
| Indole production (IND) | - |
| Acetoin production (VP) | + |
| Gelatinase production (CEL) | - |
| Acid from glucose (GLU) | + |
| Acid from manitol (MAN) | - |
| Acid from inositol (INO) | - |
| Acid from Sorbitol (SOR) | - |
| Acid from rhamnose (RHA) | - |
| Acid from sucrose (SAC) | - |
| Acid from melibiose (MEL) | - |
| Acid from amygdalin (AMY) | - |
| Acid from arabinose (ARA) | - |
| O/F test | Fermentative without gas production. |
that of *P. damsel* subsp. *piscicida* ATCC29690 (Gen Bank accession number Y18496).

The major part of investigated specimens were concomitantly found to be infested also with *C. elongatus* ectoparasitic copepods, the overall prevalence, mean intensity and mean abundance of *C. elongatus* on examined fish were 92.3%, 23.3 and 21.5, respectively. No other parasitic infestations were recorded [28] alleged that *C. elongatus* is a ubiquitous parasite among fish posing a significant problem for fish aquaculture operations all over the world by reaching high abundances that damage aquaculture.

Strong evidence links potentiated bacterial infections to copepods infestations in fish [19,29,30], either providing portals of entry by damaging fish skin [31,32], or acting as a mechanical vector for numerous bacterial pathogens [19].

Sea lice also trigger diverse detrimental changes to host fish blood including, lymphopenia, anaemia, elevated cortisol level and ion imbalance [33,34]. These outcomes damage fish immunocompetence and predispose them to array of opportunistic pathogens.

Sea lice irritate and injury fish skin by their rasping piston-like mouthparts hence increase mucus secretions thereby provide a rich source of glycoproteins which is critical for bacterial adhesions, a significant step in the pathway of infectious diseases affecting fish [33-35].

Fish ectoparasitic copepods can transmit array of bacterial and viral pathogens a result of their feeding activities on fish blood and tissues [20,36]. Additionally, fish lice boost the spread of pathogenic agents as they infest diverse host species and switch between individuals consequently, transmit these agents to new hosts [37,38].

*P. damsel* subsp. *piscicida* was isolated from *C. elongatus* infesting broodstock seabass highlighting the potential role of sea lice in disseminating photobacteriosis among cultured fish. The indistinguishable biochemical and molecular profiles of recovered isolates irrespective of their source verified the previous hypothesis. This is in accordance with the findings of [39,40], who reported that sea louse, *Lepeophtheirus salmonis* is a potential vector for *Aeromonas salmonicida*.

The significance of environmental stress in the dynamics of fish diseases is renowned. The pathways of fish disease are interrelated and variable factors relevant to invading pathogens, environment and fish should work together in synergism to define the nature of the triggered course of infection since the presence of pathogen alone not sufficient to produce disease [41-43]. The co-existence of unfavorable un-ionized ammonia levels, 0.9 mg/L exacerbated the case and put more pressure on broodstock seabass [44] recommended 0.26 mg/L as a safe long-term limit for un-ionized ammonia in seawater. High ammonia levels enhance microbial infections through suppressing the immune capacity of fish. Phagocytic and clearance efficiency are diminished. As an ultimate fate for the staggering immuno-suppression of fishes inhabiting such conditions, parasitic and bacterial invasion will be the most probable event [45].

Outbreaks of bacterial diseases in fish are induced also by dissolved oxygen (DO) deficiency [46,47] recommended, 5-8 mg/L, as optimal DO concentration for seabass which are far from that recorded in this study, 3.6 mg/L. The virulence of pathogens is exaggerated by exposure of farmed fish to reduced dissolved oxygen levels [48]. Moreover, oxygen consumption increased about twice in seabass when temperature increased from 15 to 25°C [49].

The severity and frequency of photobacteriosis infections boost at high water temperatures similar to conditions noticed in the investigated farm, 25°C, inducing fatal outbreaks in fish ultimately at water temperatures around 22°C [35]. There is also a significant seasonal variation in lice infestations with peak prevalence and intensity values during summer season [50,51].

The detected histopathological alterations were in conformity with our previous study in wild marine fishes [5,6]. The widespread multifocal granulomata in haemobiotec tissues as well as hyperactivity of melanomachrophage centers were frequently detected. These granulomas are thought to be crucial host-protective structures against virulent pathogens in attempt to restrict the expansion of infection by walling off bacteria. Pathogen proliferation and dissemination augment in infections without granulomas formation [52,53]. *P. damsel* subsp. *Piscicida* is also capable of intracellular growth in phagocytic cells. Accumulations of macrophages containing this bacterium block capillary flow resulting in local ischemia and focal necrotic changes [54].

The hyperactivity of goblet cells was also characteristic. This may indicate the dynamic involvement of these cells in the host responses [55]. Considerable evidence suggests that goblet cells have been found to act as an important barrier against many parasitic infections [56].

The recorded circulatory, degenerative and proliferative changes are attributed to the multifactorial virulence mechanisms of *P. damsel* subsp. *piscicida* including its toxic extra cellular products (ECP). These ECP were found to be lethal for different fish species including gilthead sea bream and sea bass. Phospholipases, cytotoxic, and hemolytic activities are among the (ECP) produced by this detrimental fish pathogen [9,57-59].

**Conclusion**

Our results as a whole indicate that *C. elongatus* may serve as a potential vector for *P. damsel* subsp *piscicida* and possibly enhance photobacteriosis dissemination among co-habitant fish, thus suggesting the desirability of redesigning the protocols presently used for microbial recognition during epidemiological studies not only focusing on diseased fish but should also include infesting ectoparasites to improve fish health.

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