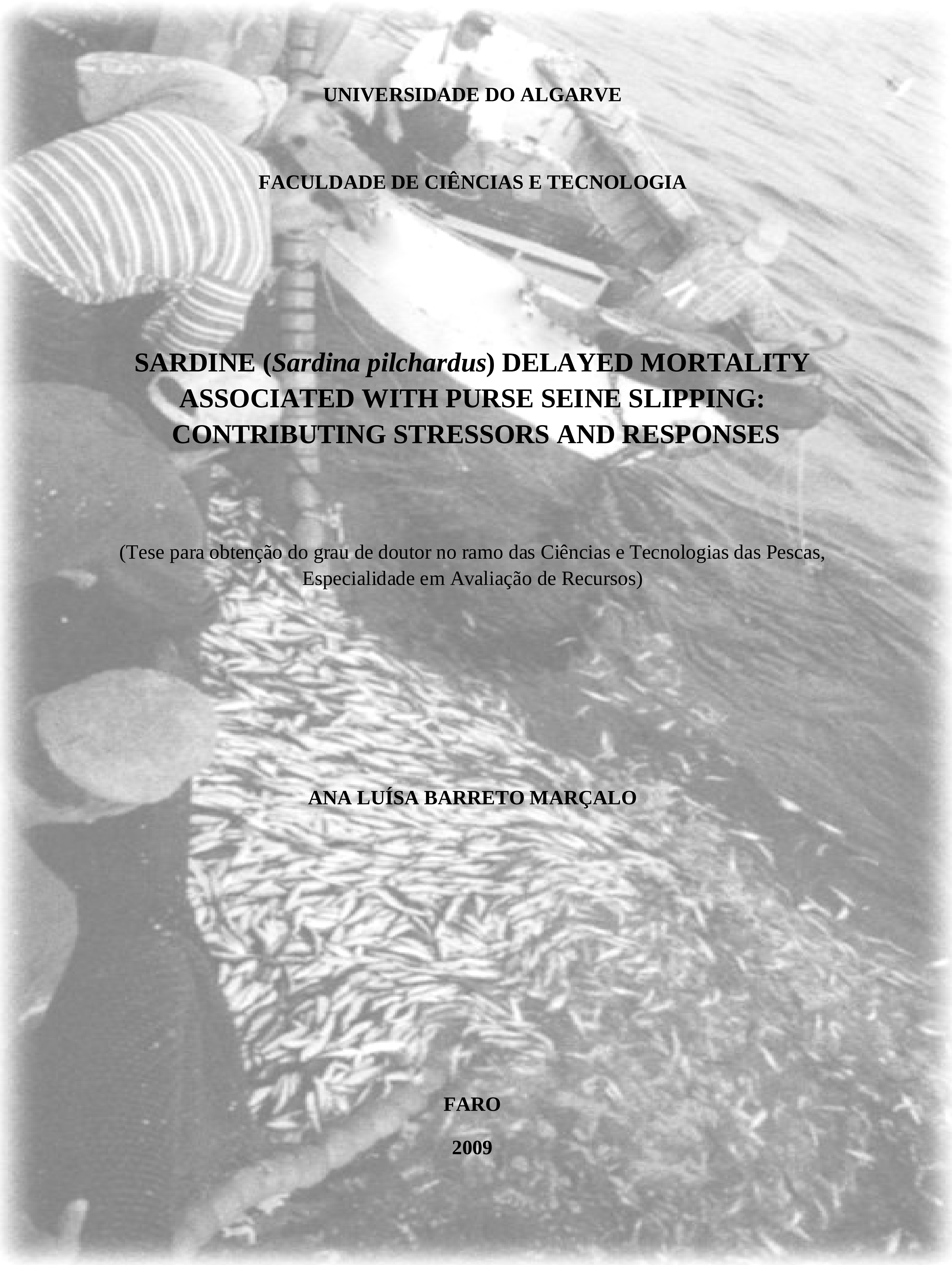

UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS E TECNOLOGIA

SARDINE (*Sardina pilchardus*) DELAYED MORTALITY ASSOCIATED WITH PURSE SEINE SLIPPING: CONTRIBUTING STRESSORS AND RESPONSES

(Tese para obtenção do grau de doutor no ramo das Ciências e Tecnologias das Pescas, Especialidade em Avaliação de Recursos)

ANA LUÍSA BARRETO MARÇALO

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Tese orientada pelo Doutor Karim Erzini, Professor Associado da Faculdade de Ciências e Tecnologia da Universidade do Algarve e co-orientada pelo Doutor Georgios Stratoudakis, Investigador Auxiliar da Unidade de Recursos Marinhos e Sustentabilidade do Instituto Nacional de Recursos Biológicos (IPIMAR-INRB/IP)

FARO

2009

O júri

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“O cardume, que foi força e vida misteriosa, que formou um só corpo e passou obedecendo não sei a que instinto ou a que inteligência superior, cai sobre Lisboa – como vem de Setúbal, do Algarve e das praias ignoradas de toda a costa lusitana, das grandes armações e dos pequenos barcos. É espalhada pelo país. Comem-na assada na brasa os trabalhadores da estrada e os homens esfaimados do campo com um pedaço seco de broa. De inverno é magra, mas pelo S. João pinga no pão. No Norte o lavrador espera-a para o jantar: é o seu melhor conduto. Os pobres fregem-na numa gota de azeite, e salgada ou saltando no cesto, fresquinha da barra, viva de Espinho, gorda, antes da desova, sem cabeça e escruchada, com a guelra em sangue, ou laivos amarelos de salmoura, constitui um manjar para pobres e para ricos. Entra em todas as casas. Há quem goste dela de caldeirada e quem a prefira simplesmente assada deixando cair no lume a gordura que rechina. Há os que só saboreiam a grande, de lombo gordo e preto, e os que acham melhor a miúda, que se chama petinga e que se devora com escama e tudo, afirmando com uma convicção respeitável que a mulher e a sardinha quer-se pequenina...”

Raul Brandão,” Os pescadores”, 1923

Para os meus pais e avós

CONTENTS

AKNOWLEDGMENTS

I want to thank Yorgos for his special support as an advisor, his friendship, credibility and giving me confidence at harsh times. I owe him the chance to fulfil my dream to return to Portugal and work in fisheries biology.

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Last but not least, I want to thank all my family. My grandparents that fulfilled my imaginary with their stories of the sea, uncles and cousins, and specially my parents and my sister, for their unconditional support, love and encouragement in pursuing my dreams all my life.

RESUMO

Esta tese tem por objectivo estudar o impacto das operações da pescaria de cerco na sardinha (*Sardina pilchardus*) deliberadamente desperdiçada através de “slipping”, procurando avaliar níveis de sobrevivência do peixe libertado e estudar os factores que levam à morte *à posteriori*. No decorrer das operações de pesca comercial foi avaliado o stress fisiológico em sangue de sardinhas. Foram realizados ensaios de captura, transporte, introdução e ambientação da espécie em cativeiro como ferramenta auxiliar para o procedimento de experiências de simulação de pesca em laboratório. Experiências de simulação testaram factores de stress operacionais (tempo de pesca e densidade da captura) e ambientais (temperatura da água) e o seu efeito nas sardinhas ao nível da sobrevivência, fisiologia, danos físicos e comportamento face a predadores. Os resultados revelaram que a pesca causa stress fisiológico a curto prazo nas sardinhas com a alteração significativa dos parâmetros analisados (cortisol, glucose, hematócrito, hemoglobina, equilibrio iónico e ATP e seus metabolitos) em função principalmente do tempo dentro da rede. O trabalho laboratorial mostrou que a sobrevivência das sardinhas desperdiçadas é inversamente proporcional ao tempo dentro da rede e que este impacto é acrescido com o aumento da temperatura da água. Os parâmetros de stress a curto prazo (fisiológicos) não se correlacionam com os níveis de sobrevivência observados para os vários graus de intensidade dos factores aplicados. No estudo dos parâmetros fisicos, comparou-se individuos vivos com os que morreram, onde os resultados demonstraram que a perda de escama é miníma em indivíduos que sobrevivem, o que leva a crer que este parâmetro fisíco é muito importante como causa directa de morte. Stress a longo prazo revela-se em todos os peixes desperdiçados, nomeadamente com a inibição do sistema reprodutor e imunitário. O comportamento das sardinhas é alterado significativamente com a pesca (diminuição de distância ao predador, capacidade de acardumar e velocidade de natação) o que aumenta a sua vulnerabilidade na presença de predadores.

Palavras-chave: Pescaria de cerco, *Sardina pilchardus*, slipping, reacções de stress, mortalidade *à posteriori*, interacções presa-predador

ABSTRACT

This dissertation aims to study the impact of commercial purse seine fishing operations on slipped sardines (*Sardina pilchardus*), accounting for the survival rates of the escapees and studying the main factors/stressors that may lead to delayed mortality. Field work onboard commercial seiners allowed to study the early dynamics of sardine (*Sardina pilchardus*) stress reactions and identify likely stressors during fishing operations. Appropriate methodology for capturing, transporting and maintaining sardines in captivity after live capture was optimized in order to use the species for laboratory fishing simulation studies. Laboratory fishing simulations were used to study some operational and environmental factors that lead to variable physical, physiological and behavior responses and result in delayed mortality after slipping. Results showed variable stress responses of sardines during purse seining operations with the significant effect of fishing duration in several physiological blood parameters (cortisol, glucose, haematocrit, haemoglobin, ionic balance, and ATP and its metabolites). Laboratory fishing simulations revealed that survival rates decrease significantly with time inside the net, with temperature having an additional effect on mortality. Fish short term stress responses (physiological) immediately after fishing do not correlate with observed delayed mortality. Scale loss is an important variable of physical injury directly related with the probability of dying. Long term stress responses such as the inhibition of the immune and reproductive systems affects all escapees (whether surviving or not). Sardine suffer behavioral impairments such as lower swimming speed, shorter predator approaches and larger nearest neighbor distances (wider school area) regardless of fishing stressor severity, what indirectly may increase their vulnerability to predation.

Key words: Purse seine fishing, *Sardina pilchardus*, slipping, stress reactions, delayed mortality, predator-prey interactions

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CHAPTER 1

Introduction

Fish discarding is one of the most important issues affecting marine fisheries management and an added source of uncertainty to stock assessments, due to the implied mortality of discarded fish or fish that escape from the fishing gears (Alverson et al., 1994). Discard quantification has been a priority mostly directed to less selective and more abrasive fishing gears, namely towed gears (Broadhurst et al. 2006), and major efforts have been made to reduce trawling discards, especially through gear modifications that improve species and size selection (Davis, 2002). However, only a few studies are available of the direct and indirect effects (leading to immediate or delayed mortality respectively) of fishing operations on fish condition and of estimates of the unaccounted mortality or survival of fish discarded or that escape (Chopin and Arimoto, 1995; Chopin et al. 1996; Suuronen et al., 1996*a*, 1996*b*; Sangster et al. 1996; Broadhurst et al., 1997). Further, detrimental effects of severe exercise caused by fishing and interactions with several environmental and biological factors responsible for delayed mortality after release, have been relatively well studied in aquaculture systems and species (Barton and Iwama, 1983), but to a lesser extent in commercial fisheries (Davis, 2002). Regarding pelagic purse seine fisheries, even fewer studies are available, mainly because of the general perception that these fisheries are highly selective (usually take hauls with low diversity of species and sizes) and with widely variable and sporadic discarding behavior (Borges et al. 2008).

1.1– Purse seine fishing

1.1.1 – General overview

Purse seining is a fishing practice particularly used to capture large (e.g. tunas) or small pelagic fish (e.g. mackerels, sardines and anchovies) that aggregate or school close to the surface (FAO). Once the school is located, mostly using acoustic electronic fish detection devices, it is quickly surrounded by a wall of netting up to 1,000m in length and over 100 m deep (Figure 1.1). Usually, a smaller auxiliary boat is used to hold one end of the net until the larger vessel (or seiner) comes around the school to complete the encircling. As the seiner encircles the fish, a process named shooting, the net is released. The net floats at the top with the use of a cork line (headline), and in the bottom has a number of lead rings (also used as weights). The rope (or purse line) that passes through the rings is then pulled, drawing the rings close to one another to form a purse with a bag shape which prevents the fish from diving and escaping (Figure 1.2). The bag is closed up alongside the boat and the net gradually dried up (a process usually referred to as hauling) reducing the volume of the purse, and crowding the fish into a smaller area until the density of the catch becomes sufficiently high to start loading the boat and transferring the fish either by brailing (dipping them out with large dip nets called brails) or with fish pumps, while the rest of the capture is maintained in the water. In some fisheries, the gear can be modified to target demersal fish species, although the basic operational/net apparatus concept is maintained (Borges et al., 2001; Erzini et al., 2002; Gonçalves et al., 2008).

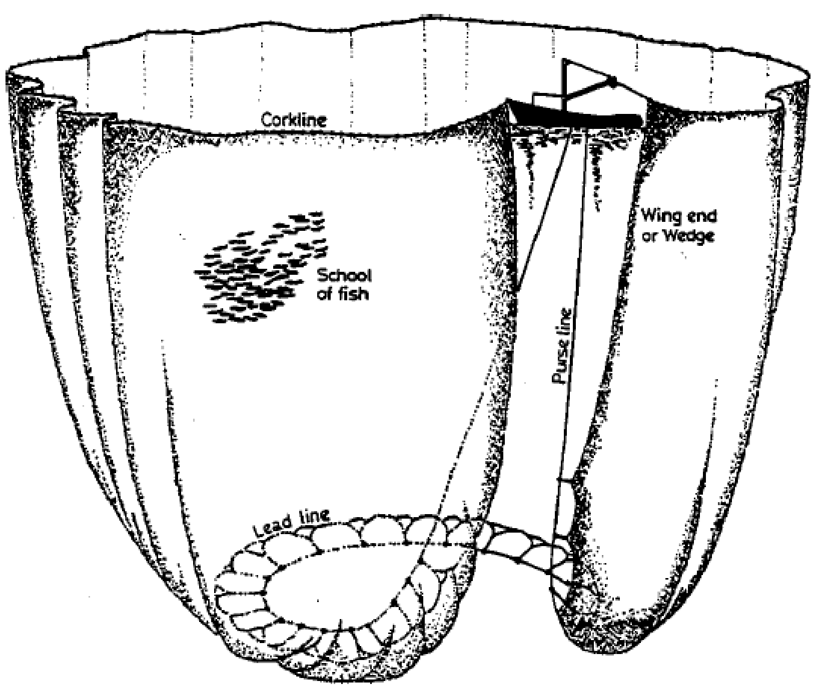

Figure 1.1 – General overview of a purse seine net after shooting and as the purse is being formed. From SEA FISH INDUSTRY Authority Industrial Development Unit, Technical report nº 288, 1986.

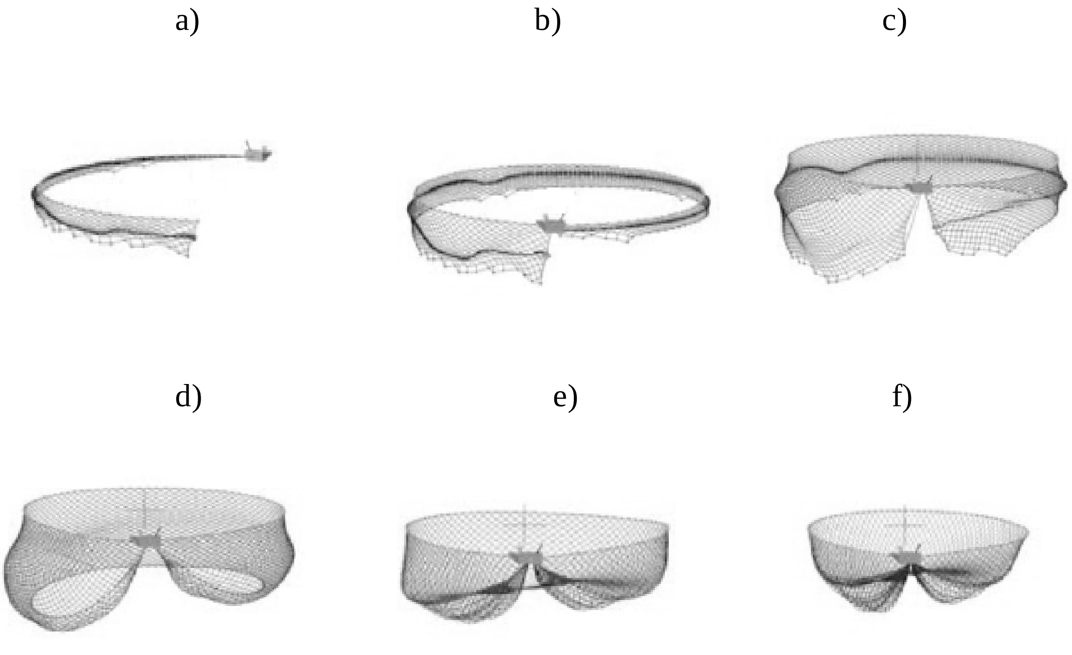

Figure 1.2 – Details on net shooting (a-b), closing-up of the net into a purse (c-d) and net hauling after the purse is formed (e-f). Adapted from Kim et al. 2007.

1.1.2 – Purse seining off the Portuguese continental coast: considerations on fleet characteristics, fisher behavior, fishing operations and catches

Off Portugal, purse seining is one of the most important commercial fishing activities (49 % and 43 % of continental only and total national annual landings respectively) (DGPA, 2008*a*). Sardine (*Sardina pilchardus*) is the main target species of the fishery (> 70 % of total landings and value; DGPA, 2008*a*), with horse mackerel (*Trachurus trachurus*), chub mackerel (*Scomber japonicus*) and anchovy (*Engraulis encrasicolus*) accounting for a smaller part of the landings (Stratoudakis and Marçalo 2002; Wise et al. 2005; DGPA, 2008*a*). The present Portuguese purse seine fleet (137 vessels in the mainland) consists of mostly old vessels (around 50 % vessels are over 20 years old) of medium size (mean total length of 17 meters, ranging from 6 to 27 meters), equipped with engines from 15 to 600 HP (mean of 265 HP) and operating with large nets (up to 1000 meters long and 120 meters deep), with a minimum mesh size of 18 mm (Parente, 2001; Stratoudakis and Marçalo, 2002; Wise et al. 2005; DGPA, 2008*a*; anonymous data). The vessels are based mainly in the ports of Póvoa do Varzim, and Matosinhos and Figueira da Foz in the northern coast, Nazaré and Peniche in the center, Setúbal, Sesimbra and Sines in the southern/west coast, and, Quarteira, Portimão and Olhão in the southern/Algarve region. The most important landing ports for species targeted by Portuguese purse seiners are in decreasing order of importance Matosinhos, Peniche, Figueira da Foz, and Sines in the west coast, and Olhão e Portimão in the Algarve (Fig 1.3; DGPA, 2003, 2004, 2005, 2006, 2007, 2008*b*). Landing data from figure 1.3 also shows that Póvoa do Varzim and Quarteira are relative important home ports but landings at both locations are not significant, while the opposite occurs in the ports of Aveiro in the northern coast and Lagos in the Algarve.

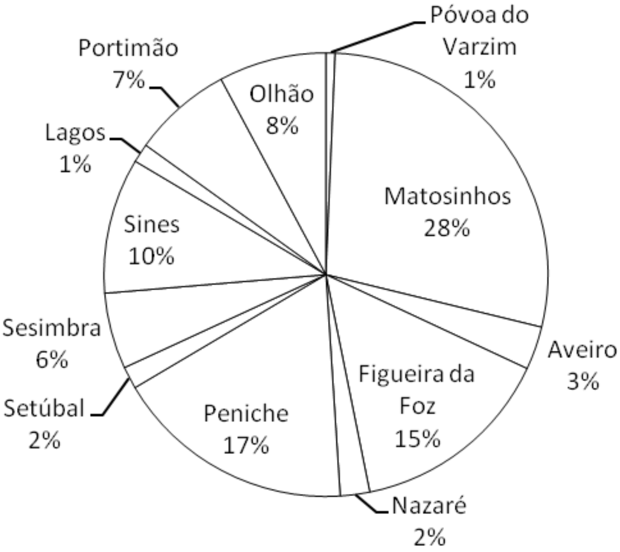

Figure 1.3 - Relative importance of Portuguese ports for landings (in tons) of the four main species (sardine, horse mackerel, chub mackerel and anchovy) targeted by purse seiners along the continental coast for 2002-2007 (DGPA, 2003-2008*b*)

Vessel characteristics differ between ports and regions. In general, the newer, larger, more potent and better equipped vessels are found in the northern and central coasts of Portugal (Fig. 1.4.; with the exception of a fleet operating off Póvoa do Varzim with smaller boats often called “Tucas”, mostly of < 10m) (Stratoudakis and Marçalo, 2002; anonymous data). The fleet in some center ports, namely Peniche and Nazaré, and in both southern regions (west coast and Algarve) is very diverse in length, with 40 % of the vessels having very small dimensions. In the Algarve region we find the oldest vessels (e.g. two of the seiners operating in the region date from 1929 and 1937) and smaller vessels called “rapas”, that often modify their gear for the capture of demersal species (Borges et al. 2001; Erzini et al. 2002; Gonçalves et al. 2008). Crew members on board differ in number depending on vessel size and can reach 24 men in the larger boats (Parente, 2000).

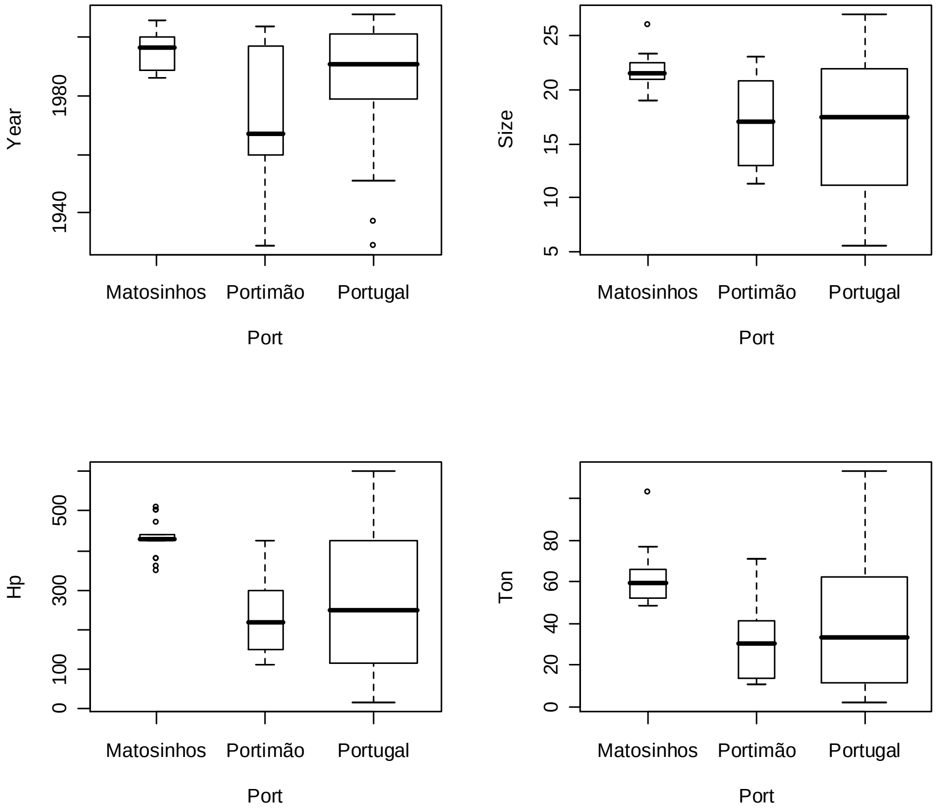

Figure 1.4 - Vessel characteristics between one port of the Northern coast (Matosinhos), one port of the Southern/Algarve coast (Portimão) and all vessels operating in Continental Portugal (anonymous data, 2009).

Operations occur within coastal areas (on the continental shelf) and daily activity is regulated by national legislation that is not rigorously enforced. The legislation tentatively comprehends a maximum of 180 fishing days per year and capture limits set annually and distributed among Producer Organizations. European legislation specifies minimum landing sizes (e.g. 11 cm for sardine, 12 cm for anchovy, 15 cm for horse mackerel and 20 cm for chub mackerel). Despite national laws determining purse seining fishing activities, there are differences in patterns among regions that are mostly related to the season (e.g. seasonal bans, departure time influenced by the season of the year/sunset time influences the behavior of the target species) or market demands. In

Wise et al. (2005) we find that regional variations are also related to the vessel characteristics, differences in species abundance and possibly socio-economic factors. Further, although in all the ports purse seine fishing is allowed for only 5 days per week, the weekend regime differs, leading to changes in the first departure of the week. In some ports the first departure occurs on Monday dawn and the last on Friday evening/dawn, (e.g. Figueira da Foz) (Wise et al. 2005), while in others the first departure occurs on Monday afternoon/Tuesday dawn and the last on Saturday dawn/morning (e. g. Olhão) (A. Marçalo, unpublished data).

In the northern and center regions, where the sardine is more abundant, catches are usually higher, and sardine is the only target and the main landed species (e.g. for 2007, sardine landed was 83.3%, 85.7%, 79.5 % of the total catch for Matosinhos, Figueira da Foz and Peniche, respectively; DGPA, 2008), trips are generally shorter and the net usually is set only once per trip (Stratoudakis and Marçalo, 2002; Wise et al., 2005). On the other hand, in the southern regions where sardines are less abundant, catches consist mainly of horse and chub mackerel and demersal species, fishing trips are longer, and the net is often set more than once per trip (Wise et al. 2005, Gonçalves et al., 2008). A fishing trip starts when the vessel leaves the port, after which searching immediately starts as the skipper turns on the echo sounder. Depending on fish intensity, echo sounder markings can be considered low, moderate or high and determine if the search should continue (e.g. low marks or inadequate fish aggregation in the water column), end (e.g. no fish detected) or if the fishing operation should start (Stratoudakis and Marçalo, 2002; Carvalho et al., 2008). Net setting occurs rapidly with the help of an auxiliary vessel (6-7 meters), a power block is used to haul most of the heavy purse seine, and final stages of hauling comprehend a largely manual operation involving all crew members alongside the seiner. Preferably, sets occur mainly around sunrise and a typical fishing operation lasts around 2 hours. In general, the number of purse seiners operating in the north and center regions is double the number of boats operating in the southern regions but the number of trips per year is less in the north and center regions. However, while fishing effort per vessel is lower in the northern and center regions, total fishing efforts are higher for these regions compared to the South. (Parente et al. 2000; Wise et al. 2005).

1.1.3 – Atlantic sardine (*Sardina pilchardus*) and the Atlanto-Iberian Stock

Sardine (*Sardina pilchardus*) is an ecologically and commercially important small pelagic clupeoid widely distributed from the northern North Sea to Mauritania/Senegal and from the Azores to the eastern Mediterranean, with the biggest populations and most important fisheries concentrated in the Atlantic coasts of North Africa and Europe (Parrish et al. 1989). It is targeted by purse-seine fisheries across most of its distribution area and by pelagic trawlers mainly in African waters. The largest sardine populations and the more productive sardine fisheries are situated off Morocco (stock biomass of 1–5 million tonnes; with landings of around 600 000 t in recent years; FAO, 2003), whereas abundance and fishing intensity are low in the Mediterranean (Silva et. al, 2008). Within Atlantic European waters, the sardine is considered to belong to a single stock for assessment purposes, the Atlanto-Iberian stock, with a stock biomass of around 450 000 t and landings of approximately 90 000 t in recent years (ICES, 2007; Silva et al. 2009). The Atlanto-Iberian sardine stock is delimited by the French/Spanish border in the north and by the Strait of Gibraltar in the south (ICES Divisions VIIIc and IXa) and is exploited mainly by the purse-seine fleets of Spain and Portugal.

