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Ecology and biology of the Antarctic giant fish *Dissostichus mawsoni* (Pisces, Nototheniidae): reproduction, trophic role and migrations in the Ross Sea region

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Abstract

Three different studies have been implemented to fill gaps in the knowledge of the Antarctic toothfish (*Dissostichus mawsoni*) life cycle in the Ross Sea. First, presence, abundance and size distribution of toothfish in the shelf, in areas periodically covered by sea ice, were investigated by means of Baited Remote Underwater Video systems (BRUVs), an emerging technology never employed before in such kind of habitat. The study was developed from 2015 to 2018 in McMurdo Sound and Terra Nova Bay, it highlighted the efficacy of the methodology for investigating the species, recording dozens of fish of different sizes. We started to make some inferences on how environmental variables influence the distribution of the toothfish in shelf areas, but even more important, we established a working protocol useful for future works. The second section has been focused on the Second Winter Survey conducted in September-October 2019 onboard of a New Zealand fishing vessel with the aim to investigate toothfish reproductive biology, its spawning period and the eggs development and dispersion. The results obtained, combined with those from the First Winter Survey conducted in June-July 2016, allowed to have for the first time a picture of Antarctic toothfish reproduction: the spawning occurs in a wide area in correspondence of the Pacific Antarctic Ridge, it happens in the middle of the winter, and eggs set during all their development in the surface layer of the water column. The last part of the work put light on the fate of toothfish after spawning, a question arose from the observation of the high percentage of sensibly debilitated individuals (named "axe handle") in the breeding ground. Samples of muscles from six individuals (two "axe handle" and two regular from the breeding area, and two regular from the shelf) were investigated with regard to lipid distribution and composition. The analysis led us to reason on lipid mobilization and usage as energy reserve and/or for neutral buoyancy for reproductive migration, how consistent is their depletion and if toothfish actively feed in the northern area. Different hypotheses on what could cause the frequent poor condition after-spawning and its potential consequences have been illustrated, with the aim to give cues for future insights.

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1. Introduction

1.1 Nototheniodei

The fish fauna of the Southern Ocean is composed of less than 400 species belonging to about 50 families (Duhamel et al. 2014), a relatively small number (2%) compared to the overall diversity of about 25,000 species (Eastman 2005), corresponding to slightly more than 1% of the world's modern fish species. It could be due to the Antarctic environment, which can be considered extreme and with unique features.

The suborder Nototheniodei have undergone specific physiological, biological and ecological adaptations to live in the icy and cold Antarctic waters.

One of the most interesting adaptation of these fish is the ability to synthesize glycoproteins in their body fluids, which prevents them from freezing and allows survival at subzero temperatures (DeVries 1971). This skill evolved about 14 million years ago (Cheng 1998). The capacity to produce antifreeze proteins is also observed in Arctic fish as adaptation to the environment, however the Antarctic fish differ from them in terms of faunistic and ecological features (Eastman 1997).

Another adaptation to aquatic life at high latitudes of the Southern Hemisphere is to reduce the amount of hemoglobin, since the viscosity and temperature are strongly negatively correlated and this leads the body fluids to become more viscous with the cold (Mintenbeck et al. 2012). Example of evolutionary modification are the icefish (family Channichthydae) which are unique among vertebrates devoid of hemoglobin; as oxygen is transported in the plasma (di Prisco 2000, di Prisco et al. 2007).

Due to the need for such a specific adaptation, the Antarctic fish fauna is highly endemic, with 88% of the species confined to the Antarctic Region south of the Polar Front (Eastman 2005). Three main ichthyofaunal subregions are recognized in the Southern Ocean, based on the distribution of the pelagic and demersal fish: the Ice-free Zone, where ice cover is absent all year, lies between the Antarctic Polar Front and the northern limit of the pack ice in winter/spring; the Seasonal Pack-ice Zone between the limit of pack ice in winter/spring and in autumn/summer, including the islands north of the maximum limit of the pack ice; the High-Antarctic Zone, the region immediately adjacent to the continent, which is covered by ice for most of the year (La Mesa et al. 2004).

The major component of Antarctic fish fauna is represented by the perciform suborder Notothenioidei. As it indicated in the updated classification (Duhamel et al. 2014, Eastman and Eakin 2015), this suborder includes 8 families, 44 genera and 139 species (107 Antarctic and 32 non-Antarctic). The Antarctic species of Nototheniodei belong to five families: Nototheniidae, Channichthydae, Artedidraconidae, Bathydraconidae and Harpagiferidae. The other three families are confined to the waters of the sub-Antarctic islands and the Patagonian region of South America.

The uniqueness of the Antarctic fish fauna with its high degree of endemism and a single dominant group is the result of a long evolutionary history of adaptive radiation in isolation at sub-zero temperatures (Mintenbeck et al. 2012). The process of isolation of the Antarctic continent from ancient super continent of Gondwana and the formation of Circum-Antarctic Ocean and Antarctic Convergence started about 30 million years ago, and led to changes in environmental conditions, oceanic circulation and climate.

The fossil record is scarce, but indicates that there has been a complete change of Antarctic fish fauna (Mintenbeck et al. 2012). The ancient, cosmopolitan and temperate last Eocene fauna had been replaced by a more modern one, highly endemic and perfectly adapted to the very low water temperatures.

The notothenioid's ancestors evolved from benthic fish which lived in temperate coastal waters of the ancient supercontinent Gondwana, they have been involved in a gradual adaptation to the increasingly extreme environments and they were able to occupy a wide variety of ecological niches, developing physiological and biochemical features. The absence of competition and trophic factors have allowed nototheniods to fill ecological niches, which in temperate oceans are occupied by taxonomically diverse fish (Eastman 1988).

They have undergone evolutionary alteration of buoyancy and morphology associated with swimming and feeding in the water column; although lacking swim bladders, some nototheniods underwent a process of diversification directed away from the ancestral benthic habitat toward pelagic or partially pelagic (Eastman and Clarke 1998, Vacchi et al. 1999). During evolution, the transition to pelagic life is accompanied by morphological and physiological changes, which enable them to have a slightly positive buoyancy, including the demineralization of skeletal apparatus with a high proportion of cartilage, which is less dense than bone (DeVries and Eastman 1978) and the storage of large amounts of lipids.

1.2 The Antarctic tootfish *Dissostichus mawsoni*

In the dominant taxonomic group of the Southern Ocean, the suborder Notothenioidea, mainly characterized by benthic medium-size fish, the two species of the genus *Dissostichus* are distinguished by their necto-benthic habits (they are able to cover even long distances swimming in the water column) and for the large size, reaching 2 m in length and more than 100 kg in weight.

Dissostichus mawsoni (Fig 1) and *D. eleginoides* play crucial rules as high level predators in the Antarctic and sub-Antarctic trophic webs. Currently, the two species represent important fishing resources in the Southern Ocean. *D. eleginoides*, the "Patagonian toothfish", which is primarily distributed in sub-Antarctic areas, has long been subject of commercial fishing. *D. mawsoni*, known as "Antarctic toothfish", is distributed in more strictly Antarctic, and less accessible, areas, and is become object of commercial fishing more recently. Both these species are commonly marketed with the commercial name of "Chilean seabass".

Fig 1. Antarctic toothfish (*Dissostichus mawsoni*)

Since its inception, the commercial fishing directed to so far little biologically and ecologically known species, such as *Dissostichus* spp., and belonging to a so particular and largely unexplored environment, raise concern with regard to sustainability and management aspects. The Commission for the Conservation of Marine Living Resources (CCAMLR), which is the body in charge to regulate and manage fishing in the Southern Ocean, has over time developed *ad hoc* models for toothfish exploitation, necessarily different from those commonly used in other areas of the world, and based on an ecosystem-based approach.

Such an approach is particularly suitable for Antarctic toothfish fishery, a high-level predator of the Antarctic ecosystems, on which different key biological and ecological aspects remain to be clarified. The research here presented fits into such context, and it has as main objective to contribute to the improvement of the knowledge on the Antarctic toothfish in the Ross Sea, a crucial area interested by resources conservation and management issues, so that in 2017 the Ross Sea Region Marine Protected Area (RSR-MPA) has been established for a period of thirty-five years. It is to date the widest marine protected area in the world.

The Ross Sea region, conventionally defined by the 60°S parallel, 150°E and 150°W meridians, and the corresponding coastline of Antarctica, is the Southern Ocean region in which Antarctic toothfish fishery is most concentrated. Such activity started in 1997, it is practiced by means of bottom longlines, and counts the annual action of more than 20 fishing vessels from about 10 different countries. Genetic analyses and tagging-recapture studies have allowed to identify in the RSR a specific stock of Antarctic toothfish (Kuhn and Gaffney 2008, Parker et al. 2013, Mormede et al. 2014), a highly relevant information for management purpose.

The stock assessment of Antarctic toothfish in the RSR has been developed in the last decade, mainly thanks to data and samples collected by means of fishing vessels operating in correspondence of the continental slope. Instead, little was known on the biology of the species in different habitats, such as the continental shelf or the northern oceanic feature, which are areas of great importance in the life cycle of the species, as feeding and growing sites for sub-adult fish and breeding grounds, respectively.

1.2.1 Life cycle and reproductive biology

Following the most reliable theories on the life cycle of the Antarctic toothfish, e.g. the one proposed by Hanchet et al. (2008), it spans a wide geographic range that cover almost the whole RSR (Fig 2). The spawning occurs during the austral winter at the northern oceanic features in correspondence of the Pacific Antarctic Ridge, from there, pelagic eggs and larvae would be transported by the Ross Sea gyres. The juveniles in the first life stages (lasting about 20 months) would live in pelagic environment, until the settlement in the eastern sector of the continental shelf, where they would change habits, passing to a demersal life. For Antarctic toothfish, the shelf would represent a growing ground from sub-adult to adult stage, a process that last about 10 years (until the fish reach a size of about 1 m), during which the species face an ontogenetic westward migration. When

adult, the toothfish would move to the slope, a highly productive area reach in prey in which they abundantly feed and fatten enough to become neutrally buoyant, a condition that allow to migrate towards the breeding ground of the northern oceanic features moving in a pelagic environment assisted by sea currents (Near et al. 2003, Ashford et al. 2012). In the hypothesized life cycle, after spawning the toothfish could return to the feeding areas to re-migrate in sequent years.
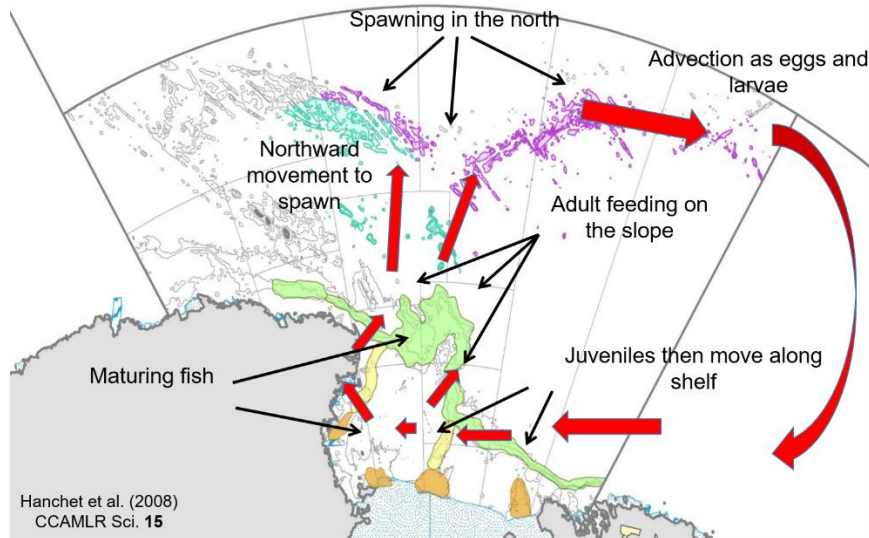
Fig 2. A life cycle model of the Antarctic toothfish Ross Sea region's population.

The proposed life cycle was overall plausible, but at the moment of the starting of the studies here proposed, some notions lacked of information and insights. For example, it was not known what were the movements of eggs and larvae along the water masses, how often an individual can breed (the high investment of energy required for migration and reproduction lead to suppose that the frequency is not annual), if after reproduction the toothfish have possibilities to return back to the slope or they remain in the north, what determines their distribution along the shelf.

1.2.2 Trophic ecology

The available information indicates that the diet varies with the variation of size and geographical area, but is always composed primarily by fish, followed by cephalopod molluscs and crustaceans (Stevens et al. 2014): in the slope toothfish prey mainly Macrouridae, Channichtyidae and Muraenolepididae, and the glacial squid (*Psychroteuthis glacialis*); in the stomachs of toothfish sampled in the northern features

still Macrouridae have been found, the violet cod (*Antimora rostrata*), and occasionally epipelagic and mesopelagic fish; it is already scanty the knowledge on the diet of Antarctic toothfish in shelf areas.

Antarctic toothfish may also behave as a scavenger (Carlig et al. 2018), in the stomachs they have also been found fragments of large cephalopods and penguin in the Ross Sea (Fenaughty et al. 2003, Roberts et al. 2011), and of seal and flying birds in the Lazarev Sea (Petrov and Tatarnikov 2011).

Stable isotopes and fatty acids analyses recently carried out on samples from the Ross Sea and eastern sectors put in evidence how Antarctic toothfish is a high level predator (Pinkerton and Bradford-Grieve 2014, Park et al. 2015). Among its predators, they are known the Weddell seal (*Leptonychotes weddellii*), the killer whale (*Orcinus orca*) and the sperm whale (*Physeter macrocephalus*), even if current information leads to think that toothfish does not represent a relevant part in their diet (Pinkerton and Bradford-Grieve 2014).

1.3 Main objectives and organization of the thesis

General aim of my work was to fill some of current gaps in our knowledge on the life cycle of the Antarctic toothfish and to contribute to an improved management of the species by CCAMLR. The research was focused on the population of the Ross Sea region, main area where the Antarctic toothfish is commercially fished.

In this area, most of the available information is derived from the fisheries, and few fishing independent data are available. This translates into seasonally biased information, mainly relative to the austral summer months, and spatially biased information, mostly from ice-free areas of the continental slope.

In present work, efforts have been directed towards obtaining novel information on the biology and ecology of the Antarctic toothfish specifically aiming at: i) enlarging the spatial coverage by including ice-covered shelf areas; ii) increasing the temporal coverage by targeting Antarctic toothfish during winter time; iii) contributing to an improved understanding of functional mechanisms underlying the toothfish life cycle. Accordingly, present thesis is organized in three sections.

The first section report on the main results of investigations carried out by means of Baited Remote Underwater Video systems (BRUVs) deployed under the fast ice in areas belonging to the RSR-MPA, close to the New Zealand Scott Base and the Italian Mario Zucchelli Station. The use of BRUVs is an emerging methodology to study the fish fauna whose application rarely interested polar waters, and never high-Antarctic environments under the sea ice prior to present work. The samplings, made during different New Zealand and Italian expeditions from 2015 to 2018, allowed to collect for the first time video footages of Antarctic toothfish in its habitat, and to investigate presence, abundance and size distribution of the species in previously unexplored shelf areas. To lay the foundation for inferences on what factors may influence the distribution of the species in shelf areas, the results have been related to environmental variables.

The second part of the thesis report on the main results of the Second Winter Survey, carried out in 2019 under the coordination of New Zealand. The survey, built on the Winter Survey 2016, was carried out on board of the New Zealand longline fishing vessel FV *Janas*. The possibility to collect sample during the austral winter, provided me with the unique opportunity to investigate and document the Antarctic toothfish reproduction. Because of the logistical constraints, reduced daylight time, low temperature and inclement weather condition, no fishing activity has ever been performed during such

period prior to the first Winter Survey, in 2016. In the first Winter survey, carried out in June-July, the collection of running ripe male and female toothfish, and embryonated toothfish eggs in the plankton, allowed to demonstrate spawning in correspondence of the northern features and to conduct trials to investigate eggs buoyancy (Stevens et al. 2016, Parker et al. 2019b). However, spawning fish were only found in one location near the end of the survey, and the eggs were early in the developmental process, thus the spatial distribution of spawning, its overall duration, as well as the distribution and buoyancy of eggs remained undetermined. In 2019, the survey was conducted three months later than the 2016 was, and extended in terms of geographical coverage. Following the classical CCAMLR ecosystem-based approach, also a series of ancillary studies were implemented to characterize the biology of Antarctic toothfish in the area during winter.

The third section of the thesis addresses an aspect of the Antarctic toothfish biology, of particular interest for the scientific community that supports the management of the species, the occurrence of "axe handle" individuals in the northern breeding ground (Fenaughty et al. 2008). "Axe handle" toothfish are individuals in very poor condition. The relatively high percentage of "axe handle" in the breeding area, led to hypothesise that the phenomenon could be somehow related to the reproduction. In particular, the poor condition of individuals might result from the combination of long exhausting reproductive migration and the high investment in gonadogenesis. However, low availability of prey in the northern area might also contribute. Here, the energy expenditure and feeding activity of Antarctic toothfish was investigated by the analysis of lipid distribution and composition along the body of the fish. Furthermore, variations among individuals in different conditions, and sampled both in the northern and in the southern areas were considered. Specifically, four toothfish, two "axe handle" individuals and two individuals in good condition, were collected during the Winter Survey 2019, and two individuals in good condition were sampled during a summer survey on the Ross Sea shelf. This study represents a first step in delineating the fate of post-spawning Antarctic toothfish.

The work was developed under the Italian National Antarctic Research Program (PNRA) in the framework of the project PILOT (Pieces in place for a research and monitoring program targeting the two key fish species of the establishing Ross Sea MPA) and with the support of the CCAMLR Scientific Scholarship for early career researchers.

2. Investigating the Antarctic toothfish under the fast ice by means of baited remote underwater video systems (BRUVs)

2.1 Introduction

The Antarctic toothfish (*Dissostichus mawsoni*) is the largest notothenioid fish inhabiting Antarctic continental waters, where it is a keystone species in the food web as a high trophic level predator. Since the late nineties, this species is targeted by licensed fisheries in the Southern Ocean operating in a regulatory framework defined and managed by the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR), according to an ecosystem-based precautionary approach (www.ccamlr.org).

Such an approach requires solid science data as foundation for adequate management decision (Long et al. 2015). For the Antarctic toothfish, biological and ecological information has mostly been collected by observers onboard of commercial fishery vessels (Mormede et al. 2014, Hanchet et al. 2015).

In the Ross Sea region, a large toothfish population has been identified based on genetics and mark-recapture studies (Mormede et al. 2014). Such a population is presumed to complete its life cycle inside the region, covering a wide geographic range from the spawning habitats in northern areas, to the feeding grounds in the slope and coastal shelf zones (Hanchet et al. 2008, Hanchet et al. 2015, Parker et al. 2019a). However, while data on size, distribution, diet and reproductive status of Antarctic toothfish from vessel-based surveys accumulated, it became evident their temporal and spatial bias.

The possibility to collect data by the use of extractive methods is indeed limited in areas where vessels cannot access, such as the case of the Ross Sea shelf region where fishing is periodically prohibited by regulations, or constrained by the occurrence of fast ice. Because of such constraints, knowledge on the Antarctic toothfish abundance and distribution in the Ross Sea shelf areas is still largely incomplete. McMurdo Sound and Terra Nova Bay are the only shelf areas where vessel-independent fishing-based sampling have been performed through vertical longline fishing from the sea ice (Eastman and DeVries 2000, Ramorino et al. 2004, Ainley et al. 2013, Parker et al. 2016, Parker et al. 2019a). Nevertheless, the making up of large holes in the sea ice, necessary to take out large toothfish, is time consuming and requires the use of large and heavy equipment,

both characteristics making such an extractive methodology logistically demanding in high Antarctica.

In order to overcome those logistic limitations, and to improve the collection of good quality data on the Antarctic toothfish in sea ice covered shelf areas, we undertook a study to test the efficacy and reliability of non-extractive methods.

Non-extractive methods for the investigation of marine fauna include acoustic and visual techniques. However, while underwater acoustics is largely used for studying zooplankton as well as pelagic and semi-demersal species, it results poorly effective for organisms that reside or move close to the bottom (Mallet et al. 2014), such as the Antarctic toothfish. Conversely, underwater video techniques allow the collection of data on abundance and distribution of target species both in the water column and close to the bottom, according to the experimental design; furthermore, they allow to collect a suite of side information on the species' behaviour, habitat, as well as intra- and inter-species relationships (see Mallet et al. 2014 for a review).

Various underwater video techniques have been set up and applied worldwide in recent years, including Baited Remote Underwater Video systems (BRUVs). The methodology itself is conceptually simple and based on a recording video camera that documents the arrival of organisms attracted to a baited lander (Cappo et al. 2006, Bassett et al. 2011). Such a technique, which minimizes observer biases and gear selectivity associated with other survey methods, is likely appropriate for fishes such as the Antarctic toothfish, characterized by good olfactory capabilities (Ferrando et al. 2019) and with benthic scavenger feeding habits (Carlig et al. 2018). BRUVs allow video documentation of species occurrence, size, arrival time, and behaviours. The systems can be used to compute relative abundance metrics (Bailey et al. 2007), provided that the bias associated with counting individuals that enter the field of view multiple times is limited (Dunlop et al. 2015). Since the mid-1990s, BRUVs have been used in temperate, tropical and subtropical areas, mostly to assess the effect of Marine Protected Areas, look at species behaviour, or assess changes in fish assemblages (Ellis and DeMartini 1995, Mallet et al. 2014, Whitmarsh et al. 2017). In cold high-latitude waters, BRUVs have been so far less employed. In northern polar environments, BRUVs were used in the marine waters of the northern Canadian territory of Nunavut (Devine et al. 2018, Devine et al. 2019). Only few baited camera deployments were performed in the Southern Ocean prior to present work, none in shelf areas under Antarctic sea ice. An autonomous lander was deployed around South Georgia and Falkland Island in the attempt to estimate the abundance of the

congeneric Patagonian toothfish (*Dissostichus eleginoides*) independently from the fishery catch data (Yau et al. 2001). A SCUBA diver positioned baited camera was set in shallow waters at Adelaide Island aimed at studying the response of scavengers to feeding cues in the area (Smale et al. 2007). A baited underwater camera system was used to examine the distribution of scavenging fauna in relation to the spatial variation in exposure to iceberg scouring within Ryder Bay, in the West Antarctic Peninsula (Dunlop 2013). In those cases, the baited camera systems were voluminous and/or needed to be set underwater by a SCUBA diver, both characteristics that make those systems unsuitable for working from the sea-ice.

Here, we report on the pilot use of a custom made simple and compact BRUV system, deployed down to more than 500 m depth from small holes in the sea ice, and provide the first data on the Antarctic toothfish abundance and distribution in ice-covered shelf areas at McMurdo Sound and Terra Nova Bay (Ross Sea, Antarctica). The results demonstrate the efficacy of the BRUV application in such areas and represent a first step towards the optimization of this non-extractive methodology for use in Antarctic field activities, especially relevant in the Ross Sea region, where a Marine Protected Area has been established in 2017.

2.2 Materials and methods

2.2.1 Study sites

The study was part of a New Zealand-Italian collaborative activity conducted in late spring from 2015 to 2018. In 2015 the survey was carried out at McMurdo Sound, near the New Zealand Scott Base; in 2016, 2017 and 2018 the activity was carried out at Terra Nova Bay and Wood Bay, near the Italian Mario Zucchelli Station (MZS) (Fig 3).
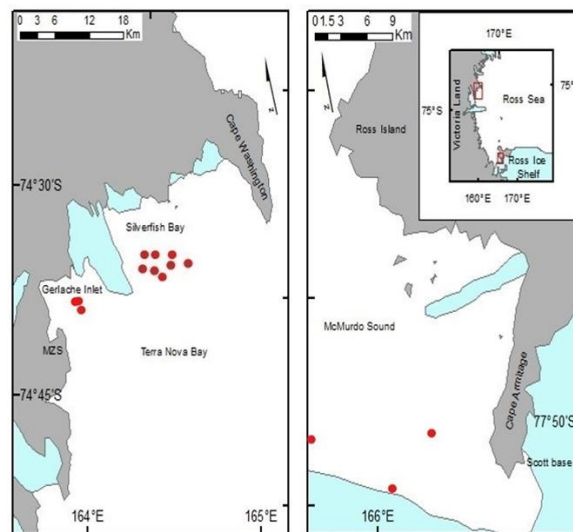
Fig 3. Maps of the studied sites in Terra Nova Bay and McMurdo Sound. Ice tongues or ice shelves marked in light-blue colour. The sampling stations (red dots), are in areas covered by fast ice during late spring.

The sampling sites were located in the continental shelf of Victoria Land, in the western Ross Sea. This shelf has a mean depth of about 500 meters, is broad and presents an undulating topography with relief and deeper zones modelled by tectonics, volcanism and geomorphological action of drift and glacial ice. The shape of the seafloor and depth control the currents and along the shelf are mainly represented by the courses of the Ice Shelf Water (ISW) and the water produced beneath the freezing sea ice (Ross Sea Shelf Water, RSSW). In warmer months, as those in which our samplings occurred, they are usually weak, less than 0.10 m/s, and flow in a north-south direction (Vanney et al. 1981). All sampling sites occurred through the fast sea ice, north of the Ross Ice Shelf. There the ice is approximately 2.5 m thick and covers the sea surface for 9-10 months of the year, from March until January. The northernmost areas were those of Wood Bay and Terra

Nova Bay, separated by Cape Washington. They are characterised by a seafloor consisting of granitic rock and softer substrates composed of coarse sands or gravels near the coast, and calcareous clay and sandy silt in deeper shelf sectors (Vanney et al. 1981, Malandrino et al. 2009). Samplings in Terra Nova Bay were performed in two different subareas, Silverfish Bay and Gerlache Inlet. The former, part of Closs Bay (Antarctic Specially Protected Area, ASPA n. 173) comprised from Cape Washington and the Campbell Glacier Tongue, has a heterogeneous seafloor along the shelf, alternating ridges and deeper areas. The seafloor of Gerlache Inlet, south of the Campbell Glacier Tongue, has the shape of a large trench. The southernmost considered area, McMurdo Sound, is located at the border of the Ross Ice Shelf, and it is comprised in the Ross Island district, consisting of a prominent volcanic complex surrounded by an almost uninterrupted peripheral depression (Vanney et al. 1981).

2.2.2 BRUV system

The BRUV system assembled for the survey consisted of two cylindrical housings made of Delrin® POM (DuPont™) with 70 mm external diameter and 250 mm in length. At the end of one cylinder was a 15 mm thick, flat acrylic camera and light sensor port. The other end a flat Delrin port with a Seacon Electrical Wet-Mate bulkhead connector. This cylinder held a full HD Mobius camera (with a 64 Gb memory card and an Arduino Micro microcontroller board), and a NiMH battery pack. The other cylinder held a commercial MR16 LED lamp (6 Watt, 12 Volt, cool white), and a dedicated NiMH battery pack. The two cylinders were fixed in parallel, and connected by cable and wet mate connectors (Seacon Electrical Wet-Mate) (Fig 4). The system was activated by plugging in the power connector. The Arduino microcontroller was programmed to switch the camera and light 'on' or 'off' following a predetermined protocol. In this configuration, the system could record and provide light continuously for seven hours, but it could be programmed with an on/off protocols to prolong the functioning time for up to three days. During field work, the BRUV system was connected with a nylon rope (3 mm) to an 8 kg steel clump weight. Small pressure-rated trawl floats were added above the BRUV to suspend it 2 m above the weight with the camera view facing vertically towards the seafloor, obtaining a field of view of about 2 m in diameter. The system was slowly deployed to the seafloor by hand or with a winch and worked for various durations ranging from about 1 to 19 hours. The camera was programmed to record at intervals to save battery life. Approximately 1 kg of arrow squid (*Notodarus* sp.) bait was used, a species also used in

the longline fishery. The bait was tied a fixed distance (2 m) below the camera and in camera view.
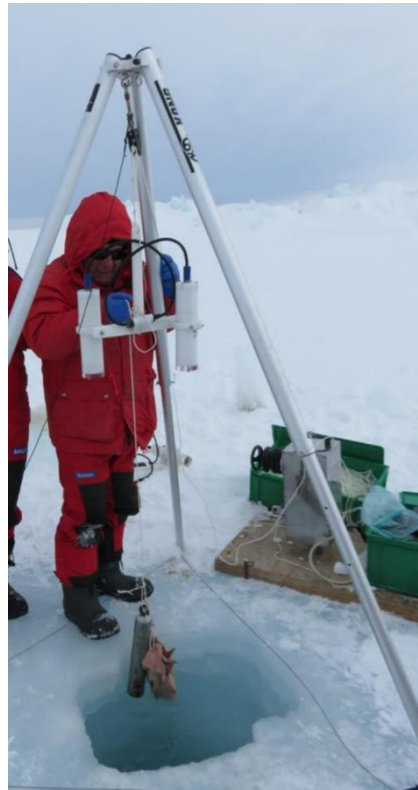
Fig 4. Essential structure of the BRUV system used in present work. The prototype was deployed through an ice hole of about 40 cm diameter.

2.2.3 Sampling design

The collection of data in 2015, 2016, and 2017 was opportunistic; in 2018 sampling followed a randomised survey design.

Each locality included different sampling stations (3 in McMurdo Sound, 3 in Gerlache Inlet, 8 in Silverfish Bay, 1 in Wood Bay). In Gerlache Inlet, one station has been replicated twice and another one three times, and in Silverfish Bay one station has been replicated three times, for a total of 20 sampling events. At each station the following environmental characteristics were taken into the account: depth, geomorphology, substrate type, benthos coverage of the seafloor. Depth was measured by means of the combined deployment of an echosounder during the video recording at each site. Three depth levels were targeted across sampling sites: <350 m, 350-500 m, >500 m. Geomorphology was defined as 'ridge' or 'trench' according to the available information

from bathymetric maps. The substrate type and the coverage by benthic organisms were evaluated through image analysis of the sea floor at the deployment site. Given the different grades of fine sediment were not easily distinguishable from video footages, the substrate types of clay, silt and/or sand were categorized as "soft" and the term 'mixed' was used when rocky formations were visible. The benthos coverage was estimated according to two % categories, '<50%' and '>50%'. Examples of seafloor features are shown in Fig 5.
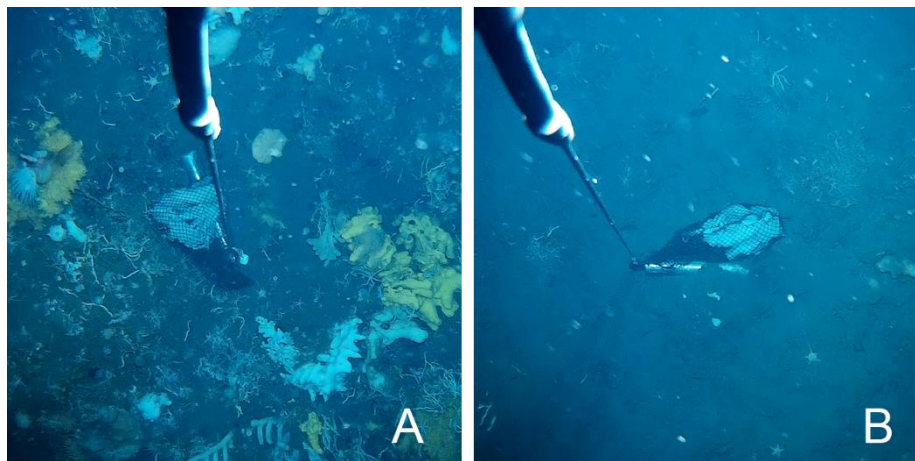
Fig 5. Examples of seafloor features. (A) mixed substrate with >50% benthos coverage; (B) soft substrate with <50% benthos coverage.

2.2.4 Video analysis

Videos were screened in full with VLC Media Player 3.0.5 Vetinari Software.
Individual identification was performed by extracting the best frames from the video, each time a toothfish was within the field of view, and establishing individual and unambiguous key features such as colour patterns, presence of scars or of other recognizable marks (e.g. parasites) (Fig 6). All frames within a video were collected in a library and compared to differentiate between individuals across deployments.
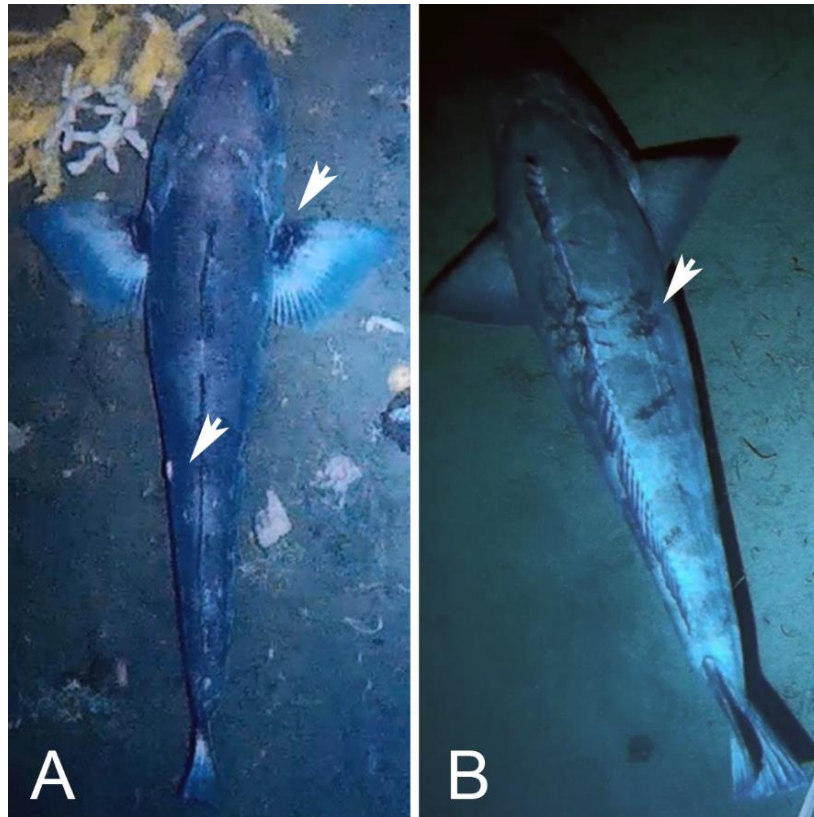
Fig 6. Examples of identifying marks on toothfish individuals. (A) distinctive colour pattern of the pectoral fins and presence of a parasite on the left side of the trunk; (B) dark spot pattern along the body.

For each individual sighted in the video, the size was estimated by comparing the length of the fish with the 33 cm long cylindrical weight lying flat on the seafloor. In order to minimize the effect of potential biased measurements, due the above approximation, we categorised the fish length into three length classes following Hanchet et al. (2015), corresponding to immature (L<100 cm), maturing (L=100-130 cm), and mature fish (L>130 cm). Behavioural traits were investigated by analysis of intra-specific interactions in the proximity of the bait as potential bias in the abundance estimates (Dunlop et al. 2015). When more than one individual was present in the camera field of view, the behaviour of each one was annotated and classed as 'neutral' or 'agonistic aggressor', or 'agonistic subordinate'. For each agonistic event, which means each kind of intentional aggressive action between individuals, the reaction of the aggressor and the subordinate fish was described as: 'stay', when the fish did not appear altered by the event and did not move; 'weak reaction', when the fish appeared disturbed by the event showing rapid movements or slight escapes but returned under the field of view after few seconds; 'escape', when the fish moved away and did not return in proximity of the bait. The size

classes of the individuals involved in the different behavioural events were considered in the analysis.

2.2.5 Relative abundance estimates

Response variables were derived from the number and the time of arrival of fishes observed in each video as proxies of relative abundance.

The Catch Per Unit of Effort (CPUE) is an index widely used in fishery research, and adapted to the BRUV analyses (Brooks et al. 2011, Barord et al. 2014). The CPUE was calculated as the total number of fish recorded, divided by the time of the video recording, and expressed as number of fishes per hour. CPUE, also named 'Attraction Rate', is not widely used in BRUV analysis, because unbiased value requires to identify each individual fish that enters the field of view of the camera during the entire set duration. This is especially difficult where large numbers of individuals are present, as it is often the case in shallow tropical or temperate waters, or for species whose individuals are not easily distinguishable. Given the relatively low number of individuals entering the field of view of the camera of our system, and the possibility to identify individuals based on size, individual colour patterns, presence of scars or of other recognizable marks, potential bias in the use of CPUE to compute the Antarctic toothfish relative abundance in current investigation are limited.

The Maximum Number (MaxN) is the most common metric used in BRUV analysis, e.g. (Langlois et al. 2012, Lowry et al. 2012, White et al. 2013, Santana-Garcon et al. 2014, Udyawer et al. 2014, Schmid et al. 2017). It is the maximum number of fish in a single frame during the entire recording, and is particularly used for dense and/or multispecies shoals of fish (Willis et al. 2003, Cappo et al. 2007, Harvey et al. 2007, Colton and Swearer 2010, Gladstone et al. 2012). The advantage of this metric is to avoid recounts of same individuals that leave the field of view of the camera and return, but it can under-estimate the true abundance of fish visiting the bait since only a portion of the fish visiting the bait contribute to MaxN (Cappo et al. 2003); for these reasons it is sometimes referred to as 'Minimum Count'.

The Time of First Arrival (TFA), in this work calculated in minutes, is the time that passes from the settling of the BRUV on the seafloor and the first record of a target species. It is considered to be related to fish abundance (Cappo et al. 2004, Stoner et al. 2008). In some reference works, abundance was calculated by means of a formula that combined TFA with other parameters, current speed and fish swimming speed, which influence plume

diffusion and the time necessary to fish to reach the attractor (Yau et al. 2001, Priede and Merrett 1996). For our work we had not any tool to measure the current speed and we have some concern to how realistically measure fish swimming speed, so we used TFA itself.

The Mean Number (MeanN) is the average of MaxN taken from set intervals (Stobart et al. 2015). To apply it, we divided the videos into 1-hour intervals, obtained the pertaining MaxN, and then calculated the average. Final segments of each video shorter than 1 hour were discarded.

The continuous Mean Count (cMeanCount) is derived from the MeanCount (Stobart et al. 2015, Campbell et al. 2015) and it is calculated as the mean number of fish observed in a series of snapshots over a viewing interval, it can be constructed using a continuous viewing protocol (viewing snapshots of every second and integrating them over the duration of the recording) or with counts that are taken at regular intervals, to reduce viewing time when the videos are long. In situations where there are few fish, infrequent snapshots could lead to an underestimate of the density of fish. Therefore, we modified the metric, transforming it to a mean of the number of fishes sighted in continuous video segments. The selected intervals were 1-hour long. Final segments of each video shorter than 1 hour were discarded.

2.2.6 Statistical analyses

To investigate any possible decay of attractive effect of the bait, and to develop optimum deployment time, when more than two toothfish were counted the cumulative number of fish was plotted throughout the set duration to examine rate of accumulation.

The relationships among the considered metrics was explored by performing a multiple Spearman rank correlation analysis.

Generalized additive models (GAM) set with Gaussian family and R^2=0.05 were used to identify which environmental factors may influence fish abundance. They were firstly applied for each response variable including all the factors, and subsequently considering only those resulting relevant in the first analyses. To fit better with Gaussian distribution, the values were *log(x+0.01)* transformed.

The same environmental factors were analysed to investigate their influence on Antarctic toothfish size distribution among the video-sampled shelf areas. A Correspondence Analysis with respective Factor Map was conducted on the percentages of individuals of the different size classes per sample.

2.3 Results

A total of 20 BRUV deployments have been considered in this pilot study, 3 in McMurdo Sound, 6 in Gerlache Inlet, 10 in Silverfish Bay, and 1 in Wood Bay. The duration of the samplings has been highly variable, from less than 1 hour to more than 19 hours, with a mean soak time of 05h 47m 29s. Most of the samplings (11) were performed in the 350-500 m depth range, 5 samplings at depths >500m, and 4 samplings at depths <350m. The seafloor at the studied stations was more frequently characterized by soft substrate (14) and benthos areal coverage <50% (14). The geomorphological features categorized as trenches were more commonly found than the ridges (14 and 6 cases, respectively).

From the deployments, 71 occurrences of Antarctic toothfish were recorded.

The analysis of the attractive effect of the bait was performed in the videos in which more than two toothfish were recorded (Fig 7). Linear regression has high R^2 values at all the seven sampling sites investigated, and no plateau appears in the rate of accumulation, possibly indicating a protracted effect of the bait over time, up to at least six hours. Therefore, the entire duration of sampling was considered in the following analyses. Only two times the counts were stopped before the end of the recordings since the bait was totally consumed by scavenging amphipods, or taken away by a toothfish.
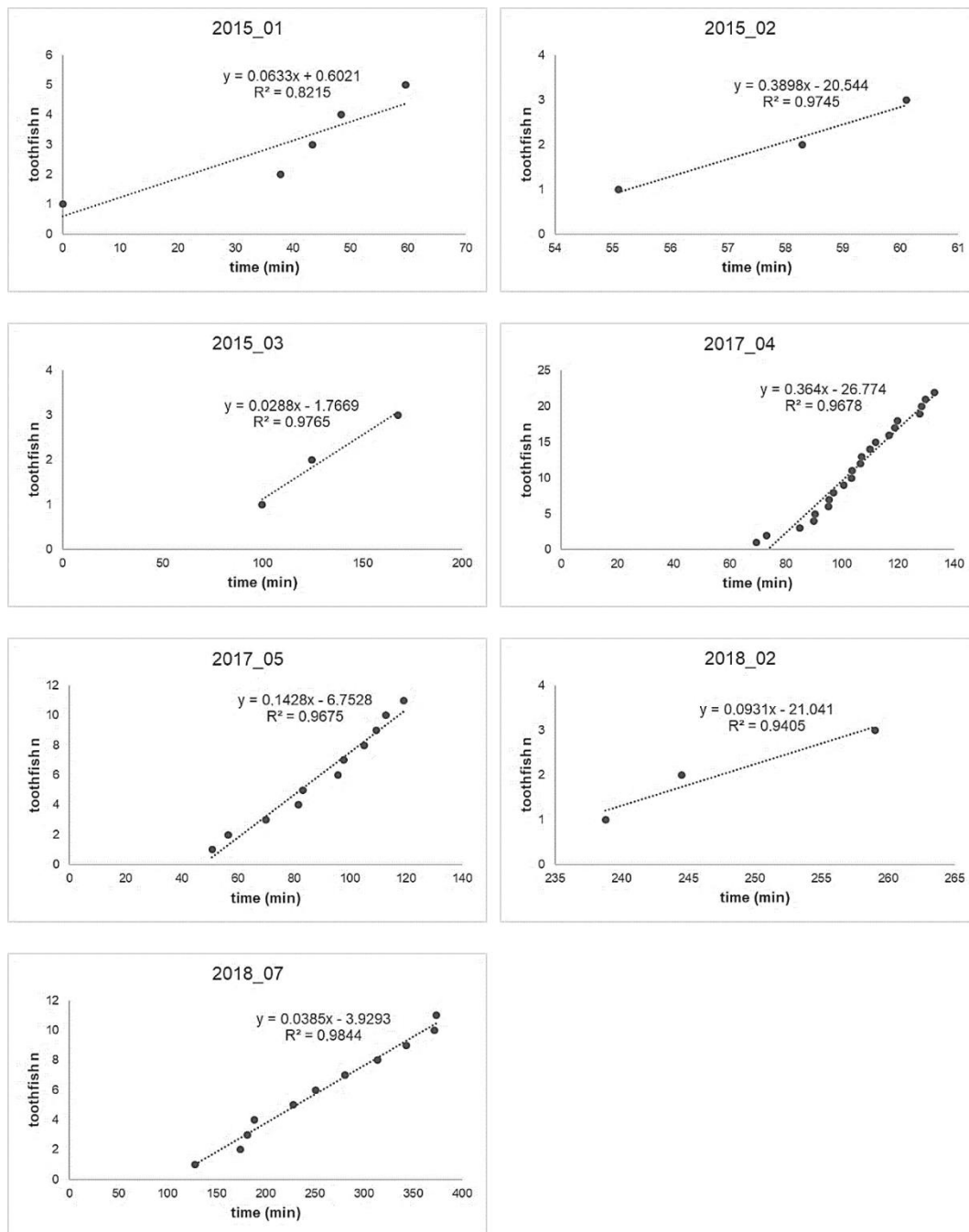
Fig 7. Plots of the cumulative numbers of toothfish over time and pertaining linear trend lines. The title of each graph reports the year (e.g. 2015_) and the sequential number of the sampling in that year (e.g. _02).

From 71 toothfish occurrences, 44 fish were recorded close to the bait together with at least another individual in the same frame. The majority of them (56.8%) were neutral to the presence of other individuals in the same area, 2 left the field of view without any intra-specific interaction, 1 was apparently involuntarily bitten by 1 other individual and had a weak escape reaction, swimming away and return a few seconds later, 2 fishes

20

increased the swimming speed in the field of view after an inadvertent contact, and in 13 cases agonistic behaviour (Fig 8) were recorded with one fish dominating over the other.

Fig 8. Sequence of an agonistic event. Two toothfish are involved (left side of the images) on the side of a third individual which is eating the bait.

A descriptive analysis was developed from the combination of the agonistic event, size classes involved, and subsequent reactions (Fig 9). In 5 cases (38.5%) the larger individuals behave as aggressors, in 5 other cases aggressors were of the same size class of the subordinates, and 3 times (23.1%) the smaller individuals were observed to aggress the large ones. The aggressors never left, they had weak reactions (regardless of the size of the fish attacked) in 3 cases, remaining near the bait in most of the interactions (10). The subordinates in 2 cases escaped and did not return (in both they were of the same size class of the aggressor), but in most cases they showed weak reactions remaining near the bait.

Agonistic behaviour: aggressors

	larger vs. smaller	same size class	smaller vs. larger
escape	0	0	0
weak reaction	1	1	1
stay	4	4	2

Agonistic behaviour: subordinates

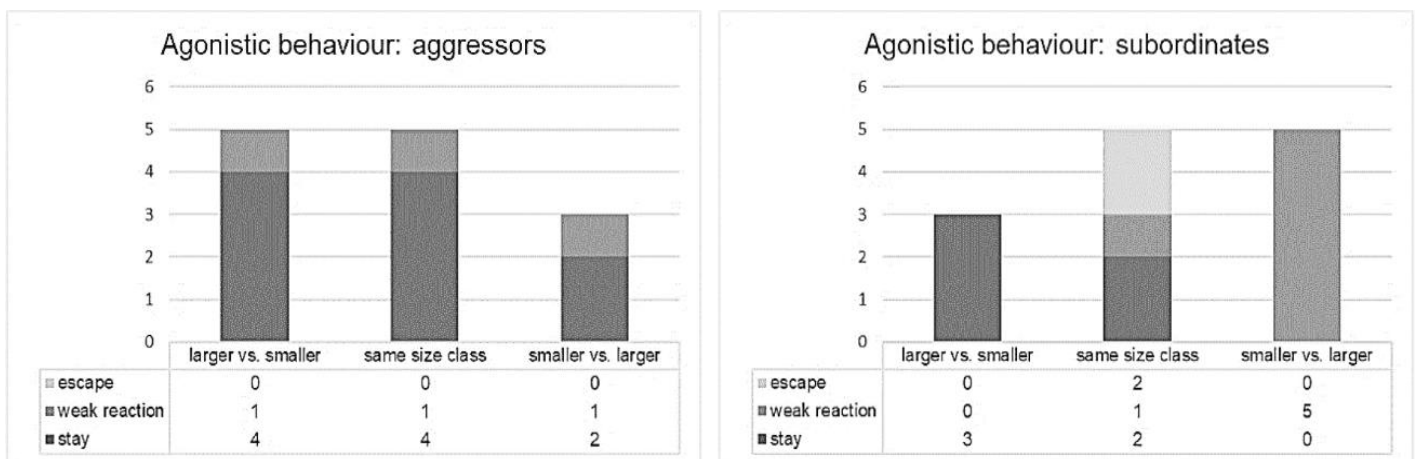|	larger vs. smaller	same size class	smaller vs. larger
escape	0	2	0
weak reaction	0	1	5
stay	3	2	0

Fig 9. Reaction of aggressors and subordinates in agonistic events, according to the size of the involved individuals.