In Portugal (ICES Division IXa), sardines represent approximately half of the annual fish landings and the bulk of purse seine catches (> 70 % of total landings), which reflects the socio-economic importance of the resource and the fishery for the country (Pestana, 1989). Atlanto-Iberian sardines are short lived (7/8 years) and experience a variety of oceanographic conditions, from temperate shelf to sub-tropical upwelling with temperatures < 10ºC and > 22ºC respectively (Coombs et al 2006). This water temperature regime influences the spawning season in different regions, although in Portuguese waters sardine spawn from the fall (September/October) to the spring (March/April), with some variation along the coast (Stratoudakis et al. 2007). Because the stock is intensely exploited, its abundance and distribution depend on good recruitment years that are directly linked to environmental factors, namely good upwelling conditions favored by North wind regimes, observed mostly in the Portuguese Northern and center coasts during summer months, leading in general to better recruitment in these areas and greater sardine abundance (Santos et al., 2001).

Historical time series data (ICES, 2007, 2008) reveal that the Atlanto-Iberian sardine stock has shown high fluctuations in abundance and high inter-annual variability in recruitment. Values for the Northern coast present a sharp decrease in 1998-2000, increasing slowly with some fluctuations afterwards, being quite stable for the next few years. Aa strong recruitment in the Northern coast in 2000 resulted in an important stock recovery that year and an increasing trend in stock abundance until 2003 (Marques et al. 2003) with a decrease in landings in 2004 and 2005 followed by an increase in 2006 and 2007. The Southern west coast population also remains relatively stable, although with some fluctuations and slight decreases for 2006 and 2007. Values for the Algarve region shows an accentuated decreasing trend since 2000 and landings in 2007 were at their lowest for the 1995-2007 period. In 2007, 35 % of the total

Portuguese commercial catches of sardines were taken off the Northern coast, 44 % off the center coast, 13,6 % in the Southern west coast, and approximately 7 % in the Algarve, (DGPA, 2008) (Fig. 1.5).

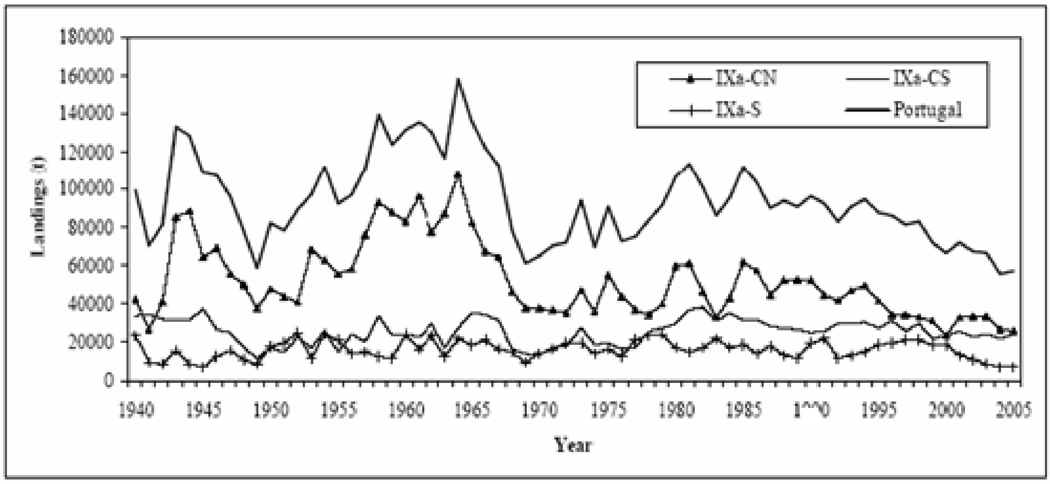

Figure 1.5 – Annual landings of sardine in ICES sub-areas IXa-CN (Northern west Portugal), IXa-CS (Southern west Portugal), IXa-S (South Portugal/Algarve), and total landings off Portugal (figure from ICES WGMHSA Report 2007). *For stock abundance estimation purposes, the center coast is included in the Northern coast.*

Official landings data for both Portugal and Spain are used for the annual stock assessment of the Atlanto-Iberian stock of sardine. Management of the stock relies largely on national measures in Spain and Portugal (time and area closures) and, in recent years, recovery plans have been established in both countries following a series of years of poor recruitment and declining stock biomass, especially since 1995 (ICES, 2002). In Portugal, landing limits for the fleet have been set annually since 1997, and distributed among Producer Organizations (POs) and the few non-associated vessels. Since 1999, POs in northern Portugal have voluntarily introduced daily landing limits on their vessels in an attempt to manage the annual quota of the PO as a function of local market-price fluctuations (Stratoudakis and Marçalo, 2002).

1.2 – Fisheries discards

1.2.1 – General overview

A widespread component and undesirable consequence of commercial fisheries is the release of a significant portion of the catch, a process named discarding (Alverson et al. 1994). In general, by-catch and subsequent discarding is inevitable, due mostly to insufficient size selectivity of different fishing gears. Reasons for discarding are highly variable, depending mainly on the gear characteristics (e.g. mesh sizes, hanging ratios) and fishing operations, and usually occur as the interaction between market (species – target/noncommercial/low value species, quality), management (minimum fish size, landings restrictions/quota limits and fishing days), operational (boat loading and processing capacity), economic (market demands), human (e. g. decision making – catches below the minimum landing size) and biological (sex, condition) factors (Hall, 1996; Stratoudakis et al., 1998; Borges et al., 2001; Davis, 2002, Gonçalves et al. 2007).

Concerns about at-sea discarding have long been recognized by fisheries scientists (Jean, 1963), as discards are seen as an important source of waste, mortality and of information bias in stock assessment of many species. There is also increasing evidence that discards can have effects at the biodiversity and community levels, through impacts on top predators, removal of individuals of many species or by elimination of prey (Hall, 2000; Kelleher, 2005). However, awareness about the extent of the discard problem and the need for mitigation is relatively recent. Alert was given by the pioneering, comprehensive study of Saila in 1983, which triggered a number of studies, especially in the last decade of the 20th century, first following reactions to the incidental capture of charismatic species such as dolphins in tuna purse seine nets (Hall, 1996), turtles in shrimp trawls (Epperly et al., 2002), and also marine mammals, birds, turtles and sharks in high seas squid driftnets (McKinnell and Seki, 1998; Rogan and Mackey, 2007), then spreading rapidly to fish species in many fisheries worldwide where discarding of unmarketable, undersized or damage fish is a common practice (Alverson et al. 1994).

Since Alverson et al. (1994) estimated global discards in commercial fisheries to be almost one third of the total catch (average of 27 million tones yearly), the consensus is that discard rates are high, variable and their estimation difficult (as illustrated by the wide range of the estimate - 17.9-39.5 million tones). Present global estimations based on the fishery-by-fishery approach are at 8 percent (proportion of the catch discarded) with an annual total discards estimate of 7.3 million tones (Kelleher, 2005). Most discards are associated with trawl fisheries targeting shrimp and demersal finfish (over 50 percent of total estimated discards), while most purse-seine, handline and small-scale fisheries are considered to have low discard rates (Kelleher, 2005, Broadhurst et al. 2006).

Development of improved methodology at the global level has contributed to the updating of discards estimates, and these are currently used in the annual stock assessment of some commercially important fish species worldwide (e.g. ICES 1996, 2000; Kelleher, 2005). However, this reported discard information is considered generalist and based on incomplete data for most fisheries, since studies have been mostly directed towards fisheries considered more detrimental or easier to sample (e.g. towed fishing gears and recreational fisheries; Ferguson and Tufts 1992; Sangster et al. 1996; Suuronen et al. 1996; Broadhurst et al. 2006; Suski et al. 2007; Gingerich et al. 2008).

Estimates are based on discard data collected by fisheries observers onboard commercial fishing vessels as part of extensive national and international sampling programmes (Borges et al. 2001, 2006; Rochet et al. 2002; Stratoudakis et al. 1999), and sampling designs generally rely on variable assumptions (e.g. discards are proportional to catch or to effort, length composition of the catch determines discard amounts and size, market prices promote discarding due to sorting criteria; quotas create incentives for discarding; Rochet and Trenkel, 2003). On a general basis, estimation of discards is difficult, stock assessments for many important commercial species in fisheries worldwide still lack discard data and studies focusing on discarding practices, discard quantification and composition continue to be scarce (Borges et al. 2005). Yet, for most of the explored stocks, the sampling level of the landings is considered adequate for assessment purposes (ICES, 2002).

Specifically, fisheries management studies carried out in the Iberian Region along the eastern Atlantic shelf (which is considered an upwelling area with high productivity and characterized by a large number of commercial and non-commercial fish species), focus on fisheries of a typically multispecies nature and deal with highly variable fish stocks. Here, different kinds of Spanish and Portuguese fleets operate, from trawlers to long liners, hand-liners to purse seiners. Consistent discard data for Portugal on most important fisheries is only available since 2002 (IPIMAR, unpublished data) and some other published studies on by-catch and discard estimates are experimental or preliminary observations. Such examples are the work on the beach seine fishery in central Portugal (Cabral et al. 2003), a few studies on fisheries in the southern (Algarve) Portuguese coast and the Mediterranean (Borges et al., 2001, Monteiro et al. 2001; Erzini et al., 2002; Gonçalves et al., 2007; Costa et al., 2008), Spanish bottom trawl fisheries (Lema et al., 2006; Días et al, 2008), and only one study on the purse seine fishery operating off the Northern Portuguese coast (Stratoudakis and Marçalo, 2002).

1.2.2 - Purse seine fishery discards

The nature of purse seine fisheries, that mostly target pelagic or schooling fish, taking hauls of low diversity of species and sizes, contributes to a more sporadic discarding behavior than in demersal fisheries (Broadhurst et al. 2006; Borges et al. 2008). Thus, reported discard rates are usually low (5.1 % and 1.6 % for purse seine fisheries targeting tuna and fisheries targeting small pelagic fish respectively; Kelleher, 2005). Discards in purse seining occur mainly as “slipping”, a term used to describe the phenomenon where part of the catch is released after drying-up of the net but without the fish being drawn aboard, by rolling the fish over the headline (floating line) of the net (Pawson and Lockwood, 1980; Lockwood et al, 1983; Mitchell et al., 2002; Stratoudakis and Marçalo, 2002). Thus, as the catch never leaves the water before being released, purse seining is considered to have a low impact on escapees, and for most fisheries, slipping is not accounted for and fish released in such condition are expected to survive (Lockwood et al. 1983).

However, increased awareness of purse seining discards occurred in tuna fisheries, mainly due to incidental catches of dolphins, although reported rates of discards of non-commercial or undersized tunas and many species of elasmobranchs are also common for these fisheries (Megalofonou et al., 2005; Kelleher, 2005; Hall, 1998). To a lesser extent, studies focusing on purse seine discard quantification have also been reported for small pelagic fisheries in northern Europe targeting mackerel (*Scomber scombrus*) and herring (*Clupea harengus*; Lockwood et al., 1983; Pawson and Lockwood, 1980; Misund and Beltestad, 1995; ICES 2000), southern and Northern Portugal (Borges et al., 2001, Erzini et al., 2002, Stratoudakis and Marçalo, 2002) targeting Atlantic sardines (*Sardina pilchardus*) and Western Australia (*Sardinops sagax*; Mitchell et al. 2002). The alert was given by the conflicting beliefs of fishermen in several of these fisheries, with some assuming that because the excess fish were swimming in the bunt of the net prior to release (slipping), all the fish would survive when discarded by slipping (Lockwood et al., 1983), while others believe that not only did most of the slipped fish die, but they also negatively affected other fish in the area, thus leading the fishers to either avoid the practice or temporarily leave the fishing grounds (Mitchell et al. 2002, A. Marçalo, unpublished data). These few studies brought attention to the variable magnitude of the slipping events, differences in survival and mortality rates and their difficult estimation within each fishery. Further, awareness was raised on the extremely delicate condition of small pelagics, and their high susceptibility to suffer physical injury and stress from handling and retention in the nets.

Consequently, attempts have been made to improve the selection in some fisheries, where minimum legal sizes are implemented by introducing the use of sorting grids. However, relatively high mortalities (>40 %) were recorded for mackerel that escaped, leading to abandonment of the idea of recommending the size-selection device in commercial fishing (Misund and Beltestad, 2000). Yet, studies focusing on more precise quantification and factors leading to unaccounted mortality of slipped fish are still scarce and in general, little is still known of the impact of purse seining discards on the marine ecosystem and at the species and population level, thus reducing the accuracy of stock assessments that are mostly based on commercial catch statistics.

1.2.3 – Preliminary observations of purse seining discards ("slipping") along the Portuguese coast

Studies focusing on estimation of slipping during purse seining activity along the Portuguese coast have been carried out only since the beginning of the century (Borges et al., 2001, Erzini et al., 2002, Stratoudakis and Marçalo, 2002, Wise et al., 2005), demonstrating firsthand the existence of the phenomenon and its variability for different years and seasons. Borges et al. (2001) reported a mean discard rate varying from 20-30 % of the total catch for purse seiners in the south coast of Portugal (Algarve), while Stratoudakis and Marçalo (2002) found that the purse seiner fleet in one port of the northern coast slipped an estimated 69 % of the total catch for the Summer/Fall 2001 season, decreasing to 24 % in the following year (Spring/Summer 2002 season). Further, slipping rates of 22 % of the total purse seining catch for Summer/Fall 2003 are reported by Wise et al. (2005) for seiners from several ports along the west coast.

For sardine, magnitude of slipping seems to correlate with seasonal and regional variations, and in particular, positive correlations are observed with years of good recruitment and increasing stock biomass, as demonstrated by the strong recruitment in 2000, which resulted in high observed slipping rates in 2001 for the Northern coast (Stratoudakis and Marçalo, 2002). In the northern and center coasts, the main factors leading to slipping are related with market demands, namely the applied quota regime, followed by fish size limitations, while in the southern west coast and the Algarve, slipping occurs mainly due to the quality of the catch (e.g. multispecies sets that decrease the price, and small catches not worth the time and crews´ physical effort - Stratoudakis and Marçalo, 2002; Wise et al., 2005).

1.3 – Stressors contributing to discarded fish delayed mortality

Several studies have demonstrated that most fishing methods lead to mortality of discarded fish or escapees (Davis, 2002). There are a variety of stressors or factors associated with capture and escape (e. g. net entrapment, mesh passage, inter-individual abrasion, contact with net walls, sustained swimming until exhaustion, air exposure, confinement and overcrowding) directly related to gear type (trawl, purse seine, hook, gill net, etc.), operation mode (fishing time, pressure, light conditions, water and air temperatures, anoxia, sea conditions, time on deck and handling procedures), environmental (water temperature, pressure, light conditions) and biological (fish behavior, size and species) conditions (Wassenberg & Hill, 1993; Chopin et al. 1996a; Olla *et al.*, 1997, 1998; Ryer & Olla, 2000; Davis, 2002; Davis & Olla, 2001, 2002; Davis & Ryer, 2003, Broadhurst, 2006, Gingerich et al. 2007). Interactions between these stressors have been identified as the direct and indirect cause of mortalities that may occur at the point of escape (immediate) or at some time after escape (delayed) due to injury and stress. How a fish reacts to one or more stressors depends on the species, fish condition and magnitude of the stressor, which in turn are responsible for the associated variable degree of physical damage (injury), fatigue, stress and mortality (Davis, 2002).

Detrimental effects of many stressors and their interaction have been studied in a number of recreational or commercial towing fisheries, and include among others, interaction of air exposure duration effects, water temperature challenges, fishing durations or differences in light conditions during capture, all known to cause impairments at the physiological, behavioral and physical level (Strange et al., 1977; Ferguson and Tufts, 1992; Olla et al. 1998; Davis et al. 2001; Farrell, 2002; Farrell et al., 2003; Davis and Parker, 2004; Davis and Schreck, 2005; Meka et al., 2005; Gingerich et al. 2007; Siepker et al. 2007; Crossing et al., 2008; White et al., 2008).

Comparing towed and purse seining fishing gear effects specifically, one can say that pelagic fish targeted by purse seining are less likely to suffer from pressure and water temperature changes or air exposure on deck than demersal fish species captured by towing gears, that are more susceptible to barotraumas and thermoclines as the fishing gear is retrieved. However, we cannot ignore the fact that considerable pelagic fish discard/escape mortality rates have been associated with increased susceptibility to scale loss when compared with some demersal species (e.g. gadids; Suuronen at al. 1996a, 1996b). Purse seine fishing targeting small pelagics, often results in catches of a considerable size (several tons) and as the net is being hauled, fish swimming behavior inside the net is characterized by frequent escape attempts, mostly consisting of bursts against the net walls (Misund, 1993; Misund and Beltestad, 1995). Moreover, when the net is completely bunted prior to fish transfer onboard, excessive fish densities are attained, leading to physical damage and stress (Pawson and Lockwood, 1980; Lockwood et al., 1983; Mitchell et al., 2001), all contributing to variable immediate mortality as well as unaccounted delayed mortality.

1.4 – Responses to stressors

Responses to stressors are generally related to the associated induction of stress by the fishing operation (Wood et al. 1983, Barton and Iwana, 1991, Wandelaar Bonga, 1997). Stress by definition is “a condition in which the dynamic equilibrium of animal organisms called homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly referred as stressors”, and it can vary from an acute behavioral or physiological response to compensate or adapt to the disturbance, enabling the animal to overcome the threat, to maladaptive or dysfunctional responses if the animal experiences chronic stress leading to mortality (Barton and Iwana, 1991). On a general basis, stress reactions affect fish in a variety of ways and at different levels based on the level of the stressors, their interaction, and depending on the level of injury (physical damage; e.g. scale loss) and the species. The most immediate or direct effects act at the individual biological and biochemical level, starting with primary responses (hormonal) such as the cortisol and catecholamine increase, that will play a role in the chain of reactions that follow, namely, moving towards secondary reactions at the metabolic (e.g. plasma glucose and lactic acid increase, depletion of muscle and liver glucogen), hematological (e.g. haematocrit and haemoglobin increase, deviation of blood cell numbers), hydromineral (e.g. ionic dysfunction) and structural (e.g. variations on condition factor) levels, and finally tertiary responses such as inhibition of growth and reproduction, disease resistance and swimming performance (Barton and Iwana, 1991; Wendelaar Bonga, 1997; Mommsen et al. 1999; Lee et al., 2003; Lupes et al. 2006). Consequently, alterations in fish behavior also occur at different levels, like increasing vulnerability of discarded fish and escapees to predators, which in turn will be an additional source of delayed mortality (Olla et al., 1992, 1995; Ryer, 2002; Masuda and Ziemann, 2003; Ryer et al. 2004, 2008).

Studies of the response to stressors during capture are scarce for small pelagics captured by purse seining. Pawson and Lockwood (1980) and Lockwood et al. (1983) mostly described the impact of the fishing process (fish density and duration) on the physical condition and mortality of Atlantic mackerel (*Scomber scombrus*). Misund and Beltestad (1995) investigated the survival of Atlantic herring after simulated purse seine bursts, which indicated that there could be a high mortality associated with the net burst, caused by severe scale loss and resultant osmoregulation difficulties. However, the only information available on the physiological and biochemical responses of small pelagics and their relationships to stressors are attempts to study gas exchange during capture (Holeton et al. 1981; Boutilier et al. 1983) and some blood physiological components (Swift, 1982, 1983) for Atlantic mackerel, or nutrient release promoted by purse seine fishing for sardines (*Sardina pilchardus*, Stratoudakis et al. 2003). Yet, none of these studies fully associated the response with the critical stressors or factors and their interactions acting during capture, and therefore do not further our knowledge of the probable causes of mortality after discarding.

1.5 – Assessment of fishing impact on discarded fish and escapees: Field vs captivity/laboratory studies

Research efforts have been directed to quantify and minimize discard rates and mortality prior to discarding, applying measures such as allowing fish to escape from fishing gear, especially in towed gears (as a result of gear modifications that improve species and size selection), or management policies to restrict fishing effort and fishing areas (Hall, 1996, 2000; Davis, 2002; Davis and Ryer, 2003; Broadhurst et al. 2006). However, less attention has been brought to the associated collateral delayed mortality of discarded fish after being brought on board or of escapees (fish that are caught and escape or are allowed to escape), which is generally unmeasured or very difficult to measure and an added source of uncertainty in stock assessments, with negative impact at the stock and population (e.g. mortality of undersized individuals or juveniles, effects on biodiversity), trophic (e.g. removal of top predators, elimination of prey) and ecosystem (e.g. transfer of biomass between water layers) levels (Hall, 2000).

In order to facilitate estimation efforts, for most fisheries it is generally assumed that all discarded fish die (Chopin et al. 1996) and all escapees survive (Pascoe and Revill, 2004). However, studies on the fate of released fish have shown that there can be a wide variation either in discarded fish survival or escapee mortality depending mostly on the species or the gear type. For organisms either discarded or escaping, estimated mortality rates range from 0-100%, although more commonly mortality is less than 20 % (Broadhurst et al., 2006). Most of these studies (> 80 %) have been conducted in the field, involving commercial fishing operations that focused on studying capture and gear effects, which can lead to variable degrees of physical injury. Subsequent monitoring of discards and escapees mortality is either directly made at sea (using net pens) or in the laboratory (Lockwood et al., 1983; McLoughlin et al., 1991; Chopin and Arimoto, 1995; Sangster et al. 1996; Suuronen et al. 1996*a*; Suuronen et al., 1996*b*; Mitchell et al. 2002). However, these studies have some limitations, providing only qualitative assessments of the discard status, they are expensive, short-term (observations limited to few days or 1 to 2 weeks maximum), and they do not include the study of the interaction of stressors with other factors operating during capture that may contribute to direct or delayed discard or escapee mortality.

Most recently, some approaches have moved completely to the laboratory. Here limitations are also present, since in most cases it is not possible to simulate the entire fishing, escape or discard practice. However, laboratory approaches can provide useful information particularly on short or long term observations after discards, by measuring the interaction of the fishing practice and associated stressors along with the species biological aspects (e.g size and condition), physiological and physical responses, and using these aspects as indicators of either recovery or delayed mortality following capture, which was not possible to measure in the field (Olla et al. 1997; Olla et al. 1998; Olla et al. 2000; Ryer and Olla, 2000; Davis et al. 2001; Davis, 2002). Understanding of the interactive role of the stressor and responses can provide fundamental knowledge on the causes that lead to delayed mortality. Only then, can adjustments be made in the field by changing fishing practices so that discarded fish or escapee mortality is decreased.

1.6 – Aims and structure of this dissertation

Seasonal and regional purse seine discarding occurring along the Portuguese coast can lead to a variable magnitude of slipped fish (fish deliberately allowed to escape at the end of the operations; Borges et al. 2001; Erzini et al. 2002; Stratoudakis and Marçalo, 2002; Wise et al. 2005). The aim of this thesis is the study of the impact of commercial purse seine fishing operations on slipped fish, while accounting for the survival rates of the escapees and studying the main factors/stressors that may lead to delayed mortality. Work was carried out on Atlantic sardines (*Sardina pilchardus*) since it is the main pelagic resource along the Portuguese coast and the target species of the fishery. To attain the objective described above, a combined field and laboratorial approach was applied, subdivided in the respective chapters described below:

1) The work described in **chapter 2** relates to the study of the early dynamics of sardine (*Sardina pilchardus*) stress reactions and identifies likely stressors during the commercial fishing operation. *In situ* observations allowed the description of fishing manoeuvers, catches, the evaluation of the scale of the slipping problem and the determination of the main reasons that lead to the event, while identifying stressors (mainly operational at this stage) during fishing operations that affected survival of the escapees. In contrast with other fisheries such as trawling or hook and line, purse seining is a fishery that allows sampling along the whole fishing operation, from early stages of net hauling to the final component of the operations when fish are transferred to the seiner. Thus, as revealed in this work, it allowed the integral study of the evolution of physiological parameters that lead to variable stress responses of sardines during purse seining operations.
2) **Chapter 3** explores a mix of field and the beginning of the laboratory approach in this thesis. The work presented here, was subdivided in two tasks. In the first, the

most appropriate methodology for transporting and maintaining sardines in captivity after live capture was optimized, followed by the second task that allowed the study of the main factors that affected early survival, physical damage and stress reactions of sardines from capture to introduction and acclimation to captivity. Several fishing trips were carried out that allowed the determination of stressors/factors related to conditions at sea, during introduction to captivity and, possibly, their interaction. The degree of physical damage, namely scale loss was related to the probability of dying, and physiological responses reestablished within two weeks in captivity, suggesting that maintenance conditions were adequate, permitting a rapid recovery from fishing and transport stress. Fully acclimated sardines could then be used in fishing experiments simulating purse seining, leading to the work performed in chapters 4 and 5.

3) **Chapters 4** and **5** are related with experiments in captivity simulating final operations of purse seining (when the fish suffers most of the stress and physical impact) in order to study some operational and environmental factors that lead to variable physical, physiological and behavior responses and result in delayed mortality after slipping. In **chapter 4**, three laboratory experiments were performed to explore the effects of operational and environmental factors. Physical damage and physiological status of sardines prior to fishing and immediately after fishing (prior to slipping) were analyzed, which together with long-term observations on physical status and survival of released/slipped fish, led to conclusions concerning the main stressors leading to mortality. Work in **chapter 5** analysed fishing effects on the behaviour of slipped sardines in the presence of predators. Parameters such as swimming speed, predator approach and nearest neighbour distances were observed and recorded through the analysis of video images.

To conclude, the final chapter (**chapter 6**) reviews and discusses the results obtained in all the experiments, in view of the global aim of this thesis which was to study the stressors acting in purse seining, analyze the responses to these stressors by the sardines, and tentatively analyze their effects on survival or delayed mortality rates after slipping. In the absence of any other work related with this kind of fishery, this thesis hopes to contribute to a better understanding of the operational, biological and environmental factors associated with purse seine fishing and possibly to suggest some measurements that decrease delayed mortality of slipped fish.

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CHAPTER 2

Sardine (*Sardina pilchardus*) stress reactions to purse seine fishing

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2.1 – Abstract

Observations from the purse seine fishery off northern Portugal are used to describe the early dynamics of sardine (*Sardina pilchardus*) stress reactions and identify likely stressors during the commercial fishing operation. Sardine blood and muscle were sampled from the onset of fishing (school identification and encircling) to the end of fish transfer onboard (90-120 minutes later). The evolution of haematocrit, haemoglobin, cortisol, glucose, ionic concentrations, ATP and its catabolites were modelled using linear mixed models as a function of time spent in the net, biological (sex, reproductive state and condition) and operational variables (catch, light level and phase of fishing operation). Significant linear trends with time were detected for most stress variables and mean concentrations after 2 h in the net were similar to literature values corresponding to acute stress reactions for teleosts. Biological variables were rarely significant and explained a small proportion of variation, while operational variables were never significant. For each stress variable, levels varied considerably between trips but the temporal evolution was common across trips. Random trip effects were uncorrelated among most biochemical variables, suggesting that distinct factors affected each stress variable during the sampled trips. However, the linear trend with time spent in the net observed for most stress variables indicates that the duration of the fishing operation is an important stressor in purse seine fishing due to the progressive water volume restriction, crowding and manipulation associated to the fishing method.

Key-words: Sardine, purse seine, fishing duration, stress, LMMs.