2.3.1 Antarctic toothfish abundance

A summary of the considered abundance metrics is provided in Table 1. In Gerlache Inlet, one station has been replicated twice (BRUV# 2016_01 and 2016_02) and another one three times (BRUV# 2017_01, 2017_02 and 2017_06), in Silverfish Bay one station has been replicated three times (BRUV# 2017_03, 2017_04 and 2017_05).

Table 1. Matrix of predictive and response variables relative to the BRUV samplings carried out from 2015 to 2018.

BRUV#	Set duration (hh:mm:ss)	Locality	depth(m)	substrate	benthos	geomorph.	CPUE	MaxN	TFA (min)	MeanN	cMean Count
2015_01	00:59:51	McMurdo S.	>500	soft	<50%	Ridge	5.01	1	37.9	1	5
2015_02	19:19:52	McMurdo S.	>500	soft	>50%	Trench	0.16	3	55.1	1	1
2015_03	03:43:13	McMurdo S.	>500	soft	<50%	Trench	0.81	2	99.9	1	1.33
2016_01	09:06:54	Gerlache I.	350-500	mixed	<50%	Trench	0	0		0	0
2016_02	04:05:50	Gerlache I.	350-500	mixed	<50%	Trench	0	0		0	0
2016_03	01:31:29	Wood B.	>500	soft	<50%	Trench	0.66	1	90.7	0.5	0
2017_01	14:46:51	Gerlache I.	350-500	soft	<50%	Trench	0	0		0	0
2017_02	09:44:44	Gerlache I.	350-500	soft	<50%	Trench	0	0		0	0
2017_03	02:16:45	Silverfish B.	350-500	soft	<50%	Trench	0.44	1	67.1	0.33	0.5
2017_04	02:25:40	Silverfish B.	350-500	soft	<50%	Trench	9.06	4	69.5	2.33	9
2017_05	02:00:45	Silverfish B.	350-500	soft	<50%	Trench	5.47	2	50.8	2	6.5
2017_06	07:08:14	Gerlache I.	350-500	soft	<50%	Trench	0	0		0	0
2018_01	01:19:38	Silverfish B.	<350	mixed	>50%	Ridge	0.75	1	8.2	0.5	1
2018_02	05:38:43	Silverfish B.	350-500	soft	<50%	Ridge	0.53	2	238.8	0.6	0.8
2018_03	06:01:05	Silverfish B.	350-500	mixed	>50%	Trench	0	0		0	0
2018_04	06:29:34	Silverfish B.	<350	mixed	>50%	Ridge	0	0		0	0
2018_05	05:29:33	Gerlache I.	350-500	soft	<50%	Trench	0	0		0	0
2018_06	01:59:25	Silverfish B.	<350	mixed	>50%	Ridge	0	0		0	0
2018_07	06:14:34	Silverfish B.	>500	soft	<50%	Trench	1.92	3	60.3	1.4	2
2018_08	05:27:00	Silverfish B.	<350	soft	>50%	Ridge	0.37	1	167.2	0.4	0.4

The multiple Spearman rank correlation analysis (Table 2) showed that cMeanCount, CPUE, MaxN and MeanN are all strongly correlated; TFA does not correlate to the other metrics. The first four metrics can be likely indicative of the same effect, that should be the fish abundance. TFA alone in our case seems not directly indicative of fish abundance, therefore it was discarded as response variable in the subsequent analyses.

Table 2. Correlations between the fish density variables used as responses in the subsequent analysis.

	cMeanCount	CPUE	MaxN	MeanN	TFA
cMeanCount	1.0000 (n=20)				
CPUE	0.9201 (n=20, p<0.001)	1.0000 (n=20)			
MaxN	0.8945 (n=20, p<0.001)	0.8811 (n=20, p<0.001)	1.0000 (n=20)		
MeanN	0.9432 (n=20, p<0.001)	0.9549 (n=20, p<0.001)	0.9657 (n=20, p<0.001)	1.0000 (n=20)	
tMaxN=1	-0.4601 (n=11, p=0.154)	-0.3273 (n=11, p=0.326)	0.0821 (n=11, p=0.810)	-0.2437 (n=11, p=0.470)	1.0000 (n=11)

GAMs conducted on log-transformed CPUE, MaxN, MeanN and cMeanCount showed that in all cases only two variables were significant in explaining abundance. In the case of CPUE, locality and benthos coverage explained together 77.1% of the deviance, the locality Gerlache Inlet had significantly lower coefficients than the others (p<0.001) and benthos coverage >50% had significantly lower value than benthos coverage <50% (p<0.001). For MaxN the model resulted in the combination of locality and substrate type, explaining together 75.5% of the deviance, with Gerlache Inlet and substrate mixed with significantly lower values (p=0.001 and p=0.004, respectively). As for CPUE, the models for MeanN and cMeanCount included the factors locality and benthos, which explained in the two cases 74.9% and 76.9% of the deviance. Gerlache Inlet resulted the most significantly different locality (p<0.001 in both cases), and for cMeanCount also Wood Bay had significantly low values (p=0.002), while the values for benthos coverage >50% resulted also in these two variables significantly lower than those for <50% (p=0.004 in MeanN and p=0.002 in cMeanCount).

2.3.2 Size classes distribution

Correspondence analysis obtained on the data in Table 3 showed that the three size classes were well separated one from each other. Three clusters were highlighted in the relative factor map, indicated by different colours, and their centroids were close to the locations of the size classes (Fig 10).

Table 3. Numbers and percentages of <100cm, 100-130cm and >130cm fish recorded divided by samplings.

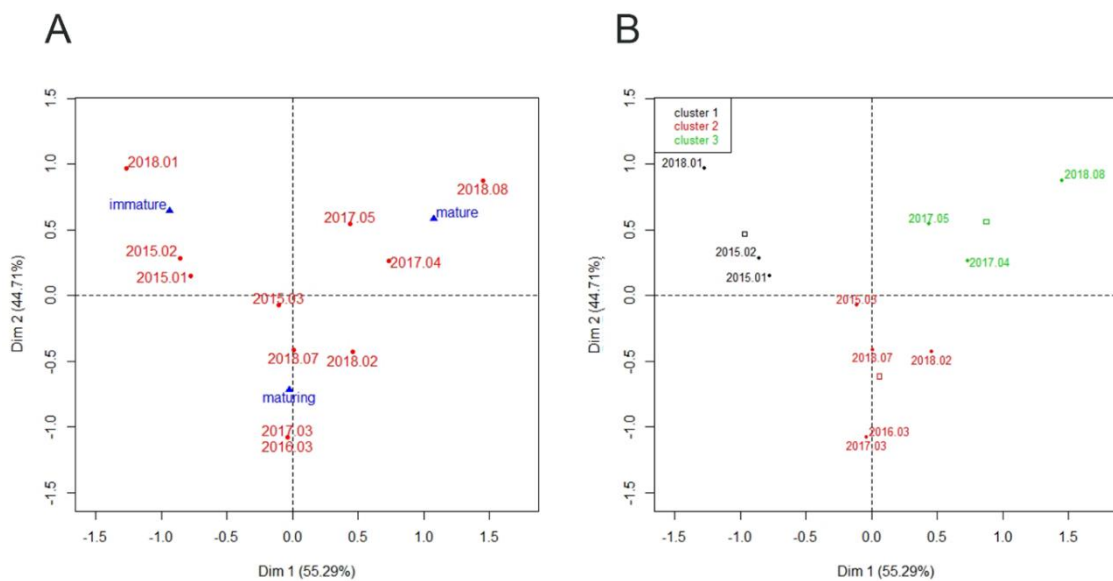| BRUV #	n <100 cm	n 100-130 cm	n >130 cm	% <100 cm	% 100-130 cm	% >130 cm
2015_01	3	2	0	60	40	0
2015_02	2	1	0	66.7	33.3	0
2015_03	3	5	2	30	50	20
2016_03	0	1	0	0	100	0
2017_03	0	1	0	0	100	0
2017_04	2	7	13	9.1	31.8	59.1
2017_05	3	2	6	27.3	18.2	54.5
2018_01	1	0	0	100	0	0
2018_02	0	2	1	0	66.7	33.3
2018_07	2	8	2	16.7	66.7	16.7
2018_08	0	0	2	0	0	100

Fig 10. Results of the CA. Sampling events associated to size classes and clustering of the samplings, represented with different colours.

A higher percentage of large fish were associated with depths less than 500 m and in Silverfish Bay. Most toothfish, irrespective of size, were recorded in areas with soft seafloors and low benthos coverage. Mid-size fish were associated with depths deeper than 350 m. Of the five samples where mid-size fish dominate, four were in trench habitats, and one on a ridge. Immature toothfish were not associated with any environmental factor investigated, however, the only sample point with mixed substrate was present in the cluster corresponding to immature fishes.

2.4 Discussion

We investigated the performance of the use of a purpose-built BRUV system conducted through the fast sea ice to study the Antarctic toothfish in under-sampled and otherwise inaccessible areas of the Southern Ocean.

As a first remarkable result, we confirmed that the essential design and small size of our purpose-built underwater image-capture system made it suitable for deployment through holes in the sea ice of relatively small diameter (40 cm), thus significantly reducing the workload and logistic efforts required to perform the survey from the sea ice. Moreover, the BRUV was easily transportable and light enough to be set and hauled by hand. The system was deployed at significant depth, down to more than 500 m, and allowed to collect high-quality video footages recording the occurrence of 71 Antarctic toothfish in the Ross Sea shelf area.

As expected on the basis of current information on its olfactory capability (Ferrando et al. 2019), the Antarctic toothfish seem attracted by the odour plume from the bait, thus supporting the potential for the use of BRUV to study and survey this species. The effects of other potential attractants related to the BRUV system, such as light, noise, or presence of other organisms around the lander, were considered not influential. Such assumption derives from two video samplings (not included in present work), carried out without bait in 2015 in McMurdo Sound and in 2017 in Silverfish Bay: no toothfish were recorded from the camera without the bait in areas where Antarctic toothfish differently occurred. Similarly, in the two cases in which the bait was consumed by amphipods, or taken away by a toothfish, no fish was recorded after such events.

The linear trend lines with high R^2 resulting from the analyses of videos indicate a continuous and persistent attractive effect of the bait, which seems to last up to six hours. Accordingly, soak times from two to six hours are highly recommendable in future works. Nevertheless, at the low sea water temperature of high Antarctica (about -2°C), long duration of the batteries is challenging, pointing to the need to find alternative solutions that would allow data collection within an appropriate time-frame. From our experience, we suggest a protocol based on video records at short intervals, allowing to extend the soak time while saving energy of the batteries. In theory, such a discontinuous recording might affect the count of occurrences, altering the derived values of abundance and distribution. However, we observed that individuals coming to the BRUV usually stay near the bait for a time longer than that of a single footage.

In general, the quality of the video was high, allowing to collect a unique documentation of the Antarctic toothfish in its habitat. To the best of our knowledge, only brief images of a single fish, incidentally acquired during a video survey in the Ross Sea (Eastman and Barry 2002), and sparse observations of fish from cameras attached to Weddell seals (Fuiman et al. 2002), were available prior to present work. Owing to the good quality of the videos and the low number of individuals occurring in the field of view of the camera simultaneously, identification was possible for all the individuals, and biases related to re-counted individuals were avoided. The presence of scars and unique colour patterns were effective natural markings to distinguish individual toothfish from images taken from the video footages. Although the persistence over time of those markings is unknown, they may be used as reliable and effective markers within a single location or deployment to avoid duplicate counts and over-estimation of abundance. In present work the identification of each toothfish was performed by a human operator. Such a procedure, effective on a small dataset, such as the one generated in present pilot study, is time-consuming, and largely based on the expertise of the annotators. The development of automated individual identification methods, including the more advanced machine learning techniques, would greatly improve the methodology, reducing the labour cost while allowing greater video data processing per time unit and expansion of the analysis to large datasets.

Investigation on the effect of intra-specific interactions around the bait resulted in neutral behaviour as the most common response. Moreover, even when agonistic interactions occurred, the individuals involved typically remained or returned to the bait. Taken together, these results seem to indicate the likely lack of effect of intra-specific interactions in determining the number of fish entering the field of view of the camera, thus supporting the use of BRUV as tool to estimate the abundance of toothfish.

The comparative analysis of various abundance metrics led to discard the time of first arrival (TFA) as reliable proxy for the Antarctic toothfish abundance. This metric, in a formula developed by Priede and Merrett (1996), and adopted by Yau et al. (2001) for the congeneric Patagonian toothfish, is integrated in a formula with data on the current speed and fish swimming speed. In present work, we tested TFA alone (as proposed by Cappo et al. (2006) and Stoner et al. (2008)) having not associated tools to measure the current speed, and concerned by the fact that the real speed of a fish attracted to the bait still remains uncertain, making this metric difficult to be standardized.

The performance of other video abundance estimation techniques (cMeanCount, CPUE, MaxN and MeanN) was highly correlated, indicating that there was little change in the information between those indices, and supporting the reliability of those metrics to estimate the abundance of toothfish.

The small dataset considered herein prevent us from drawing any broad conclusion on the Antarctic toothfish abundance and distribution in the area. However, the research provided interesting cues for future work. For instance, differently from what resulting from the commercial fishery data (Hanchet et al. 2015), a high percentage of large fish were found associated with depth lower than 500 m. Further investigation is needed to determine whether this is a bias associated to the small size of the sample, or a new information highlighting the peculiar fish size classes distribution in the coastal sea-ice covered areas.

A significant effect of the factor 'locality' out came from the models providing, in some cases, elements to improve the method in future studies. For example, the absence of toothfish recorded in 'Gerlache Inlet' is an interesting data that might be related to an environmental factor herein not included, that is the presence in the close vicinity of Antarctic toothfish predators. Indeed, numerous Weddell seals (*Leptonychotes weddellii*) were spotted in the area, close to cracks in the sea-ice. Weddell seals are known predators of the Antarctic toothfish and their abundance might be negatively related to that of its prey (Ross et al. 1982, Testa et al. 1985, Ponganis and Stockard 2007). Detailed information on the presence and abundance of Weddell seals is to be included in elaborating future predictive models to infer the Antarctic toothfish distribution.

Other predictive variables could be combined with those used here to obtain a more realistic model of Antarctic toothfish distribution and abundance under the sea ice. For example, water temperature, tidal stage or current speed and direction are all environmental factors potentially affecting the dispersion of the odour plume from the bait, or could be important in determine wide movements of the fish among different areas.

3. Antarctic toothfish reproduction and other information from the Second Winter Survey

3.1 Introduction

The life cycle of the Antarctic toothfish (*Dissostichus mawsoni*) population present in the Ross Sea is extended in a wide area that cover about 15 degrees of latitude and tens of degrees of longitude (Hanchet et al. 2008, 2015, Ashford et al. 2012, Mormede et al. 2014). In such area, identified as FAO Area 88, research on the species has been mainly associated to the exploratory longline fishery that started in 1997. More than 20 years of activity allow to have now a good understanding of many biological and ecological aspects of the Antarctic toothfish (Parker and Marriott 2012, Hanchet et al. 2015). However, due to the temporally restricted annual fishing period, some knowledge gaps have been remained not clarified for a long time.

One of the major shortcomings has concerned the reproduction and the early life history of the species. It has been due to the period of the spawning, which is temporally far from the period in which almost the whole fishing activity – and associated research – is annually carried out (Hanchet et al. 2008, Parker and Grimes 2010, Parker and Marriott 2012). Due to logistic facilities linked to environmental condition, toothfish fishery is indeed concentrated in the austral summer and early autumn, while the life cycle hypothesis suggested that the Antarctic toothfish spawn during winter in correspondence of the seamounts, ridges and banks present in the northern region of the Subareas 88.1 and 88.2 (Hanchet et al. 2008).

Spawning in the north had been previously hypothesized based on movements from recaptures of tagged toothfish and gonad development in the region observed during the summer (Hanchet et al. 2008). Oceanic circulation, in particular the movements of the Ross Sea gyres, would after play a crucial rule in the dispersion of eggs, larvae and juveniles (Hanchet et al. 2008, Ashford et al. 2012, Dunn et al. 2012). The most reliable population model consists in a two-area model, the southern feeding area and the northern breeding one, with migrations between them. Other than from tagged individuals, such hypotheses derived from stable isotope data: already in Bury et al. (2008) found similar isotope signatures in specimens from the southern slope and the north, in both cases homogeneous with prey that the species can find in the south, suggesting the division in

the two areas with different functions in the life cycle of the species, precisely feeding grounds in the south and breeding grounds in the north. Such conclusion would also explain the worst condition found in toothfish in the north compared to that of fish caught in the south (Fenaughty 2006, Fenaughty et al. 2008, Parker and Marriott 2012).

These hypotheses needed to be confirmed and completed, considering the relevance of improving knowledge on basic life history and movement patterns in better determine population structure, improve spatial population models and understand how the ecosystem role of the Antarctic toothfish vary spatially, and consequently to implement adequate conservation measures (Hanchet et al. 2015). To obtain information to address uncertainties in toothfish life-cycle movements and spawning dynamics it has been necessary to go out from the classic data collection associated to the routine summer fishery and to implement dedicated winter voyages. For this reason, the Commission for the Conservation of Antarctic Marine Living resources (CCAMLR) approved in 2016 the first Winter Survey, successfully carried out in the northern Ross Sea region during June and July (Stevens et al. 2016, Parker et al. 2019b).

The 2016 Winter Survey was planned to be the first of a 2-3 surveys series, required to fully establish the spatial and temporal extent of spawning, and all the biological information associated, in a large area such as that of the northern seamounts, ridge and banks. Also, a multi-survey program has been considered because the spawning season had to be identified in a window of different months and it could be extended for a period larger than that possible for a single voyage in the prohibitive condition of the Southern Ocean during the winter months.

The first Winter Survey was carried out in correspondence of the northern seamounts of Subarea 88.1 SSRUs B-C, associated to longline fishing activity as usual (Stevens et al. 2016, Parker et al. 2019b). Following the ecosystem-based CCAMLR approach, with a major focus on aspects of reproductive biology of the species, a wide series of biological and ecological data was collected. The primary objectives of the survey were to document the timing and location of spawning activity, to collect biological information about reproductive status during the spawning season, to look for temporal signals in size and age structure, condition factor, and sex ratio during the winter in the north, and possibly to infer residence time on the spawning grounds.

A further research activity was dedicated to the study of eggs buoyancy. Such information, for which no data were available since no Antarctic toothfish eggs or larvae have been captured before in the Ross Sea or elsewhere, is relevant for the egg and larval

circulation model, as the depth in the water column affects their resulting entrainment ad subsequent transport and distribution in the wider Ross Sea region.

During the survey, the presence of ripe running male and female Antarctic toothfish was reported, and a peak in gonadosomatic index (GSI) of near 20% for females was recorded at the end of June, with some indication of a decrease in July due to an increasing proportion of spent fish (Stevens et al. 2016, Parker et al. 2019b). Eggs were successfully fertilized on board and the buoyancy of the early stages of embryonic development was measured, resulting strongly buoyant so that even with surface mixing they would accumulate within the first 5 m of the water column. Such a characteristic let to deduce that Antarctic toothfish eggs would set under the sea ice over most of the supposed spawning area. Also, 19 eggs were collected from the plankton sampling (Stevens et al. 2016, Parker et al. 2019b).

However, spawning fish were only found in one location at the western edge of the survey area (SSRU 88.1 B) near the end of the survey. Thus, the spatial distribution of spawning and its timing have been remained unknown. Furthermore, eggs collected were early in the developmental process, and the fertilized eggs dead after few days because of warming of the incubator system while moving north to come back, so buoyancy and distribution of Antarctic toothfish eggs and larvae throughout the development remained to be determined.

As planned, other surveys proved to be necessary to obtain a more complete overview of all the aspects linked to reproduction and early life stages of the species. Therefore, a second Winter Survey was implemented 2019. The new voyage extended the survey area eastward to SSRU 88.2 A-B, to expand the investigation to a wider area where spawning might occur. It was conducted three months later than the 2016 survey, in September and October, to avoid the period of sea ice maximum, which occurs in August, that likely would have prevented to enter with the fishing vessel in the interested areas. Carrying out the voyage in the end of the austral winter would have enhanced the chances of determining the end of the spawning season and so estimate the spawning duration, as well as sampling eggs and/or larvae immediately after the spawning season, investigating the potential linkage to the sea ice and how their buoyancy change during the development.

Summarizing, the primary objectives of the second Winder Survey have been incremental respect to those of the previous one, precisely: to investigate the potential dispersion areas of Antarctic toothfish eggs and larvae studying their characteristics, especially in

association with sea ice; to investigate the distribution of Antarctic toothfish in Subareas 88.1 and 88.2 in relation to spawning, to further refine the developmental cycle and likely residence time on the spawning ground; and to investigate the timing of the movement of Antarctic toothfish to and from the spawning grounds through an analysis of length and age composition of the catch, sex ratio and condition factor (Parker and Di Blasi, 2020).

3.2 Materials and methods

3.2.1 Survey design

Subarea 88.1 SSRUs B–C and Subarea 88.2 SSRU's A–B were divided into eight strata to spread the research effort and tagging, and to cover as wide an area as plausible to locate areas where spawning might take place, in correspondence of the Pacific Antarctic ridge (Fig 11 A, B). Each stratum spanned 5° of longitude (~250 km at 63°) and 6° of latitude (more than 600 km) to enable research sets to be completed in each stratum while accommodating the variable northern extent of winter sea ice, knowing that sea ice during winter can extend to latitudes lower than 65° S and consequently much of the northern Ross Sea region is covered during the winter months (Markus et al. 2018). The strata were bounded by 60 – 66° S latitude and 170° E – 150° W longitude. The first four strata, from 170° E to 170° W, matched those of the first Winter Survey 2016.
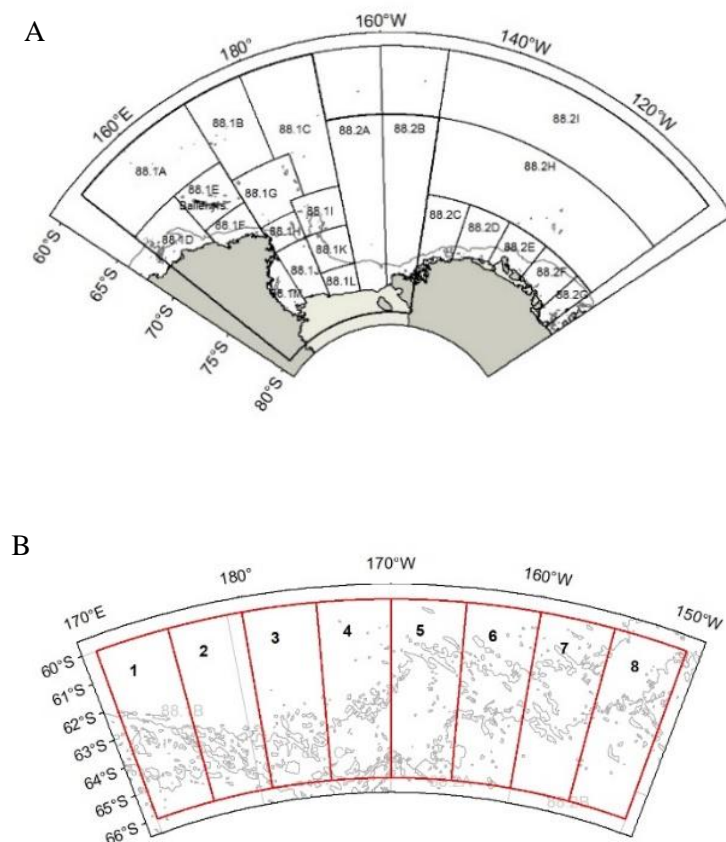

Fig 11. (A) Subareas of the FAO area 88; (B) the 8 strata in which the survey area was divided. Depth contours of 1000, 2000, 3000 m are plotted in grey colour.

Longline sampling locations targeted seamount features shallower than 2000 m where spawning of Antarctic toothfish was predicted more likely to occur. Sampling was conducted as far south as possible with exact sampling locations determined by the sea ice coverage encountered.