2.2 – Introduction

Responses to stressful events are well documented for several fish species, where unavoidable manipulation (capture, handling, netting, crowding or transport) and poor water quality under aquaculture conditions can affect commercial production or re-stocking programs. Stress is related to a cascade of physiological reactions that range from immediate hormonal responses to long-term changes in growth and reproductive potential (Mazeaud et al. 1977, Wood et al. 1983, Barton and Iwana 1991). In the attempt to regain homeostasis, responses are grouped into primary (hormonal), secondary (metabolic, hematological, hydromineral and structural) and tertiary (whole organism and population). Primary responses are characterized by the activation of the hypothalamo-pituitary interrenal axis and the release of catecholamines and cortisol into the bloodstream (Wendelaar Bonga 1997), whereas secondary responses arise from the action of these hormones at the blood and tissue level (e.g. hyperglycemia, increases of cardiac output, oxygen uptake, erythrocyte release and swell, rise in haematocrit and haemoglobin and disturbance of the hydromineral balance). Long-term (tertiary) reactions range from suppressed appetite, growth and reproduction to immunodeficiency and, eventually, death (Mazeaud et al. 1977, Bourke et al.1987, Barton and Iwana 1991, Schreck et al. 2001).

In several fisheries, concerns about the potential survival of released or escaping components of the fish catch have highlighted the importance to identify the main stressors, quantify the stress reactions and understand the consequences of stress caused during fishing (Pawson and Lockwood 1980; Swift 1983; Bourke et al. 1987; Chopin and Arimoto 1995; Olla et al. 1997; Olla et al. 1998; Davis et al.

2001). Studies are usually based on laboratory simulations of the fishing practice, where the additional effect of behaviour factors (e.g. swimming performance, predator avoidance) and environmental conditions (e.g. temperature, light intensity) can be tested experimentally (Olla et al. 1997; Olla et al. 1998; Davis et al. 2001; Davis 2005). Some studies question the correlation of stress variables with ensuing mortality (Davis et al. 2001; Davis 2002), although it is generally considered that physiological responses provide an accurate indicator of the magnitude of stressors experienced during the fishing process (Davis 2005).

Observations of fish stress reactions to commercial fishing are very scarce and problems usually arise in the determination of the duration and nature of the stressor related to the fishing operation (Chopin and Arimoto 1995; Manire et al. 2001; Davis 2002). In addition, uncontrolled additional stressors (e.g. changes in environmental conditions during fishing – Olla et al. 1998; Davis et al. 2001) can have a confounding effect in field studies, while the consequences of stress reactions to subsequent survival cannot be easily monitored (Davis 2002). Some of these problems seem to be less restrictive in purse seine fishing for small pelagics, where the operation takes place in the relatively stable upper part of the water column, the duration of fishing can be measured precisely for all fish and sampling can take place throughout the operation. In addition, purse seine fisheries provide an interesting case for fish stress studies since existing observations have demonstrated that the deliberate release of a component of the catch (slipping) is frequent (Lockwood et al. 1983; Misund and Beltestad 2000; Mitchell et al. 2002; Stratoudakis and Marçalo 2002) and potentially stressful events associated to the fishing operation have already been detected (Stratoudakis et al. 2003). Here, we use field samples from the Portuguese purse seine fishery to describe the early dynamics of stress variables in sardine (*Sardina pilchardus*) during commercial fishing operations. Linear mixed models are used to analyse stress variables as a function of the time elapsed since the original application of the fishing stressor (detection of the school and encircling), to identify biological and operational variables with a measurable impact on sardine stress reactions and to provide realistic estimates of mean levels of primary and secondary stress responses during and at the end of commercial fishing operations at sea.

2.3 – Material and Methods

The study took place between May and July 2002 onboard commercial purse seine vessels operating off the port of Figueira da Foz in northern Portugal. Sampled trips were short (total duration up to 12 hours), took place within half a degree of latitude from the home port (39°46´ - 40°43´N) and usually consisted of a single setting of the net before or around sunrise. Fishing followed the typical purse-seine operation (Stratoudakis and Marçalo 2002), involving the rapid deployment of a long net (up to 800 m long and 150 m deep) around marks of pelagic fish identified by the electronic fish-finding equipment, the closure of the bottom of the net (trapping the fish in a purse) and the drying-up of the net (gradually reducing the volume of the purse until the density of the fish becomes sufficiently high to start the transport onboard). Once a target school was detected and the net was set, fishing operations usually lasted about 2 hours (90 - 160 min). Hauling time was usually around 1 hour, while the transfer period varied depending on catch size (Figure 2.1).

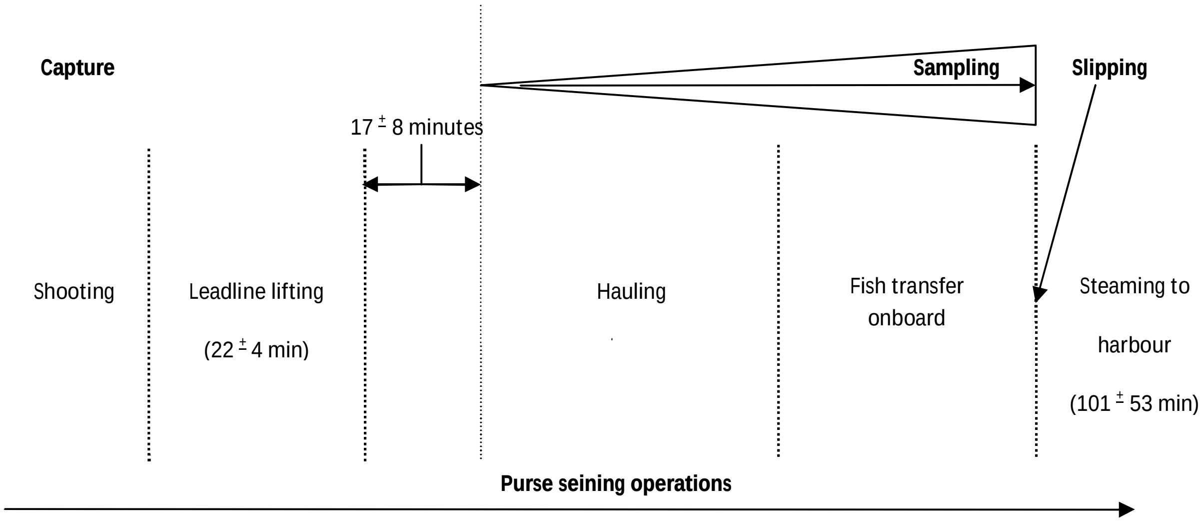

Figure 2.1 - Diagram showing sequence of events during purse seining operations and sampling. Average operation time (minutes) and standard deviations for the ten trips are indicated in parenthesis. Increasing sampling intensity is indicated at the top of the diagram as net volume decrease facilitated the capture of fish.

Catch and slipping estimates in each trip were obtained by visual evaluation provided by the skipper (Stratoudakis and Marçalo 2002). A trained observer followed 19 fishing trips and collected blood and muscle samples from fish individually collected from the encircled area. In 9 trips no fish were caught (failure to detect sufficiently dense schools, gear failures, bad weather, etc.), so fish samples were only collected in 10 fishing trips (Table 2.1). One set was sampled in each trip, although in two trips the net was set a second time before returning to port.

Table 2.1 - Summary of operational and environmental characteristics of the sampled purse seine trips off northern Portugal in 2002. Luminosity is the proportion of the fishing operation after sunrise. Catch is the biomass caught per set (tons) and landings the biomass transferred on deck (the difference is the biomass slipped per set).

Trip	Month	Depth (m)	Wave height (m)	Luminosity	Hauling duration (min)	Transfer duration (min)	Catch (Landings)
1	May	21.2	1.5	1	47	46	13.2 (6.0)
2		19.8	2	0.86	55	30	5.3 (5.1)
3		47.6	2.5	1	28	34	4.4 (4.4)
4		51.2	2.5	1	52	42	10.3 (5.9)
5	June	28.4	1	1	47	15	1.8 (1.7)
6		34.8	1.5	1	55	29	4.2 (4.2)
7		44.1	1.5	0	62	25	1.1 (1.1)
8	July	27.6	1	0.25	53	63	16.9 (10.3)
9		28.5	1	0.67	70	61	12.5 (10.3)
10		47.6	0.5	1	50	15	1.4 (1.3)

Fish with similar swimming performance (regular movements and presenting no signs of lethargic behaviour) were individually collected with a hand net from the encircled area from the start of hauling to the end of fish transfer aboard. Overall, 174 fish were collected in the 10 trips, ranging from 7 to 24 per trip. Time of sampling ranged from 30 to 138 minutes after net deployment, covering approximately equally the phase of hauling and fish transfer. Blood was taken immediately after capture by caudal vein puncture with a hypodermic needle. Depending on fish size, 400-700 μl of blood were retrieved and immediately transferred to heparinized tubes. Blood samples were kept at 0 ºC for roughly 24 hours for subsequent haematological analysis. Fish tissue (3-5 g of muscle from the dorsal area) was also collected from some individuals (total of 72 sardines), cleaned from skin and scales and immediately frozen in liquid nitrogen for adenine nucleotide determination. There was insufficient time to sample both blood and muscle from all individuals, so muscle sampling was restricted to a few fish (3-4 fish) at the beginning and at the end of the sampling period. Handling to obtain both blood and muscle samples never exceeded 3 minutes per fish. Sampled fish were frozen for subsequent biological analysis. In the laboratory, fish were thawed, measured and weighed. Standard biological parameters such as sex, maturity state, fat index, gutted weight and gonad weight were also obtained. Fish condition was estimated as the ratio of total weight over the cube of total length (x1000) and the gonadosomatic index (GSI) estimated as the ratio of gonad weight over gutted weight (x100). Table 2.2 summarises the sampling effort and the biological measurements in each trip.

Within a day from collection, blood samples were processed at the Veterinary Medicine Laboratory of the Technical University of Lisbon. Blood samples were initially homogenized at room temperature for 15 minutes for haematocrit (Hct) and haemoglobin (Hb) analysis. Hct values (%) were obtained by centrifuging blood samples for 5 minutes at 5000 rpm in microhaematocrit tubes and determined using a microcapillary reader. Whole blood Hb (g/dL) was measured using a Boehringer Mannhein Reflotron II chemistry set analyzer. The Mean Cell Haemoglobin Concentration (MCHC) was calculated using the formula Hb / (Hct/100). Plasma from the blood samples was separated by centrifugation at 2500 rpm for 10 minutes and frozen at –20º C for subsequent analysis of glucose, ionic concentration (sodium, potassium and chloride) and cortisol. Glucose was measured using a Boehringer Mannhein Reflotron II chemistry set analyzer. Glucose consumption by blood cells was inhibited with blood refrigeration prior to analysis, although subsequent analyses showed that the 24 hours between sampling and processing resulted in a reduction of glucose levels up to 10% (A. Marçalo, personal observations). Sodium, chloride and potassium concentration were determined in a Vet Lyte electrolyte analyzer (IDeXX Laboratories, Inc., USA). Plasma cortisol was assayed in duplicate and measured by a validated solid RIA assay, without extraction, using a commercially available kit (Coat-A-Count Cortisol kit, Diagnostic Product Corporation, Los Angeles, CA, USA). The intra-assay coefficient of variation of all samples was 5.1%, calculated according to Rodbard (1974).

All muscle samples were stored in liquid nitrogen onboard and maintained at – 80 ºC until laboratory analysis (on average one month after collection). Extracts for analysis were prepared by blending 3-5 g of sardine muscle with 25 ml of 0.6 M perchloric acid at 0ºC for 1 min in a Polytron PT3000 homogenizer according to Ryder (1985). They were analyzed using a simple reverse-phase separation with a commercially available column (Hewlett Packard LiChrosorb RP-18, 10 μm, 200 x 4.6 mm) and a Hewlett Packard 1050 Series liquid chromatograph equipped with a multiple wavelength UV detector. Nucleotides were identified by comparing retention time with prepared standards as recommended by Ryder (1985). Samples were analyzed in duplicate and the means calculated.

The early dynamics of biochemical stress variables in sardine during purse seine fishing were investigated using linear mixed models (LMMs). LMMs provide a general framework for modeling unbalanced nested data (variable number of fish sampled at variables times within trips) that can incorporate information from continuous and categorical explanatory variables, while capturing the randomness inherent in field data by the concept of the random effect (see Pinheiro and Bates (2000) for statistical principles, Lai and Hesler (2004) for a fisheries application). Model selection for each biochemical variable was as follows. First a ‘full’ model was fitted with the fixed component set to the main effects of time (since net deployment), biological variables (maturity, sex and fat) and operational variables (log (catch), light level and phase of fishing process). Trip was included as a random effect, with the mean response in each trip (for any given values of the fixed explanatory variables) assumed to be distributed normally with zero mean and constant variance. The within-trip errors were assumed to be independent of the random effects and also distributed normally with zero mean and constant variance (for nucleotide adenosine variables, 1-2 outliers where removed to approximate the distributional assumptions). The model was then simplified by stepwise elimination of the main effect with the lowest (non-significant) value of the Wald test statistic (in the results, the Wald statistic is reported as the equivalent *F*-statistic on the appropriate degrees of freedom). More complex models were also considered but gave no additional significant results. In particular, there was no evidence of any interaction between the time effect and the biological or operational variables or that the time effect varied between trips.

2.4 – Results

The sampled trips took place over a range of environmental and operational conditions that are typical of purse seine fishing off Portugal (Table 2.1) and similar to those reported in southern Australia (Mitchell et al. 2002). Purse seining for small pelagics usually takes place at the inner continental shelf (20-60 m depth), where abrupt pressure and temperature differences are unlikely to have an impact on fish survival. Sea surface temperatures revealed that the variability within the three months was small (average = 15.7 ± 0.4 ºC, range from 15.1 to 16.1 ºC) and its effect on stress in the fish should be negligible. Although there is a preference for net setting around sunrise (when distinct pelagic schools start to re-emerge after the night dispersal), the entire fishing operation can take place either at night or during daylight. Wave height can vary considerably among trips (<0.5 m to > 2 m of swell) and may affect stress reactions during hauling and fish transfer by increasing the probability of fish collision with the net walls or cause mutual abrasion. Of the 10 sets sampled, 6 were on moderate echo-sounder marks and only 2 on dense marks, resulting in catches that varied from 1 to 17 tones. However, in three sets many fish escaped during the closing of the net. Depending on the catch composition and the daily landing limit, slipping varied from practically nothing to 7 tones per set. Only variables that changed within the trip and could be measured at each sampling time (presence/absence of light and phase of the fishing operation) were considered for modeling, together with the log (catch) in each trip.

Table 2.2 shows that there was little variation in sardine size, but there was a change in mean condition along the study period. The mean total length of the 174 sampled sardines was 16.9 cm (range 14.7- 19.5 cm), with a mean total weight of 39.6 g (range 24.0- 74.1g).

Table 2.2 - Summary of sampling effort and biological data (mean and range) for the ten purse seine trips followed by an observer off northern Portugal in 2002 (Nb: blood samples; Nm: muscle samples).

Trip	Nb		Nm		Fish length (range) cm	Fish weight (range) g	Condition factor (range)	Gonadosomatic index (range)
	Hauling	Transfer	Hauling	Transfer				
1	5	15	5	4	17.0 (16.3 -17.9)	38.3 (29.8 - 50.1)	8 (7 - 9)	0.8 (0.0 – 2.8)
2	4	10	4	2	17.0 (16.2 - 17.6)	35.2 (28.7 - 44.2)	7 (7 - 8)	0.6 (0.2 – 1.0)
3	1	6	1	4	16.8 (15.7 - 17.5)	35.8 (30.4 - 42.2)	8 (7 - 8)	0.7 (0.0 – 1.1)
4	11	11	5	5	16.7 (15.3 - 17.7)	36.9 (27.9 - 46.3)	8 (7 - 9)	0.9 (0.0 – 4.0)
5	5	7	4	3	16.0 (14.9 - 17.2)	32.8 (24.0 - 39.8)	8 (7 - 9)	0.2 (0.1 – 0.5)
6	8	13	4	4	17.3 (15.0 - 19.5)	43.4 (30.5 - 73.6)	8 (7 - 10)	0.4 (0.0 – 1.0)
7	9	8	4	3	16.7 (15.5 - 18.1)	40.6 (30.0 - 48.1)	9 (8 - 9)	0.4 (0.0 – 1.3)
8	8	16	4	4	16.8 (15.2 - 18.1)	44.8 (30.8 - 52.9)	9 (8 - 13)	0.4 (0.0 – 1.3)
9	13	11	4	4	17.9 (17.1 - 19.0)	56.2 (45.2 - 74.1)	10 (8 - 12)	0.3 (0.0 – 1.0)
10	5	9	4	3	15.9 (14.7 - 17.1)	35.0 (28.2 - 42.4)	9 (8 - 10)	0.2 (0.0 – 0.7)

There was no evidence of systematic variation in fish size within or between trips, although female sardine were larger/heavier than males. Despite the relatively large size of fish (which should correspond to a modal age of 2 years), sex could only be determined in 165 fish (77 males and 88 females). Sampling took place in late spring/summer, coinciding with the transition from the late spawning to feeding season. This transition is particularly evident in changes in the condition factor and gonadosomatic index through the study period; sardine condition factor is significantly lower in the first four trips (that took place in May) than in the remaining ones ($F_{1,8}$ =12.1; p=0.008), while the converse is true for GSI ($F_{1,8}$ = 26.6; p<0.001). The biological transition from spawning to feeding is well depicted by the macroscopic data on maturity stage and fat content. Given that maturity and fat data can be easily summarized by 2-level categorical variables (maturity - active/inactive; fat – not visible/visible) these two variables were used as proxies for reproductive activity and condition respectively in the LMMs.

Reliable haematological data were not obtained in 11 of the 174 fish. Preliminary exploration also showed a potentially significant effect of sex, so data for the 9 unsexed fish were removed, reducing the total number of observations to 154 fish. Haematocrit (observations ranging between 21.9 and 51%) decreased significantly with time ($F_{1,\ 143}$= 17.0; p<0.001). The estimated rate of decrease was 3.8% per hour, resulting in mean heamatocrit (over fish and trips) of 39.7% one hour after the onset of fishing (Table 2.3 and Figure 2.2a). Haemoglobin concentration (observations ranging between 6.9 and 16.1 g/dL) also decreased significantly with time ($F_{1,\ 142}$ = 12.5; p<0.001). The estimated rate of decrease was 11.5 g/dL per hour, resulting to a mean haemoglobin concentration of 12.52 g/dL for male fish after one hour in the net (Table 2.3 and Figure 2.2b). Females had slightly higher haemoglobin levels than males ($F_{1,\ 142}$

= 4.7; p=0.031). Neither time nor any of the other variables had any effect on MCHC. The decrease of both haematocrit and haemoglobin concentration over time and the temporal stability of MCHC indicate that the observed physiological changes most likely resulted from an increase in the volume of plasma over time and not from haemolysis under stress.

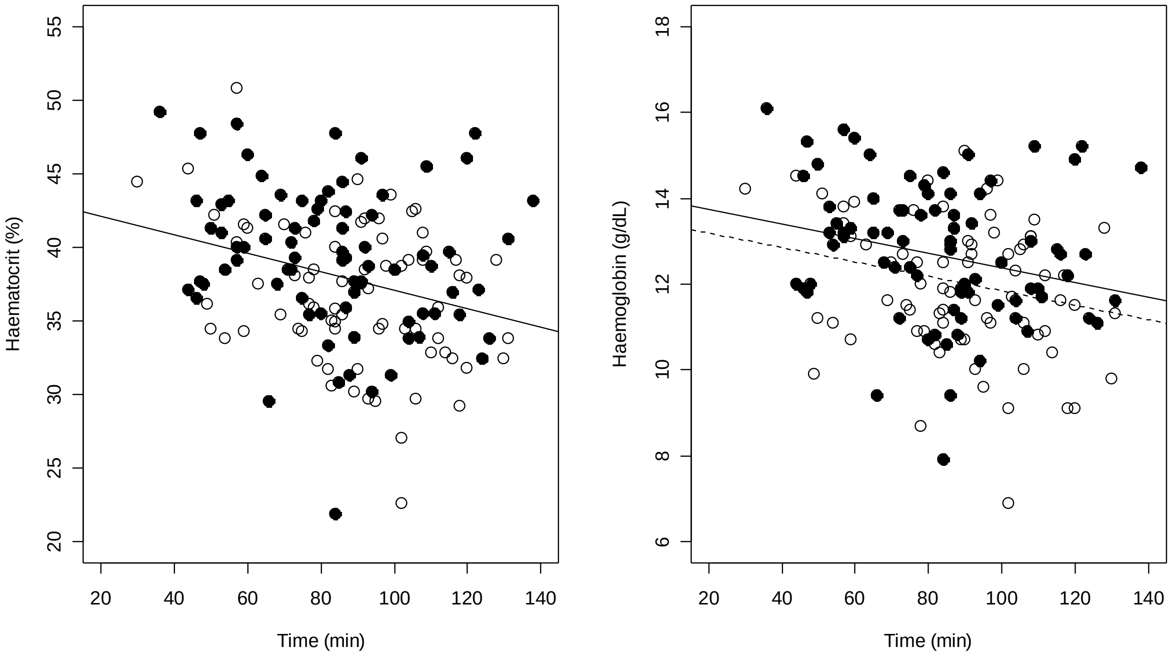

Figure 2.2 - Observed (points) and fitted (lines) effect of time from onset of fishing on the haematocrit (left) and haemoglobin (right) of sardine in the purse seine net (filled circles: females; open circles: males). Sex was marginally significant in the haemoglobin model (solid line: fitted effect for females; broken line: fitted effect for males).

Glucose (observations ranging between 46.7 and 192 mg/dL) and cortisol (observations ranging between 0.2 and 31.1 µg/dL) levels (n = 174; data not available for one fish) both increased linearly with time (glucose: $F_{1,161} = 63.9$, $p<0.001$; cortisol: $F_{1,162} = 59.8$, $p<0.001$). Glucose levels were greater in fatter fish ($F_{1,161} = 11.7$, $p<0.001$). Glucose mean concentration after one hour in the net was 99.7 and 120.9

mg/dL for lean and fat fish respectively, with an estimated increase in both cases by 35.2 mg/dL per hour. Cortisol mean concentration was 8.9 µg/dL after one hour in the net, increasing by a rate of 6.9 µg/dL per hour.

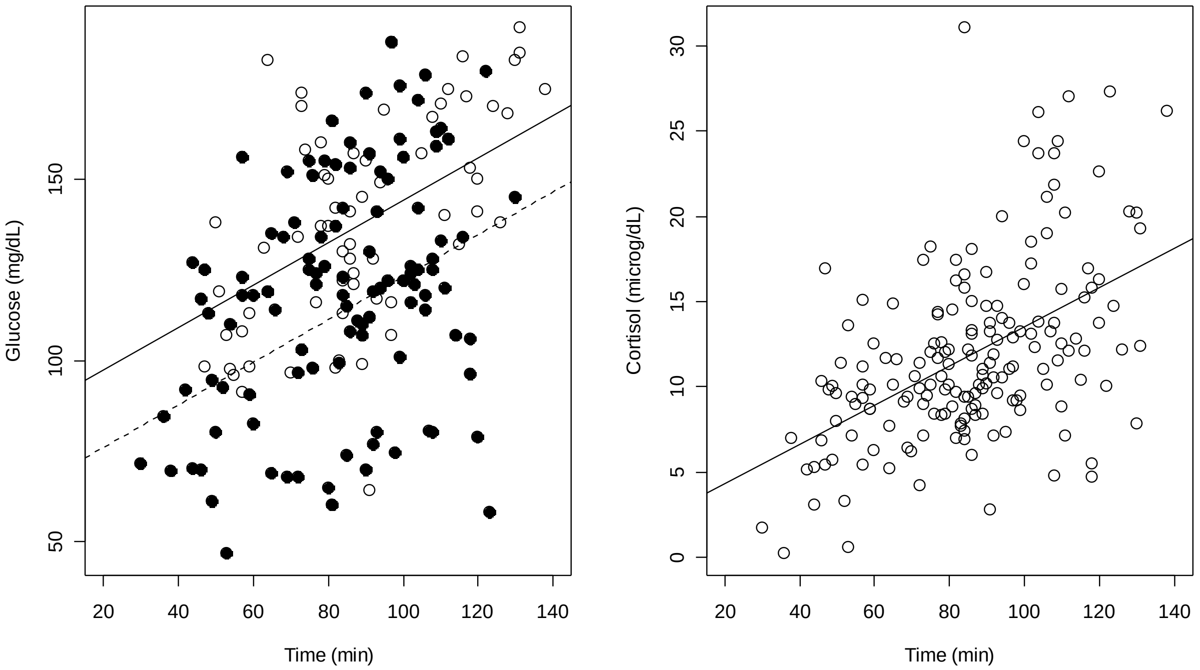

Figure 2.3 - Observed (points) and fitted (lines) effect of time from onset of fishing on glucose (left) and cortisol (right) levels in the plasma of sardine blood. Fish with larger fat deposits (open circles) had a significantly higher level of blood glucose than fish with lower fat deposits (solid line: time effect for fat fish; broken line: time effect for thinner fish).

Of the three ionic concentrations measured in the blood plasma (sodium - Na$^+$, potassium - K$^+$ and chloride - Cl$^-$) only Cl$^-$ (observations ranging between 119 and 200 mmol/L) was modelled (n=130, ionic concentrations not measured for 44 fish). This is because Na$^+$ (observations ranging between 133 and 205 mmol/L) and K$^+$ (observations ranging between 1.5 and 15 mmol/L) concentrations often exceeded the higher and lower limits of calibration respectively (Figures 4 c and d). Cl$^-$ concentration increased with time ($F_{1,119}$ = 14.4; $p<0.001$) by an estimated rate of 10 mmol/L per hour, reaching 144.3 mmol/L after an hour in the net (Figure 2.4a and Table 2.3).

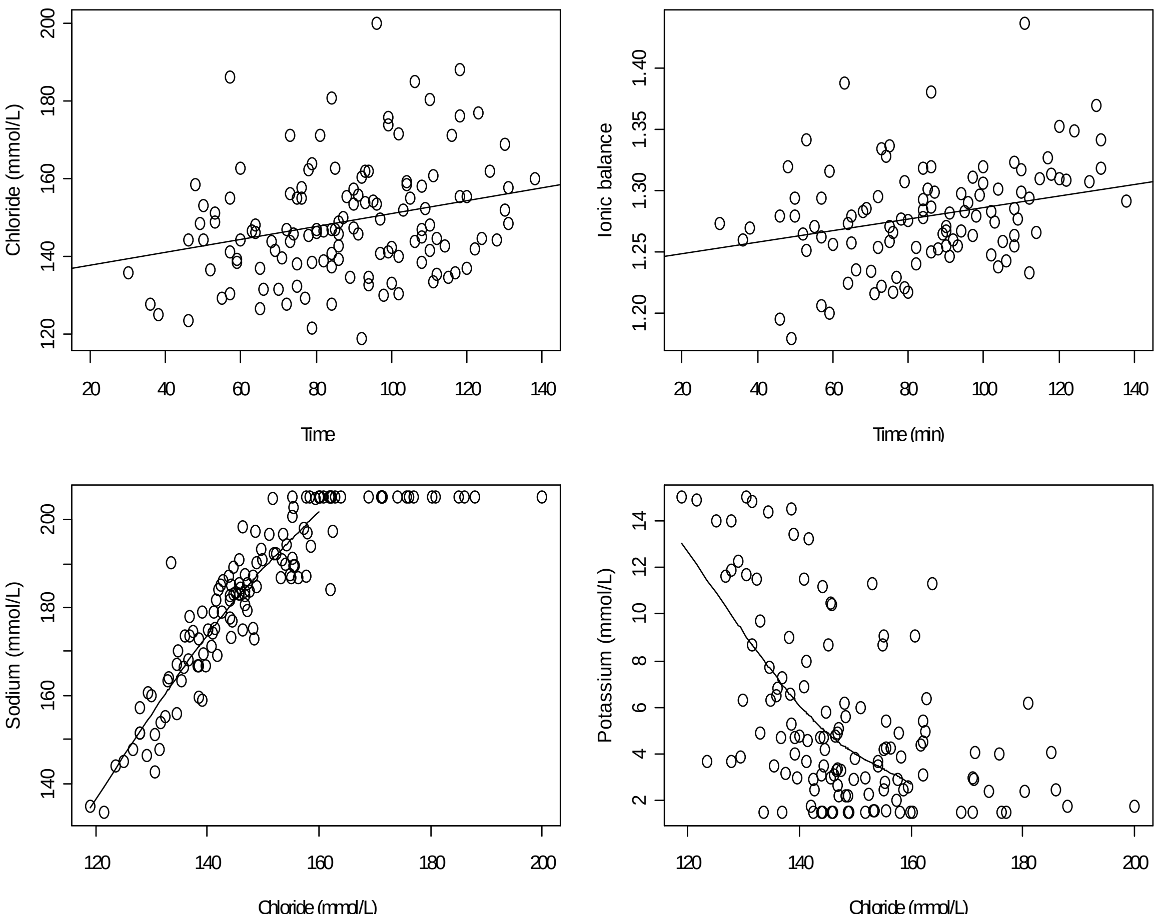

Figure 2.4 - Top panels: Observed (points) and fitted (line) chloride concentration (left) and ionic balance (right) in the plasma over time spent in the net. Bottom panels: chloride vs sodium (left) and chloride vs potassium (right) concentrations in the sampled fish. Lines in panels c and d correspond to smooth splines on 3 degrees of freedom.

No other variable had a significant effect on Cl^-. The high correlation between Cl^- and Na^+ (positive, Fig. 2.4c) and Cl^- and K^+ (negative, Fig. 2.4d) suggest that Na^+ and K^+ increased and decreased respectively with time, although we cannot assess the statistical significance of this. Further, if we only consider ionic concentrations for $Cl^- < 160$

mmol/L (where saturation for Na^+ seems to have occurred), ionic balance seems also to have changed over time (Fig. 2.4b). This is because the increase in Na^+ was more rapid than the decrease in K^+ and increase in Cl^- combined, leading to an increase in the ratio of positively: negatively charged ions in the plasma with time spent in the net.

Table 2.3 - Summary of the fitted linear mixed model for each biochemical variable, separately for the fixed and random parameter estimates. Intercept of the fixed model effect refers to the mean concentration after one hour spent in the net and slope to the rate of change per minute. Random effects are summarized by the standard deviation (SD) between trips (random trip effect accounted by the model) and between fish (residual standard deviation).

Variable (units)	**Fixed**		**Random (SD)**	
	Intercept	**Slope**	**Trips**	**Fish**
Ht (%)	39.65 (0.92)	-0.063 (0.015)	2.38	4.21
Hb (g/dL)	Male: 12.52 (0.30) Female: 13.06 (0.39)	-0.017 (0.005)	0.59	1.45
Glucose (mg/dL)	No fat: 99.65 (7.40) Fat: 120.90 (9.65)	0.586 (0.076)	21.17	22.20
Cortisol (μg/dL)	8.92 (0.66)	0.115 (0.015)	1.33	4.41
Chloride (mmol/L)	144.32 (4.06)	0.167 (0.043)	11.84	10.93
ATP (μmol/g)	3.17 (0.32)	-0.015 (0.007)	0.54	1.84
ADP (μmol/g)	1.26 (0.11)	-0.004 (0.002)	0.28	0.48
HxR (μmol/g)	0.10 (0.02)	0.001 (0.0003)	0.05	0.08

In the case of adenine nucleotides and related compounds (adenosine tri- bi- and mono-phosphate (ATP, ADP and AMP respectively); inosine monophoshate – IMP; inosine – HxR and hypoxanthine Hx) muscle concentrations (μmol/g wet weight) were only measured in 74 fish. Concentrations were highest for IMP (observations ranging between 6 and 24.2 μmol/g) and lowest for Hx (observations ranging between 0 and 0.7 μmol/g) and HxR (observations ranging between 0 and 1.7 μmol/g). Marginally significant time effects (Table 2.3 and Figure 2.5) were detected for ATP (linear decrease with time – $F_{1,61} = 4.4$; $p = 0.040$) and ADP (linear decrease with time – $F_{1,61} = 4.78$; $p = 0.033$) and a highly significant time effect was found for HxR (linear increase with time – $F_{1,61} = 9.74$; $p = 0.003$). No other variables were significant, although in the case of inosine monophosphate only marginally so (linear increase with time - $F_{1,61} = 3.1$; $p = 0.083$).

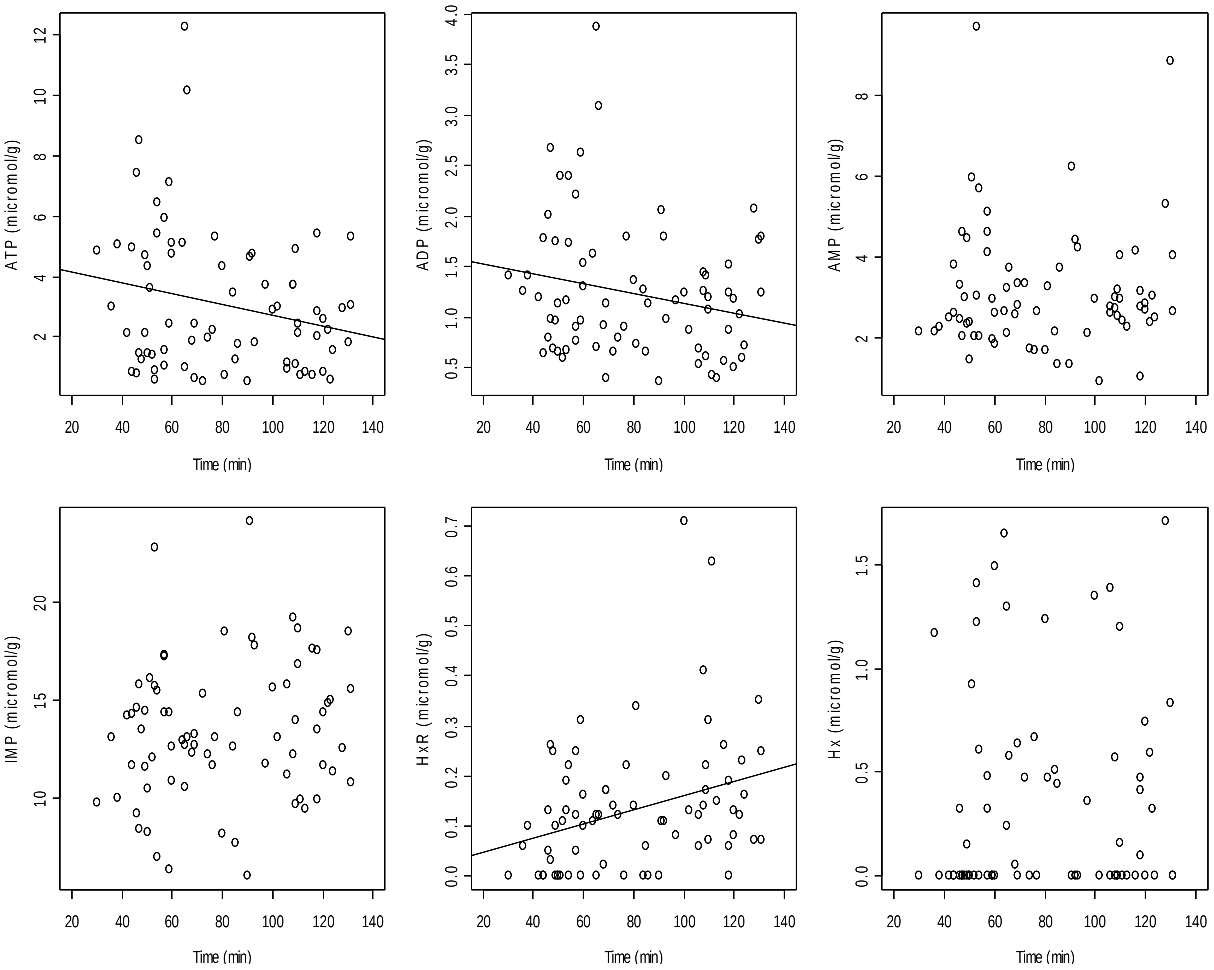

Figure 2.5 - Evolution of ATP and its catabolites in the sardine muscle along the duration of the purse seine fishing operations. Lines correspond to the fitted effect on mixed models with a significant time variable. Points correspond to observations.

To compare the rate of change with time among biochemical variables, the fitted slope (Table 2.3) was multiplied by 60 min (to represent an hourly change) and divided by the intercept (mean concentration after an hour spent in the net). Percent hourly change in concentration was lowest for chloride and haematological variables (<10%), intermediate for glucose, ATP and ADP (20-35%) and highest for cortisol and hypoxanthine (>75%). Table 2.3 also shows that the random standard deviation between trips ranged from less than half of the residual standard deviation between fish (haematological variables, cortisol, ATP and its catabolites) to approximately the same (glucose and chloride). Highly significant correlations (positive) of random trip effects were only found for haematocrit and heamoglobin and for ATP and ADP, while marginally significant correlations (negative) were also found for chloride with ATP and ADP.

2.5 – Discussion

The present study demonstrates that purse seine fisheries can provide an interesting case study for improving the understanding of fish stress reactions to fishing. Purse seining off northern Portugal induced significant stress responses in sardines, with mean values at the end of the fishing operation similar to peak values reported in the literature after acute stress. Biological and operational variables had a minor impact on stress responses, while a highly significant linear trend with time spent in the net was observed for most variables. The lack of a significant catch effect (fixed) and the absence of significant between-trip variation in the time effect (random) indicate that time spent in the net is a more important operational variable than fish abundance (total catch in the net). Also, the lack of a significant operation phase effect (hauling or transferring) suggests that the observed pulse in nutrient release during the end of hauling (Stratoudakis et al. 2003) is not associated with a sudden increase in stress variables, while the lack of correlation between the random trip effects of groups of biochemical variables indicates that distinct (unaccounted) factors such as wave height, luminosity or handling during operations (all increasing vulnerability to abrasion and physical damage) may influence the mean concentrations (but not the temporal evolution) of each variable. Variables like change in sea water temperature and pressure that have been shown experimentally to be important stressors in other fisheries (Olla et al. 1998; Davis et al. 2001; Davis 2002) are less likely to be important in this study, since vertical or between-trip differences are less pronounced. Finally, deck exposure (Davis et al. 2001; Davis 2002) is a stressor that is usually not relevant in purse seine fisheries, where slipping occurs through the lowering of the head rope of the net in the water (Lockwood et al. 1983; Mitchell et al. 2002; Stratoudakis and Marçalo 2002).

The most significant and consistent systematic effect in this study was the linear trend in most stress variables related to the time spent in the net. Although this trend might be considered to reflect the time delay in attaining peak values after the application of a stressor at the onset of fishing, available evidence suggests that the stressor operates throughout the hauling and fish transfer process and therefore that the fish stress reaction is proportional to the duration of the fishing operation. This is most evident in cortisol, for which plasma concentration typically rises to peak levels within a few minutes after the application of an acute stressor (Wendelaar Bonga 1997). If capture itself (net encircling the fish school) was the only fishing stressor, then a linear model should not provide an adequate fit to the cortisol data (Fig. 2.3b) since peak values should be reached early within the fishing operation. The duration of the fishing operation has already been shown experimentally to be an important fish stressor (Olla et al. 1997), while observations in the Portuguese and Australian purse seine fisheries for sardine (*Sardina pilchardus* and *Sardinops sagax* respectively) indicate that extreme behavioural reactions (gulping for air, jumps out of the water, disoriented or lethargic swimming) become progressively more frequent up to the transfer of the fish onboard (Stratoudakis and Marçalo 2002, Mitchell et al. 2002). We therefore suggest that the duration of the fishing operation is an important stressor in purse seine fishing due to the progressive water volume restriction, crowding and manipulation associated to the typical operation of this fishing method.

Cortisol showed the highest rate of increase during the sampling period among the biochemical variables tested, reaching a mean concentration of 15.8 µg/dL after two hours of confinement in the net. Glucose also increased with time (although at a lower rate), reaching 156.1 mg/dL after two hours and being consistently higher in fatter fish. These results agree with the known patterns of cortisolemia and hyperglycemia associated to acute fish stress (Barton and Iwana 1991; Wenderlaar Bonga 1997). For most teleosts, cortisol base levels are below 3-4 µg/dL with peak post-stress values up to 20 µg/dL or higher (Barton and Iwana 1991), while post-stress glucose peaks around 150-200 mg/dL or above are often reported (Barton and Iwana 1991; Olla et al. 1997; Davis et al. 2001; Manire et al. 2001). Experiments simulating the confinement of Atlantic mackerel (*Scomber scombrus*) during the drying-up of purse seine nets showed rapid post-stress increase in cortisol (>20 µg/dL) and significant concentration differences between moribund and healthy fish (Pawson and Lockwood 1980). However, a range of subsequent trials (including simulation of purse seine slipping) failed to demonstrate post-stress increase in cortisol and glucose or differences between moribund and healthy mackerel (Swift 1983). Although more recent experiments with other fish species have also failed to demonstrate a correlation between delayed discard mortality, cortisol and glucose (Olla et al. 1998; Davis et al. 2001; Davis 2002), it is worth noting that concentrations reported from the simulated slipping operation for mackerel (Swift 1983) are considerably lower to those observed for sardine in the field after two hours of confinement in the purse seine net.

The analysis of adenine nucleotides and related compounds in sardine muscle showed a reduction in ATP and ADP concentration and a significant increase in inosine with time spent in the net. These results are in agreement with the known degradation sequence of muscle ATP during catabolic reactions resulting from fish exhaustion and stress (Wood 1991). At the onset of fishing, mean ATP concentration was 4.07 µmol/g, reducing by more than 50% within two hours of confinement. These values confirm the suggestion of Mendes et al. (2001) that the low baseline ATP values (<1 µmol/g) reported for several teleost species immediately after trawl capture, were due to ATP breakdown resulting from stress reactions during fishing. More difficult to explain is the temporal stability (the linear increase observed was marginally non-significant) of inosine monophosphate (the main early product of ATP degradation in teleosts), although the highly significant increase of inosine confirms the standard degradation sequence.

Sardine haematocrit and haemoglobin significantly decreased with time spent in the net, with female fish having higher haemoglobin concentrations than males. The unexpected sex effect is likely due to an underlying relation of haematological parameters with fish weight (Nespolo and Rosenmann 2002), depicted by sex differences in weight distribution (female sardines were significantly heavier than males). Indeed, adding gonad-free weight as an explanatory variable in the LMMs, led to a significant (positive) relation with fish weight both for haematocrit and haemoglobin, turning the sex effect non-significant. The decrease of the two haematological parameters with time contradicts most existing literature, where haematocrit and haemoglobin usually rise during exposure to a stressor to increase oxygen transportation, facilitate acid/base regulation and reduce cardiac work costs (Swift 1982; Wood et al. 1983; Wells et al. 1986). In Atlantic mackerel, significantly higher haemoglobin concentration was found in stressed fish, with haemoconcentration being likely due to osmotic imbalance and dehydration resulting from injuries and skin loss (Swift 1982). In the only study demonstrating results similar to those found here for sardine, Bourke et al. (1987) demonstrated a considerable reduction in the haematocrit of skipjack tuna (*Katsuwonus pelamis)* in the first hours after capture, suggesting that extreme haemodilution (either due to increased drinking rate or, more likely, from inter- or intra-cellular tissue fluids) could be the major source of delayed tuna mortality.

Ionic concentrations in sardine plasma changed with time spent in the net, either increasing (sodium and chloride) or decreasing (potassium), although measurement limitations only permitted the modeling of chloride. The relation with time spent in the net was highly significant, although the rate of chloride change was much lower than that of cortisol, adenosine nucleotides and glucose and only similar to that of haematocrit and haemoglobin. Marked ionic and fluid volume disturbances are known to be caused by exhaustive exercise and stress (Wood et al. 1983; Burke et al. 1987; Wood 1991), but common patterns of stress response are difficult to establish as gas exchange, hydromineral control, acid/base balance and nitrogen metabolism are all closely linked through processes mainly located in the gills (Wendelaar Bonga 1997). Pawson and Lockwood (1980) suggested that the increase in sodium concentration observed in stressed mackerel was caused by dehydration due to skin damage and scale loss and was related to subsequent mortality. Burke et al. (1987) also reported osmotic dysfunctions for skipjack tuna in the first hours post-capture, with the transient increase in serum osmolarity being mainly due to increase in chloride, potassium and lactate. The simultaneous increase in blood osmolarity and volume led the authors to suggest that this could result from fish swallowing and absorbing seawater and salts from the gut faster than the ion-exchange mechanism in the gills can excrete the added salt load. Although the hypothesis was not supported by early weight loss in skipjack tuna, the above mechanism seems plausible for the ionic and haematological early dynamics observed in sardine.

Overall, the physiological stress variables of sardine monitored during commercial purse seine fishing off northern Portugal seem to fall into two categories. Cortisol, glucose and adenine nucleotide dynamics agree with reported adaptive stress reactions for many teleosts. Mean concentrations at the end of fishing provided clear evidence of an acute stressor resulting from the fishing practice over the few hours, starting from the identification, chase and encircling of the target school and progressing with the gradual reduction of water volume and increase in crowding and manipulation. On the other hand, maladaptive responses such as changes in plasma ionic concentrations and haematological variables provided results that are less in line with existing stress literature. These variables have been less frequently monitored and there are indications that they can be related to fish death resulting from post-capture stress (Pawson and Lockwood 1980; Wood et al. 1983; Bourke et al. 1987). The hypothesis that excessive drinking and assimilation of salts by sardines can cause extreme hydromineral disturbances during purse seining and affect subsequent survival needs to be further explored. The field results obtained in this study can be used in future laboratory experiments to guarantee that the simulated fishing impact is realistic. Laboratory experiments can then be used to explore the links between magnitude of stressor, stress reactions and subsequent fish survival and to evaluate behavioral impairments related to the stress caused by purse seine fishing (Olla et al. 1997; Ryer 2004; Davis 2005).

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CHAPTER 3

Sardine early survival, physical condition and stress after introduction to captivity

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3.1 – Abstract

The main factors that affect early survival, physical damage and stress reactions of sardine (*Sardina pilchardus*) after live capture and introduction to captivity were examined. A total of 2800 sardines were captured alive from commercial purse seiners in five trials off southern Portugal and monitored for 4 weeks in aquaculture tanks. Survival rates varied considerably between trials (from <20 to >80% after a month), with most deaths occurring in the first 5 days. Sardine early survival was affected by factors related to conditions at sea (catch composition, sea temperature and transportation density), during introduction to captivity (magnitude of thermal shock, land transportation duration and use of antibacterial treatment) and, possibly, their interaction. Physical damage was related to the probability of dying, with fish that died during the first week showing significantly higher scale loss and larger caudal fin erosion that those that were alive in the same period. For all stress variables measured (blood haematocrit, cortisol, glucose and ions in the plasma), the most extreme values were attained during introduction to captivity or in the first hours after. After 2 weeks in captivity, most variables had returned to levels close to those observed at the onset of purse-seine fishing, suggesting that maintenance conditions were adequate to permit a rapid recovery from fishing and transport stress.

Key words: *Sardina pilchardus*; live capture; mortality; stress reactions; scale loss; fin erosion

3.2 – Introduction

Sardines and anchovies form an important component of world fish catches, being the target of large purse seine fleets in the productive coastal waters of the California, Humboldt, Japan, Benguela and Canary current systems. Most knowledge on the life history and behaviour of these small clupeoids is obtained indirectly from the analysis of long series of biological data from catch samples (e.g. Parrish *et al.*, 1986; Silva *et al.,* 2006) or from survey observations (e.g. Freon & Misund, 1999). In a few cases, successful maintenance of such species in captivity has provided valuable information on feeding behaviour (van der Lingen, 1994; Garrido *et al.*, 2007), reproduction (Olmedo *et al.,* 1990; Matsuyama *et al.,* 1991; Shiraichi *et al.*, 1996) and bioenergetics (van der Lingen, 1995). Schooling small pelagic fishes, however, are sensitive to capture and handling (Stratoudakis *et al.*, 2003; Marçalo *et al.*, 2006), since escape reactions within the crowded nets can cause extensive scale loss and abrasions that lead to bacterial infections and high mortality rates (James *et al.*, 1988; Mitchell *et al.*, 2002). Despite the potential for biological insight provided by the maintenance of small pelagics in captivity, capture, handling and acclimation methods have been poorly reported and very few studies have attempted to identify factors that influence sardine and anchovy survival after introduction to controlled environments (James *et al.*, 1988; Olmedo *et al.*, 1990; Peleteiro *et al.*, 2004).

Fish maintenance under controlled conditions can also help to estimate the collateral mortality resulting from fishing (Broadhurst *et al.*, 2006), disentangle the anthropogenic and environmental factors that influence post-capture survival (Davis, 2002) and understand the physiological reactions that lead to fish death (Wood *et al.*, 1983; Kojima *et al.*, 2004; Portz *et al.*, 2006). Such studies may be based on post-fishing monitoring operations in the field (Main & Sangster, 1990; Misund & Beltestad, 2000; Kerstetter & Graves, 2005) or on laboratory experiments that simulate fishing practices (Olla *et al.*, 1998; Broadhurst *et al.*, 1999; Kojima *et al.*, 2004). In the case of purse seine fisheries for small pelagics, unaccounted fishing mortality can result from the deliberate release of excess/unwanted catch by the lowering of the head rope of the net at the end of the fishing operation (Lockwood *et al.*, 1983; Mitchell *et al.*, 2002; Stratoudakis & Marçalo, 2002). From the few field and laboratory observations available, it can be deduced that delayed mortality of small pelagics after purse seine fishing can be high and variable (Pawson & Lockwood, 1980; Lockwood *et al.*, 1983; Misund & Beltestad, 2000; Mitchell *et al.*, 2002), while the reduced capacity for osmoregulation resulting from stress reactions to capture, crowding and handling may be among the factors contributing to delayed death (Marçalo *et al.*, 2006). Operational constraints in the field and limited husbandry experience in captivity, however, have conditioned so far the study of collateral mortality in small pelagic fishes.

In the present study, observations from five mixed field/laboratory trials were used to describe appropriate capture and husbandry methods for maintaining sardine *Sardina Pilchardus* (Walbaum) in captivity, identify the main factors that affect its early survival and quantify the physical and physiological reactions in the first weeks after capture. Post-capture survival of sardine was monitored for a month after capture from a commercial purse seine, transferred to land by a small research vessel and installation to large aquaculture tanks. Variation in survival rates between trials was related to conditions at sea and husbandry practices in the laboratory. Biological sampling (including scale loss and fin damage measurements) was performed to all fish that died during the first two weeks in captivity to describe the post-capture evolution of physical condition. Similar information was obtained from random samples of live fish in the same period to test for differences in physical condition that might cause fish death. Finally, exploratory analysis of post-capture temporal trends in several stress variables (haematocrit, cortisol, glucose and ionic concentration in the plasma) is described in two trials and compared to the values reported at the beginning and end of purse seine fishing operations in the field (Marçalo *et al.*, 2006).

3.3 – Material and Methods

3.3.1 – Capture and transfer of live fish

Live sardines were obtained from commercial purse-seiners operating off western Algarve in southern Portugal during five trials of the RV "Puntazzo" (Table 3.1). All fishing operations occurred during daylight (net set around sunrise) with good weather conditions (wave height ~0.5 m), apart from trial 3 that took place at night and with slightly rougher weather (wave height ~1.5 m). Commercial fishing followed the typical purse-seine operation (Stratoudakis & Marçalo, 2002), although drying-up of the net was interrupted at its final stage to avoid additional damage due to the transfer of fish aboard. Approximately an hour after the setting of the net, the RV approached the auxiliary vessel of the purse-seiner and temporarily attached to it sideways to facilitate fish transfer. Crew members of the two vessels collected fish swimming within the netted area and transferred them from the net directly to the transport tanks of the RV using hand nets and 25 l buckets. In trials 4 and 5, a specifically designed vinyl scoop (15 l) with a removable 1.5 m wooden arm was used.

Small quantities of fish were transferred in each operation (5-20 fish per bucket or scoop) to minimize physical contact among fish and avoid damage through abrasion (James *et al.*, 1988). Fish transfer was generally fast (less than 20 minutes), and operations were facilitated in trials with good weather (minimal wind and wave action and weak currents) and when sardine was the dominant or only species in the catch. When the catch consisted of a mixture of pelagic species, attempts were made during netting to minimize the capture of other species, since sardine was usually found closer to the surface than chub mackerel *Scomber japonicus* (Houttuyn), jack and horse mackerel *Trachurus picturatus* (Bowdich) and *Trachurus trachurus* L. respectively and anchovy *Engraulis encrasicolus* L.. However, all specimens caught in the buckets or the scoops were transferred to the transport tanks to avoid unnecessary handling of sardines during transfer.

On the research vessel, fish were placed into 300 l circular tanks (trials 1 and 2) or 1000 l rectangular tanks (trials 3 to 5) previously filled with oxygenated seawater (80-120 % saturation). Fish stocking densities were visually adjusted during the transfer operations, aiming at less than 0.3 fish l^{-1} in each tank (apart from a circular tank in trial 2 that density was deliberately higher). Deviations from intended transport densities occasionally occurred, especially during trials with species mixture in the catch, but in most cases densities below 16 kg m^{-3} were achieved (Table 3.1).

TABLE 3.1 - Summary information of field operations (from fishing to arrival at the station) in the 5 trials used to provide sardine for maintenance in captivity. *N*, number of fishes collected; SST, sea surface temperature; NA, not analysed.

Trial	Year	Month	SST (ºC)	Depth (m)	Catch (tons)	Species	*N*	Density (Kg m^{-3})	*N* death	Sea transport (hours)	Land transport (hours)
1	2003	July	18.8	NA	1.5	Sardine	356	12.6	30	~2:00	~1:00
2	2004	May	15.3	NA	1.0	Sardine	492	15.6	12	~2:00	~1:00
3	2005	October	21.0	35	0.5	Sardine	599	16.3	39	< 1:00	~2:00
					6.0	Chub Mackerel	~10		0		
4	2006	March	14.9	73	1.4	Sardine	980	9.8	5	2:15	3:20
5	2006	July	19.2	35	0.3	Sardine	927	15.8	47	3:30	2:05
					0.6	Chub Mackerel	112		0		
					0.3	Jack Mackerel	484		0		

After the conclusion of transfer operations, tanks were covered with dark plastic nets and the aeration system was re-adjusted. During the return trip to the fishing port of Olhão (ranging from less than an hour to over three hours depending on distance from the fishing grounds), water in the tanks was renovated every hour to eliminate excretory products and maintain an approximately constant temperature. Oxygen levels were regularly inspected with a portable oxygen meter (Oximar, F. Ribeiro, Lda, Barcarena, Portugal and Sinergia, WTR, Venice, Italy) and the flow of the aeration pump was frequently adjusted to maintain approximately stable saturation levels in the tanks. Dead fish were rapidly removed and placed in individual plastic bags for subsequent biological analysis. Organic matter and loose scales (forming a layer of foam at the surface of the tanks) were regularly removed with a hand net. At arrival to the port, water level was lowered to approximately half the tank volume in order to facilitate offloading. Each tank was offloaded with the help of the vessel crane, placed on a truck and transported to the Aquaculture Research Station of IPIMAR in Olhão (5 min trip^{-1}).