3.2.2 Longline gear configuration

The New Zealand flagged longline vessel FV *Janas* was used for the survey. The vessel used a standardised autoline longline gear enabling comparison with other vessels using the same gear type and deployment procedures. Integrated weighted line was used for all longlines - this has 50 g of lead weight per metre of mainline. The snoods were multifilament and 300 - 400 mm long. The hooks used (code 69, size 21) have been proven capable of catching toothfish of the target length when fish are available (Fig 12). Hooks were baited using an automatic baiting machine with arrow squid (*Notodarus* spp., ~35 g per hook). The percentage of successfully baited hooks per line was monitored on each deployment to allow effort standardisation among lines.

Fig 12. Preparation of the longline before the baiting and the deployment. The system automatically brings the longline there after the hauling and move it for the baiting and deployment, the fishermen just change lost or damaged hooks.

Longlines were configured from magazines, each with 950 hooks spaced at 1.4 m along the 11 mm mainline. The spacing of hooks and snoods was a fixed value as these are connected to rotors and swivels that are permanently attached to the mainline. A total of up to 18 magazines could be set on a single seamount comprising a cluster of lines. To spread sampling effort, clusters were separated by at least 10 nm. A soak time of 18 ± 6

hours was targeted, subject to environmental conditions (e.g. ice, weather) and operational requirements.

3.2.3 Research implemented

A range of routine and opportunistic measurements were taken to characterise the toothfish and bycatch composition of each line, and environmental parameters were recorded in accordance to the ecosystem-based CCAMLR approach. Tissue samples and bycatch specimens were collected for a number of biological analyses to be conducted at a later date.

Here it will be report exclusively the work strictly focused on the characterization of the Antarctic toothfish in the northern area and all the aspects linked to its reproduction.

3.2.3.1 Tootfish biological data

Length, weight, sex, gonad stage, and gonad weight were recorded for at least the first 100 Antarctic toothfish captured on each line. Fish were measured to the nearest centimetre at or below the total length. Most toothfish were weighed using a digital Marel scale (to 0.1 kg), but a hanging spring balance (to 0.5 kg) was used when the Marel scale became non-functional. Smaller toothfish or bycatch species were weighed using a digital Marel scale to the nearest 10 g. Sex and macroscopic reproductive status were determined using the CCAMLR 5-stage system (CCAMLR Observer manual). Stomach weight, stomach contents, and liver weight were recorded for up to at least 40 Antarctic toothfish per haul, using the digital Marel scale. Stomach fullness and digestion state were recorded, and macroscopic identity of prey items was determined to the finest possible taxonomic resolution.

Each individual was also characterized as "regular condition" or "axe handle", these last are poorly conditioned Antarctic toothfish, typically present in the northern region, so named by the vessel crews because they appear shrunken behind the head, with a notably thinner trunk than fish in regular condition (Fenaughty et al. 2008).

For each fish recorded, a series of biological parameters were calculated. The gonadosomatic index (GSI) was calculated as the gonad weight (g) / 1000 / total weight (kg) * 100 for both males and females. Changes in weight for fish of a given length were compared using the exponents from the sex-specific length-weight relationship to generate the index for each sex (Anderson and Neumann 1996, Hanchet et al. 2015).

Other indices were taken into consideration as proxies of energy status (Chellappa et al. 1995). The index of condition was calculated as total weight (kg) / length (cm)^x * 100 000, where x was 2.965 for females or 3.108 for males (Hanchet et al. 2015, Parker et al. 2019b). The somatic index (SI) was calculated as the total weight minus the combined weights of the gonad, stomach and liver, and divided by the length, males and females were divided to obtained sex-specific growth exponents. Hepatosomatic index (HSI) was calculated as the ratio between liver weight and gutted weight, and its variation between sexes and between "axe handle" and "regular condition" individuals was investigated.

3.2.3.2 Conventional and Popup Satellite Archival Tags (PSAT) tagging

The survey provided the unique opportunity to tag toothfish in the north during the spawning season. This allowed to mark fish that in future if recaptured will provide high value information on the route of post-spawning movements and residence time of toothfish in the northern region. Therefore, a standardised CCAMLR tagging program was put in place. Tagging with conventional CCAMLR Hallmark tags (two per fish, placed in the two sides at the base of the anterior part of the second dorsal fin, and fish length recorded) was conducted at a rate of three Antarctic toothfish per tonne caught (Fig 13). As fish populating the areas of the survey was likely to be large, it was expected that a right tagging rate of three fish per ton should have equated to 1 in 9 fish tagged (if mean weight = 32.7 kg) compared to the usual rate of 1 in 27 tagged in the Ross Sea as a whole.

Fig 13. The right position in which the conventional CCAMLR tags were placed.

The overlap between the size frequency distribution of the tagged and released individuals and size frequency distribution of the landed ones had to be as higher as possible. A plan

of tagging the first three fish each ton was carried out at first, while the continuous monitoring of the tagging overlap could lead to corrections to improve the overlap.

Furthermore, all captured toothfish were scanned for the presence of tags. Tag recovered would help to describe toothfish population in the northern Ross Sea and provide insights about winter spawning migration. In addition to standard biological measurements, muscle, liver, stomach contents, and otoliths were sampled from each recaptured tagged toothfish.

Pop-off satellite archival tags (PSAT) were provided by the US AMLR program in San Diego to be deployed on Antarctic toothfish during the survey. Such kind of tags, to be set on a side at the base of the base of the first dorsal fin, have the function to record horizontal and vertical movements of the fish for a planned period, after which the tag would crush by means of a programmed explosion and go to the surface to send the collected data to the ARGOS satellite.

Fish chosen for tagging with a PSAT were also tagged following classical CCAMLR approved procedures described above. PSAT tagging targeted large (possibly > 150 cm TL) fish in excellent condition (as fish larger than 150 cm are more likely to be female) (Fig 14). PSAT-tagged fish were spread geographically depending on composition of catches and fish suitability.

Fig 14. Placing of a pop-off satellite archival tag (PSAT) in a high-condition tootfish.

3.2.3.3 Eggs collection and buoyancy measurement

The Antarctic toothfish eggs have been sought in the water column using a 60 cm diameter bongo net (200 and 500 µm mesh sizes) (Fig 15). Bongo was deployed at depths ranging between the first meters close to the surface to 250 meters, regulated by different lengths of an 11 mm polyester sinking "down line". The depth reached by the net was regulated also by means of different configurations of the system, varying the weight of the depressor from 15 kg to no weight, and on three occasions associating it to a "windy buoy" (an A5 red inflated polyethylene float) linked to the line from 3 to 10 meters before the net to maintain the net at a shallow depth. Tows were made for 1-2 h by drifting with the wind at 0.75-1.5 kn using the net as a sea anchor. Flow meters recorded the volume of water filtered by each net and a depth logger were attached to each deployment to record the depth profile achieved.

Fig 15. The bongo net used during the survey.

Any toothfish eggs were immediately removed for buoyancy experiments or fixed in 10% formalin or 70% ethanol for later analyses.

Buoyancy of eggs was measured using a system of three density-gradient columns (Coombs 1981), each one with the same salinity gradient spanning from denser than seawater at the bottom to less dense than seawater at the top ranging from 1.015 to 1.028 $g*m^{-3}$. The gradients were constructed in calm conditions upon departure using Millipore

distilled water and aquarium salt following the procedure of Coombs (1981) (Fig 16A). In each column seven density calibrated standard floats were set as density references for the eggs (Fig 16B).
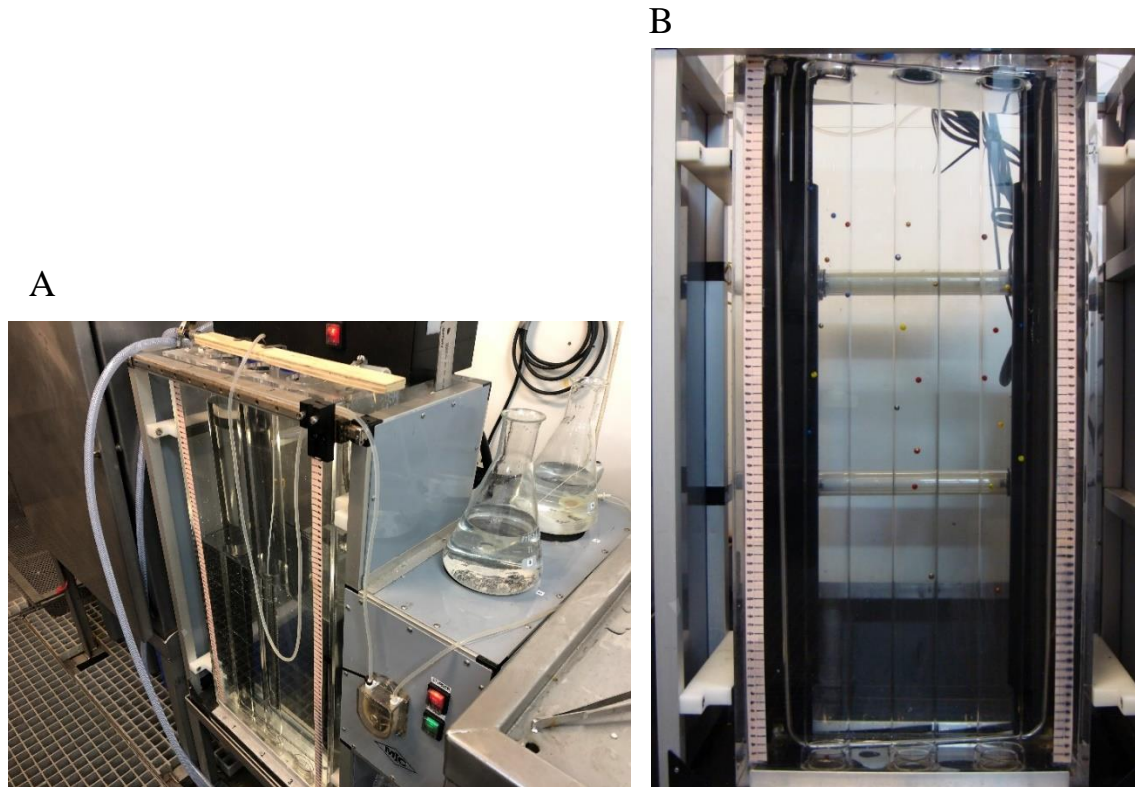

Fig 16. (A) Creating the salinity gradient in the buoyancy chamber. The machine mixes water of different salinities creating a new water gradually more and more dense, which is introduced in the columns from the bottom to the top. (B) Density gradient chamber with the calibrated floats in the columns. The floats provide a known standard height to measure relative to the height of eggs placed in the chamber.

Live eggs captured in the plankton were identified under a dissecting microscope and immediately placed into a density gradient cylinder to sink to its equilibrium density (Coombs 1981). Temperature and locations of eggs and standard floats in each column were recorded daily and photographed using a Canon SLR with macro lens. Developmental stages of embryos were determined through examination of egg photographs in the buoyancy chambers taking into account the reported developmental stages of the congeneric Patagonian toothfish (*Dissostichus eleginoides*) (Mujica et al. 2016). The buoyancy experiments have been carried out as long as possible to investigate potential changes in Antarctic toothfish egg buoyancy through their development.

3.3 Results

3.3.1 Longline sampling

Longline sampling commenced on 16 September in SSRU 88.2 B survey stratum 8 and finished on 14 October in SSRU 88.1 B survey stratum 1. Sampling activity progressed westward, generally following the sea ice extent. The plan to carry activities south to the edge of the pack ice was sometimes not possible to realize, because of repeated storms followed by SW winds that brought temperatures down to -16° C, causing the ocean surface to freeze rapidly and the formation of new grease ice or pancake ice (Fig 17).

Fig 17. Navigating in an area of sea ice formation with the typical pattern of pancake ice. In such situation the navigation was borderline, and fishing and research activity was completely precluded.

As SSRUs 88.2 A and B have only rarely been fished (only 66 longlines to date on just a few features), a significant part of the survey was dedicated to identifying, mapping and sampling uncharted seamount features that were north of the sea ice edge.

A total of 24 longlines were set in strata 1, 6, 7, and 8 (Fig 18). Soak times during the survey ranged from 9–33 hours, with a mean soak time of 19.3 hours. The long soak times were mainly due to inclement weather precluding hauling and/or lines becoming stuck on rough bottom for extended periods. Depths fished ranged from 867 to 2016 m. overall, nine bathymetric features have been sampled, of which only two had been fished

previously. Most of lines 4, 6, and 21 were lost, with no catch associated with lines 6 or 21. Eleven clusters were fished, with a maximum of 18 magazines set on a single feature.
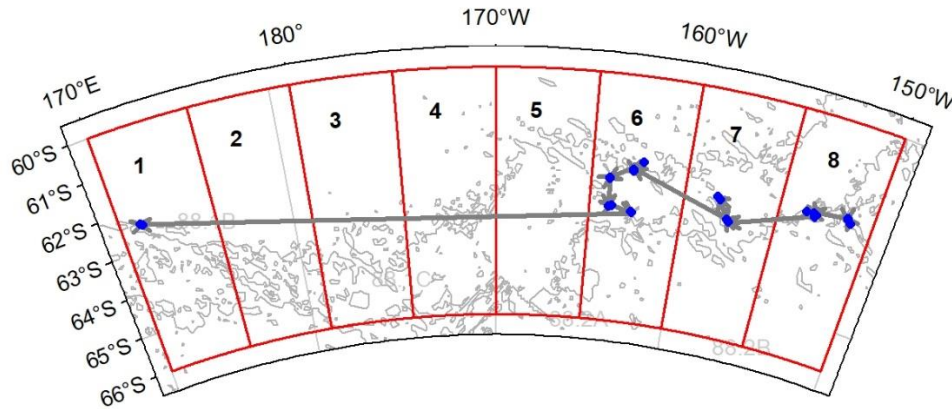

Fig 18. Locations of the longline samples (blue dots). The 1000, 2000, and 3000 m depth contours are shown in grey. Grey arrows indicate the sequence of longlines set during the survey generally moving from west to east.

3.3.2 Biological data

3.3.2.1 Catch composition

Antarctic toothfish dominated the catch, especially in strata 6, 7, and 8, both in terms of number (67.0%) and weight (96.6%). In total, 2228 specimens were hauled with a total weight of 66.4 t. Of these, 300 were tagged and released, resulting in 1928 retained fish with a landed weight of 57.5 t (Table 4).

The congeneric Patagonian toothfish was rare in strata 6, 7, and 8, whilst it was the main catch on the seamount sampled in stratum 1, coherently to what reported for the 2016 First Winter Survey (Stevens et al. 2016, Parker et al. 2019b). In total, 86 Patagonian toothfish were hauled (83 retained and 3 tagged and released), resulting in 1.3% of the catch in number and 2.6% in weight (859 kg).

The bycatch was composed mainly of violet cod (*Antimora rostrata*, 16.1% in number and 1.1% in weight), followed by macrourids (*Macrourus* spp., 12.9% in number and 0.9% in weight). Among macrourids, in strata 6, 7, and 8, the most common were *M. caml* and *M. whitsoni*, but occasionally also specimens of *M. holotrachys* and *M. carinatus* were caught. For the seamount in stratum 1, bycatch was mostly *M. holotrachys*. Less

common bycatch species, each representing less than 1% of the total catch both in number and weight, were giant lepidion (*Lepidion* spp.), eel cods (*Muraenolepis* spp.), Lithodidae (king crabs), Nototheniidae, and Channichthydae.

Table 4. Total retained catch of each species in weight (kg) and number (N), total hauled catch in weight and numbers (including tagged fish), and proportion of catch by weight and number. Species codes are those used by CCAMLR: TOA, *Dissostichus mawsoni*; TOP, *Dissostichus eleginoides*; ANT, *Antimora rostrata*; GRV, *Macrourus* spp; LEV, Lipidion sp.; MRL Muraenolepis sp; KCX, Lithodidae (king crabs); NOX, Nototheniidae; and ICX, Channichthyidae. Species are sorted by total retained catch

Code	Catch (kg)	Catch (N)	Hauled (kg)	Hauled (N)	% (weight)	% (N)
TOA	57474.61	1928	66419.16	2228	96.54	67.03
TOP	859.65	83	888.96	86	1.29	2.59
ANT	753.35	536	753.35	536	1.09	16.13
GRV	593.45	428	593.45	428	0.86	12.88
LEV	127.98	22	127.98	22	0.19	0.66
MRL	12.43	12	12.43	12	0.02	0.36
KCX	3.22	9	3.22	9	0	0.27
NOX	1.2	2	1.2	2	0	0.06
ICX	0.4	1	0.4	1	0	0.03

3.3.2.2 Antarctic toothfish catch observations and data collection

Length distributions indicated that the catch was generally of large fish of both sexes, with mean lengths of 142 and 153 cm for males and females respectively (Fig 19). A small number of Antarctic toothfish were less than 100 cm, and most of those were males. Length distributions were similar among strata (Fig 20).
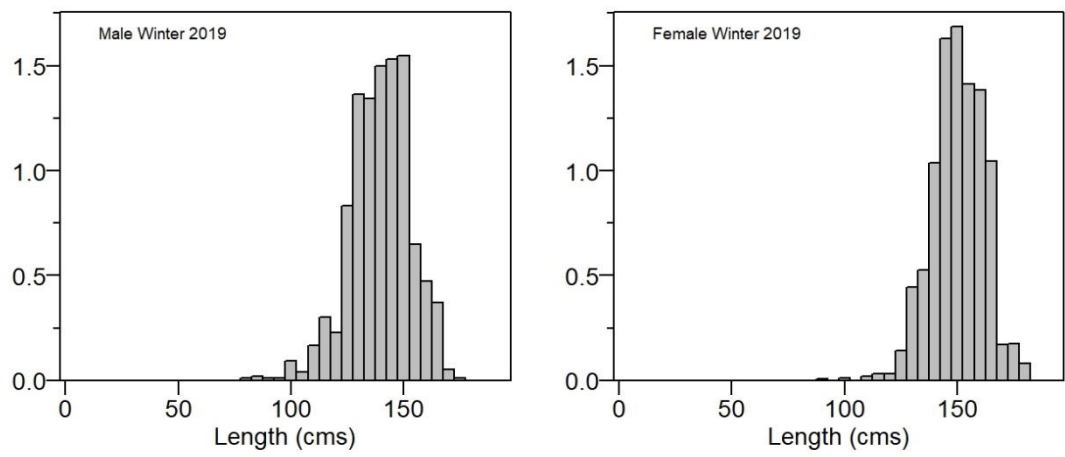
Fig 19. Scaled length frequency distributions male (left) and female Antarctic toothfish (right) from each stratum. Data are summarised in 5 cm length increments.
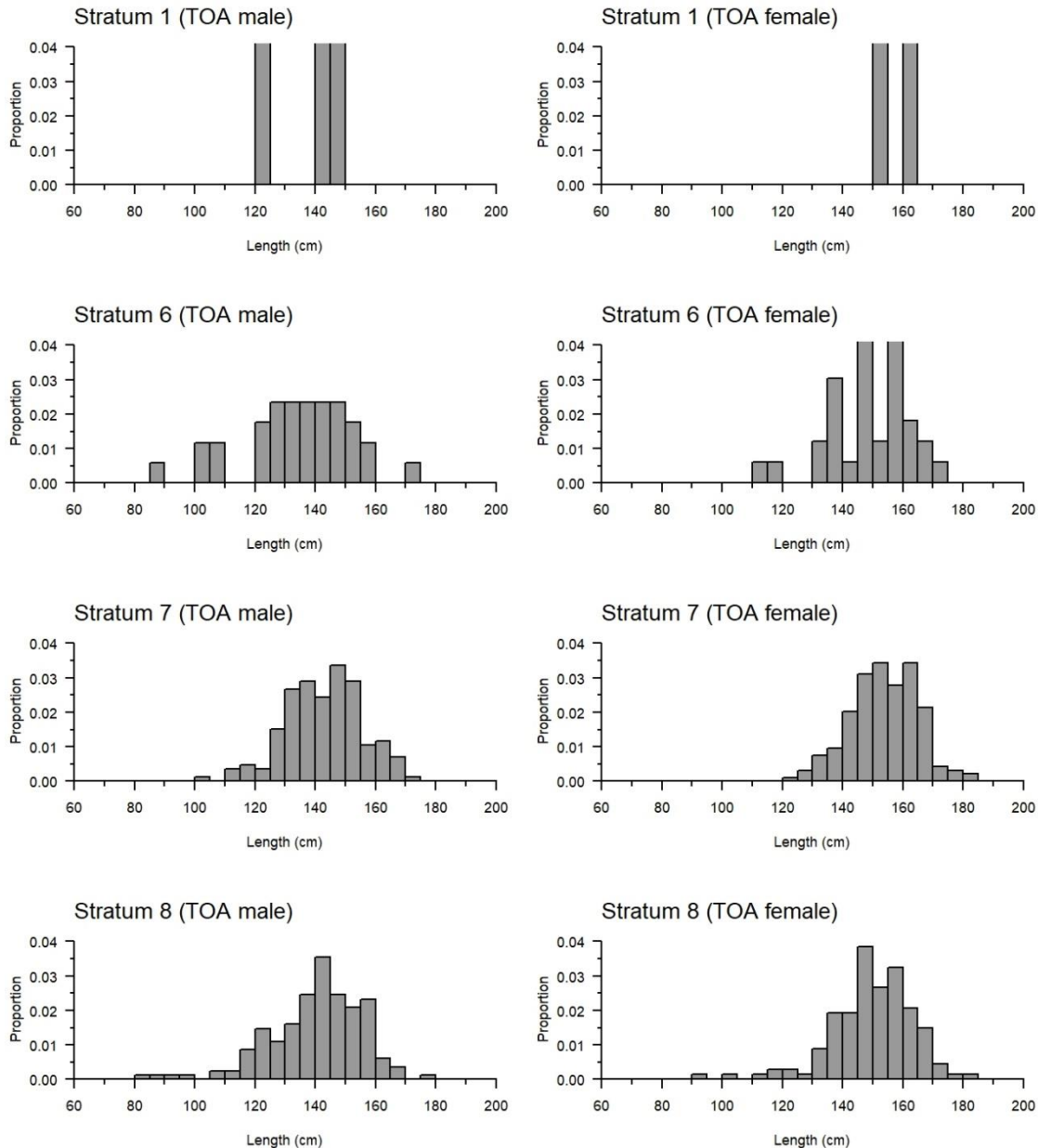
Fig 20. Length distributions by sex for Antarctic toothfish sampled in each stratum during the 2019 Winter Survey.

The sex ratio varied between 40 and 60% males. This was lower than observed in the northern Ross Sea in summer (68%) and in the 2016 winter survey within strata 1-4 in June-July (Stevens et al. 2016).

A total of 732 Antarctic toothfish were sampled. Total weight and gonad weight were recorded for 728 of the 732 sampled Antarctic toothfish; organ weights were recorded for 377 specimens. In addition, fish fitting the criteria for "axe handle" or "skinny fish", which are fish with much less muscle mass than others, were annotated. These fish were

easily identified by a depression in the amount of musculature behind the skull, a long thin fish appearance, and a wrinkled appearance to the skin (Fenaughty et al. 2008).

3.3.2.3 Reproductive status

Almost all Antarctic toothfish were spent or in early resting condition; only one female and two males were staged as immature. Testes were ruddy, small, sometimes convoluted, and firm with no expression of residual milt, even in cross section. Among those sampled, only two males presented residual milt. Ovaries were often bloodshot and flaccid, but even if seemingly full, they contained no visible eggs or developing eggs and only lamellar connective tissue. No difference in gonad appearance was observed among the different survey areas. One female was observed with some residual eggs, which were atretic and all appeared with a discrete white atretic area inside.

The gonadosomatic index (GSI) was typically $< 2\%$ in males and $< 5\%$ in females, approaching the values typically observed in summer (Fig 21). Two females were staged as ripe although only one had ag GSI over 10% (i.e., 21%, but was not ready to spawn).
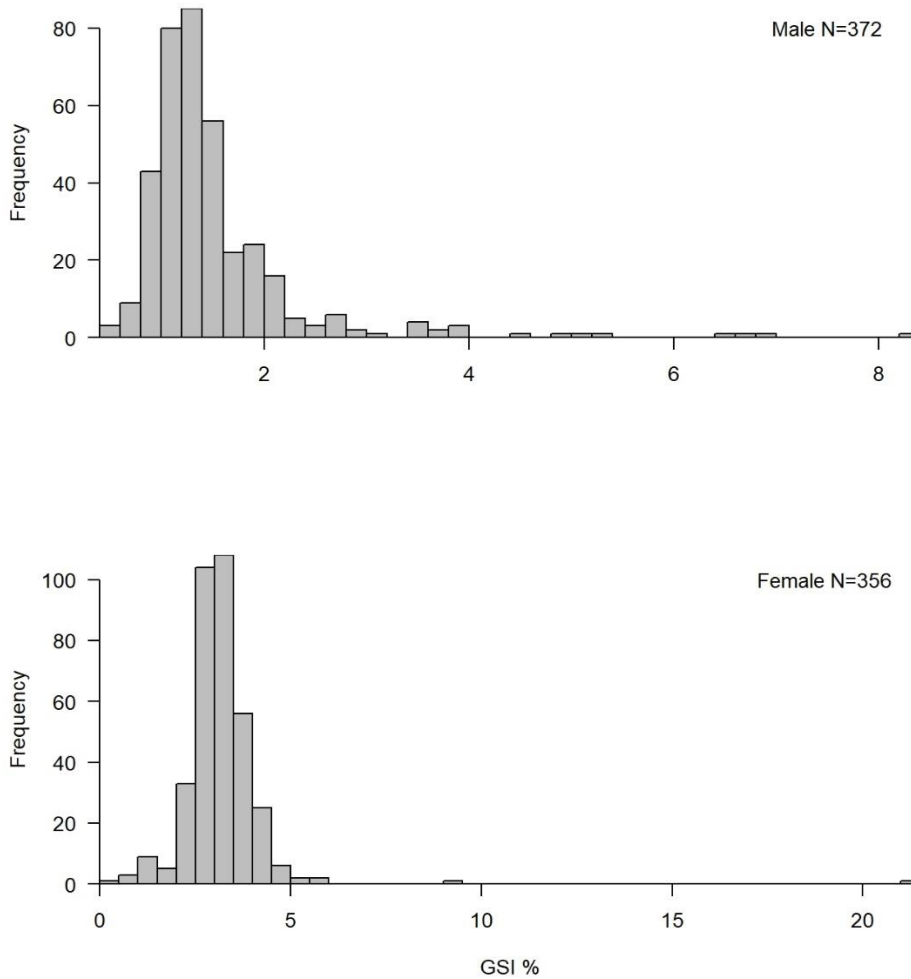
Fig 21. Histograms of gonadosomatic index (GSI) for male and female *D. mawsoni* collected during the 2019 Winter Survey.

Inserting our results in graph of boxplots obtained with data collected in previous surveys conducted in other periods, it is possible to appreciate the monthly variation of female's GSI. As already explained, data of June, July, September and October are available only for the northern area and come exclusively from the two Winter Surveys carried out to date. In this area it is possible to observe significant higher values compared to those recorded in the shelf or in the slope, and furthermore a clear trend in gonads development, which reaches its peak in early July and immediately decreases, leading us to infer a potential continuous decrease after to reach the values measured in the Second Winter Survey in September and October (Fig 22).
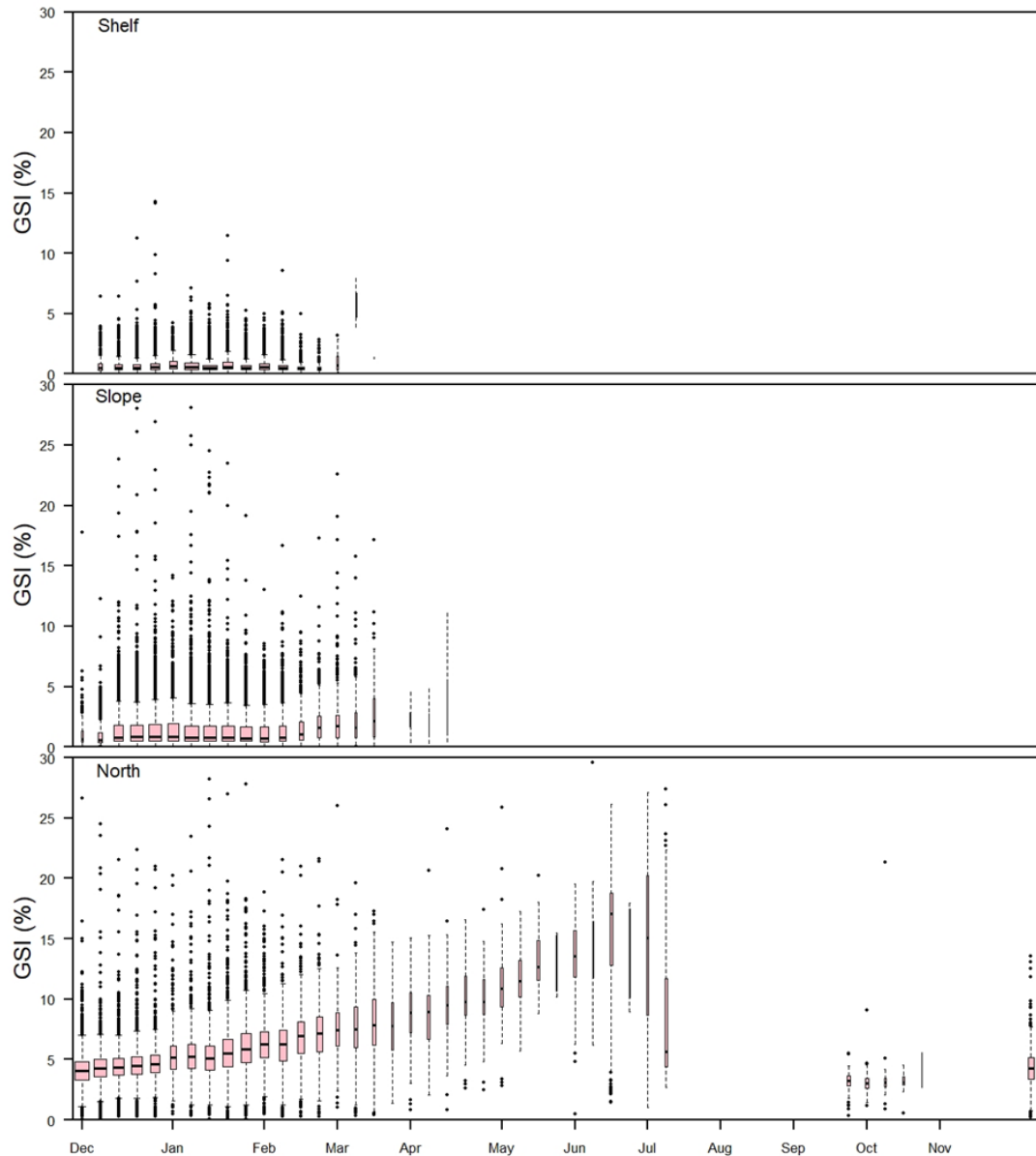
Fig 22. Progression of female's GSI during the year in different zones.

3.3.2.4 Condition indices

Sex-specific condition factors were lower than those observed in the summer or pre-spawning winter periods, and much lower than those observed on the Ross Sea slope during the summer fishery (Fig 23). "Axe handles" were the thinnest of these fish, comprising only 10% of the females and 16% of the males. "Axe handle" fish comprise an even smaller proportion of the toothfish in summer when condition is higher, and tends to be more prevalent in males.
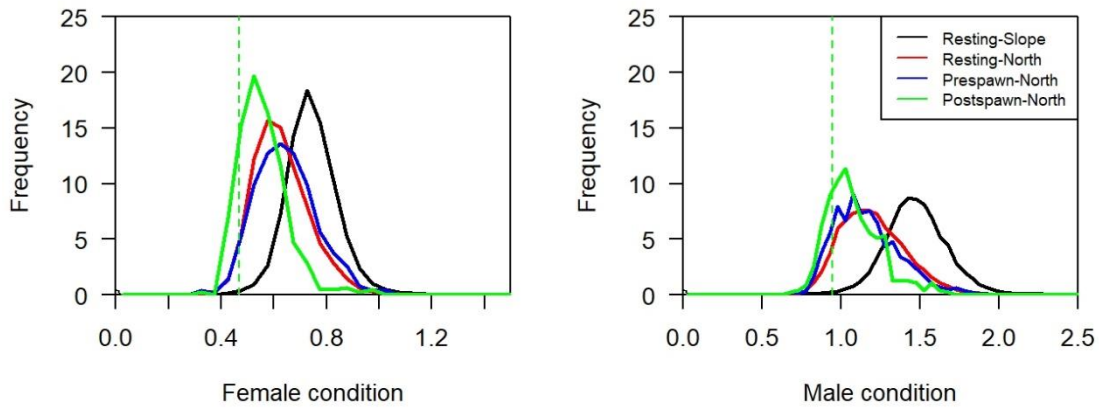
Fig 23. Sex specific condition factors for male and female Antarctic toothfish in different situations. Distributions of samples from the summer fishery, the 2016 winter pre-spawning survey, the 2019 winter post-spawning survey, and the summer slope fishery. Green vertical line indicates the mean condition factor of fish considered to be "axe handles".

One effect of measuring condition post spawning is the loss of the weight of gametes, which can be more than 20% of the total body weight (Parker et al. 2019b). Somatic weight, estimated after removing gonad weight, stomach weight, and liver weight, can be used to calculate a sex-specific somatic condition factor. The somatic weight index still showed "axe handle" fish comprised about the same proportion of the lower tail of the distribution, indicating that the effect was not strongly related to the loss of gametes or reduction in liver weight (Fig 24).
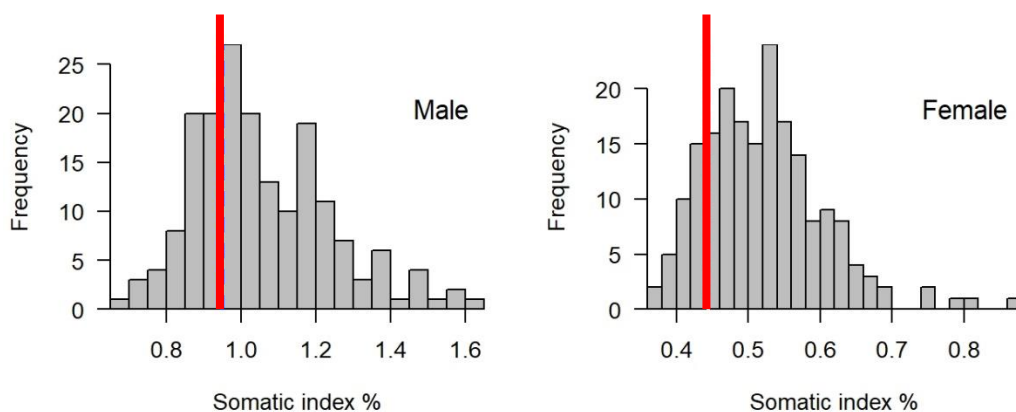

Fig 24. Somatic index (SI) distribution by sex. Vertical red lines indicate the mean SI values for axe-handle fish.

Liver weights were highly variable among Antarctic toothfish, with hepatosomatic indices (HSI) ranging from 0.25% to 4% (Fig 25a). HSI differed by sex, with female values about twice that of males. In addition, fish with poor body condition ("axe handles") had values about 30% less than fish with higher condition (Fig 25b). While HSI tended to increase with fish size for females, it tended to decrease with fish size in males (Fig 25 c, d).
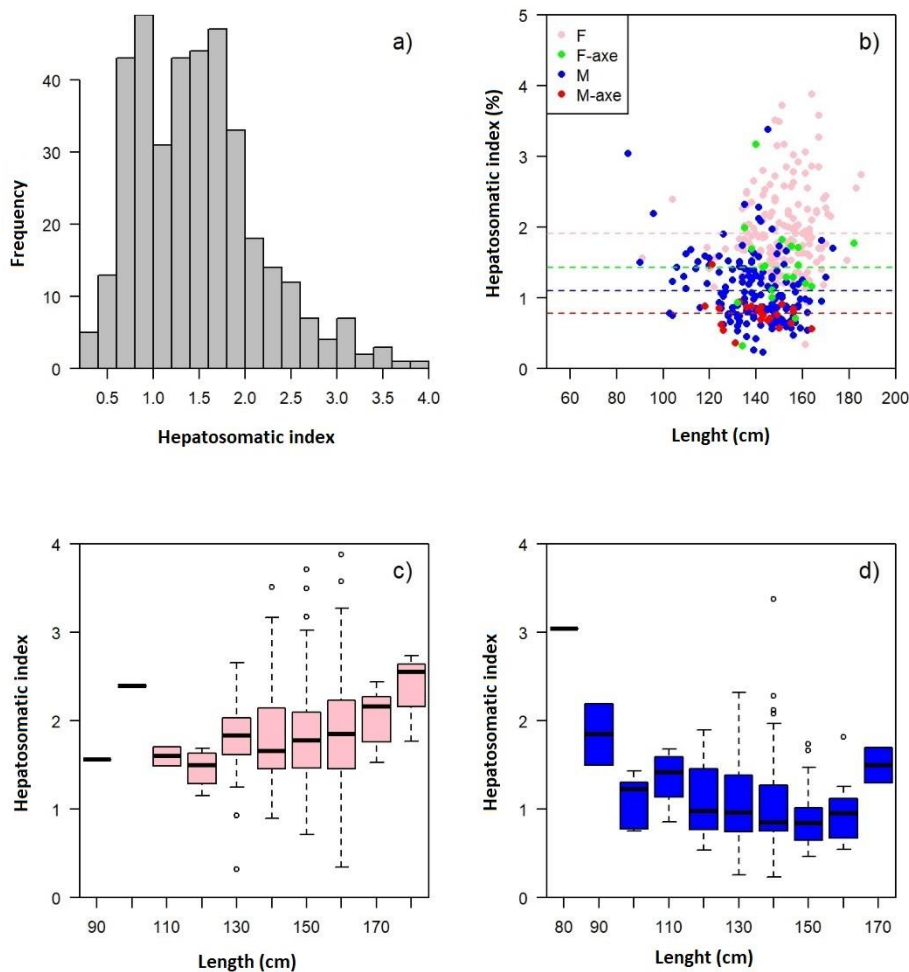

Fig 25. Statistics developed on HSI values calculated in the survey. a) Histogram of hepatosomatic index, b) scatter plot of HSI for males, females split by condition being normal or axe handle. Horizontal lines indicate mean for each group. c) and d) box whisker plots of the HSI for females and males in 10 cm length classes.

3.3.2.5 Stomach contents

As typical for northern seamount toothfish, a large proportion (86.2%) of the stomachs were empty. When food was present, digestion state was variable, indicating that fish were feeding at various times and there was no strong pulse in the timing of food ingestion. Stomach contents of the 62 fish with prey items present followed the patterns observed in the summer fishery and in the previous winter survey (Stevens et al. 2016), which was dominated by bony fishes, often macrourids and violet cod, squids, and various other demersal species (Table 5).

Table 5. Frequency and percent occurrence of stomach contents in non-empty Antarctic toothfish stomachs (62 of 450 examined).

Prey	Frequency	Percentage
Osteichthyes	26	36.62
Bait	15	21.13
Macrourus spp.	7	9.86
Antimora rostrata	6	8.45
Lithodidae	3	4.23
Squid	3	4.23
Unidentified	3	4.23
Penguin feet	2	2.82
Crustacean	1	1.41
Holothurian	1	1.41
Muraenolepis spp.	1	1.41
Rajidae egg capsules	1	1.4
Rock	1	1.4
Sponge	1	1.4
Total	71	100

3.3.3 Biological data

3.3.3.1 CCAMLR tags

Antarctic toothfish were tagged at a rate of 3 fish per ton of catch. However due to the stratum-level catch limit of 50 t, a total of 179 were tagged on a single line while following CCAMLR tagging protocols. The overall tagging rate was therefore 5.22 tags per tonne and a total of 300 Antarctic toothfish were tagged throughout the survey. Fish

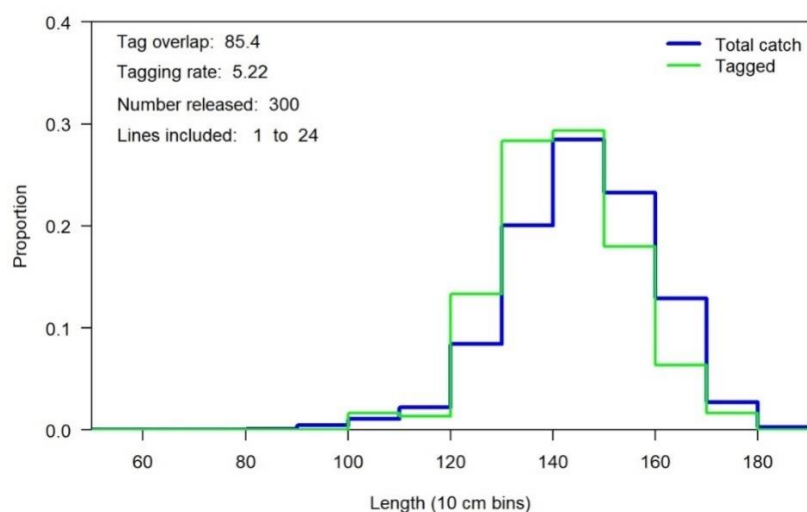were tagged continuously throughout hauling to maintain a good tagging overlap statistic of 85.4% (Fig 26).

Fig 26. Tagged fish size overlap diagram showing the size frequency distribution of landed Antarctic toothfish and the size frequency distribution of tagged and released fish across survey hauls.

During sampling of a newly mapped seamount, 5 tagged fish were recaptured. One was a fish released just two days earlier. The other four fish were from different areas of the Ross Sea slope, travelled more than 2300 km, and were at liberty for at least four years (Table 6, Fig 27). Biological data was recorded for each recapture and tissue samples were taken.

Table 6. Details of release and recapture of four toothfish recaptured during the winter survey.

			Release				Recapture
Year	SSRU	Vessel	Length	SSRU	Length	Liberty (y)	Distance (km)
2015	88.1 H	San Aotea II	164	88.2 B	138	5	2719
2012	88.1 K	Tronio	119	88.2 B	160	8	2808
2014	88.1 H	Seljevaer	132	88.2 B	138	6	2647
2014	88.1 I	Janas	134	88.2 B	142	6	2326

Note, one fish was likely incorrectly measured at release given the smaller size at recapture
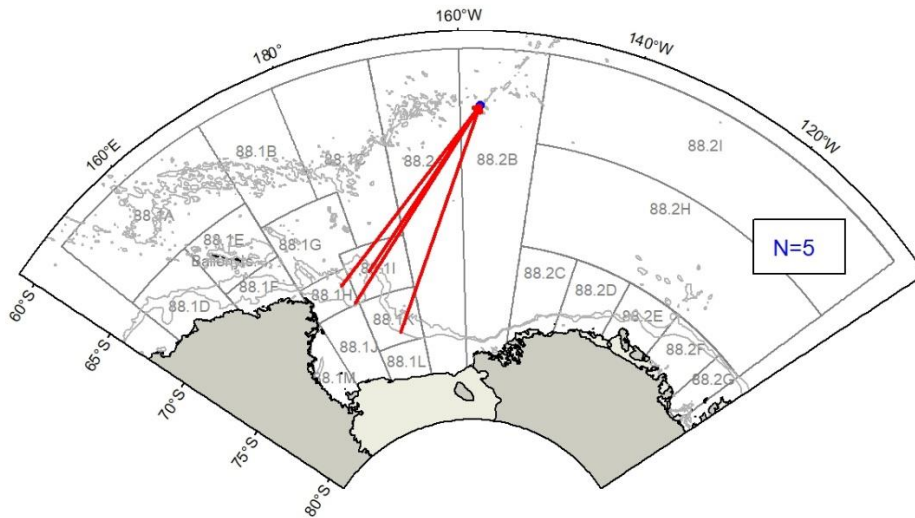
Fig 27. Locations of release and recapture for 5 fish recaptured during the winter 2019 survey. Note one fish was released and recaptured at the same location during the survey.

3.3.3.2 Pop-off satellite archival tags (PSAT)

Four PSATs were deployed on Antarctic toothfish in excellent condition that had lengths of 149, 150, 160 and 155 cm and were also tagged with CCAMLR tags (Table 7). The first PSAT was deployed in stratum 7 and was programmed to detach June 1st 2020. Two PSAT tags were deployed in stratum 6 and were programmed to detach March 1st 2020 and June 1st 2020. The last tag was deployed in stratum 1 and was programmed to detach on November 1st 2020 (Fig 28). An underwater video was made of the release of the last PSAT tagged fish. The fish was detected at 183 m depth several minutes post-release by the echosounder (Fig 29). None of the tags have been detected by the ARGOS satellite.

Table 7. Release details for PSAT deployments.

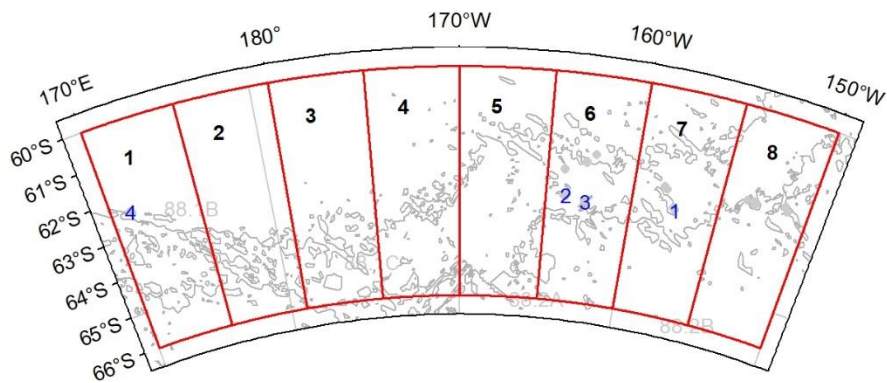| PSAT ID	Programmed pop off date	Line n°	Latitude (D M.M)	Longitude (D M.M)	Depth (m)	Length (cm)	CCAMLR Tag1	CCAMLR Tag2
2095	1 June 2020	8	63°08.17'S	157°25.68'W	1738	149	A318763	A318764
2074	1 March 2020	20	63°12.71'S	163°49.31'W	1115	150	A351844	A351845
2085	1 June 2020	22	63°18.73'S	162°39.40'W	1443	160	A351846	A351847
2092	1 November 2020	23	62°22.38'S	171°08.17'E	1700	155	A351854	A351855

Fig 28. Locations of release for Antarctic toothfish tagged with PSAT tags during the 2019 winter survey.
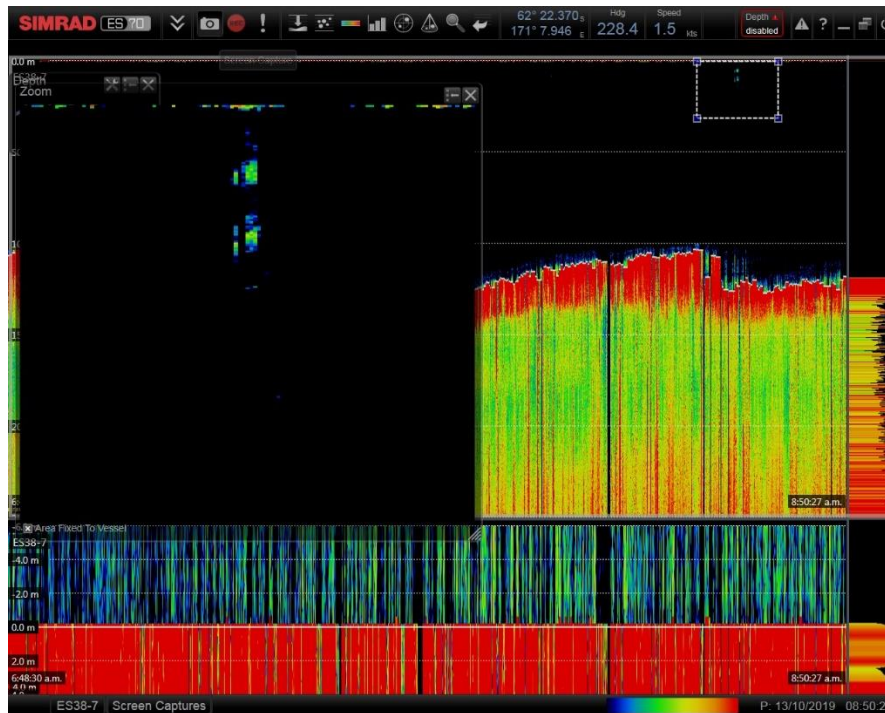
Fig 29. Screen capture of the monitor connected to the echosounder after the release of the last PSAT-tagged toothfish. In the little frame on the right it is possible to see the signal of the fish. On the left, the image is magnified.