Total duration of transport and offloading to the aquaculture tanks ranged from one to over three hours per trial, depending on the number of tanks transported (a single 1000 l tank could be transported at a time) and their species composition (more time was spent when sardine had to be sorted from other species during introduction to the storing tanks of the aquaculture station).

3.3.2 – Maintenance in captivity

At the Aquaculture Research Station, sardines were separated from other pelagic species and rapidly transferred to storing tanks with the help of hand nets and buckets. In each trial, most (or all) fish that survived the transport operations were transferred to a main storing tank for short-term monitoring and sampling (Table 3.2). In trials 1 to 3, the main storing tank (circular with 9 m^3 volume) was situated indoors, while in trials 4 and 5 an outdoors tank (rectangular with 10 m^3 volume subdivided into four equal compartments by removable lateral panels) was used. In both cases, an open-system water circulation and variable water flow (minimum of 1.8 m^3 hr^{-1}) was used, with aeration placed at the center of the tank (or tank compartment) to facilitate the circular movement of fish (shoaling usually resumed within the first day in captivity). Plastic nets were used to cover the tank to avoid accidental deaths from jumps. In the indoors tank (trials 1 to 3), a solution of 45 mg l^{-1} of oxytetracycline (OTC treatment) was added and water stopped for 5 hours during the first seven days in captivity to minimize bacterial infection (James *et al.*, 1988). This procedure was not followed in the outdoors storing tank (trials 4 and 5) and some smaller indoors tanks (circular with 1.5 m^3 volume) that were used in trials 2 and 3 for additional monitoring and blood sampling (Table 3.2). In all cases, fish were kept under a natural light regime and photoperiod. Although refrigeration or heating systems were activated during the summer or winter months respectively, daily and seasonal variations in water temperature were observed (Table 3.2).

TABLE 3.2 - Summary of maintenance conditions in captivity and observations during the short-term monitoring of sardine in the five trials. Mean values for temperature and oxygen saturation, ranges are presented in parentheses. *N*, number of sardines stocked; T, temperature; O$_2$, oxygen saturation in the water; ppm, parts per million; OTC, oxytetracycline.

Trial	Storing (Tank design)	*N*	Density (Kg m^{-3})	T Day 0 (ºC)	T (ºC)	O$_2$ (ppm)	OTC Treatment	Physical damage sampling	Blood sampling	Additional monitoring
1	Indoors (Circular)	326	1.5	23	21.3 (18.9-23.8)	6.7 (5.8-8.1)	Yes	No	No	No
2	Indoors (Circular)	303	1.3	17.8	21.6 (18.0-23.9)	6.4 (4.6-6.0)	Yes	No	Yes (3 days)	Density effect (3 days, 1.5 m^3 tanks)
3	Indoors (Circular)	182	1.4	18.9	19.2 (18.0-20.4)	6.8 (5.7-11.0)	Yes	Scales	Yes (15 days)	Treatment effect (15 days, 1.5 m^3 tanks)
4	Oudoors (Rectangular)	980	4.0	16.7	18.2 (14.5-20.2)	6.9 (5.6-7.8)	No	Scales, Tail	Yes (15 days) Not analysed	Land transport duration
5	Outdoors (Rectangular)	927	4.1	24.3	23.1 (22.5-26.0)	6.9 (5.6-7.8)	No	Scales, Tail	Yes (15 days) Not analysed	Land transport duration

After the conclusion of the short-term monitoring study period (two weeks for biological observations, one month for survival), fish were maintained in the main storing tank for long-term acclimation and subsequent experimental work (e.g. Peleteiro *et al.*, 2004; Garrido *et al.*, 2007). In most cases, sardines appeared adapted to captive conditions by the end of two weeks. Fish were fed with dried pellets (1mm diameter pellets, Aquasoja-Sorgal, S.A., Portugal) at a daily rate of 1-2 % biomass (wet mass). Feeding started with a few individuals initially leaving the shoal and exploring the pellets, usually within the second week in captivity. Regular provision of food with an automatic feeder was initiated when the whole school was feeding and quantities were adjusted according to fish mass gains. Fecal and uneaten food pellets were regularly siphoned from the bottom of the tank and good water quality was maintained through daily water purges of a quarter of the tank. Significant mass gains, gonad maturation and unaided spawning in the storing tanks along the captive period indicate that maintenance conditions in the aquaculture station of IPIMAR were adequate. Most fish survived in captivity for more than 1 year (the last fish from trial 1 were sacrificed 2.5 years after capture), with mortality rates close to zero.

3.3.3 – Short-term monitoring

After the conclusion of capture and transfer operations, a random sample of 50 individuals was collected, sacrificed with a toxic concentration of 2-phenoxyethanol, placed in individual plastic bags and frozen for biological analysis (Table 3.3).

TABLE 3.3 - Summary of biological data (mean) for a random sample of sardines at the day of capture in the five trials. Values in parentheses are the range. L_T, total length; M_T, total mass; F_C, condition factor; I_G, gonadossomatic index; NA, not analysed.

Trial	L_T (cm)	M_T (g)	F_C	I_G
1	17.6	43.7	8.0	NA
	(16.0-18.7)	(30.0 –54.2)	(6.5 – 9.0)	
2	17,4	38.0	7.2	4.2
	(16.0-21.0)	(27.0-64.0)	(6.3 – 9.1)	(0.2 – 9.4)
3	19.8	70.7	9.0	6.3
	(15.7-22.4)	(32.2-97.5)	(7.9 – 10.6)	(0.8 – 11.1)
4	17.7	40.2	7.1	5.1
	(14.5-20.2)	(21.5-58.1)	(5.7 - 7.9)	(1.7 – 10.2)
5	17.3	42.3	8.1	0.5
	(15.9-19.2)	(32.6-50.4)	(6.8 - 9.6)	(0.1 – 3.8)

In trials with mixed catches, total length (L_T) and mass data were also collected for the other pelagics to estimate final transport densities (kg m^{-3}). The main holding tank was monitored daily in the first month after capture, registering water temperature, fish behaviour and the number of deaths. Dead fish were removed from the tank, placed in individual plastic bags and frozen for subsequent biological analysis. Apart from the above monitoring operations that were performed in all trials following identical procedures, additional observations were made in each trial either aiming to address a specific issue or reflecting the accumulating experience from previous trials. As a result, the range of monitoring activities increased over the study period and identical procedures were only followed in trials 4 and 5 (Table 3.2).

In trial 1, no further monitoring activities were undertaken due to the lack of previous experience. In trial 2, the impact of transfer and stocking density was monitored in a short experiment (3 days). At sea, 180 and 108 sardines were placed in two circular transport tanks (corresponding to transport densities of 21.8 and 13.1 kg m^{-3} respectively) and the survivors of each tank were transferred at the aquaculture station to 1.5 m^3 circular tanks. Dead fish were retrieved twice daily from the tanks and used to calculate daily mortalities. Blood samples from five fish were taken 1, 6, 16, 29, 37 and 53 hours after capture to describe early post-capture evolution of stress variables for each stocking density and compare with observations during purse seine fishing (Marçalo *et al.,* 2006). In trial 3, 295 fish were distributed in five 1.5 m^3 at similar densities to the main storing tank but without antibacterial treatment. Two tanks were used for the monitoring of stress variables while the other three were used for monitoring survival over the first 15 days from capture. Blood sampling was performed on days 1, 7 and 15 (10 fish per tank). Dead fish were retrieved from each tank twice daily, placed in individual plastic bags and frozen for subsequent scale loss measurements and biological sampling (see section on Laboratory Analysis). In trials 4 and 5, only the main storing tank was used (outdoors, without antibacterial treatment) and the fish from each of the four rectangular transfer tanks were offloaded to a separate compartment of the storing tank. In both trials, all dead fish were daily removed and sampled (included scale loss and tail damage), while sampling of live fish (for biological parameters and blood sampling) took place in days 1, 2, 5, 10 and 15 (10 fish per compartment on day 1 and 5 fish per compartment on the following days). In trial 5, very high post-capture mortality obligated the termination of short-term monitoring on day 10.

3.3.4 – Laboratory analysis

All sampled fish (random sample at day of capture, all dead fish and fish randomly collected alive for blood sampling within the monitoring period) were measured (total length, L_T) and weighed (total and gutted mass, M_T and M_G respectively), while other standard biological parameters (sex, macroscopic maturity state, fat index and gonad mass) were also recorded (for details on sardine biological sampling see Silva *et al.*, 2006). Fish condition (condition factor, F_C) was estimated as the quotient of total mass over the cube of total length (x 1000) and the gonadosomatic index (I_G) as the quotient of gonad mass over gutted mass (x 100). Dead fish from trial 3 were used to develop a procedure for measuring scale loss by adapting the method of Main & Sangster (1990). Each flank of the fish was divided into eight regions that could be delimited visually, and scale loss in each region was evaluated on a level of zero to ten (corresponding to 0 to 100% scale loss). Mean level among regions and flanks was used to describe fish scale loss (expressed as percentage). Individual observations by region and flank (379 fish) showed no significant differences between flanks but some significant differences between regions (mainly between dorsal and caudal areas, results not shown). As a result, routine evaluation of scale loss in trials 4 and 5 (both for dead and alive fish) was based on the eight regions of a single flank. In trial 5, a fin condition factor was also calculated as the quotient of caudal fin length over total fish length (x 100) to monitor the degree of caudal fin erosion (Latremouille, 2003).

Blood sampling was performed on live fish individually collected from the tanks with a hand net and immediately placed to a small sedation tank with 2-phenoxyethanol (300 ppm). Once fish were fully sedated (1-2 min after introduction), blood was collected from the caudal vein (usually 0.6-1 ml) with a heparinized needle and fish were sacrificed by severing the spinal cord behind the head. Biological sampling was subsequently performed. Blood was immediately refrigerated and haematocrit values determined within 24 hours by placing the blood sample into capillary tubes and centrifuging for five minutes at 5,000 rpm in an autocrit centrifuge (Hettich EBA 20). The remaining blood samples were centrifuged at 2,500 rpm for 10 minutes and the plasma stored at -20ºC for subsequent analysis of cortisol, glucose and ions (Na^+, Cl^-

and K^+) at the Laboratory of the Lisbon Faculty of Veterinary Medicine. In trial 2, chloride and sodium concentrations occasionally exceeded the higher limits of calibration (205 mmol l^{-1}), but this was rectified in trial 3 by diluting further the original sample of plasma. Blood samples from trials 4 and 5 have not yet been analysed. More details on blood sampling and plasma processing can be found in Marçalo *et al.* (2006).

3.3.5 – Data analysis

The evolution of physical (scale loss and fin damage) and physiological (cortisol, glucose and chloride concentration in the plasma) variables with time in captivity and the possible impact of other variables were analysed, separately for each trial, with generalised linear and additive models (GLMs and GAMs). A GLM with an over-dispersed Poisson distribution and a log-link was used to model the effect of time in captivity on scale loss and compare between fish that were dead or alive at each sampling day. A GAM with a normal distribution and an identity link was used to model the early dynamics (first 48 hours in captivity) of stress variables in trial 2, also considering the effect of fish density in the tank (high/low factor level). A similar model was used to describe the early dynamics of fin erosion (first week in captivity) and to explore differences between live and dead fish. In all cases, a full model was initially fitted considering the interaction between the continuous variable (time) and the two levels factor and final model selection was based on partial t-tests for the GLMs and GCV scores for the GAMs (Wood, 2006). All models were fitted in R and model adequacy was visually inspected through residual plots.

3.4 – Results

In the five purse seine trials sampled, 3.7 tonnes of sardine were caught (ranging between 0.3-1.5 tonnes per trial) and 3394 fish (approximately 155 kg) were transferred alive to the transport tanks onboard the research vessel. There was little between-trial variation in sardine size (mean L_T = 180 mm; mean M_T = 47 g), except for sardines in trial 3 that were larger (Table 3.3). Mean condition and gonadosomatic index varied between trials depending on the main activity during the sampling period (spawning from autumn to late spring; feeding in the summer). In three trials sardine was the only species caught, while other pelagic species (mainly chub mackerel) constituted the bulk of the catch in the other two. Although the transfer of other pelagic species was avoided, approximately 220 specimens of chub mackerel (17-24 cm), 500 of jack mackerel (12-15 cm) and a few specimens of horse mackerel and anchovy were introduced to the transport tanks. Transport mortality was practically zero for horse, jack and chub mackerel, low for sardine and high for anchovy. In trials 4 and 5 that sea/land transport were monitored separately, zero and six (0.6 %) sardines died during the sea transport and five (0.5 %) and 47 (5.1 %) died during the land transport respectively. Fish density in the transport tanks was shown to have a pronounced effect on sardine mortality (experiment in trial 2). About 8.3 % of the fish transported at a high density (21.8 kg m^{-3}) died during the three hours of sea and land transport, while only 0.9 % of the fish that were transported at a lower density (13.1 kg m^{-3}) died in the same period. The effect of density was further demonstrated in the first two days in captivity, with cumulative mortality reaching 50 % in the high-density tank and 4.6 % in the low-density. In trials 4 and 5, a significant but less pronounced effect was shown to be associated to the duration of the transport on land. Despite the large difference in mean survival rates between these two trials (Fig. 3.1), significant within-trial differences in cumulative mortality were observed between fish that were removed first and last from the research vessel. A difference of two hours in exposure to the sun without water renewal in the harbour led to higher cumulative mortality over the study period (5 % difference) and a more lethargic behaviour (slower swimming and reduced capacity to school) in the first few days.

Of the 2718 sardines that were introduced to the main storing tank over the five trials, 1421 fish (52 %) were still alive a month after capture (excluding the 216 fish that were used for blood sampling in trials 4 and 5). Considerable between-trial variation in survival rates was observed (Fig. 3.1), but in all cases most deaths occurred within the first five days, followed by very low daily mortality rates in the remaining period.

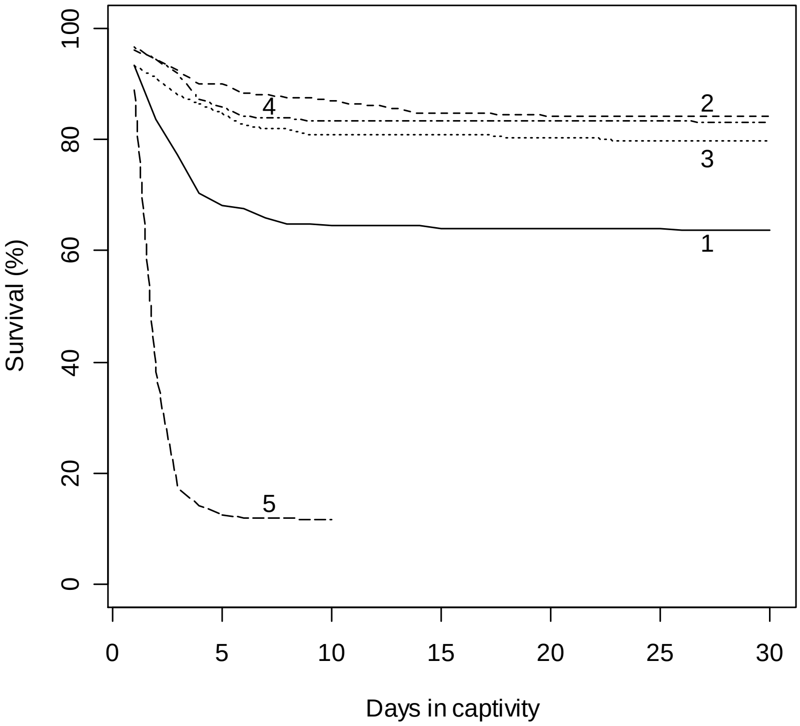

Figure 3.1 - Sardine cumulative survival in the main storing tank during the first month in captivity. A different line type is used for each trial (numbered 1 to 5). A description of sea and land operations for each trial are provided in Tables 1 and 2 respectively.

Cumulative survival rates during the first month in captivity were very high for trials 2, 3 and 4 (>80 %), reasonable for trial 1 (~65 %, despite the lack of previous experience) and very low for trial 5 (<20 %). Survival was highest in trials that occurred at lower sea temperatures (< 16 ^{o}C), when sardine was the only species in the catch and when the thermal shock from introduction to the storing tanks was minimal (< 2 ^{o}C higher than sea temperature). Under these conditions, the effect of storing tank (circular or rectangular) and OTC treatment (provided in trial 2 but not in trial 4) were negligible. Survival was lowest when sub-optimal conditions for the above factors (catch mixture, high sea temperature and large thermal shock) were combined with a lack of OTC treatment (trial 5). The importance of OTC treatment was also evident in the experiment performed in trial 3, where treatment in the main storing tank attenuated the adverse effects of catch mixture and high sea water temperature (80 % survival rate), while its absence led to an average survival of 23.6 % (range 14.6 – 33.7%). The importance of the thermal shock was also demonstrated in trial 1, where an increase of 5 ºC led to a 65 % survival rate under otherwise optimal conditions.

Physical damage was associated to the probability of dying after live capture and transport to captivity, with dead fish having a significantly higher level of scale loss (Table 3.4, Fig. 3.2) and caudal fin damage (Fig. 3.3) than fish sampled alive during the first week in captivity. The significantly lower level of scale loss in fish that died during transport than those that died in the first day in captivity (last panel; Fig. 3.2) suggests that scale loss through abrasion does not occur only during the operations at sea but can be prolonged until the introduction of fish in the storing tank. From then onwards, the consistent reduction of mean scale loss with time in captivity (both for dead and alive fish; Fig. 3.2) indicates that fish with larger losses die earlier.

TABLE 3.4 - Summary statistics of GLMs fitted to fish scale loss as a function of time in captivity (days) and state (dead or alive) for each sampled trial (in trial 3, scale loss was only evaluated in dead fish). The exponent of the intercept corresponds to the mean percentage of scale loss on the first day in captivity while the slope indicates the mean daily reduction (proportion) in scale loss. In the models for trials 4 and 5, significant differences in the intercept were found between dead and alive fish, while in trial 5 the difference in slope was also significant. S.E., standard error.

Trial	Explained deviance (%)	Fish state	Intercept (S.E.)	Slope (S.E.)
3	23.8	Dead	3.477 (0.072)	-0.335 (0.043)
4	54.2	Dead	4.144 (0.092)	-0.290 (0.030)
		Alive	2.430 (0.169)	-
5	16.7	Dead	3.059 (0.070)	-0.088 (0.040)
		Alive	2.531 (0.268)	-0.324 (0.119)

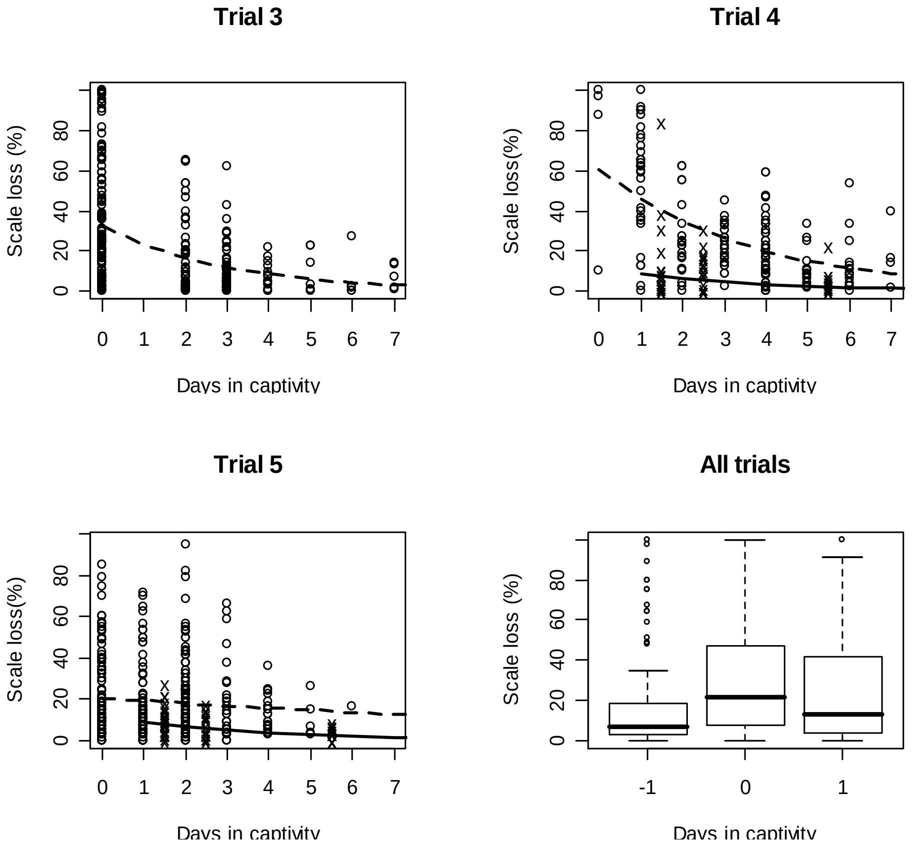

Figure 3.2 - Sardine scale loss (%) during the first week in captivity, separately for fish sampled dead (open circles) and alive (crosses) in trials 3 to 5 (first three panels). Sampling day for live fish is shifted by 0.5 days to improve clarity. In trial 3, scale loss was only evaluated on dead fish. Lines indicate fitted GLM (shown in Table 4; solid line for live fish, broken line for dead fish). Last panel shows distribution (boxplots) of scale loss for all sardines that died during transport (day -1) and the first two days in captivity (days 0 and 1) in the three sampled trials.

The significant between-trial differences in peak scale loss (mean value of approximately 60 % for fish that died during the first day in captivity in trial 4, 30 % in trial 3 and 20 % in trial 5; Table 3.4) are not related to ensuing survival (highest in trial 4 and lowest in trial 5), suggesting that fish condition during capture (higher in trials 3 and 5 than trial 4; Table 3.3) may also affect the extend of damage caused by abrasion. By comparison, maximal erosion of the caudal fin (attained in the 4th-5th day in captivity) seems to be related to cumulative survival, given the considerably higher levels of erosion observed in trial 5. The apparent increase of the fin factor during the second week in captivity (when mortality was very low or null) indicates that recovery of the caudal fin can be fast once maintenance conditions return to normality.

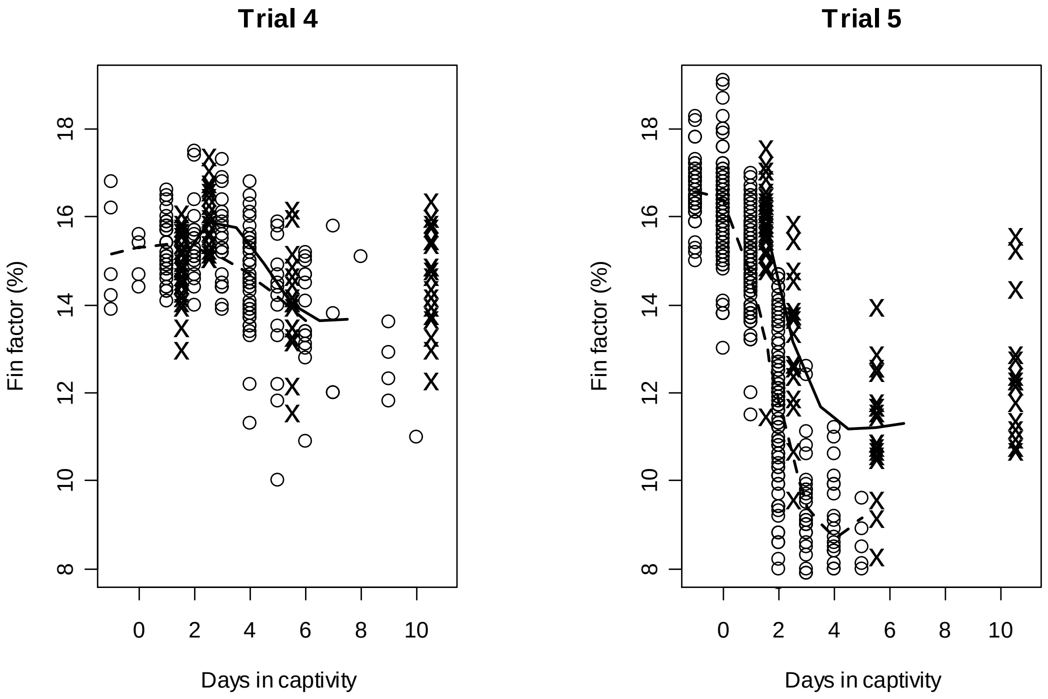

Figure 3.3 - Sardine fin damage factor (%) during the first 10 days in captivity, separately for fish sampled dead (open circles) and alive (crosses) in trials 4 and 5. Sampling day for live fish is shifted by 0.5 days to improve clarity. Lines indicate fitted GAM (solid for live fish, broken for dead fish).

The experiment in trial 2 (high/low transport and storing density) also allowed to monitor the post-capture evolution of stress variables over the first 48 hours in captivity and compare between tanks with more than an order of magnitude difference in cumulative mortality (Fig. 3.4). Cortisol concentration in the plasma was highest during introduction to the storing tanks and decreased thereafter, with no significant differences between fish in high and low density. Glucose concentration also decreased significantly with time, although in this case there was a significant time x density interaction, with higher initial mobilisation and more rapid consumption in fish at the high density tank. Chloride concentration in the plasma followed the same temporal evolution in the two tanks (i.e. non- significant time x density interaction), peaking within the first day in captivity and declining thereafter, being significantly lower in the low density tank throughout the study. In the case of chloride, the differences between densities are likely to be even higher than those reported, due to a number of early observations in high density that exceeded the calibration limit.

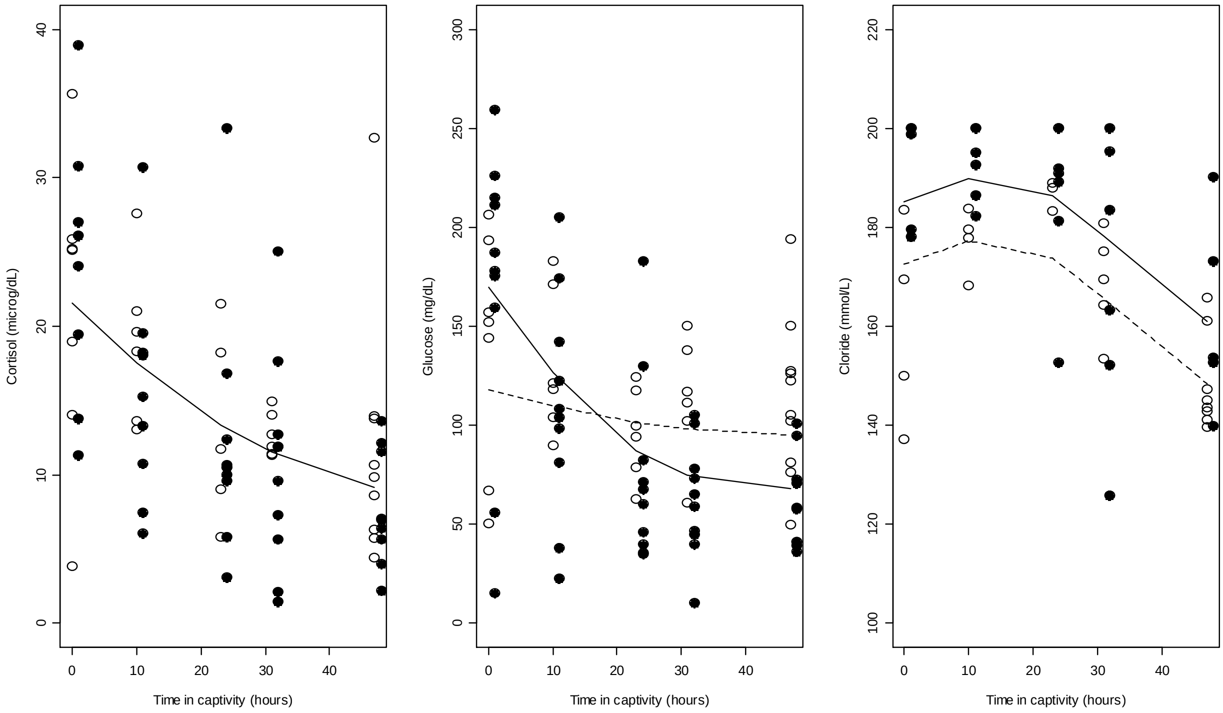

Figure 3.4 - Sardine early dynamics of cortisol, glucose and chloride in blood collected in the first two days in captivity (trial 2). Lines indicate fitted GAM with a normal distribution also considering the effect of fish density in the tank (solid line for high density, broken line for low factor level).