3.3.4 Eggs collection, development and buoyancy studies

3.3.4.1 Eggs culture

Fertilization of egg on board, which was in the plan of the survey, was not possible because no ripe or running Antarctic toothfish were caught. The only chance to get embryonated eggs was then to collect them in the plankton. From 16 September to 13 October 2019, 19 plankton tows were carried out.

No larval toothfish were identified in the plankton, only Antarctic toothfish eggs were present but rare in the areas sampled with the bongo nets, captured in six tows in strata 6, 7, and 8 (Fig 30). When present in SSRU 88.2 B, eggs resulted in the plankton with a density of 1 egg per 1800 m^3 of water sampled. Eggs were only collected when sampling to the south of the fracture zone. None occurred during the three tows made with the "wind buoy", but this was not a standard part of the design and was done to permit towing the nets closer to the surface to target the buoyant eggs. A total of 8 eggs were collected, 6 were immediately put in the buoyancy chamber and 2 were fixed in 10% formalin for future analyses, which will follow those carried out on the eggs collected in 2016 (Ghigliotti et al. 2018).
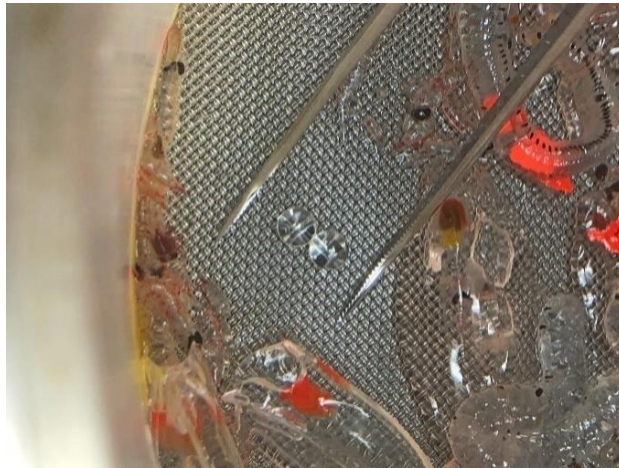
Fig 30. A couple of Antarctic toothfish eggs sorted in a plankton sampling.

The eggs in the buoyancy chamber were the only eggs available to follow embryo development and document changes through daily photographs. The eggs were in remarkably good condition despite the long tow durations and remained alive inside the columns. Development stage in almost all cases corresponded to day-15 of Patagonian toothfish embryo development reported by Mujica et al. 2016, with embryos having a distinguishable head with eye lenses but no-pigment, vertebral column, myomeres, and developing tail fin rays (Fig 31 A). During the time the embryos were held in the buoyancy chambers, development continued with pigment forming in the eyes of at least two embryos (Fig 31 B). Development appeared slow compared to that of the Patagonian toothfish (Mujica et al. 2016), but the development they documented was at a higher temperature of 5°C.
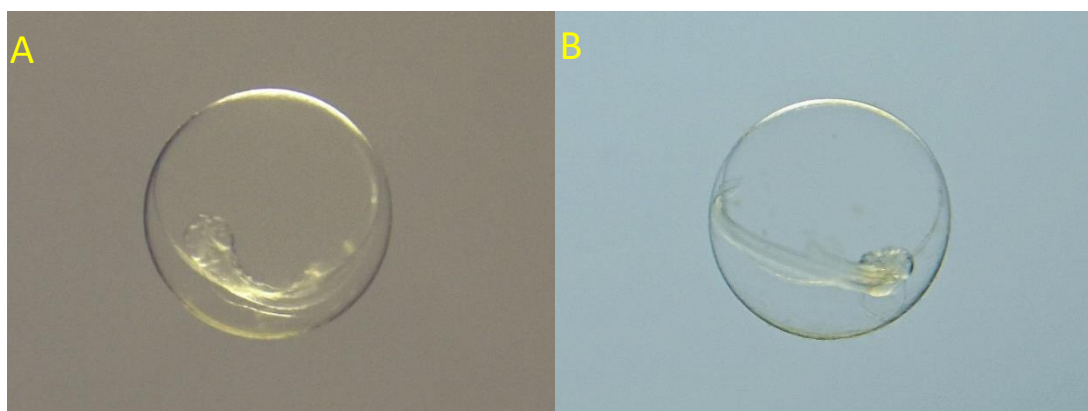

Fig 31. Macro-photos of eggs inside the buoyancy chamber. (A) an egg after a few days from its collection; (B) an embryo developed in the column.

3.3.4.2 Buoyancy measurements

The first two eggs were placed in the chamber on September 16th and buoyancy measurements started 2 days later. Daily measurements were taken of their relative position with respect to density-calibrated glass spheres; water temperature in the density gradient columns was also recorded. Four additional eggs were placed in the chambers: 2 on the 26th September, 1 on the 28th of September, and 1 into chamber 2 on the 6th of October.

The eggs were at neutral buoyancy between the density standard spheres at 1026.2 and 1028.1 kg/m^3 (Fig 32), indicating they would be less dense than the sea water in the area and accumulate near the surface (mean of upper 100 m = 1027.7 kg/m^3). These results are similar to the results from the 2016 Winter Survey based on newly fertilised eggs (Stevens et al. 2016).
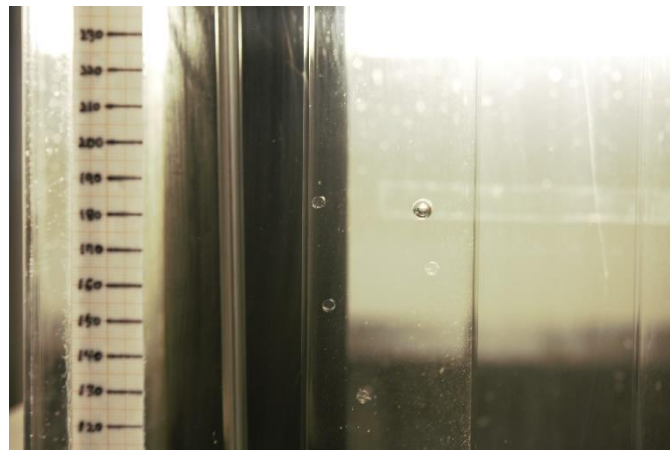

Fig 32. Eggs near a density-calibrated sphere inside the buoyancy chamber.

A malfunction with the circulation-chiller unit caused water temperature to increase 15 degrees over a 24-hour period, resulting in mass mortality of the eggs on October 7th. Eggs were preserved in formalin for structural analyses.

3.4 Discussion

This second Winter Survey has been a part of a planned multi-annual survey for the investigation of timing and location of spawning, and early life history of the Antarctic toothfish. The first Winter Survey, carried out in June and July 2016 demonstrated the feasibility of winter studies in the northern Ross Sea during the winter months, even if they are conditioned by the sea ice extent.

Overall, the survey of 2019 has to be considered very successful, a huge quantity of information can be deduced from the data collected and can be added to those acquired in 2016 to obtain a more complete picture of the aspects linked to the reproduction of the Antarctic toothfish.

All the biological evidences supported the conclusion that the northern seamounts, ridges and banks are right places for the spawning of the Antarctic toothfish Ross Sea population, and that in September and October the spawning period has been already finished. The majority of the fish landed were large adults. From the observation of the gonad developmental stage and the calculation of the gonadosomatic index resulted that almost all of the individual were spent and the values of GSI were much lower than those recorded in 2016 a few months before, indicating that spawning had ceased at least several weeks prior. This suggests that the spawning season, at least in the area sampled, occurs in early and mid-winter and it is short, lasting presumably about a month, with the peak occurring in July (Stevens et al. 2016, Parker et al. 2019b).

The confirmation that the northern Ross Sea is predominantly a breeding area for the Antarctic toothfish came from the analysis of the stomach contents. Even if fish approached the baits, then the feeding activity cannot be considered completely null, the proportion of empty stomach was relevant, as typically found there (Stevens at al. 2014), coherently with the results of stable isotope analyses that found mainly signals of prey from slope areas (Pinkerton et al. 2014). Such information has to be flanked by the poor condition factors of both females and males, highly lower than those observed in the slope during the summer, and also lower than those observed in other months in the north. The loss of condition, further than to the reduced feeding activity, is likely due to the energy consumption for the reproduction and the loss of the mass of gametes, that for the species can represent a significant percentage of the total body mass (Parker et al. 2019b). In this contest, notably the proportion of so called "axe handle" individuals resulted higher than in other seasons. What causes a so poor-condition fish is to date unknown, the fact that

during the winter period the proportion is higher gives us clues that it could be linked to the expenditure of energy related to the breeding activity, maybe the migration or the spawning, even if, because not all post-spawning individual are "axe handle", we cannot consider them simply as reproductively spent adults (Fenaughty et al. 2008). Also, it is unknown what is the destiny of these fish, if they can restore or there is a post-spawning natural mortality. To obtain information on this, during the survey some samples of muscle, liver and gonads of both axe-handle and regular-condition fish have been collected for sequent analyses.

As for each CCAMLR-managed toothfish fishing activity, a tagging program was implemented. For a species such as the Antarctic toothfish which life cycle is extended in an extremely wide area (Hanchet et al. 2008, 2015, Ashford et al. 2012, Mormede et al. 2014) tagging results particularly important to investigate the movements among zones. During the survey, four Antarctic toothfish tagged in previous years in slope areas were recaptured. These fish were tagged 5-8 years before in different zones of the Ross Sea slope, thousands of kilometres far, during summer fishing, and ours represents the first cases of recaptures in SSRU 88.2 B. With other recaptures previously occurred in other subareas of the northern Ross Sea region, it strengthens our knowledge on the linkage between slope and seamount features during the life cycle of the Antarctic toothfish, confirming the migration hypothesized by Parker and Marriott (2012).

While we can now assume with a good likelihood the movements of at least a part of adult population from the south to the north features, we have not evidences on what happens after the spawning in the north. The theory on the life cycle supposes a return to the slope of adult fish following Ross gyre circulation (Hanchet at al. 2008, Ashford et al. 2012). Such hypothesis is based on analyses of stable isotopes and mercury levels detected in the muscle tissues. The isotopic signals mainly attributable to preys from the slope and the resulted low mercury levels, contrasting the high values of the main preys found in the seamounts, indeed pointed out the probability of a short residence time in the north (Hanchet et al. 2015), Pinkerton et al. 2014). A certain level of uncertainty remains from these clues, the results are based on muscle tissue samples and could be biased because mature Antarctic toothfish may partition energy to liver and gonad tissues for spawning activities. Another weak indication of a slow dispersal from there come from the shorter residence time in the north observed in tagged toothfish compared with those on the slope (Hanchet et al. 2015).

Actually, to date we have not robust information on the post-spawning migration, we don't know if and how it occurs, what is in the case the timing of the migration to return in the feeding zones, and if fish resides for more of a spawning season on the northern seamounts. For this reason, the implementation of the tagging program during the winter surveys carried out in the northern areas is particularly relevant. The pop-off satellite archival tags (PSATs) were used with the same aim of clarify post-spawning history in the life cycle of the Antarctic toothfish, being furthermore able to record the horizontal and vertical movements of the fish for the time in which they remain set. The fact that none of them have been traced by the satellite could be imputed to the high depth that the fish reached after the release, even if the 'crash prevention release' was programmed to occur at 2000 m depth, where the pressure was supposed to not damage the PSAT. The system has to be improved for future applications.

Another primary objective of the survey was the collection of Antarctic toothfish eggs and/or larvae from the plankton, and if possible to fertilize and farm eggs on board to follow their development. Further then presence and distribution, a particular aim was to investigate how change their buoyancy during the development, which is functional to their dispersion carried by the movements of the water masses. Such objectives resumed those of the survey of 2016, when for the first time Antarctic toothfish eggs were collected and eggs from ripe females were successfully fertilized with milt of running ripe males (Stevens et al. 2016, Parker et al. 2019b). Unfortunately, in the first Winter Survey the eggs were collected by means of a plankton net with a rough action that damaged them and made them useless for buoyancy studies. At the same time, those fertilized and farmed occurred at the end of the survey and the buoyancy was measured only during the first days of the embryonic development. The 2016 voyage was anyway a great success from this point of view, because no Antarcitc toothfish embryos were even seen before, and put the basis for the planning of subsequent studies.

This new Winter Survey has given the opportunity to extend the search of eggs in the plankton to later months and in other areas potentially associated to the spawning, to investigate their distribution over time and space, and to have the chance to observe their development and eventual variation in density in multiple developmental stages. The use of a more delicate bongo net allowed to collect intact and alive eggs. Those collected in the plankton were the only eggs used for buoyancy and development observations, because the timing of the survey did not allow to find running ripe fish and to carry out fertilization experiments on board. Although much of the likely spawning habitat was

supposed to be under the sea ice, and in different occasions the formation of new sea ice in the form of grease ice or pancake ice prevented the deployment of the bongo net or its right work even where the vessel was able to move, some plankton tows were done. Embryos, though rare, were caught on multiple tows within 150 m of the surface, mainly in Subarea 88.2. This, flanked to the results of the 2016, indicated at least two things: the Antarctic toothfish eggs potentially set in the higher stratum of the water column for a long period, and the northern Ross Sea features in correspondence of the Pacific Antarctic ridge should represent in a great part of their extension a proper reproductive area for the Antarctic toothfish (considering that in 2016 the eggs were collected hundreds of kilometres west, in Subarea 88.1). The embryos were all in advanced developmental status, confirming that the spawning ceased some weeks before, as revealed by other biological data. Buoyancy studies indicated that the density of the embryos do not vary with the development, being strongly positive in these developed eggs, and during their further development in the chamber, as in the early-fertilized ones measured in 2016. It is so likely that until the hatching they could remain close to the surface and, being a large part of the spawning area covered by the sea ice during the winter period, the ice could have the function to protect them and to slowing down their dispersion with the water currents. Such information has to be carefully taken into account in the modelling of advection and dispersion, which are crucial for a complete and accurate stock structure examination (Dunn et al. 2012).

Attempts to observe Antarctic toothfish embryos under the ice were done using a mini-ROV. The original plan was to arrive to the margin of the pack ice and drive the ROV under it, actually instead it was not possible, because the pack was divided from the open water by a "buffer zone" characterized by grease ice and pancake ice. The ROV was then deployed in correspondence of an area characterized by large pancakes ice, but on one hand it results very difficult to operate the ROV connected to the cable among the pancakes and on the other hands the eggs, although large, being more than 3.5 mm of diameter, results too much hyaline to be detected with the adopted camera.

As sawn, the development of the embryos was followed in the eggs set in the buoyancy chamber. From a comparison with what reported for the congeneric Patagonian toothfish (Mujica et al. 2016), the developmental stage of the eggs at the time of the catch corresponded to the day-15 Patagonian toothfish embryo, and about other two weeks of development were followed in the chamber. The observation revealed a slower developmental rate of the Antarctic toothfish embryos compared to that of the Patagonian

toothfish. Considering that the temperature of the water in the chamber was some degrees higher than the natural water temperature of the surface stratum in the period of the sampling, we can deduce that the development should be even slower and that at the moment of the sampling the embryos should be quite older than 15 days, than the spawning occurred some weeks before as other results led to conclude.

In conclusion, thanks to the amount of results from this new Winter Survey now it is possible to have a quite exhaustive picture of different aspects linked to the reproduction of Antarctic toothfish in the northern features of the Ross Sea, something that until 2016 was exclusively hypothesized. The gap represented by the core months of the winter is and probably will remain unbridgeable, because of the logistical constraints in operate in the area in that period, but thanks to the information obtained until the beginning of July and in September-October, we can infer the information necessary to fill our knowledge on the succession of the reproductive events and the associated variation of biological characteristics of the Antarctic toothfish in the northern features of Ross Sea during winter. What remains by now unsolved is what is the actual distribution of the embryos under the sea ice, when does the hatching occurs, and how does the sea ice intervene in the models of dispersion of eggs and larvae. Also, further studies are needed to deepen our knowledge on what occur to adult toothfish after the spawning. Filling these gaps will be crucial for a right stock assessment and the implementation of an adequate management plan for a species such as the Antarctic toothfish that is ecologically relevant and represent a fishing resource.

4. The fate of an Antarctic toothfish: clues from lipid distribution and composition

4.1 Introduction

The wide life cycle of the Antarctic toothfish (*Dissostichus mawsoni*) is extended for thousands of kilometres, from the shelf and slope areas, in which settlement and maturation to adult fish occur, to the northern seamounts that represent the breeding areas (Hanchet et al. 2008, 2015, Parker et al. 2019). The movements between southern and northern areas and *vice versa* are presumed to take place in pelagic environment (Near et al. 2003). In particular, when adult, the species undergoes a long, potentially exhausting, reproductive migration from the slope to the seamounts, about which little is known.

Migration is presumed to be an ancestral life trait for notothenioid, although nowadays many notothenioids have sedentary life styles, such feature persists in the *Dissostichus* species, in the Antarctic silverfish (*Pleuragramma antarctica*) and in the non-Antarctic notothenioid *Eleginops maclovinus* (Near et al. 2004, Fenaughty et al. 2008).

Reproductive migration confers the benefit to spawn in an optimal area for eggs dispersion and early life stages survival, but it also has high costs and subjects fish to a number of ecological needs, such as the necessity to accumulate a surplus of energy, the capacity to survive possible predators, changing environmental conditions and starvation, and to optimize geographical position for the migration itself and the subsequent life stages (Hinch et al. 2005, Del Raye et al. 2013).

Among the biological implications of migrations, there is the suppression or inhibition of responses to stimuli that would arrest movements, such as feeding. It means that many migrants do not feed or feed sparsely during their route, and in some cases cease feeding activity prior to the migration (Hinch et al. 2005). Furthermore, the breeding grounds, once reached, are not necessarily suitable areas for preys. This bring to the requirement for the involved individuals to store a huge amount of energy before facing the migration and subsequent spawning. Such energy storage needs ads on others, such as gonadal development, energy to support competition for territories and spawning, etc. (Larsson et al. 1990, Jonsson et al. 1997, Del Raye et al. 2013, Bayse et al. 2018, Hertz et al. 2019).

As in other vertebrates, in fish energy is stored mainly in the form of lipids, which distribute in various body districts (Kaneko et al. 2016, Khieokhajonkhet et al. 2019), and

have diversified biochemical composition (Clarke et al. 1984, Fenaughty et al. 2008, Tocher 2003), depending on the species. The accumulation of lipids necessary to sustain an event so demanding as the reproductive migration, trigged the need for an adequate overabundant feeding activity in advance (Midwood et al. 2016). Further than the immediately close-to-migration huge feeding, the reproductive output is related to lipid accumulation during juvenile life stages (Martin et al. 2017).

For the Antarctic toothfish, the slope high-productive feeding ground is a fundamental area in which to achieve the optimal condition prior to the migration, but also the lipid accumulation that occurs in the shelf areas, in which juveniles spend about ten years before reaching adulthood result highly important for the future reproductive success. The amount of lipids accumulated in the slope have also another function strictly related to the Antarctic toothfish migration, that occurs in the pelagic environment, assisted by currents that should limit energy consumption (Near et al. 2003, Hanchet et al. 2008, 2015). As a nototheniod, indeed, the Antarctic toothfish is devoid of swim bladder, and to move far from the bottom it needs to counterbalance its negative buoyancy. Having evolved from a bottom-living ancestor (Clarke et al. 1984), neutral buoyancy is uncommon in notothenioids, and the clade includes mainly strictly demersal species. In the rare cases of pelagic notothenioid species, neutral buoyancy is obtained by means of adaptations that allow a lightening of the body, such as reduced skeletal ossification (Voskoboinikova et al. 2017), and accumulation of neutral lipids within the white axial musculature and in subcutaneous tissue (DeVries and Eastman 1978, Eastman and DeVries 1981, 1982, Eastman 1993, Fenaughty et al. 2008). Differential accumulation of lipids is considered the mean mechanism changing the buoyancy of the Antarctic toothfish during its life cycle (Near et al. 2003, Fenaughty et al. 2008).

The lipid stored during the slope feeding might then have the double function of energy reserve and somatic trait for neutral buoyancy. However, to date this is not completely proved, and a question remains whether the same lipids can effectively play this double role.

In Antarctic toothfish, triacylglycerols are the dominant type of lipids and are concentrated mainly in the white axial muscles and in subcutaneous tissue (Clarke et al. 1984, Fenaughty et al. 2008). Triacyglycerols provide less lift than other types of lipids utilized by other fish groups for achieving neutral buoyancy (e.g. wax esters) (Clarke et al. 1984), so the use of triacylglycerols in the Antarctic toothfish could be a clue of the double function of the lipid storage in this species. Triacylglycerols are indeed good

energy reserves, particularly the component of unsaturated fatty acids (UFA), which are more easily metabolized than saturated fatty acids (SFA). Thus, in fish species that store mainly triacylglycerols, UFA might have a principal metabolic function, while SFA would provide the buoyancy aid (Hazel and Sidell 2004).

Antarctic toothfish stores lipids within adipocytes, and does not possess the specialized lipidic sacs described in the pelagic notothenioid Antarctic silverfish (*Pleuragramma antarctica*). These sacs seem to be permanent less metabolic active structures, with the only function of regulate general fish density (Clarke et al. 1984, Eastman and DeVries 1989). Differently, the adipocytes in which are stored lipids in the Antarctic toothfish make them metabolically available.

If the huge part of lipids that the Antarctic toothfish accumulate are available both for buoyancy purpose and as energy reserve, it means that in this species the neutral buoyancy is ephemeral and fluctuate over the course of life cycle in response to the accumulation and use of energy (Fenaughty et al. 2008). Fluctuations in energy stores are common in fish, they can be related to seasonal variation of food availability or reproduction, when energy is normally mobilized from somatic and visceral tissues for the reproductive development (Jonsson et al. 1997, Hinch et al. 2005, Martin et al. 2017). However, in long-range migrant species such as the Antarctic toothfish the consumption of energy reserves is more drastic. The energy depletion due to the combination of protract fasting, highly expensive migration, gametogenesis and reproduction, lead to a very high consumption of lipid stores, and a consequent variation of buoyancy, which in the northern seamounts should return negative.

In fish, lipid reserves are not the sole source of energy, when they are mostly depleted, proteins of the white muscle can be metabolized (Hinch et al, 2005), this could result in a sensible consumption of the axial muscle mass (Fenaughty et al. 2008). The presence in the northern seamounts of the Ross Sea (Pacific sector of the Southern Ocean) of a huge percentage of Antarctic toothfish in particularly poor condition is probably symptomatic of such mechanism of lipids and proteins depletion (Fenaughty 2006, Fenaughty et al. 2008). Antarctic toothfish in such a poor condition are commonly named "axe handle" by the crew of the fishing vessels because of their thin trunk, and the shrunken appearance behind the head. The "axe handle" condition might be caused by the high energy consumption during migration and reproduction, but the fact that large numbers of "axe handle" individuals have been sight in months far from the spawning period, with gonads in early developmental stages, suggests that a relevant contributing

cause of such condition could also be the low productivity and food availability of the northern areas (Fenaughty 2006). If this holds truth, a number of questions on the fate of the Antarctic toothfish present in the northern seamounts arises. Are all toothfish destined to reach the poor "axe handle" condition? Can a toothfish undertake a post-reproductive migration from the breeding northern areas to the feeding slope areas (which would mean reacquire good neutrally-buoyant condition)? What cause underlie the difference between toothfish in regular body condition and the "axe handle" individuals? And, taking the reasoning towards its extreme, is the Antarctic toothfish an iteroparous or a semelparous species?

Semelparity, i.e. the characteristic of an animal to spend a very high percentage of its energy for a single reproductive event and die after it, is not uncommon in long-range migrant fish. It happens for example in the southern population of the American shad (*Alosa sapidissima*) (Leggett and Carscadden 1978, Leonard and McCormick 1999, Bayse et al. 2018), in the rainbow smelt (*Osmerus mordax*) (Schaefer et al. 1981), and in the Atlantic and Pacific salmons of the genera *Salmo* and *Oncorhynchus* (Jonsson et al. 1997, Hinch et al. 2005, Crossin et al. 2009). To date, there is no information on the fate of Antarctic toothfish individuals after reproduction. Such information would be of highly relevant interest for improving the populations assessments and consequent management of the species by the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR).