Complementary information on the post-capture evolution of stress variables was obtained in trial 3, where blood sampling was performed at weekly intervals (Fig. 3.5).

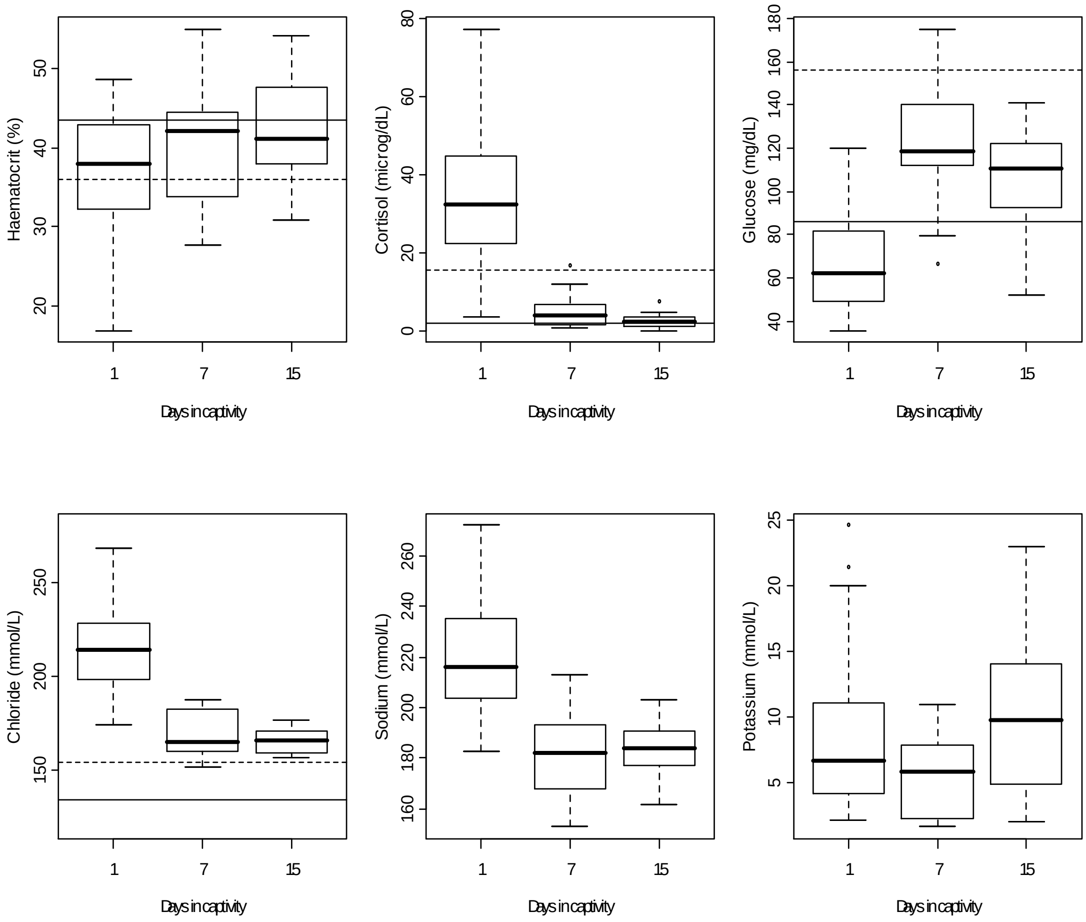

Figure 3.5 - Boxplots of haematocrit, glucose, cortisol, chloride, sodium and potassium distribution in the plasma of sardine blood collected weekly during the first two weeks in captivity (trial 3). Mean observations at the beginning (solid line) and at the end of purse seine fishing operations (broken line) are calculated from Table 3 in Marçalo *et al.* (2006).

Of the six variables measured, only potassium showed non-significant differences between the first and last sampling period (corresponding to the 15 day monitoring in captivity). Cortisol, chloride and sodium had significantly lower concentrations two weeks after capture, while glucose and haematocrit were significantly higher. Finally, the evolution of the gonadosomatic index during the same period, indicates the temporary recession of reproductive activity (tertiary stress response) resulting from the acute stress related to capture and transfer operations (Fig. 3.6).

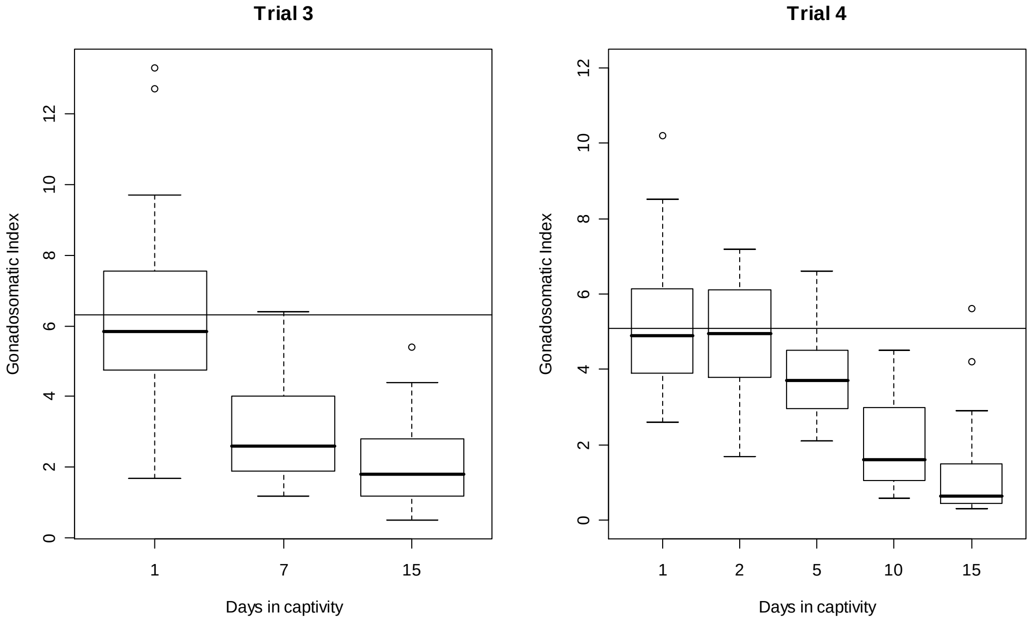

Figure 3.6 - Sardine gonadosomatic index (GSI) distribution (boxplots) along the first two weeks in captivity for two trials that took place during the early (October – trial 3) and late (March – trial 4) 2005/2006 spawning season. Lines indicate mean GSI for fish sampled during the day of capture.

3.5 – Discussion

The results of the present study demonstrate that sardine live capture from purse seine nets and transport to captivity can be very successful under optimal capture and husbandry conditions, with survival rates that can exceed 80 % in the first month. In all trials, fish resumed their schooling behaviour within one day in captivity and, within two weeks, mortality rates were reduced practically to zero and normal feeding behaviour restarted. This pattern is similar to the few existing studies on sardine introduction to captivity, where most deaths occurred in the first days and were related to the trauma of capture and transportation to laboratory conditions (James *et al.*, 1988; Olmedo *et al.*, 1990; Mitchell *et al.*, 2002). Survival rates during the transfer operations and the early post-capture period (especially the first four-five days in captivity), however, varied considerably in the present study, according to a suit of factors that can be related to catch, transport and maintenance conditions and, possibly, their interaction (Davis, 2002; Portz *et al.*, 2006). Despite the inability to control initial conditions at sea (due to the reliance on a commercial fishing operation) and the between-trial variation in some transport and maintenance conditions in captivity (either intentionally introduced to test the effect of a particular factor or imposed by operational limitations), the observed contrast in survival rates among the five trials allows some first conclusions on the relative importance of several factors that may influence sardine survival in captivity to be drawn.

The experiment in trial 2 showed that, similar to what is known for the impact of fish stocking density under culture conditions (Wexler *et al.*, 2003; Portz *et al.*, 2006), transport density can have a pronounced effect on sardine survival. Fish that were transported at a higher density (> 20 kg m^{-3}) showed a mortality rate in the following two days that was almost an order of magnitude higher than for fish that were otherwise treated identically but maintained at a lower density. The standard capture and transport procedures adopted in the five trials, however, seem to have a limited impact on subsequent survival, with duration of the land transport being the only other factor that measurably affected survival. The variation in cumulative survival observed among the 5 trials (Fig. 3.1) should thus be mainly attributed to the influence of variable initial conditions at sea and their interaction with the maintenance conditions in captivity. Among these factors, the most important seem to be water temperature during capture, magnitude of thermal shock during introduction to captivity and (to a lesser extend) other conditions at sea (catch composition, luminosity and weather). With good conditions at sea (i.e. relatively low water temperature, good weather and sardine the only species in the catch) and a limited thermal shock (<2 ºC increase) cumulative survival rates in trials 2 and 4 were similarly high, despite using an inshore circular tank with OTC treatment in the former and an outdoors rectangular tank without treatment in the latter. Under sub-optimal conditions at sea (trials 1, 3 and 5: higher water temperature in all and species mixture in the latter two), however, the magnitude of the thermal shock and the provision of an OTC treatment seem to have a major influence on subsequent survival. Although survival in these three trials was lower than for the two performed under better conditions at sea, the absence of OTC treatment associated to a high maintenance temperature and a large thermal shock led to an additional increase in mortality of more than 40 % during trial 5. These results are in line with existing evidence on the impact of ambient temperature and thermal shock to fish survival after capture stress (Olla *et al.*, 1998; Davis & Olla, 2001; Suuronen *et al.*, 2005) and indicate that OTC treatment is of paramount importance when acclimation is performed under sub-optimal thermal conditions (Portz *et al.*, 2006; A. Marçalo unpublished data).

The comparison of physical condition between fish that died or were alive during the sampling day indicates that scale loss and caudal fin erosion are significantly associated with the probability of dying within the first week in captivity. Despite the differences in cumulative survival rate between trials 3, 4 and 5, fish that died earlier in each trial had a higher rate of scale loss than fish that died later or than fish that were alive during the same day (Fig. 3.3). The decline of the mean level of scale loss in live fish with time probably indicates that even low levels of scale loss can eventually lead to death, while the very high initial rate of scale loss for fish that died in trial 4 (where cumulative mortality was lowest but also fish condition was lowest) possibly indicates that the degree of scale loss induced by injuries or abrasion may be related to fish condition. The significant reduction of scale loss with time after introduction to captivity also indicates that abrasions due to collisions with tank walls or fights with other fish in captivity are unlikely to be the reason for the observed erosion in the caudal fin of sardine and that bacterial infections related to immuno-deficiencies is the most likely cause (Latremouille, 2003). In both dead and alive fish, significant fin erosion was only observed after the second day in captivity and lowest fin factor values occurred several days later, although live fish showed signs of partial recovery during the second week. Overall, the significant differences in fin erosion between dead and alive fish (especially in trial 5) possibly reveals that tertiary stress responses related to immuno-deficiency may be linked to the probability of sardine survival.

All stress-related variables measured in the blood samples during trials 2 and 3 showed inverse temporal trends to those reported for sardine during purse seine fishing operations at sea (Marçalo *et al.,* 2006). The early post-capture observations in trial 2 indicate that the most extreme values for all stress variables were attained either during introduction to captivity or in the first hours after (Fig. 3.4), while the longer monitoring in trial 3 (Fig. 3.5) indicates that after two weeks in captivity most variables had returned to levels close to those observed at the onset of fishing (Marçalo *et al.,* 2006). In particular, the early maxima in cortisol (primary response to a stressor; Barton & Iwana, 1991), and the significant decrease thereafter indicate that maintenance conditions in captivity were adequate to permit recovery from the acute stress introduced by fishing and transport. Opposite temporal trends were observed for haematocrit (i.e. early post-capture minima and late maxima), confirming the findings of Marçalo *et al.* (2006) showing that, unlike most teleosts, acute stress leads to a reduction in sardine haematocrit, probably as a result of haemodilution. This is further supported by the temporal trend in the concentration of chloride and sodium in the plasma, which is compatible with the hypothesis of reduced capacity for hydromineral control after excessive influx of seawater during acute stress (Bourke *et al.,* 1987; Marçalo *et al.*, 2006).

The significant difference in chloride concentration between fish kept at low and high density during trial 2 (Fig. 3.4) provides some indication that secondary stress reactions related to hydromineral balance may be related with the probability of sardine survival after acute stress. Despite the non-significant differences in the primary stress reaction (cortisol), mean chloride concentration was consistently higher in the high density tank, where higher mortality of an order of magnitude was observed, in comparison with the lower density tank in the same period. A marginally significant difference was also observed in the pattern of initial glucose mobilisation in the two tanks, but the pattern of glucose reaction to stress is more complex and has been shown to be affected by other biological variables in sardine (Marçalo *et al.,* 2006).

Finally, long term (tertiary) stress responses in sardine were reflected by the significant decrease of the gonadosomatic index from the second day in captivity onwards (Fig. 3.6). Inhibition of reproduction is commonly observed in stressed fish, due to the suppression of hormones inducing spermiation and ovulation (Wendelaar Bonga, 1997; Wood, 1991) and the reallocation of energy resources to regain homeostasis. However, our observations indicate that stress-induced inhibition in sardine is relatively short-lived, since fish from the same trial naturally spawned two months later (A. Marçalo unpublished data).

3.6 – References

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CHAPTER 4

Fishing simulation experiments for predicting effects of purse seine capture on sardines (*Sardina pilchardus*)

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4.1 – Abstract

To study the impact of purse seine fishing on deliberately released sardines, two laboratory experiments were performed to explore the effect of net confinement for 10, 20, 40 and 60 minute at 18ºC and 23º C. A third experiment considered two levels of fish density at 20 and 40 minute confinement at 16^{o} C. Analysis of cortisol and haematocrit, demonstrated that stress immediately after simulated fishing was milder than in commercial fishing and did not correlate with observed delayed mortality. Scale loss was related with the probability of dying (mean values of 16.3 % and 2 % for dead fish and survivors respectively), while fin erosion was a long-term stress response affecting both dead and surviving fish. Time of confinement was an important stressor with survival rates significantly decreasing with increasing periods in the net, with temperature having an additional negative effect. Density effects were less conclusive, while there was some indication that survival correlates with biological condition (heavier fish more likely to survive). These results indicated that delayed mortality after purse seine slipping can be substantial, although death is not guaranteed and appropriately modified fishing operations and favourable environmental conditions may enhance the probability of sardine survival.

Keywords: delayed mortality; physical condition; purse seine fishing; *Sardina pilchardus*; stressors; stress reactions

4.2 – Introduction

The fate of released or slipped pelagic fish at the end of purse seine fishing operations is poorly studied. Although the problem has been considered for fisheries targeting mackerel (*Scomber scombrus*; Lockwood et al., 1983), herring (*Clupea harengus*; ICES, 2000) and sardines (*Sardinops sagax*, Mitchell et al., 2001; *Sardina pilchardus*, Stratoudakis and Marçalo, 2002), mortality rates are difficult to estimate because immediate mortality is generally low and the consequences to the surviving escapees are difficult to evaluate (Mitchell et al., 2001; Stratoudakis and Marçalo, 2002; Stratoudakis et al., 2003). The few observations on post-fishing fate of small pelagic fish carried out in the field have demonstrated that mortality can be high and variable, mostly caused by physical damage through scale loss resulting from abrasion during final stages of the operations prior to slipping (Pawson and Lockwood, 1980; Lockwood et al., 1983; Misund and Beltestad, 1995; Mitchell et al., 2001). However, these studies were of short duration and faced operational constraints (e.g. capture followed by transfer to net pens and transport to observation sites) that may have caused additional stress and physical damage to the fish.

Controlled laboratory experiments have been suggested as the most efficient and reliable way to understand principles controlling collateral fishing-related mortality (Davis and Olla, 2001; Davis, 2002), since delayed death can occur for extended periods after capture which would be difficult to assess in the wild. Injuries and changes in plasma physiology or behaviour can easily be observed in the laboratory for longer periods and correlated to delayed mortality resulting from capture (Davis, 2005). However, most studies of delayed fishing mortality so far have focused on the effects of towed fishing gears or recreational fisheries in cases that fishing operations lead to considerable changes in environmental conditions, such as air exposure or crossing of steep thermoclines (Sangster et al., 1996; Suuronen et al., 1996; Broadhurst et al., 2006; Suski et al., 2007; Gingerich et al., 2008), thus being easier to isolate and simulate the main stressor associated to fishing. In contrast, purse seining operations are more selective and target schooling fish not so easy to maintain and manipulate in captivity, added to the fact that the catch load never leaves the water, leading to the general perception that these fisheries are less detrimental to the fish.

Here, advantage was taken of knowledge of commercial purse seine fishing operations for sardine off Portugal (Stratoudakis and Marçalo, 2002; Stratoudakis et al., 2003; Marçalo et al., 2006) and recent advances in sardine live capture and maintenance in captivity (Marçalo et al., 2008*a*), to use, for the first time, fully acclimated, unstressed fish in controlled experiments where final stages of purse seining operations were simulated. Three experiments were performed to assess the role of operational (holding time and density) and environmental (water temperature) factors on the survival of slipped (released) sardines and short and long term physiological and physical reactions described. Fish were observed for up to 10 days after simulated fishing, while a sub-sample of fish was sacrificed immediately before and after the net confinement to evaluate the level of physiological stress reaction in comparison to field observations from the Portuguese purse seine commercial fishery for sardine (Marçalo et al., 2006).

4.3 – Material and Methods

4.3.1 - Collection of fish for experiments

Live sardines were captured by commercial purse-seiners off western Algarve in southern Portugal following methods described in Marçalo et al. (2008*a*). Sardines used in experiment 1 and 2 were captured in February 2007 during night and under relatively rough sea conditions (wave height ~1.5 m - 2m), while sardines for experiment 3 were captured in April 2007 during daylight (net set around sunrise) and under good sea conditions (wave height ~ 0 - 0.5 m). Whole stock survival rates after two weeks in captivity were of ~ 40 % and > 80% for fish captured in February and April respectively. Fish were allowed to acclimate for a period of at least two weeks prior to any experiment, based on previous evidence that capture and transfer related mortality practically ceases within a week and physiological equilibrium after fishing and transport stress is re-established by the second week (Marçalo et al., 2008*a*).

4.3.2 – Experimental set up

Fishing simulation experiments were performed at the Aquaculture Research Station of IPIMAR in Olhão between February and June 2007 using one rectangular 10 m^3 tank subdivided into four equal compartments by removable lateral framed panels (see Fig. 4.1A; Marçalo et al., 2008*a*). All compartments were supplied with seawater (at ambient temperature) with an open-system water circulation and variable water flow, aerated using air-stone diffusers placed at the centre of each tank compartment. Since a conventional purse seining set could not be performed in captivity, a netting device was constructed with the same mesh (18 mm) used by commercial purse seiners operating off the Portuguese coast was designed (Fig. 4.1B) to allow the simulation of the commercial dry-up time (Marçalo et al., 2006). The fishing net device was made with enough height and width to allow the encirclement of the fish shoal present in each compartment. Two plastic tubes holding the net allowed the whole device to be placed in the back end of each compartment. Each tube was held by a technician, responsible for sliding the tube against the lateral compartment wall and slowly pushing the whole netting device and consequently the shoal to the front. Next, the bottom half of the net, which was attached with loose plastic binders, was lifted using four ropes attached to the bottom and pushed from the outside, allowing the net to be lifted and the fish confined in a purse.

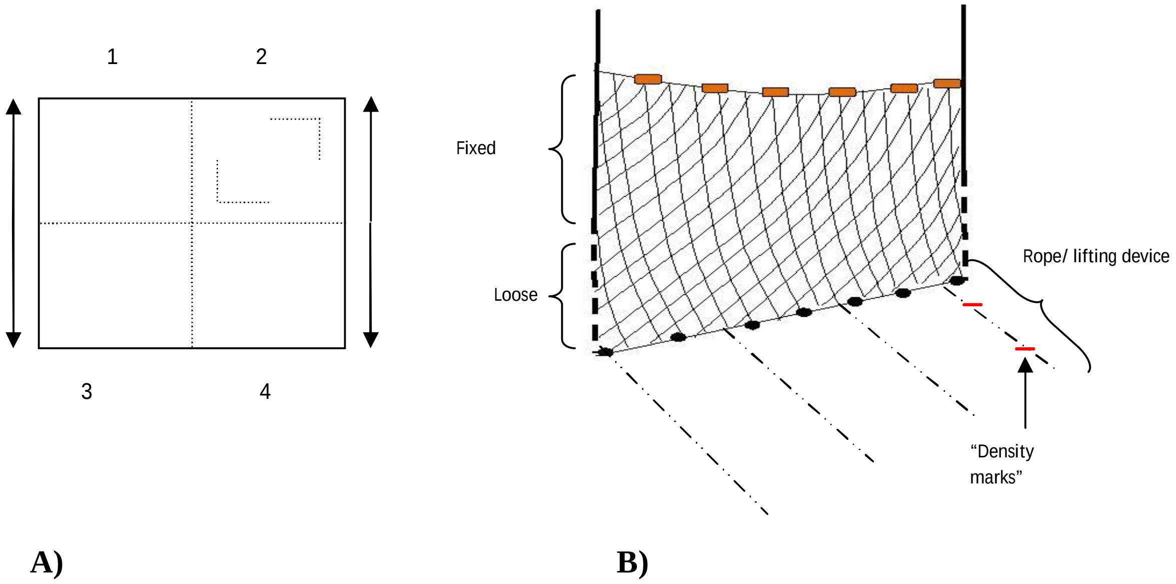

Figure 4.1 - Diagram showing (A) the 10m^3 subdvided tank; arrows represent direction of the operations.(B) Netting device

Fish for experiment 1 were immediately introduced into each compartment of the experimental tank after arrival at the station, and the remaining fish held at a stocking tank located nearby. For experiment 2, a new batch of fish were transferred from the stocking tank, subdivided equally per compartment and allowed to acclimate for two weeks. Reduced availability of fish led to a smaller number of fish per compartment in this experiment (Table 4.1). The absence of controlled water temperature in the experimental tank led to small daily variations between experiment 1 and 2 (± 2ºC). Sardines used in experiment 3 and obtained from the second fishing trip (April 2007) were also immediately introduced into the experimental tank, but the acclimation period was longer (1.5 months), thus allowing for the seasonal increase in water temperature (more than 5ºC) to provide sufficient contrast to test the respective environmental effect.

Counting mistakes on the day of fish arrival from the sea (experiment 1 and 3), or sardine jumping from one compartment to another caused some deviations from the initial number of individuals per compartment for all experiments and treatments (Table 4.1). Also, for all treatments during each experiment, some sardines escaped under the simulation device (see Table 4.1). The following sections refer in turn to each of the three experiments performed. For readability the factors tested are referred to as "time", "density" and "temperature".

4.3.3 – *Experiment 1: Effect of simulated fishing duration at low temperature*

Sardines were confined in a net for 10, 20, 40 and 60 min based on final net dry-up times (where the smaller volume of the purse is attained to allow fish density sufficiently high to start the transport onboard) observed during purse seine commercial fishing operations (Marçalo et al., 2006). Each compartment was treated individually, and the net device operated so that fish were herded and held inside the purse for the respective time, after which they were slipped back into the tank (allowed to swim freely over the "head rope" of the simulation device). Fish confined within the net were calculated indirectly from the total number of fish inside the tank compartment and the number of fish that escaped the net (approximate visual estimate). At the day of the experiment (day 0), prior to netting (pre-stress) and prior to slipping (post-stress), 6 fish were randomly sampled for blood (caught by a hand net while swimming freely – pre-stress; caught by a hand net from inside the purse – post-stress) and sacrificed for further biological analysis (more details in Marçalo et al., 2008*a*). Fish mortalities were recorded daily for 10 days. Average temperature was 18 ºC.

4.3.4 – *Experiment 2: Effect of simulated fishing duration and density.*

Fish were confined in a net for 20 and 40 min. Fish densities were visually established according to prior observed commercial conditions. Density effects were tested by bunting (bunt corresponds to the bagging part of a fishing net) more (allowing minimum swimming space available) or less (allowing some swimming space) the purse, creating a smaller (high density) or a higher (low density) water volume respectively. Fish mortalities were recorded daily for 9 days. Fish sampling and net device operation procedures followed the methods described in experiment 1.

4.3.5 – *Experiment 3: Effect of simulated fishing duration at high temperature.*

All the methodology was performed according to experiment 1, although only 5 fish were randomly sampled for pre and post-stress analysis and acclimation was longer to obtain the environmental effect (average temperature 23 ºC).

4.3.6 – Laboratory sampling

Fish collected for blood sampling were retrieved from each compartment with a hand net and sedated with 2-phenoxyethanol (300 ppm). Once fish were fully sedated (1-2 min after introduction into the small “sedation” container), blood was collected from the caudal vein (usually 0.6-1 ml) with a heparinized needle, and fish were immediately sacrificed by severing the spinal cord behind the head. Blood was immediately refrigerated and haematocrit values determined within 24 hours by initially homogenizing at room temperature and placing the blood sample into capillary tubes and centrifuging for five minutes at 5,000 rpm in an autocrit centrifuge (Hettich EBA 20). Although blood cells are known to become anoxic and swell if blood is not processed immediately, in the absence of the best sampling techniques (e.g. equipment availability and sampling time), refrigeration of blood samples for up to 24 hrs is a common routine in veterinary laboratories and shown to inhibit other mechanisms (e.g. glucose consumption by blood cells; Marçalo et al. 2006). After haematocrit analysis, the remaining blood samples were centrifuged at 2,500 rpm for 10 minutes and the plasma stored at -20ºC for subsequent analysis of cortisol at the Laboratory of the Lisbon Faculty of Veterinary Medicine following procedures described in Marçalo et al. (2006).

Biological parameters, scale loss and fin erosion were recorded for all fish sampled alive for blood analysis and for all dead fish, while at the end of the observation period (day 9 or 10), all survivors were collected from each compartment with a hand net, sedated and checked only for weight, total length and physical parameters (scale loss and fin erosion). For biological sampling, fish were measured (total length, L_T) and weighed (total and gutted mass, M_T and M_G respectively), while other standard biological parameters (sex, macroscopic maturity state, fat index and gonad mass) were also recorded (for details on sardine biological sampling see Silva *et al.*, 2006). Fish condition was estimated by the condition factor ($F_C = 1000\ M_T / L_T^3$). Scale loss assessment was performed by adapting the method of Main and Sangster (1990), while evaluation of the degree of caudal fin erosion followed the fin condition factor presented in Latremouille (2003), both described in Marçalo et al. (2008*a*).

4.3.7 – Statistical analysis

Exploratory data analysis for the fish sampled at day 0 (comparisons pre- and post- fishing and effect of fishing on cortisol and heamatocrit) revealed some abnormally high cortisol values. Due to small sample sizes, removing these data points by considering them as outliers would be inadequate, so a log(x+1) transformation was used for stabilizing variances. To evaluate the overall effect of fishing on cortisol, hematocrit, scale loss and fin erosion the data from the three experiments were pooled, and differences in values before and after fishing were tested using an unequal variance t-test (Ruxton, 2006). One sided tests were used as the direction of change was expected a priori (Marçalo et al., 2006). To evaluate the effect of fishing time and temperature on cortisol and haematocrit we considered the post-stress samples from experiments 1 and 3. An ANOVA table was built on a linear model of each relevant variable as a function of fishing time and temperature (both as factor variables) and the interaction term. A similar analysis was conducted on the data from the 2nd experiment to evaluate the effect of density and fishing time on cortisol and haematocrit. Terms not statistically significant were removed and corresponding simpler models analyzed.

For the analysis of survival data we conducted both an exploratory analysis via Kaplan-Meyer (KM) survival estimators (Kaplan and Meier, 1958) and implemented a Cox Proportional Hazards regression model (CPH; Cox, 1972). The KM survival estimators allow to compare visually survival over time as a function of the different treatments, and are presented both as a function of experiment and of fishing time (the latter in order to visualize the temperature effect). To avoid any parametric assumptions, a non-parametric bootstrap procedure (see Efron and Tibshirani, 1993 for details about the bootstrap) was implemented, re-sampling fish within experiments and treatments, hence obtaining 95% confidence intervals for the Kaplan-Meyer survival estimates. The interpretation of the CPH model is analogous to a regression model, but in this case the parameters relate to a higher or lower hazard associated with the corresponding variable. We considered the model with time of death (a censured time for fish alive at the end of the experiment) as the response variable and fishing time, density, temperature and fish weight as explanatory variables. Schonfeld, Martingale and score residual analysis (Therneau and Grambsch, 2000) indicated no evidence of failure of the Cox model assumption of proportional hazards. Fin erosion and scale loss were pooled across experiments and compared for all fish that died during the experiment and those which were alive at the end of the observation period, using similar t-tests as described above. Although we tried to model these physical parameters as a function of the different treatments there were no consistent results, and these are not presented. In general, and for simplicity, we refer to statistical significant results for tests with P-values smaller than 0.05. Statistical analyses were performed using the open source software R (version 2.7.0; R Core Development Team 2008) and the Cox model was implemented using the dedicated R library “survival”.

4.4 – Results

A total of 969 (365 in experiment 1; 247 in experiment 2; 357 in experiment 3) sardines were used in the three experiments. Uncontrolled fish escapes were observed during the fishing operations, ranging (per tank compartment) from 13-24 %, 8-24 % and 21-32 % respectively for experiments 1 to 3. There was no variation in sardine mean length across experiments (mean length= 18.7 cm) and mean weight for the first two experiments (mean weight= 54.0 g). However, a considerable percentage (~30%) of fish from experiments 1 and 2 (first wild stock) had an uncommonly high condition factor for the season (spawning season), while sardines used in experiment 3 (second wild stock), had a slightly lower and less variable weight, leading to a lower mean condition factor for the individuals used in the 3rd experiment (Table 4.1).

Table 4.1 - Summary information of field and captivity operations to provide sardines used in the 3 experiments. Biological data presented as mean (values in parentheses indicate the standard deviation). L_T, total length (cm); M_T total mass (grams); F_C, condition factor; T, temperature; LD, Low density; HD, High density; Treat, treatment; Accl, acclimation; Obs, observation.

Experiment	**Date of capture**	**Date of experiment**	**Accl (Days)**	**Obs (Days)**	**T (ºC)**	**Factor**	**Treat**	**n total**	**n confined**	L_T	M_T	F_C
1	February 2, 2007	February 26, 2007	17	10	17.7	**Time; Low T**	1-10 min	104	91	18.7 (1.5)	54.4 (19.3)	7.9 (1.4)
							2-20 min	85	65			
							3-40 min	81	62			
							4-60 min	94	73			
2	February 2, 2007	March 14, 2007	13	9	15.6	**Time; Density**	1-20 min/ LD	63	55	18.7 (1.8)	53.9 (21.4)	7.8 (1.6)
							2-20 min/ HD	54	49			
							3-40 min/ LD	67	61			
							4-40 min/ HD	63	48			
3	April 19, 2007	June 5, 2007	47	10	23.0	**Time; High T**	1-10 min	70	55	18.7 (0.9)	46.3 (7.2)	7.0 (0.7)
							2-20 min	108	73			
							3-40 min	89	64			
							4-60 min	90	68			

Boxplots of the two physiological parameters (log transformed cortisol, and haematocrit) and the two physical parameters (scale loss and fin factor) pooled across experiments and treatments are shown in Figure 4.2 to show the impact of simulated fishing, while table 4.2 presents the ANOVA analysis used to test the effect of operational (time and density) and environmental (temperature) factors in the patterns of physiology parameters.

Physiology patterns:

Both physiological parameters (Figure 4.2) showed significant changes immediately after simulated fishing and the direction of change was in agreement with trends over time observed in the field (Marçalo et al., 2006). A statistically significant ($P < 0.001$) increase in cortisol was observed after simulated fishing, log transformed data in the figure corresponding to a real median pre-stress value of 1.1 µg/dL (n = 68) and a median post-stress value of 7.1 µg/dL (n = 68). However, while median concentrations at pre-fishing were only slightly lower than the mean value observed at the onset of fishing at sea (2 µg/dL, Marçalo et al., 2006), median simulated post-fishing concentrations were about half the mean value observed during the final stages of commercial fishing (15.9 µg/dL, Marçalo et al., 2006). Reliable haematocrit data were not obtained for 3 of the 136 fish. Simulated fishing had a milder effect on haematocrit, which nonetheless showed a statistically significant ($P < 0.01$) decrease from a pre-stress mean value of 45.1 % (n = 65) to a post-stress value 40.6 % (n = 68). As for cortisol, the haematocrit levels observed during final fishing operations at sea (mean value of 35.9 %, Marçalo et al., 2006) were not attained, indicating that simulated fishing was sufficient to elicit a significant stress reaction, but its impact was possibly less acute than that of a commercial fishing operation.

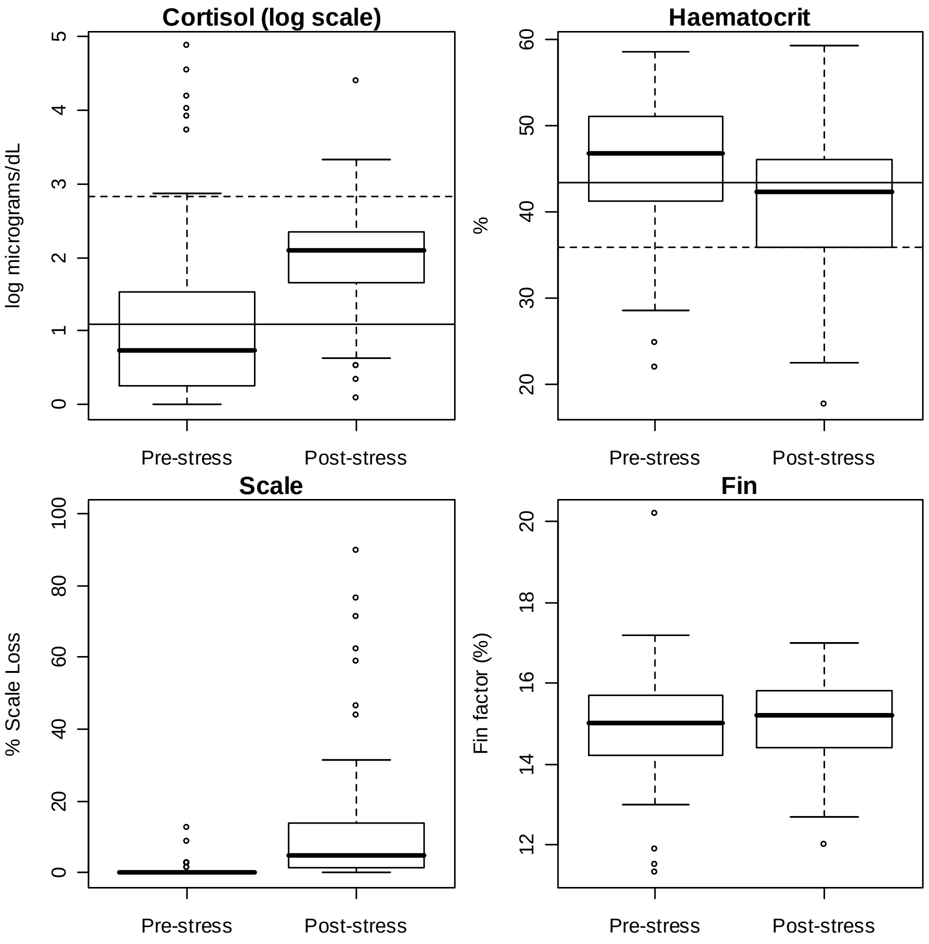

Figure 4.2 - Boxplots of physiological parameters (cortisol and haematocrit - top row) and physical parameters (scale loss and fin factor – bottom row) as a function of pre or post stress condition, pooled across experiments and tanks (treatments). The box stretches from the 25th percentile to the 75th percentile. The line across the box represents the median. The ends of the vertical line indicate the minimum and maximum data values. Individual points are considered outliers. Mean observations at the beginning (solid line) and at the end of purse-seine fishing operations (dashed line) for the physiological parameters are calculated from Marçalo et al. (2006).

Stressor effects on physiology:

1. *Simulated fishing duration and temperature*

The ANOVA analysis (Table 4.2) used to test the effect of operational (time and density) and environmental (temperature) factors showed that for the data of simulated fishing duration at low temperature (experiment 1) and simulated fishing duration at high temperature (experiment 3) combined, simulated fishing duration significantly increased cortisol (*F* = 9.75, *P* < 0.001) but had no effect on haematocrit (*F* = 2.01, *P* = 0.13), while temperature had no effect on either of the physiological parameters (*F* = 1.40, *P* = 0.24 for cortisol; *F* = 1.66, *P* = 0.20 for haematocrit).

2. Simulated fishing duration and density

The ANOVA also revealed that when compared to low density, high density of sardines in the net led to a significantly higher haematocrit (*F* = 8.54, *P* = 0.008) and no apparent effect on cortisol (*F* = 2.31, *P*= 0.14), while simulated fishing duration had no significant effect on any of the physiological parameters (*F* = 0.08, *P* = 0.77 for cortisol; *F* = 0.05, *P* = 0.83 for haematocrit).

Table 4.2 - ANOVA values for operational and environmental variables affecting physiological parameters at pre- fishing and post- fishing conditions. Mean squares (MS), F-statistic value (F-value), degrees of freedom (df) and P-value.

			MS	**F-value**	**df**	**P-value**
Cortisol	1 & 3	Interaction	0.230	0.865	3,36	0.468
		Temperature	0.368	1.400	1,39	0.244
		Time	7.764	9.751	3,40	<0.0001
	2	Interaction	1.302	2.742	1,20	0.113
		Density	1.141	2.314	1,22	0.142
		Time	0.046	0.084	1,22	0.774
Haematocrit	1 & 3	Interaction	44.381	0.766	3,36	0.521
		Temperature	101.426	1.658	1,42	0.205
		Time	116.470	2.007	3,40	0.128
	2	Interaction	1.307	0.019	1,20	0.892
		Density	533.927	8.540	1,22	0.008
		Time	3.227	0.049	1,21	0.826

Physical injury:

In figure 4.2 it is also shown that fishing led to a statistically significant increase in scale loss (*P* <0.001) from a mean value of 1% scale loss for pre-stress and mean value of 5% scale loss for post-stress. No significant differences were observed for the fin factor (mean value of 15.0 % for both pre and post stress fish; *P* = 0.99). Unfortunately, as for the physiological parameters discussed before, no similar study was performed so far in order to account for the physical impact during live commercial fishing, thus no reference values are available to established any comparison within physical injury results attained in captivity and live capture. Looking at the physical parameters pooled across all dead sardines and survivors at the end of all the experiments (Figure 4.3), dead fish show a significantly higher scale loss than survivors (*P* ~ 0). Survivors have minimum scale loss (mean value of 2%), which is close to the scale loss observed for fish sampled prior to simulated fishing (mean value of 1 %, although with some extreme cases of up to 13% loss). The observed differences in scale loss (mean value of 13 % for fish sampled post stress, 27 % for dead fish and 2 % for survivors) further support that scale loss is associated with the probability of dying after capture, in agreement with previous observations presented in Marçalo et al. (2008*a*). Effects on fin factor were not significant between dead fish and survivors (mean value of 13.6 % for dead fish and 13.7 % for survivors; *P* = 0.68). However, fin factors for dead fish and survivors are smaller than values attained at day 0 (mean value of 15% for both pre and post sampled fish), indicating a delayed fishing effect. Gradual post capture fin erosion for dead fish and fish sampled alive within two weeks in captivity was also observed in Marçalo et al. (2008*a*), mainly from day 1 onwards.

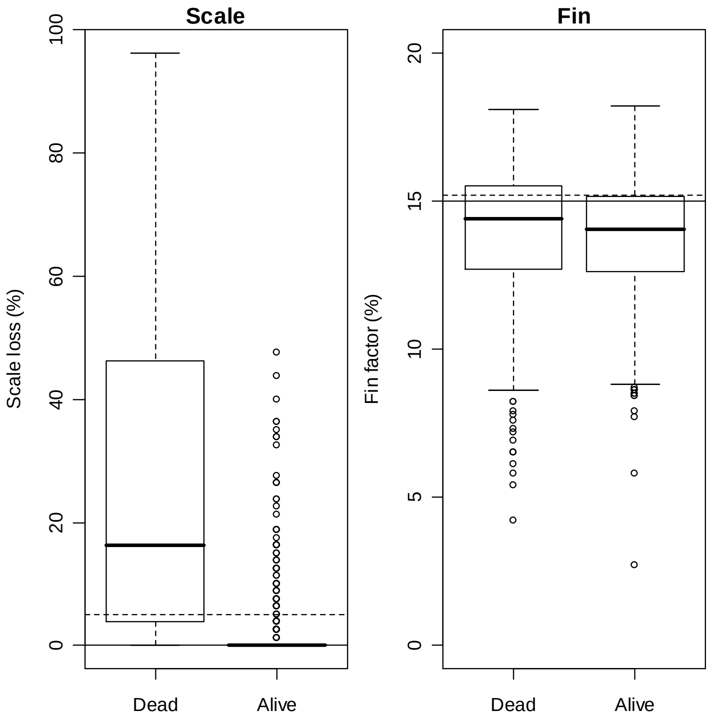

Figure 4.3 - Boxplots of physical parameters of dead fish and alive fish (survivors at termination day), pooled across experiments and tanks (treatments). The box stretches from the 25th percentile to the 75th percentile. The line across the box represents the median. The ends of the vertical line indicate the minimum and maximum data values. Individual points are considered outliers. Also shown are median observations for pre stress (solid line) and immediate post stress (dashed line).

Survival:

Sardine survival in each experiment as a function of observation days and separately for each treatment is shown in Figure 4.4. For all experiments and treatments most mortality occurred within the first 5 days, followed by low daily mortality rates in the remaining period, confirming prior observations after commercial fishing (Marçalo et al., 2008*a*). Also, in all experiments, simulated fishing duration had a significant impact on sardine survival rate, with >70% survival at the end of the experiment in all treatments with short simulated fishing duration (10 or 20 minutes in the net) and significantly lower survival for longer simulated fishing duration (40 or 60 minutes when survival could go down to 30% after 10 days). Simulated fishing duration at low temperature (experiments 1) and simulated fishing duration at high temperature (experiment 3) indicate that there was no statistically significant difference in survival between 10 and 20 min durations, whereas mortality for 60 min was higher than for 40 min, albeit statistically non-significant for the sample sizes and levels of variation observed. Similarly, simulated fishing duration and density (experiment 2) demonstrated that density in the net had no effect at shorter duration (20 min), while mortality was higher for the denser bunting at longer duration, although once more the difference was non-significant.

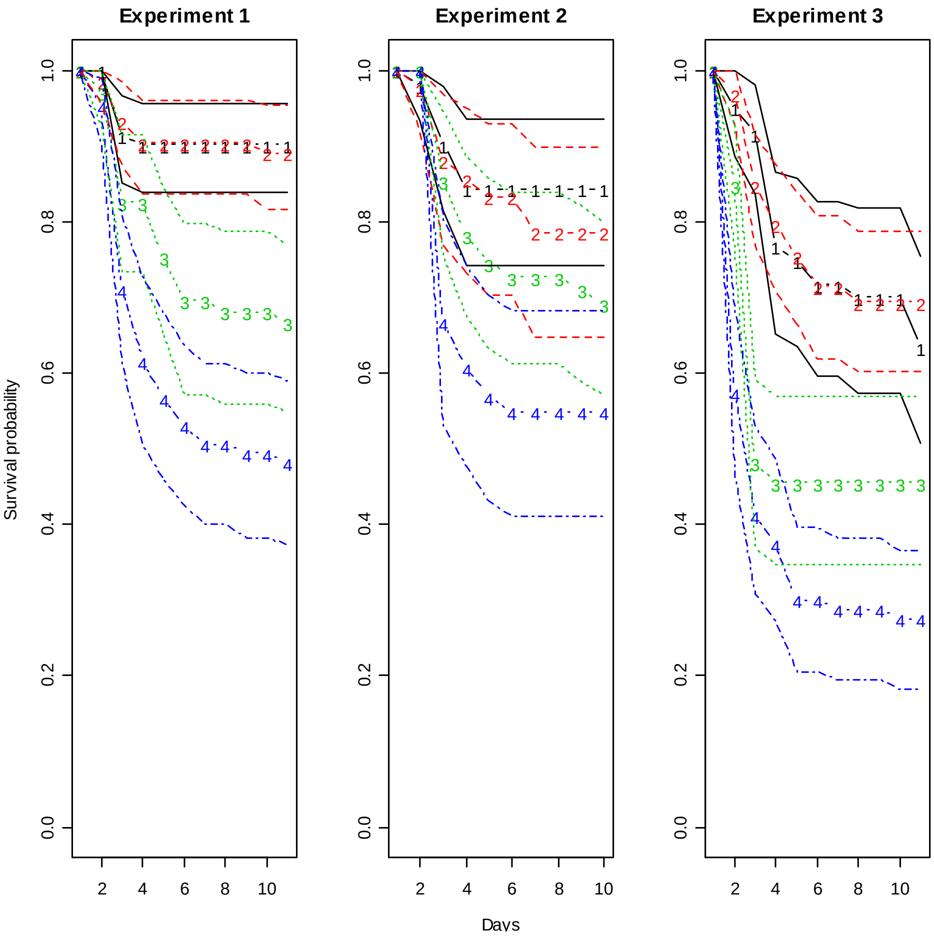

Figure 4.4 - Treatment survival (Kaplan-Mayer survival estimators) as a function of observation day for each experiment. Solid line for 1 and dashed lines for 2-4 represent 95% confidence intervals for the survival curves obtained by a nonparametric bootstrap. Numbers refer to treatments in each experiment (more detail in table 4.1)

Finally, the survival curves plotted separately for each simulated fishing duration across experiments (Figure 4.5) show a significant temperature effect on survival that is consistent across durations; survival at 23^{o}C is approximately 20% lower than at lower temperatures (16 or 18^{o}C) at any given simulated fishing duration treatment level.

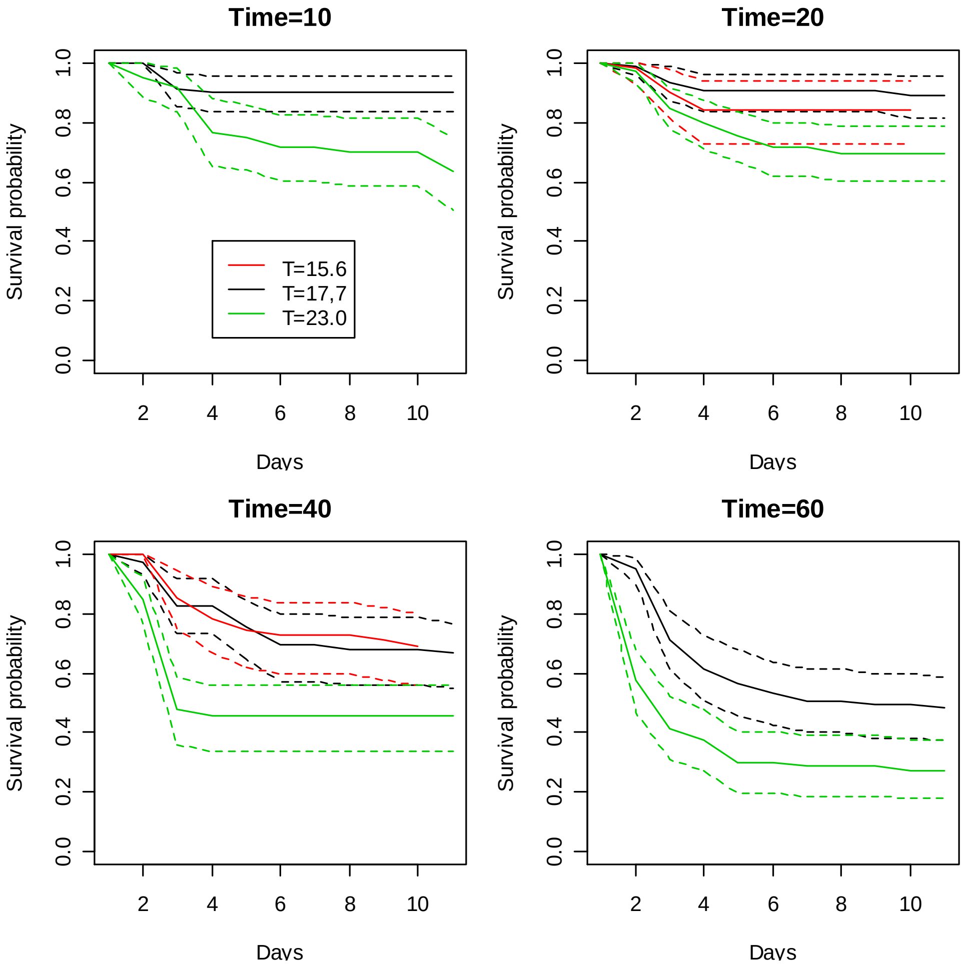

Figure 4.5 - Experiment survival (Kaplan-Mayer survival estimators) as a function of observation day for each fishing time treatment level, showing a temperature effect. Dashed lines represent the 95% confidence intervals for the survival curves obtained by a nonparametric bootstrap.

Support information on the factors influencing survival was obtained by modeling survival as a function of simulated fishing duration (net confinement), water temperature, fish density and fish weight (Table 4.3). This analysis confirms that increasing simulated fishing time and water temperature increase the risk of dying, but also that high density leads also to increased risk (nonetheless a statistically non-significant risk, likely due to the small sample size used in this treatment).

Table 4.3 - Cox proportional regression model output for sardine survival data from the three experiments as a function of operational (fishing time and fish density), environmental (water temperature) and biological (fish total weight) variables. “exp(coef)”=1 indicates absence of change in hazard due to a given variable, a value larger than 1 means a larger risk and lower than 1 a lower risk (or protection factor) associated with the corresponding variable.

Factor	**coef (S.E.)**	**exp(coef) (95% CI)**	**p-value**
Time (20)	-0.068 (0.229)	0.934 (0.596, 1.464)	0.77
Time (40)	1.012 (0.215)	2.750 (1.804, 4.192)	$<10^{-5}$
Time (60)	1.460 (0.206)	4.304 (2.873, 6.448)	$<10^{-5}$
Temperature (17.7)	-0.407 (0.244)	0.666 (0.412, 1.075)	$<10^{-5}$
Temperature (23.0)	0.693 (0.228)	2.000 (1.280, 3.125)	0.0023
Density High	0.443 (0.267)	1.558 (0.923, 2.629)	0.097
Twt	-0.061 (0.006)	0.941 (0.931, 0.951)	$<10^{-5}$

Further, there is also evidence that heavier (larger and more robust) fish have increased survival probabilities (Figure 4.6). The difference between the mean weight of dead fish (n = 297) and alive fish at termination (n = 536), 40.9 and 51.9 grams respectively, is highly significant ($P \sim 0$). This is further supported by analyzing condition factor over time (all fish pooled across treatments and experiments, results not presented) which shows that sardines sacrificed at day 0 (which were a random sample, n = 136) had significantly higher condition factors than those fish that died in the first day (n = 131; *P* ~ 0), suggesting that physically more fit sardines are less likely to die.

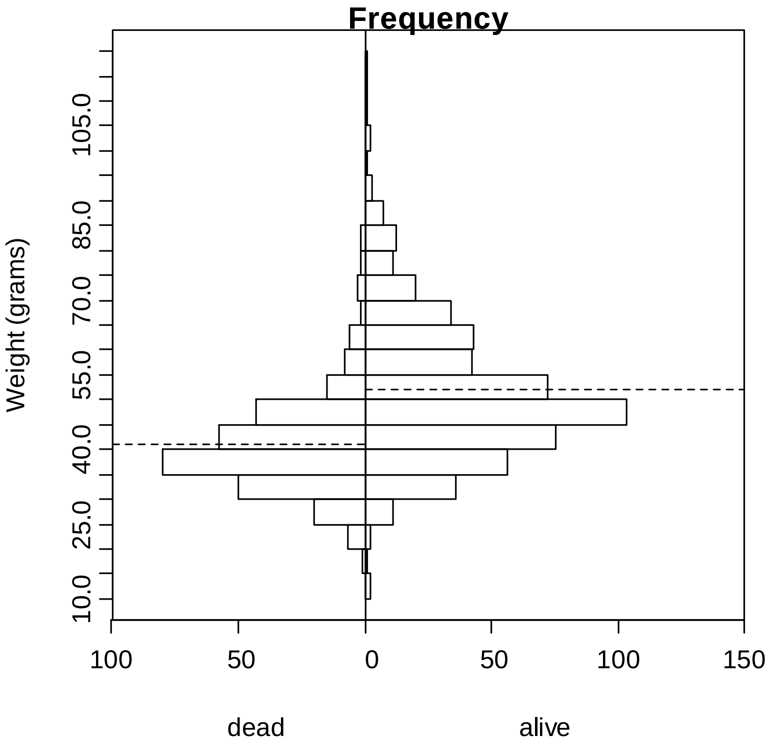

Figure 4.6 - Weight frequency distributions of fish dead and alive at termination day. Vertical dashed lines represent mean weight.

4.5 – Discussion

The results of this study demonstrated that using acclimated sardines in purse seine fishing simulations can allow the assessment of the impact of operational, environmental and biological variables on the survival, physiological and physical condition of slipped pelagic fish. Off Portugal, commercial purse seine fishing operations prior to bunting (e.g. school pursue, net shooting and hauling) constitute a stressful event that can exceed 1.5 hours (Marçalo et al., 2006). In these laboratory experiments, only the final part of the operations, when the net is fully bunted, were simulated, since previous studies have indicated that this is when most stress and physical damage occur due to increased crowding, abrasion and handling (Pawson and Lockwood, 1980; Lockwood et al., 1983; Mitchell et al., 2002; Stratoudakis and Marçalo, 2002; Stratoudakis et al., 2003).