In this work, we tried to answer some of the above questions on the Antarctic toothfish post-spawning destiny by approaching the study of lipid distribution and composition in individuals collected in the shelf and in the northern seamounts, in both regular and "axe handle" conditions. Such an investigation included: i) assessment of lipid distribution in various parts of the body; ii) analysis of the lipid quantity and distribution among the specimens in different conditions; iii) determination of fatty acids composition; iv) comparative analysis of differences in fatty acids composition among specimens in different body conditions.

4.2 Materials and Methods

4.2.1 Study areas and fish samples

Samples were collected during two surveys, led by New Zealand, in different areas of the Ross Sea, where different moments of the life cycle of the Antarctic toothfish take place (Fig 33A).

In September-October 2019, the Winter Survey was conducted onboard of the New Zealand flagged fishing vessel *FV Janas* in Subareas 88.1 SSRUs B-C and 88.2 SSRUs A-B, a wide area of the northern Ross Sea extended from 60° to 66° S, and from 150° W to 170° E (Fig 33 B). Sampling locations targeted seamount features shallower than 2000 m as potential locations for Antarctic toothfish spawning. Late winter-early spring period of sampling allowed to catch adult post-spawning specimens (Parker and Di Blasi 2020).

In January-February 2020, the Ross Sea Shelf Survey was conducted onboard of the New Zealand flagged fishing vessel *FV San Aotea II* in Subareas SSRUs 88.1 J, 88.1 L and 88.1 M, shelf areas of the southern Ross Sea comprised from 76.5° to 78° S, and from 175° W to 165° E (Fig 33-C). The survey targeted sub-adult and adult Antarctic toothfish in feeding areas, which presence had been ascertained in a series of annual shelf surveys started in 2012 (Parker et al. 2020).
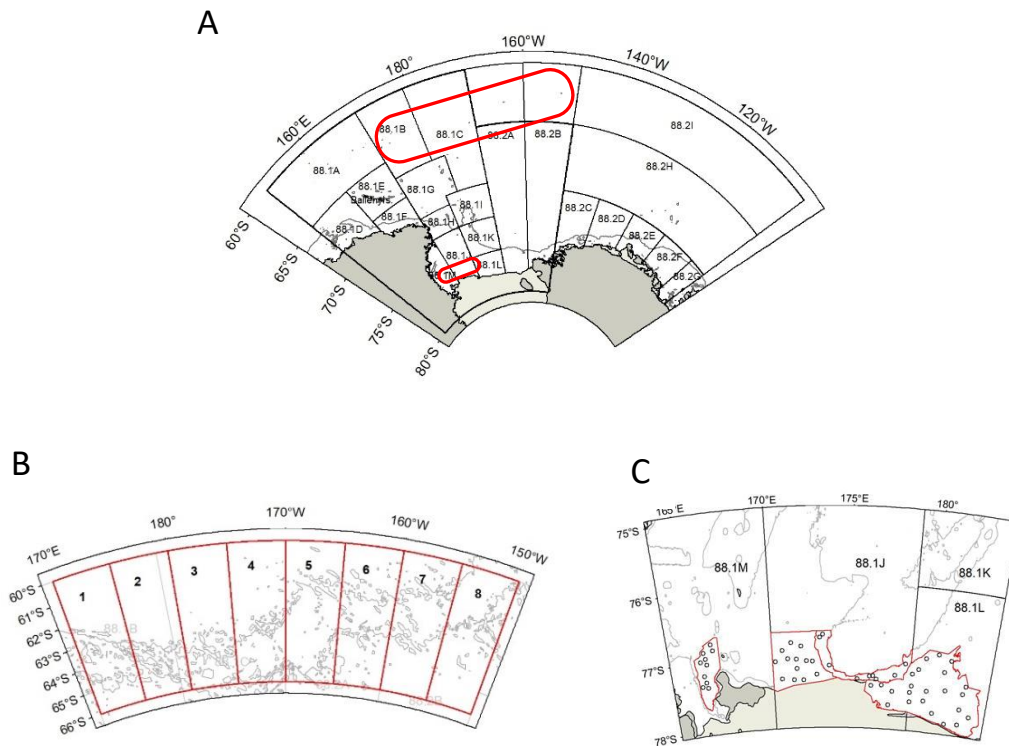
Fig 33. Areas in which samplings were carried out. A general view of the Ross Sea, with the sampling area of the seamount, on the north, and the sampling area of the shelf, on the south, are highlighted by red contours (A). Detail of Winter Survey area, the red blocks correspond to the statistical division used by CCAMLR for fishing management and investigation (B). Detail of Ross Sea Shelf Survey area, red contours correspond to the CCAMLR blocks, white dots correspond to planned sampling points (C).

Scientific sampling was in both cases associated to commercial fishing, implemented by means of autoline longline gears. Integrated weighted line, with ca. 50 g of lead weight per metre of mainline, was used for all longlines. The snoods were 300-400 mm long, spaced 1.4 m, and connected via rotors and swivels that were permanently attached to the backbone. Hooks were size 14/0 or 15/0, baited using automatic baiting machine with 35-45 g of squid (Parker and Di Blasi 2020, Parker et al. 2020).

Six Antarctic toothfish, representative of three different conditions, were sampled for our study. Four post-spawning adult specimens were collected during the Winter Survey in a breeding area, two in visibly poor condition, categorized ad "axe handle", and two in apparently good condition. Two close-to-adult specimens were collected during the Ross Sea Shelf Survey in a feeding ground. K condition factor was calculated as Weight (kg) / Total Length (cm)^3 * 10^5 for each individual. All individuals sampled were of the same sex (males) to avoid gender-related differences in the lipid accumulation. For each fish,

two slices of trunk, about 3 cm wide, were sampled for lipids investigation. One was obtained in correspondence of the ventral cavity, cut at the posterior end of the first dorsal fin, the other was cut more posteriorly, about at the middle of the second dorsal fin. Samples were preserved frozen at -20°C.

4.2.2 Lipid distribution and quantitative analysis

Each slice was cut into two complementary specular halves. One half was utilized for the investigation of lipid distribution and quantity through staining with oil red O solution (Khieokhajonkhet et al. 2014, 2019, Kaneko et al. 2016).

The oil red O stock solution was prepared by mixing 0.5 g of oil red O powder in 100 ml pure isopropanol, dissolving at 37° C using a magnetic stirrer. The solution was filtered using No. 2 filter paper. The oil red O working solution was then prepared diluting a rate of stock solution in filtered sea water (3:2, v/v), and filtering it again using No. 2 filter paper. Each sample was defrosted at room temperature, and soaked in filtered sea water for 30 min. Then it was incubated in 60% isopropanol for 30 min, and subsequently incubated in oil red O working solution for other 30 min. The sample was after gently washed with 60% isopropanol, before a final wash with filtered sea water. Images were captured at a metric reference using a Nikon D5600 digital camera with Nikon AF-S Micro Nikkor 40 mm macro lens.

Images analysis was performed by means of Fiji ImageJ software (Schindelin et al. 2012). After a first general observation of lipid distribution in the surfaces of the cross sections, a series of measures was performed. For each half slice, the thickness of the subcutaneous lipid layer was calculated as the mean of 5 randomly selected measures replicated from the dorsal to the ventral area, and the thickness of lipid accumulation in myosepta was calculated as the mean of 10 randomly selected measures replicated on the whole surface of the section. The percentages of the dorsal and the ventral surfaces of lipid accumulation respect to the entire surface of the section were separately calculated. The percentage of total lipids area respect to the entire section area was calculated by means of the threshold technique, putting in evidence the pixels correspondent to the red-coloured lipids respect to the total pixels of the section. Furthermore, in the sections obtained in correspondence of the ventral cavity, the thickness of the lipid layer around the cavity was calculated as the mean of 5 randomly selected measures. Linear measures were standardized to the total length of the fish.

Principal Component Analysis (PCA) on the measures was developed by means of R 4.0.2 software (R Development Core Team 2020) to investigate how the specimens differentiated on the basis of lipid distribution and quantity, and what areas of lipids storage contributed most to the variation.

4.2.3 Lipid composition

In the remaining not-stained half slices, an analysis of composition of lipids was conducted. Considering that triacylglycerols represent the major lipid component in the notothenioids (Clarke et al. 1984, Fenaughty et al. 2008), we focused our investigation on fatty acids.

From each slice, we cored a portion (1.0-2.5 g) in the central part of the section, paying attention to collect both subcutaneous and intramuscular lipid accumulations. Each core was then processed following Grasselli et al. (2014). The portion was inserted in a potter with PBS 1x in the proportion 1g:10ml and homogenized with a glass dounce homogenizer on ice. Lipids were then extracted in chloroform/methanol according the procedure of Folch et al. (1957). 0.5 ml of sample were added to 5 ml of chloroform, 2.5 ml of methanol and 2.5 ml of milli-Q water and mixed in a 50 ml funnel for 90 min.

To separate phases, the mixture was centrifuged at 1000xg for 5 min at room temperature. The lower lipid phase was collected and evaporated at room temperature under nitrogen. The lipid phase was saponified with 5 ml of methanolic KOH (1M) for 60 min at 80°C. After cooling, the non-saponifiable lipids were extracted by two washings with diethylether, and the aqueous phase was acidified with 5 ml of HCl (6M) and extracted with hexane. The upper phase was then evaporated under nitrogen. The evaporated fraction was subjected to acid-catalyzed esterification (Morrison and Smith 1964). It was resuspended in 1 ml of 14% BF_3/Met-OH for 5 min at 100°C, cooled, and extracted with a mixture of hexane: water (2:1). After centrifugation (1000xg for 5 min at room temperature), the upper phase was collected and evaporated.

Samples resuspended in hexane were injected in a HP5890 series II gas chromatograph coupled to a HP5970 mass spectrometer equipped with an electron impact ionization source (Agilent). Separation was performed on a DB5MS capillary column (Phenomenex, 0.25 mm × 30 m); the helium gas flow was 1 ml/min. The oven temperature gradient was as follows: initial temperature of 100°C, isothermal at 100°C for 3 min, 100 to 300°C (rate, 15°C/min) and isothermal at 300°C for 5 min. The MS analysis was performed in fullscan mode. FAME (fatty acid methyl ester) quantification was performed using a

calibration curve obtained injecting different FAME standards referring to selected ions. The most abundant and specific ions were used for the quantification of FAMEs: m/z 74 was used for saturated FAMEs and m/z 55 for monounsaturated FAMEs. The regression curves were linear in the range of the FAME concentrations used for the analysis.

On the spectra obtained for each sample, fatty acids were identified and their relative percentage was calculated on the basis of the subtended area of each peak. Principal Component Analysis (PCA) on the fatty acids percentages was developed by means of R 4.0.2 software (R Development Core Team 2020) to investigate how the specimens differentiated on the basis of lipid composition, and what fatty acids contributed most to the variation. Ratio between saturated and unsaturated fatty acids was calculated (SFA/UFA).

4.3 Results

4.3.1 Sampled individuals

A total of six specimens have been used for our analyses. Four Antarctic toothfish were sampled during the Winter Survey 2019. The lengths of these individuals, spanning from 124 to 141 cm, and the presence in a breeding ground, make them attributable to adult stages. The specimens were selected to be two in poor condition "axe handle" and two in regular condition. The visual estimate was confirmed by the differences in K condition factor. Two Antarctic toothfish were sampled during the Ross Sea Shelf Survey 2020. These may be defined as close-to-adult individuals, being 96 and 116 cm in length, and were selected to be in regular condition, as confirmed by the high K condition factor values (Table 8).

Table 8. Main characteristics of the Antarctic toothfish individuals analysed in our work. Each specimen is indicated by the sequential number of the longline (SET) of the respective survey, and the sequential number of the Antarctic toothfish caught in such longline (TOA, the CCAMLR acronym for the species). For simplicity, in the next tables and graphs individuals will be indicated with numbers from 1 to 6, as they are in the "#" column.

#	Specimen	Length (cm)	Weight (kg)	Condition (K)	Classification
1	SET 5 TOA 109	141	22.7	0.8098	Axe handle (post-spawning)
2	SET 24 TOA 1	124	17	0.8916	Axe handle (post-spawning)
3	SET 12 TOA 4	139	28.2	1.0500	Regular (post-spawning)
4	SET 23 TOA 1	141	30	1.0702	Regular (post-spawning)
5	SET 137 TOA 2	116	17	1.0891	Regular (feeding)
6	SET 137 TOA 3	96	11	1.2433	Regular (feeding)

4.3.2 Lipid accumulation

4.3.2.1. Lipid distribution

When slices were stained with oil red O, the distribution of lipid in fish skeletal muscle of the Antarctic toothfish was visible. Regardless of the quantity, the pattern of lipid distribution appeared similar among all the studied specimens. Lipid were mainly

distributed in the subcutaneous region and myosepta. There were also both dorsal and ventral surfaces in which it was observed a relevant accumulation of lipid between muscular bundles (Fig 34 A, B). In the slices cut in correspondence of the ventral cavity, a lipid layer around the cavity was also identified (Fig 34 A).
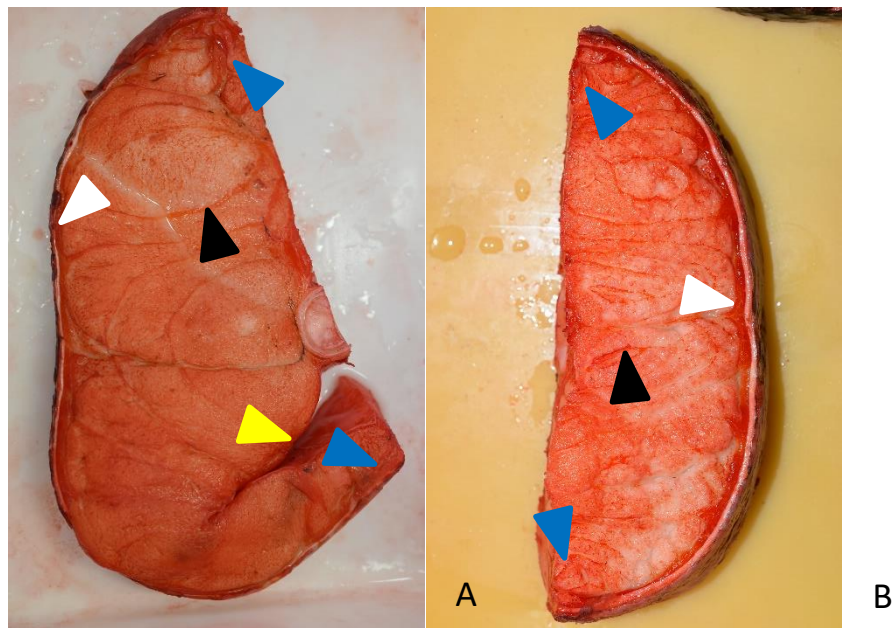

Fig 34. Lipid distribution in skeletal muscle in slices of Antarctic toothfish highlighted by oil red O staining. The slice cut in correspondence of the ventral cavity (A), and the posterior one (B) belong to the specimen #4, a post-breeding specimen in good condition. White arrowheads indicate subcutaneous lipid layers, black arrowheads indicate myosepta, blue arrowheads indicate dorsal and ventral lipid accumulation between muscular bundles, and yellow arrowheads indicate lipid layer around the ventral cavity.

4.3.2.2 Differences in lipid accumulation among individuals

Eleven values were obtained from each individual in the areas were lipids resulted most concentrated (Table 9). The matrix obtained served to investigate how lipid quantitative distribution varied among the individuals, as representative of three different conditions.

Table 9. Values of the measurements considered in the analysis of lipid accumulation relatively to the specimens. SC = mean subcutaneous layer thickness for the posterior sestion; SCc = mean subcutaneous layer thickness for the section in correspondence to the ventral cavity; AC = layer around the ventral cavity thickness; %VS = percentage of the ventral area of lipid accumulation between muscular bundles for the posterior section; %VSc = percentage of the ventral area of lipid accumulation between muscular bundles for the section in correspondence to the ventral cavity; %DS = percentage of the dorsal area of lipid accumulation between muscular bundles for the posterior section; %DSc = percentage of the dorsal area of lipid accumulation between muscular bundles for the section in correspondence to the ventral cavity; M = mean myopsepta layer thickness for the posterior section; Mc = mean myosepta layer thickness for the section in correspondence to the ventral cavity; %TOT = total percentage of lipid identified in the surface of the posterior section with the threshold technique; %TOTc = total percentage of lipid identified in the surface of the section in correspondence of the ventral cavity with the threshold technique.

#	SC	SCc	AC	%VS	%VSc	%DS	%DSc	M	Mc	%TOT	%TOTc
1	0.078	0.136	0.083	1.478	1.924	0.376	0.433	0.018	0.048	12.455	16.859
2	0.065	0.059	0.019	0.972	0.846	0.736	1.308	0.022	0.030	20.778	16.509
3	0.106	0.235	0.068	1.551	1.540	1.508	2.012	0.028	0.064	23.767	22.466
4	0.104	0.160	0.048	1.312	1.278	0.859	1.282	0.029	0.054	24.697	22.290
5	0.127	0.184	0.079	1.391	1.962	1.052	0.811	0.033	0.043	22.575	15.930
6	0.127	0.226	0.115	1.575	2.263	0.913	1.195	0.032	0.056	21.677	16.073

The plot of the PCA (Fig 35) reveals a clear distinction among individuals 1 and 2 ("axe handle") and the others along the axis of Dim1. Individuals 3 and 4 (regular from the breeding area) are in turn well separated from individuals 5 and 6 (regular from the feeding area) along Dim2.

Dim1 and Dim2 explain together 86.638% of the variance (Table 10). Dim 1 is driven by the thickness of the subcutaneous layers in both the sections of the trunk, the quantity of lipids accumulated in the ventral surface, and the thickness of the lipids among the myosepta in correspondence of the ventral cavity. Albeit with a slightly lower contribute, also the thickness of lipid accumulation among myosepta in the posterior part of the body appears relevant in the variation among individuals. Specifically, all the values of the variables that contribute to the differentiation along Dim1, were lower for the "axe handle" specimens. Dim2 is driven mainly by the percentage of the dorsal surface in which lipids are accumulated. The values were higher for individuals in regular condition collected in the northern breeding ground.
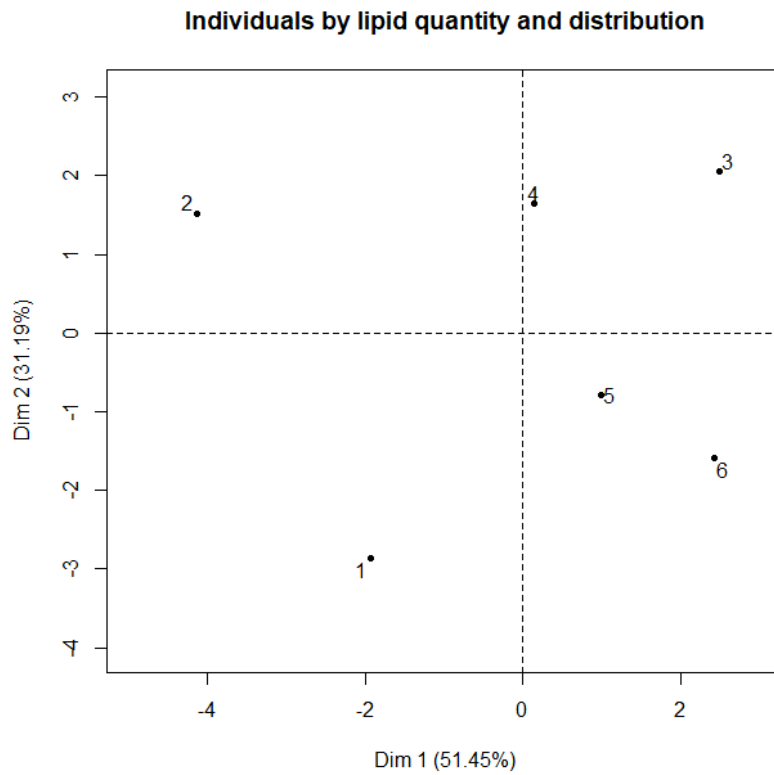
Individuals by lipid quantity and distribution

Fig 35. PCA plot developed on the variable selected to investigate variations of lipid distribution and quantity among the Antarctic toothfish individuals. Individuals are identified by numbers following Table 8.

Table 10. Explanation of variance of the first two dimensions of the PCA on the lipid distribution and quantity, and contribution of each variable.

	Dim 1	Dim 2
Variance	5.659	3.431
% of Variance	51.448	31.189
Cumulative % of Variance	51.448	82.638
Subcutaneous	**0.896**	-0.133
Subcutaneous c	**0.988**	-0.082
Around cavity	0.696	-0.699
% Ventral surface	**0.826**	-0.434
% Ventral surface c	0.630	-0.773
% Dorsal surface	0.697	0.606
% Dorsal surface c	0.388	**0.830**
Myosepta	0.784	0.182
Myosepta c	**0.849**	0.061
% Total	0.489	0.750
% Total c	0.329	0.690

Coefficients < - 0.800 and > 0.800 are in bold

4.3.3 Lipid composition and differences among individuals

The lipid extraction and the gas chromatograph analysis carried out in each subsample of the sections of the trunks, allowed to obtain the fatty acids composition. Based on the peaks of the chromatograms, 13 saturated fatty acids (SFA) and unsaturated fatty acids (UFA) were distinguished, and most of them were identified (Table 11).

Table 11. Relative percentages of the fatty acids identified in the samples. In column "#", letter "c" near the identification number of each individual indicates that the sample was collected in the section cut in correspondence of the ventral cavity, where the number is alone it refers to the section of the posterior part of the trunk. "Pn" indicate unidentified polyunsaturated fatty acids.

#	12:0	14:1	14:0	15:0r	15:0	16:1	16:0	18:1	18:0	P1	20:1	P2	P3
1c	0.00	0.28	26.04	0.87	0.60	10.60	21.55	30.16	3.04	1.59	2.63	0.39	2.26
1	0.00	0.00	24.98	0.00	0.00	8.57	33.36	23.14	7.29	0.00	2.66	0.00	0.00
2c	0.00	0.31	21.08	0.64	0.47	9.65	22.83	32.09	4.37	1.04	3.39	0.33	3.79
2	0.00	0.39	24.11	0.57	0.52	9.28	23.33	30.65	3.97	0.87	3.25	0.00	3.07
3c	0.00	0.33	18.62	0.41	0.49	11.84	27.56	27.61	4.23	1.85	3.26	1.06	2.75
3	0.98	0.34	15.79	0.00	0.63	9.38	45.66	12.22	11.65	2.00	0.66	0.69	0.00
4c	0.00	0.42	29.70	0.79	0.93	14.52	24.86	21.09	2.52	3.13	1.02	1.01	0.00
4	0.00	0.43	28.54	0.90	0.95	14.15	26.99	20.46	2.48	2.90	1.26	0.93	0.00
5c	0.00	1.02	34.15	0.70	0.64	13.80	23.58	18.16	2.86	4.15	0.94	0.00	0.00
5	0.00	0.76	30.98	0.91	0.67	14.70	25.49	19.10	0.52	4.93	0.85	1.07	0.00
6c	0.00	0.74	32.76	0.65	0.61	13.80	26.08	18.36	1.85	3.26	0.79	1.10	0.00
6	0.00	0.50	24.49	0.52	0.53	11.66	28.68	23.25	3.23	3.04	1.81	1.09	1.20

Differences in relative abundance of fatty acids among individuals, and in different parts of the trunk, were investigated by means of PCA developed on the percentages of each fatty acid revealed by the gas chromatograph (Fig 36).

Along Dim1 axis, individuals 1, 2 and 3 (two "axe handle" and one regular from breeding ground) result clearly distinct by individuals 4, 5 and 6 (all regular, one from the breeding ground and two from the feeding ground), regardless of the trunk section. Dim2 divides only the two trunk parts of the individuals 1 and 3, one "axe handle" and one regular from breeding ground, respectively. The two trunk parts of all the other toothfish result very close for each individual respect to Dim2, indicating that in these cases there is very low variation in lipid composition along the body of the fish.

Dim1 and Dim2 explain together 79.488% of the variance (Table 12). Dim1 is driven mainly by myristic acid (14:0), myriosteloic acid (14:1), palmiteloic acid (16:1), and by one unidentified polyunsaturated acid, with values > 0.800 or < -0.800, but there are also other SFA and UFA that resulted relevant, with values > 0.700. For all the most relevant fatty acids that determined the differentiation, the percentages were higher in the individuals 4, 5 and 6. Dim2 is driven by lauric acid (12:0), which was revealed only in the posterior section of the individual 3, palmitic acid (16:0) and oleic acid (18:1). palmitic acid (16:0) resulted higher in percentage in the posterior section of the

individuals 1 and 3, while oleic acid (18:1) resulted lower in the posterior section in the same individuals.
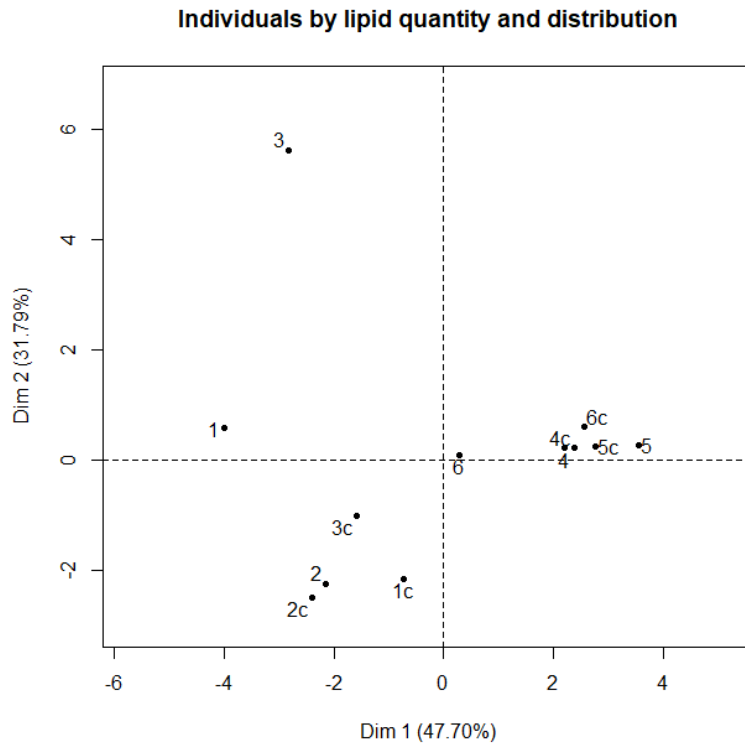

Fig 36. PCA plot developed on the relative percentages of fatty acids in the Antarctic toothfish individuals. Individuals are identified by numbers following Table 1.

Table 12. Explanation of variance of the first two dimensions of the PCA on the lipid composition, and contribution of each variable.

	Dim 1	Dim 2
Variance	6.201	4.132
% of Variance	47.703	31.758
Cumulative % of Variance	47.703	79.488
12:0	-0.341	**-0.835**
14:1	**0.810**	0.082
14:0	**0.819**	-0.194
15:0r	0.755	-0.538
15:0	0.712	0.108
16:1	**0.966**	0.032
16:0	-0.422	**0.895**
18:1	-0.404	**-0.899**
18:0	-0.757	0.627
P1	**0.922**	0.226
20:1	-0.688	-0.701
P2	0.499	0.243
P3	-0.508	0.696

Coefficients < -0.800 and > 0.800 are in bold

The ratios between relative percentages of SFA and UFA indicates a prevalence of SFA in all sample (Table 13). In almost all cases the value of the ratio resulted < 2.00, only in the posterior section of the individual 3 there was an apparently abnormal quantity of SFA respect to UFA. This sample presented different anomalies, it was the only one that presented lauric acid (12:0), and had overabundant quantities of palmitic acid (16:0) and stearic acid (18:0), and very low oleic acid (18:1) respect to all the other samples.

The most abundant fatty acid resulted the monounsaturated oleic acid (18:1) in the "axe handle" individuals and in the regular number 3, with the exception of the posterior section of 1 and 3, where the saturated palmitic acid (16:0) was prevalent. In individuals 4, 5 and 6, which resulted close together considering the first two dimension of the PCA, the saturated myristic acid (14:0) was the prevalent fatty acid, with the only exception of

the posterior section of individual 6, where palmitic acid (16:0) resulted the most abundant one.

Table 13. Relative percentages of saturated fatty acids (SFA) and unsaturated fatty acids (UFA), and their ratio in each sample. Prevalent fatty acid is also indicated.

#	Condition	SFA%	UFA%	SFA/UFA	% dominant FA
1c	Axe handle	52.11	47.89	1.09	18:1
1	Axe handle	65.63	34.37	1.91	16:0
2c	Axe handle	49.40	50.60	0.98	18:1
2	Axe handle	52.50	47.50	1.11	18:1
3c	Regular	51.31	48.69	1.05	18:1
3	Regular	74.71	25.29	2.95	16:0
4c	Regular	58.80	41.20	1.43	14:0
4	Regular	59.86	40.14	1.49	14:0
5c	Regular	61.93	38.07	1.63	14:0
5	Regular	58.58	41.42	1.41	14:0
6c	Regular	61.95	38.05	1.63	14:0
6	Regular	57.45	42.55	1.35	16:0

4.4 Discussion

The lipid distribution, quantity and composition have been investigated in six Antarctic toothfish specimens selected ase representative of different stages of the life cycle of the species, which is known to cover a very broad area (Hanchet et al. 2008, 2015). Focus of the study was to investigate the connection between the southern feeding ground and the northern breeding ground. The reproductive migration is thousands of kilometres long (Parker and Di Blasi 2020), it is likely highly energy expensive, and there no information is available on the fate of Antarctic toothfish individuals after such a potentially exhausting breeding migration.

The migration is supposed to be assisted by midwater currents, to take advantage of which the toothfish has to reach neutral buoyancy (Near et al. 2003). In absence of swim bladder, the neutral buoyancy is supposed to be obtained by lipid accumulation during the permanence in the highly productive areas of the continental slope. The abundant lipid storage may also serve as energy reserve for the subsequent dispendious activities (migration and reproduction), further considering that the breeding ground of the toothfish in the northern seamounts is supposed to be a resource-poor area (Fenaughty 2006). However, the amount of energy devoted to each of the activities related to the reproduction is unknown, and so is the fate of Antarctic toothfish individuals in such a low productive area.

To date it is unknown if individuals after spawning can return to the feeding ground. For this to happen, it is conceivable that the toothfish have to re-store a conspicuous quantity of lipids for neutral buoyancy and energy reserve, which is unlikely in a such low productive area. The presence in the northern seamounts of a relevant percentage of individuals in poor condition, named "axe handle", raised further doubts about the possibility to acquire there the amount of energy necessary to return to the slope.

To improve the knowledge on the usage of lipids by Antarctic toothfish, how they are stored and what is the entity of quali-quantitative depletion for reproduction, what is the possibility to reacquire condition after spawning, we carried out the analyses on four specimens from the breeding ground, sampled in post-spawning period, both regular and "axe handle" ones, and two specimens from the continental shelf, which is a feeding ground. All the selected individuals were adult or close-to-adult males, to avoid intersex variability, and because the "axe handle" condition is more frequent in males (Fenaughty et al. 2008, Parker and Di Blasi 2020).

The study on lipid distribution confirmed a pattern already known for the species, with main storage areas represented by the subcutaneous layer and the myosepta (Eastman and DeVries 1981, Clarke et al. 1984, Fenaughty et al. 2008). We found also other areas of lipid accumulation, namely dorsal and ventral surfaces where lipids were stored between muscular bundles, and a thin layer sorrounding the ventral cavity. The thickness of the areas of storage varied among the specimens, sometimes visibly, but the described pattern was constant in all of them.

The major factors of variation between "axe handle" individuals and individuals in better condition resulted the thickness of subcutaneous layer along all the trunk, the volume of the posterior ventral area of lipid storage, and the myosepta, in particular the ones in the anterior part of the trunk, which are in general thicker than the rear ones, coherently with what reported in a previous work conducted on well-nourished Antarctic toothfish (Clarke et al. 1984). In "axe handle", all these structures resulted thinner than in the other specimens, indicating how these might be the areas of storage for energy reserve purpose. The percentage of lipid surface identified respect to the total surface of the anterior section appears to give the most controversial result (Table 9). The values of "axe handle" individuals and those of regular individuals from feeding ground are indeed very similar, which is unexpected. It could be interpreted in two ways. First, the individuals from the shelf were not yet completely adult, and were in an area where energy acquired by feeding are invested in growth rather than in lipid storage. They were far to be ready for a long migration, because lipid for energy and neutral buoyancy were not yet adequately stored. Second, "axe handle" individuals were in a such debilitated condition that even the muscular mass was consumed, leaving a bit of residual lipids, so that the percentage ratio between lipid surface and total surface did not show evident low value because not only the numerator but also the denominator was decreased. Drawing to muscle proteins as energy reserve when lipids are almost exhausted because of consuming migration is a common strategy in fish, it is for example the case of the sockeye salmon *Oncorhynchus nerka* (Kiessling et al. 2004) or the American shad *Alosa sapidissima* (Bayse et al. 2018). Even long periods of starvation may lead to muscle consumption, as it happens in the Atlantic cod *Gadus morhua* (Lemieux et al. 2004).

The individuals in regular condition resulted distinct on the basis of their provenience. The main element involved in the differentiation was the size of the dorsal surface in which lipids are accumulated between muscular bundles. Such surface resulted larger for post-spawning specimens from the northern seamounts. It can be interpreted as a good

lipid storage maintained by these two individuals, and confirmed how lipid accumulation in the Antarctic toothfish from the shelf was incomplete for migration. The presence of relevant lipid accumulation in the dorsal surface can indeed be explained as a residual of the storage that was utilized to reach the neutral buoyancy functional to the reproductive migration. If true, it means that Antarctic toothfish stores lipids for buoyancy in a body area analogous to that described for the Antarctic silverfish (*Pleuragramma antarctica*), a neutral buoyant notothenioid fish (DeVries and Eastman 1978). In the case of the Antarctic silverfish, lipids are in large connective tissue sacs, and presumably not available for metabolic purposes. In Antarctic toothfish, even if located in a similar area, lipids are not contained in sacs, but in adipose cells, which make them potentially available for energy requirements (Clarke et al. 1984), making the buoyancy variable during the life cycle.

Lipid composition was investigated through the characterization of the fatty acids in the samples. Triacylclycerols represent the main lipid classes present in nototheniods as easily catabolizable reserves (Clarke et al. 1984), for this we concentrated our investigation on their fatty acids components. From the gas-chromatographic analysis, the complete set of saturated and unsaturated fatty acids (SFA and UFA) was obtained. Overall the presence of long chain saturated and monounsaturated fatty acids, composed by 14 to 20 carbon atoms, resulted for each one of the individuals investigated. A shorter chain lauric acid (12:0) was identified only in one sample, correspondent to the posterior section of a regular individual from the breeding area. Three different polyunsaturated fatty acids were also revealed in the chromatograms, but their degradation due to long permanence at -20°C did not allowed their precise identification.

The relative percentage of each fatty acid resulted sensibly variable among the individuals, and in two cases between the anterior and the posterior samples obtained from the same specimen. The principal component analysis well divided "axe handles" from regular individuals from the shelf. Such result was expected, because of the very different conditions of the two groups. What was unexpected was the positions in the plot of the two individuals in good condition from the breeding area: one was associated with the "axe handle", the other with the individuals from the shelf. To the differentiation between the two clusters contributed mainly unsaturated fatty acids, with the exception of the myristic acid (14:0). The three individuals on the positive side of the Dim1, which are those in better condition, resulted with homogeneous distribution of fatty acids along the body, as revealed by the proximity between the two points corresponding to the

anterior and posterior section of the trunk relatively to both the two principal dimensions. Different pattern was the one obtained for two of the other three individuals, in particular one of the "axe handle" and the regular one showed a different distribution of fatty acid types in the two samples of the trunk, as highlighted by the separation between the anterior and the posterior samples along the Dim2. The most evident and less expected inhomogeneity was once again that of the regular individual, which posterior section was the only sample that presented lauric acid and had sensibly higher stearic acid (16:0) and lower oleic acid (18:1). On the basis of our small dataset, this individual appeared anomalous and the pattern of its fatty acids composition resulted difficult to understand. High level predatory fish, such as the Antarctic toothfish is (Pinkerton et al. 2014), are not able to biosynthesize fatty acids and their lipid component entirely derive from their diet (Tocher 2003). For this, fatty acids can be considered as a "memory" of the diet of the fish. In particular, UFA, which are preferentially oxidized by the Antarctic fish (Sidell et al. 1995, Hazel and Sidell 2004), may represent a shorter-term memory than SFA, which could be stored for a longer time and also for purposes different than energy reserve, including buoyancy. Conversely, SFA may represent a long-term memory of the fish diet.

It is possible to hypothesize that the individual from the breeding area, clustered with the two from the shelf, could have migrated from that feeding area, while the regular individual clustered with the two "axe handle", irrespective to the condition, could have originated by the same area in the southern Ross Sea. This is just a hypothesis, weakly supported by the reduced number of samples. If it is true, it might have two implications. First, not only the slope, but also the shelf can be considered as a ground in which the Antarctic toothfish can reach adulthood and the fatty condition proper to the migration towards northern seamount. The presence of large adults in the shelf (Parker et al. 2019, Di Blasi et al. 2021) may support such consideration. Second, if the relative percentages of fatty acids of individuals in the breeding area do not differ from those of the original feeding area, it means that the Antarctic toothfish is able to catabolize in equal proportion all the fatty acids stored, without differentiation between UFA and SFA, contrary to what happens in other species (Sidell et al. 1995, Tocher 2003). This would highlight the flexibility of lipid usage for both somatic (i.e. buoyancy variation) and energetic purpose by means of the species.

If the previous hypothesis is not true, we can deduce that Antarctic toothfish consumes preferentially some types of fatty acids and that their composition is the result of oxidative

processes. Specifically, we can consider that the fatty acids that most determine the differentiation between our two clusters are those preferentially depleted as energy reserve. In our case, we found that "axe handle" individuals had lower relative quantity of stearic acid and higher relative quantity of oleic acid, the first could be the most used. The regular individual clustered in the "axe handle" group presented the same pattern in the anterior part of the trunk, but the opposite (i.e. with higher percentage of stearic acid and lower percentage of oleic acid) in the posterior part. Does it mean that such individual is going towards a "axe handle" condition, and that the depletion starts from the anterior part of the trunk, while the posterior one is more conservative? Surely to confirm or confute it futher investigations on a larger number of specimens is necessary.

The most abundant fatty acid in each individual, and the ratio SFA/UFA, were considered as good indicators of fatty acids usage. The monounsaturated oleic acid had been reported to be the most abundant fatty acid component in notothenioid fish (Nachman 1985, Fenaughty et al. 2008), this was not confirmed by present study. Oleic acid resulted to be the most abundant fatty acid in the majority of the "axe handle" samples, and in the anterior section of a regular individual from the breeding area. The other samples resulted dominated by the saturated myristic and stearic acids.

Overall, with the only exception of one sample from an "axe handle" individual, SFA resulted in all the considered conditions more abundant than UFA. In particular, for the individuals from the shelf the proportion of SFA respect to UFA was observed to be higher in the anterior part of the trunk, possibly the barycentre of the buoyancy. If SFA have primarily a somatic function, it is possible that they were in a phase of storage of lipids to reach the future neutral buoyancy. This would be another clue for the shelf to be considered a feeding ground for Antarctic toothfish that fatten to approach the northward migration.

In all the individuals from the northern seamounts, conversely, the ratio SFA/UFA resulted higher in the posterior part of the trunk. It is conceivable that the Antarctic toothfish take advantage of the dual functionality of the fatty acids very well, and during and at the end of the migration, consume first the somatic fatty acids from the barycentre of buoyancy, because they are no more useful for buoyancy once the seamounts have been reached. It could be partly confirmed by the major tryacilglycerols constituent that remained in the toothfish in poor condition, which was in most cases the UFA oleic acid, differently from the fish from the shelf or in better condition, which tryacilglycerols were dominated by somatic SFAs.

Where SFAs resulted the major constituent, we presume that fish were storing fat for migration, or there were some residuals after the migration occurred. Where an UFA was the most abundant constituent, it is possible that fish were not storing, but it is also deductible that they are not fasting. Because the UFA are potentially easy-to-consume fatty acids, their presence may indicate a quite recent assumption, but few possibilities of storing for somatic purposes. "Axe handles" should be so fish that are feeding, but the resources of the area do not allow to accumulate somatic lipids. Among our specimens, potentially the individual in regular condition which lipid composition is similar to the "axe handle" ones could go towards such poor condition, because of the resources available in the northern seamounts.

The previous reasoning brings back a question already arosen by Fenaughty et al. (2008): are the Antarctic toothfish present in the seamounts able to restore the lipids necessary for energy and neutral buoyancy to re-migrate towards the south after spawning? And furthermore, how long they will be able to survive in the north? Do they die after spawning? Three different scenarios seem possible.

A group of toothfish might die while another group of individuals, with better fitness, will survive and continue their life cycle. These latter may have a better condition, and have accumulated a higher amount of lipids before the exhausting reproductive migration. Differentiation in success in reaching their reproductive objectives of individual of the same species, caused by the different condition at the start of the migration, is reported for other migrant species. For the sockeye salmon the initial gross somatic energy is reported to be a relevant factor in reproductive success (Crossin et al. 2009). In the case of the toothfish, the success should be not simply the possibility to spawn, but the iteroparity, i.e. the survivorship after spawning, which would allow to spawn in sequent reproductive seasons and to continue to spread their genes. From this point of view, the capability to not fall in a potentially irreversible "axe handle" condition could provide greater fitness. The successful Antarctic toothfish should arrive to the seamounts in good condition and reserves enough to spawn and continue life activity to maintain their health status.

As a second hypothesis, all Antarctic toothfish might share the same fate at the breeding ground. The reproductive migration is energy consuming, but when they arrive to the spawning ground, they might still have a good amount of reserve. Part of such residual energy would be used for reproduction (i.e. spawning and all related reproductive behaviours). If after the spawning event the energy storage is still not drastically depleted,

the major problem is then the permanence in the northern seamounts, a habitat poor of resources, unable to provide toothfish with sufficient amount of fatty acids. This would be consistent with the low lipid content and different proportion of components of tryacilglycerols in the poor-condition individuals of our work. SFA, the somatic fatty acids, seem to be difficult to obtain there. This translates into the impossibility of individuals at the seamounts to re-acquire good condition, and enough lipid storage to sustain metabolism and neutral buoyancy required for a returning migration towards south. Antarctic toothfish might remain in the northern seamounts for different years, weakening gradually more and more, and leading to a "slow death". The reason why both individuals in good condition and "axe handle" are found in the northern breeding area is due to the different time elapsed from the arrival of the single individuals to the seamounts. This particular life cycle could be defined as "semi-semelparity", in which the fish never return to the feeding ground, but does not die after the first reproductive event, a strategy that guarantees expanded genetic dissemination to each individual.

A third scenario is conceivable that would explain the presence of both regular and "axe handle" toothfish in the same spawning ground. If energy storage is performed by different individuals in different feeding areas, a degree of heterogeneity among fish at the breeding ground might occur. In this case, what determines such distinction would not be the individual fitness and capability to accumulate the adequate lipid amount for migration and reproduction, but the energy storage allowed by the area where the migration start. Groups from different feeding areas could reach different rates of energy accumulation to face migration and reproduction, and only those that depart with higher condition will overcome the efforts maintaining an overall good condition that allow the individuals to survive after the spawning. Following this theory, "axe handle" would be the individuals that depart from poorer feeding areas, spent a high percentage of their lipids, and consequently draw on the proteins of the white muscle. Poor conditions cannot be restored in the scanty-resource habitat of the northern seamounts, and those fish are destined to die in the short term. We can so hypothesize that a part of the Ross Sea Antarctic toothfish population could be iteroparous and the other part could be semelparous, depending from area where the migration begins, similarly to what happens for the American shad (Bayse at al. 2018). Because iteroparous and semelparous toothfish may mix their gametes during spawning, genetic analysis probably cannot help to clarify whether this theory is correct, but the continuation of the tagging program already

implemented by the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) would help to have relevant information in the future.

Further insights are needed to test the hypotheses, on a wide range of samples, a higher number of replicates, and including females and samples from other areas. For example, it will be extremely interesting implement the same analyses on individuals from the slope, considered to be the main feeding and fattening ground for the Antarctic toothfish (Hanchet et al. 2008, 2015).

5. Conclusion

At the start of the work, lacks of knowledge on the life cycle of Antarctic toothfish existed. The interest in the species is particularly relevant for two reasons: it is an ecologically important species as high-level predator of the trophic web of the Southern Ocean, and it represents one of two mainly exploited resources in Antarctic waters, together with Antarctic krill (*Euphausia superba*). In particular, it is the most targeted resource in the Ross Sea. The classical methods of data collection for toothfish, associated to commercial fishing activity, which is restricted in time (it is open only few summer months per year) and in space (it is constrained by sea ice extension), do not allow to cover all the life cycle. To fill some of the existed gaps, it was necessary to overcome the conventional sampling methods.

It was little known what was the distribution, in terms of abundance and size, of Antarctic toothfish in shelf areas, especially in periods in which it is covered by fast and pack ice. The development of a purpose-built baited remote underwater video system (BRUV) that was tested from the sea ice, in under-sampled and otherwise inaccessible areas, provided encouraging results. The Antarctic toothfish was attracted by the bait and not disturbed by the system, supporting the potential for the use of BRUV to study and survey the species. The analysis of relations between environmental variables and toothfish abundance metrics was undertaken. While the small dataset available prevented to reach robust conclusions, the pilot study allowed to point out shortcomings, and to draw an operative protocol effective for toothfish investigation from the sea ice. Furthermore, the non-extractive nature of the sampling makes it suitable for areas under protection, such as Marine Protected Areas (MPAs) and Antarctic Specially Protected Areas (ASPAs).

Other missing information was related to the reproduction of Antarctic toothfish. The current life cycle model hypothesized that spawning in the Ross Sea may occur in winter in the northern features in correspondence of the Pacific Antarctic Ridge (Hanchet et al. 2008, 2015), but that was not documented until the Winter Survey 2016. A precious amount of data and samples had been collected, the spawning in the northern features was demonstrated, eggs were collected in the plankton, and buoyancy of eggs was analysed for studies of dispersion. Anyway, since the voyage was in June-July and the most interesting results were obtained at the end of the survey, many gaps remained. The Second Winter Survey in 2019, took place three months later than the previous one, and in a wider area, to obtain complementary information and determine timing of the

spawning and extension of the breeding area, where planktonic embryos are and how vary their buoyancy during development. All these objectives have been successfully achieved. Toothfish were found to be post-spawning, suggesting that the spawning season should occur in mid-winter and be short, lasting about one month. Eggs collected in the plankton were in advanced developmental stage, they were found in the highest stratum of the water column, suggesting that they would remain positively buoyant during all the development. That was also confirmed by the buoyancy analysis. All this information, added to the one obtained in 2016, allows now to have a quite exhaustive picture of various aspects related to the reproduction of Antarctic toothfish, including timing, spawning areas, and embryo developmental features.

Another information related to reproduction was unknown prior to present work and is the fate of toothfish after spawning. The occurrence, in relatively high percentage, of Antarctic toothfish in very poor condition in the north (the so called "axe handle" individuals) led to the hypothesis that the individuals, or part of them, might not be to restore the condition after the energies expended for migration and reproduction, particularly in a low productive area such as the northern breeding ground (Fenaughty et al. 2008). An investigation on lipid distribution and composition, and their variation in toothfish in different condition and from different areas provided insights into the lipid mobilization and usage as energy reserve and/or for neutral buoyancy for reproductive migration, how it is consistent their depletion, and if toothfish actively feed in the northern area. The results provided clues to draw a range of hypotheses on the fate of post-spawning toothfish. Far to be definitive conclusions, they provide cues for future studies and insights.

Overall, the objective to improve knowledge on toothfish life cycle has been successfully achieved. In some cases, the gaps were filled, in others, pilot studies allowed to lay the foundations for future investigation and paved the way to further studies.

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Acknowledgments