Mean concentration levels of plasma cortisol for sardines prior to fishing were within the range of general resting or baseline levels reported for most teleosts (Barton and Iwama, 1991) and below the values reported for the species in the field at the onset of fishing operations (Marçalo et al., 2006), demonstrating the good physiological condition of the fish used and their adequate acclimation. Levels of cortisol increased significantly immediately after simulated fishing but did not attain the concentration observed at the end of purse seining operations in the field. Similar results were observed for haematocrit, with average concentrations decreasing significantly after simulated fishing, but both pre- and post- stress levels were higher than those observed in the wild. These changes in sardine physiological condition due to the simulated fishing operation indicated that experimental fishing was sufficient to elicit a significant stress reaction, but its impact was probably less acute than that of a commercial fishing operation at sea. It is plausible that excluding from the simulation the earlier parts of net setting and fish manipulation during transfer to the vessel with a scoop led to a milder stress reaction in captivity.

The direction of post-fishing changes in cortisol and haematocrit were in line with patterns previously observed for the species in the wild (Fig. 4.2), where a significant increase of cortisol and decrease of haematocrit during the commercial fishing operation was reported (Marçalo et al., 2006). Cortisol rise is a common primary response under stressful conditions in fish, while haematocrit usually increases for most teleosts to facilitate oxygen transportation and reduce cardiac costs (Wood et al., 1983;

Barton and Iwana, 1991; Bonga, 1997), namely small pelagics such as Atlantic mackerel (Swift 1982, Boutilier et al. 1984). The contradictory observed decrease in haematocrit in sardines (as in Marçalo et al., 2006; Marçalo et al., 2008*a*) goes in line with observations in Bourke et al. 1987 for skipjack tuna that suffered increased blood volume and osmolarity in the first hours post capture. For sardines, we can suggest at this stage that the main cause for haematocrit decrease is related to the potential for fluid (water) shifting between the tissues and the blood (here osmotic shift of water out of the muscle intracellular compartment into the extracellular fluids/blood plasma compartment), which always happens and is caused by increased permeability of the gills with increased stress (Wood et al. 1983; Wood 1991). Further, this mechanism can be the result of Na and Cl entering from the external seawater faster than they can be pumped out, raising plasma osmotic pressure, and creating in turn an internal osmotic imbalance which gets water out of muscle intracellular compartment into the extracellular fluids/blood plasma compartment and blood plasma diluting the red blood cells and decreasing the haematocrit.

Post-fishing stress levels in sardines as measured by cortisol and haematocrit were not proportional to the magnitude of the stressors (operational and environmental factors tested in the experiments). Sardines exposed to simulated fishing duration at low temperature (experiment 1) and simulated fishing duration at high temperature (experiment 3) did experience a duration effect for cortisol but not for haematocrit, while there was not a temperature effect on any of the physiological variables. Concerning simulated fishing duration and density (experiment 2), high density affected significantly haematocrit and not cortisol, while fishing duration had no effect on any variable in this experiment The absence of correspondence within physiological stress levels and stressor intensity and taking into account the observed mortality levels, indicates that physiological stress induced by net confinement duration, density and elevated water temperature were not useful predictors of potential delayed mortality, only indicating the stress reaction. As supported by Davis et al. 2001, here caution should also be taken when using physiological variables to evaluate stress that ultimately results in mortality. Nevertheless, failure to induce post-stress levels in the laboratory comparable to those attained for the same parameters in the field (here mostly due to the absence of full simulation of field conditions or absence of predators), or lack of correlation between the magnitude of physiological measures and delayed discard mortality have often been reported in experimental studies, without undermining their utility for understanding and finding the main factors that drive unaccounted fishing mortality (Olla et al., 1998; Davis et al., 2001; Davis, 2002). Other processes such as long term stress effects on ecological and biological aspects of the species (e.g. predator avoidance, feeding behavior, reproductive and immune system effects) should be further investigated in order to understand delayed mortality.

In all experiments, most sardine delayed mortalities were observed in the first 3-4 days after fishing, a pattern similar to that described in previous studies on the species during capture, transport and preliminary observations in captivity (Olmedo et al., 1990; Mitchell et al., 2002; Marçalo et al., 2008*a*). However, uncertainty over the magnitude of the simulated fishing impact and the fact that some fish were not subjected to the experimental treatment, by escaping the netting device, possibly diluted the treatment effects. As a result, the survival rates reported from this study should not be considered as absolute values that can be applied to the field, but as indicators of relative differences in survival rates for the three factors tested (time, density and temperature).

A significant decrease in sardine survival was observed as holding duration inside the net increased in the three experiments. Some mortality was observed even for a very short holding duration (10 min), although most deaths occurred for holding times of 40 or 60 minutes. Prior to the fishing simulations, sardines in the tank compartments were mortality-free for several days prior to the simulations, consequently, they could be considered as a control. Although the long, traumatic and stressful operation of drying up of the net during commercial operations was absent in these experiments, vigorous escape reactions (e.g. snouts pointing to the net walls) and severe abrasion were observed and can be pointed as the main causes of stress build-up and physical damage, thus resulting in the mortality rates observed as holding time increased. Experiment 2 did not reveal a significant density effect for a given holding time, although at 40 min holding time mortality was higher in the fishing operation with the higher fish density.

Fishing simulations at 23 ºC reduced survival rates by about 20 % for all treatments when compared to experiments performed at 16 or 18ºC. High water temperatures lead to physiological changes such as increase in cardiac outputs and decline in animal performance, being also characterized by decreased dissolved oxygen, which combined with exercise and stress, impairs post release recovery (Davis, 2002; Farrell, 2002; Gingerich et al., 2007; Crossin et al., 2008). Here, the 5 ºC rise in water temperature was not intended to determine the upper lethal water temperature for the species, but rather to observe the influence of extreme sea surface temperatures. Such temperatures may be experienced during some fishing events occurring in Southern regions of the sardine habitat range, namely during summer months in Southern Portugal, the Mediterranean Sea and the Northwestern African coast (Coombs et al., 2006; Ganias et al., 2007).

The Cox proportional hazards model indicated that heavier (physically fitter and in better condition) fish had lower probability of dying after fishing. This is in line with the idea that smaller fish fatigue faster and are more susceptible to suffering injuries from the pressure and abrasion associated with handling of the catch during fishing manoeuvres (Chopin and Arimoto, 1995; Davis, 2002; Broadhurst et al., 2008). However, the evidence that fitter fish (fish with higher condition factor) are less likely to die, may also be considered a factor confounding the results observed in experiment 3. Although higher water temperature is indicated as the most probable cause of decreasing survival rates, we cannot eliminate the hypothesis that lower condition factor (favoured by decreased weight) of the individuals used in this experiment may have led to lower survival. Despite this fact, earlier observations during capture, transport and maintenance of the species in captivity indicated that water temperature during operations was among the most important variables affecting survival rates (Marçalo et al., 2008*a*), while other laboratory observations indicated that sardine acclimation at lower water temperatures reduced post capture immediate mortality and delayed mortality (A. Marçalo, unpublished data).

The comparison of the physical condition (scale loss and fin erosion) between fish before and after fishing (Fig. 4.2) indicated that simulated fishing significantly increases scale loss, an expected process due to the abrasion suffered by fish during final stages of purse seining. The limited scale loss of survivors (mean value of 2 %) at the end of the observation period compared to the large scale loss of fish that died after slipping (mean value of 27 %), indicated that this physical parameter is strongly associated with the probability of dying after release. Further, the importance and trends of scale loss after capture were previously discussed during experiments of introduction of the species in captivity (Marçalo et al., 2008*a*), where the observed reduction of scale loss with time in captivity indicated that fish with greater scale loss die earlier. Looking into the daily evolution of the fin factor pooled across all fish (pre and post stress, dead and survivors; data not shown), we can observe that fin factor decreased up to day 6 and then increased, but never reaching the values observed at simulated pre-fishing. This pattern was also observed both for dead and alive fish in Marçalo et al. (2008*a*), with dead fish having lower fin factor at all times. Thus, fin erosion trends showed that this parameter is a long term stress response only revealed days after the fishing stressor, most likely related to immune suppression and caused by bacterial infections (Latremouille, 2003).

Our results showed that longer duration of the final purse seine fishing stages (when net is completely bunted) and operations at higher sea surface temperatures reduce the probability of survival of slipped fish. Although primary physiological stress reactions did not seem to correlate with the probability of survival, future work should focus on tertiary stress reactions (like decreased resistance to disease) or indirect stress effects (like behavioural impairment) to explore their importance for delayed mortality. For example, work on predator-prey interactions after slipping revealed impairments related to sardine shoaling formation, swimming speed and predator-prey distances - Marçalo et al. 2008*b*; A. Marçalo, unpublished data. Physical injuries, especially scale loss, were among the most important causes of death, hence modifications in the commercial fishing practice that may reduce abrasion could lead to higher probabilities of slipped fish survival.

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CHAPTER 5

Behavioral responses of sardines facing predators after simulated purse-seine fishing

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5.1 - Abstract

To evaluate the effect of holding time and density on sardine (*Sardina pilchardus*) behavioural changes and mortality caused by fishing and predation, three laboratory experiments simulated purse seining fishing operations, fish slipping (fish deliberately allowed to escape) and subsequent contact with potential predators (sea bass, *Dicentrachus labrax*). Experiment 1 compared the effect of a mild fishing stressor (20 minute fishing and lower sardine density) with a control (fishing not simulated). The second and third experiments compared a mild stressor with a severe stressor (40 minute fishing at higher density) at two sardine sample sizes (small and large sample for experiment 2 and 3 respectively). Behavior response data were collected on sardine predator approach, nearest-neighbor distance (school cohesiveness) and swimming speed. Results indicated a significant effect of fishing time and density on the survival of slipped sardines, but no conclusive results on predation mortality. However, fish after simulated fishing suffered significant behavioural impairments such as lower swimming speed, shorter predator approaches and larger nearest neighbor distances (wider school area) than control fish, regardless of stressor severity. These results suggest that, added to the delayed mortality caused by factors related to fishing operations, slipped pelagic fish can also suffer behavior impairments that may increase their vulnerability to predation, thus potentially being an additional source of unobserved mortality after deliberate release from purse seine nets.

Key words: Behavior responses, delayed mortality, purse seine fishing, *Sardina pilchardus,* slipping, predation

5.2 - Introduction

Delayed mortality of fish discarded from commercial fishing vessels or fish escaping from fishing gears is an important issue for fisheries management and conservation (Alverson et al. 1994; Davis, 2002; Broadhurst et al. 2006). Stressors that contribute to fish mortality have been studied both in the field and in the laboratory for many species and fisheries, especially those using demersal towed gears such as trawls (Davis, 2002). Most studies have concentrated on evaluating causes of death due to fishing, such as physiological disruptions or physical injury resulting from extensive handling (Olla and Davis 1989; Olla et al. 1992), alteration of light conditions (Olla et al. 1997) or temperature or pressure shock (Olla et al. 1998, Davis et al. 2001, Davis and Olla 2001; Davis and Parker 2004). Some studies have brought attention to an important component of unobserved mortality, caused by sub-lethal effects that may still cause indirect mortality due to behavioral impairments. For instance, deficient capacity for schooling, orientation, predator evasion or swimming after escape or release from fishing gears and during aquaculture manipulation have been observed in the laboratory for some species, all resulting in a reduced ability to avoid predation and therefore contributing to mortality (Olla et al. 1995; Mesa 1994; Davis 2002; Ryer 2002, 2004).

Delayed mortality has been less explored in pelagic purse seine fisheries, although observations have demonstrated that the deliberate release of a component of the catch (slipping) may be frequent and problematic, leading to high and variable mortality of the escapees (Lockwood et al. 1983; Misund and Beltestad 2000; Mitchell et al. 2002; Stratoudakis and Marçalo 2002). Although some stress reactions of small pelagic fish during purse seine fishing have been described (Stratoudakis et al. 2003; Marçalo et al. 2006), estimates of unaccounted mortality rates have been difficult to obtain, due to operational constraints in the field and limited husbandry experience for studies in captivity. However, recent advances in acclimating sardines (*Sardina pilchardus*) in captivity led to laboratory studies that revealed that survival rates of escapees are affected by a mixture operational (e.g. holding time and density) and environmental (e.g. water temperature) stressors, which are responsible for triggering primary and secondary physiological stress responses causing physical damage, leading to variable mortality rates (Marçalo et al. 2008; A. Marçalo unpublished data).

Like other small pelagic fish, sardine schooling is a dynamic process (e. g. change of shape, relative position in the water column, interaction with other schools) aiming to increase feeding potential, minimize predation risk, maximize reproductive success or support migratory behavior (Pitcher et al. 1996; Misund et al. 1998, 2003; Zwolinski et al. 2006, 2007), where higher activity rates have been demonstrated to be caused by the presence of predators (Misund et al., 2003). Thus, it is legitimate to consider that the physiological and physical disruptions suffered during the purse seine capture process may have detrimental consequences on sardine behavior responses and survival after release from purse seine nets. In this study, three experiments were performed where acclimated sardines in four treatment groups were subjected to simulations of the final stages of purse seining operations followed by slipping and immediate contact with potential predators (sea bass, *Dicentrachus labrax*), in order to evaluate the roles of holding time and density on the immediate and delayed mortality caused by fishing and predation, and to determine the behavior of escapees compared to a control group (exposure to predators but no simulated fishing). Behavior parameters (predator approach, nearest neighbor distance and swimming speed) were described daily during 8 hours up to 6 days after fishing with the help of video analysis.

5.3 - Materials and Methods

5.3.1 - Collection of fish for experiments

Live sardines were captured in January 2008 by a commercial purse-seiner off western Algarve in southern Portugal during daylight (net set around sunrise) and under good sea conditions (wave height ~ 0 - 0.5 m) following methods described in Marçalo et al. (2008). Whole stock survival rate after one month in captivity was > 80 %. Sardines were allowed to acclimate for a minimum period of at least two weeks prior to each experiment, based on previous evidence that capture and transfer related mortality practically ceases within a week and physiological equilibrium after fishing and transport stress is re-established by the second week (Marçalo et al., 2008). Aquaculture reared sea bass *Dicentrachus labrax* were used as predators. Four adult sea bass (3-4 years old) were obtained from an outdoor earth tank at the Aquaculture Research Station of IPIMAR in Olhão, weighed and measured and immediately placed in two compartments of the experimental tank (two per compartment).

5.3.2 - Experimental set-up

Experiments were performed between February and April 2008 using a rectangular 10 m^3 tank subdivided into four equal compartments by removable lateral framed panels. All compartments were supplied with seawater (at ambient temperature) with an open-system water circulation and variable water flow, aerated using air-stone diffusers placed at the centre of each tank compartment. Sardines for experiment 1 were immediately introduced into the two compartments of the experimental tank not containing sea bass after arrival at the station, and allowed to acclimate for one month. The remaining fish were held at a stocking tank located nearby. For experiments 2 and 3, new batches of sardines were transferred from the stocking tank, subdivided equally in two compartments and allowed to acclimate to the experimental tank for two weeks. The same sea bass were used for all experiments, although acclimation and starvation periods in between experiments were established to decrease experimental bias from one experiment to the next (Table 5.1). The absence of controlled water temperature in the experimental tank led to small daily variations between experiments (± 1ºC increase from one experiment to the next). Sardine jumping from one compartment to another caused some deviations from the initial number of individuals per compartment for all experiments and treatments (Table 5.1).

Table 5.1 - Summary information on acclimation operations and fishing and predation survival effects for the three experiments. T, temperature; n, number of sardines

Experiment	**Date**	**Observation (Days)**	**Acclimation of prey (Days)**	**Starvation of predator (Days)**	**Factor (code)**	**Treatment**	**n initial**	**T (ºC)**	**% Mortality (Predation)**	**% Mortality (Control & Fishing)**
1	19 February,	6	31	15	1- No stress	Control	175	16.7	2,9	1,1
	2008				2-Mild stress	20 minutes/ low density	169		3,6	3,0
2	11 March,	6	14	14	3-Mild stress	20 minutes/ low density	137	17.6	4,4	7,3
	2008				Severe stress	40 minutes/ high density	138		8,7	69,6
3	1 April,	7	14	14	4-Mild stress	20 minutes/ low density	62	18.4	17,6	6,5
	2008				5-Severe stress	40 minutes/ high density	61		14,9	42,6

5.3.3 - Experiments

For each experiment, mild or severe stressors considering time or density effects were established according to observations taken from fishing simulation experiments previously performed (A. Marçalo, unpublished data). Density effects were tested by bunting more (allowing minimum swimming space available) or less (allowing more ample swimming space) the purse, creating a smaller (high sardine density) or a higher (low sardine density) water volume respectively. For readability the treatments performed are referred as “control” for non-fishing simulated sardines and “fished” for fishing simulated sardines.

a) Experiment 1: Control and effect of a mild stressor

Sardines used as control were not manipulated (treatment 1), while a mild stressor considering fishing time (20 minutes) and density (low density) effects was applied to the “fished” compartment (treatment 2). Simulated purse seine fishing was performed using a netting device using the same mesh size (18mm) used by commercial purse seiners operating off the Portuguese coast was designed to allow the simulation of the commercial dry-up time (Marçalo et al. 2006). The net device was operated so that fish were herded and held inside the purse for the respective time, after which they were slipped back into the tank (allowed to swim freely over the “head rope” of the simulation device). Framed lateral panels for both control and fished compartments were immediately lifted and removed, allowing the interaction of the sardines with the two predators. Behaviour observations were recorded daily for 6 days with the exception of day 4 due to bad weather conditions (heavy rain and wind) that did not allow good image quality for analysis. Dead fish were removed daily prior to the start of video recording.

b) Experiment 2: Effect of a mild and a severe stressor with high prey density.

Two degrees of severity were considered: a mild stressor of fishing for 20 minutes at low density (treatment 3) and a severe stressor of fishing at 40 minutes at high density. Simulated fishing operations were performed one tank compartment at a time. High mortality rates within two days in the severe stressor tank led to the elimination of this treatment from the analysis. Behaviour observations were recorded daily for 5 days. Net device operation procedures for each compartment, predator and prey combination, dead fish removal and video recording followed the methods described in experiment 1.

c) Experiment 3: Effect of a mild and a severe stressor with lower prey density.

All the methodology was performed according to experiment 2, although sardine numbers were decreased based on the high mortality observed in the 40 minute high density simulation of experiment 2. Behaviour observations were recorded daily for 6 days. The mild and severe stressed tanks are considered respectively treatments 4 and 5.

5.3.4. - Behavioural observations

Experiments were recorded using an Ikelite 6002 EV Cam HRz strobe mounted 2 m above the tank, connected to a Sony DVD recorder (model RDR-HX910). A grid with 10 x 10 cm squares made out of plastic tube and ropes was designed and introduced at the bottom of each compartment (Figure 5.1) to be used as a reference for video recording (see below). At the start of each experiment the water and air supply to the tank was switched off (experiments were performed with no water flow), and the water level decreased to about half the tank height in order to decrease shortcomings derived from using 2-dimensional data to characterize 3-dimensional data (Ryer, 2002). The fish were filmed for 15 min prior to fishing and exposure to the predators to be able to measure sardine behavior prior to stress. Analyses of video recordings taken during experiments were performed to determine predator approach and sardine nearest-neighbor distance and swimming speeds. Video recordings were continuous for at least 2 hours after predator and prey were placed together, after which we recorded only 15 minutes in each hour during daylight or up to 8 straight hours daily.

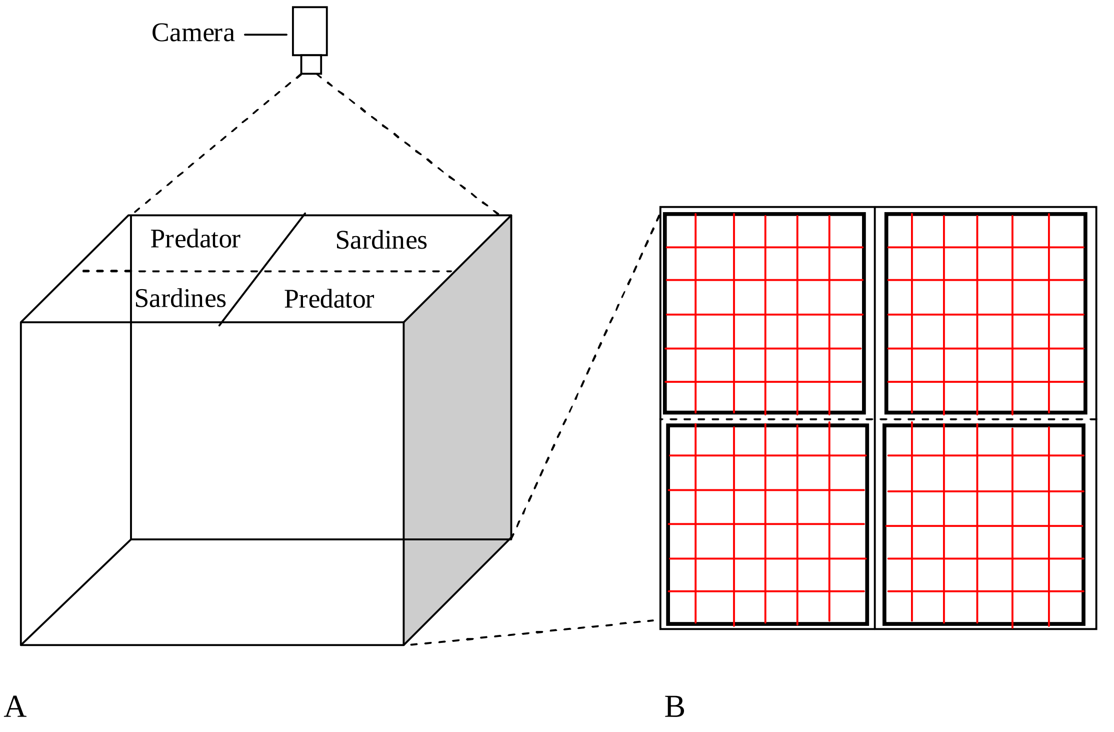

Figure 5.1 – Experimental tank design and filming apparatus with predator-prey disposal before combination (A). Removable separation panels (dashed lines) were lifted after fishing simulation allowing the interaction of predators and prey. Grids with 10 x 10 cm squares placed at the bottom of each compartment for video recording reference (B).

Predator approach and nearest-neighbor distance were measured using the public-domain image processing program ImageJ and calculated from random frames obtained from the videos. For experiments 1 and 2, we took 3 frames for each half hour of the 2 hr continuous video of day 0 (onset of experiment), and 3 frames in each 15 minute interval for the remaining hours. For experiment 3, frame sampling doubled in each time interval. For each frame, predator approach was obtained by measuring the distance from the predator that was closest to the sardine school. Predator-prey distances were averaged for each hour segment. Nearest-neighbor distances were recorded by randomly selecting a 3x3 square from the “grid box” on the floor, measuring distances between 3 pairs of sardines within the square and averaging them for each hour segment. Swimming speed was determined by counting the number of frames an individual sardine took to completely cross a grid line, and was recorded as centimeters per second (cm s^{-1}). Sardines whose path deviated more than 20° during a swimming speed measurement were excluded. 5 measurements of swimming speed were taken for each hourly time interval.

5.3.5. - Measuring lethal predation

For all treatments, at the end of observation period, sardine survivors were collected from each compartment with a hand net and counted (final survivor count). Numbers of fish eaten by the predators were estimated indirectly by subtracting the sum of the survivors and the sardines that died during the course of the experiment from the initial number of fish placed in the tank compartment.

5.3.6 - Statistical analysis

Given the unbalanced nature of sampling (varying total number of recording days among experiments, no recording on day 4 in experiment 1), a comparative analysis was only performed up to day 3. The evolution of predator approach, nearest neighbor distance and swimming speed parameters with days prior and after fishing simulation and exposure of sardines to the predators, were analyzed separately. Correspondent data for each parameter was analyzed with Two-Way ANOVA, using treatment and day as independent factors, followed by Tukey *à posteriori* comparisons. Homogeneity and normality were not severely deviated since the software allowed proceeding with parametric analysis. In general, and for simplicity, we refer to statistically significant results for tests with P-values smaller than 0.05. Statistical analyses were performed using the software program SigmaStat version 3.5.

5.4 - Results

A total of 742 sardines (344 in experiment 1; 275 in experiment 2; 123 in experiment 3) were used in the three experiments. There was no variation in sardine mean length across experiments (mean length= 19.5 cm), although a variation in condition factor was observed, with a decrease of condition factor from one experiment to the next (Table 5.2).

Table 5.2 - Summary of biological data (mean ± S.E) for sardines (prey) and sea bass (predator) used in the 3 experiments.

Experiment	**Treatment**	**Prey**			**Predator**		
		Length (cm)	**Weight (grams)**	**Condition factor**	**Length (cm)**	**Weight (grams)**	**Condition factor**
1	1	19.0 ± 0.5	50.0 ± 6.5	7.3 ± 0.9	52.0 ± 1.4	1692.5 ± 113.8	12.1 ± 1.8
	2	19.9 ± 0.9	56.8 ± 5.8	7.3 ± 1.3	48.7 ± 4.0	1531.5 ± 372.6	13.2 ± 0.0
2	3	19.5 ± 0.8	50.2 ± 6.2	6.7 ± 0.4			
3	4	19.3 ± 1.3	45.4 ± 8.8	6.3 ± 0.4			
	5	19.5 ± 0.8	46.8 ± 7.7	6.3 ± 0.7			

Sardine survival rates at the end of the observation period for each experiment compared with the control (treatment 1, experiment 1) confirmed that fishing time and density have a significant impact on sardine mortality (Table 5.1). Higher survival rates were observed for treatments with short fishing time and lower density (treatments 2 , 3 and 4; 20 minutes at lower density) and significantly lower survival for longer fishing time at higher densities (40 minutes at higher density in experiments 2 and 3). School size (n) had no effect at shorter fishing time and lower density, but was significant at higher fishing time and density leading to higher mortality rates at the severe stressor tanks, in agreement with previous findings (A. Marçalo, unpublished data). Also, a larger school size in the treatment using 40 minutes fishing time and higher density in experiment 2 resulted in very high mortality rates within two days, which lead to elimination of this treatment from the analysis. Mortality estimation due to predation is not conclusive. Although increased predation rates were observed for fished treatments when compared with the control, and there was a proportional increase in predation with time and density in experiment 2, results from experiment 3 are not in agreement. Further, predation rates increase from one experiment to the next, possibly indicating gradual predator habituation.

Of the three behavior parameters measured, observations in predator approach are the least clear. Although there is a significant treatment effect (ANOVA: F = 2.85, df = 4, $p < 0.05$; Table 5.3), multiple comparisons revealed that this effect only occurred during experiment 1 when comparing the fished tank (treatment 2) with the control. However, it is clearly observed that in general, sardines allowed closer predator approaches during the day the experiment started (day 0), with predator distances in the fished tanks approximately half those of the control (Figure 5.2). Immediately after direct exposure to the predators, control sardines tended to stay on the opposite side of the tank, while fished sardines often appeared unaware of the predator´s approach, allowing them to pass within a few centimeters. Recovery rates (capacity to increase distances to the predator) was identical for both control and fished sardines. Within 24 hours control and fished sardines had similar average distances to the predator, which were maintained up to the end of the observation period.

Table 5.3 - ANOVA and tukey test values for treatments affecting predator approach, nearest-neighbor distance and swimming speed up to 3 days after purse seining simulation.[a]

	df	**SS**	**MS**	***F***	**p**	**Tukey (Multiple Comparison)** **Groups**
Predator approach ANOVA						
Treatment	4	11828,7	2957,2	2,85	0,024	*
Day	3	70489,9	23496,6	22,65	<0,001	1 x 2; 1 x 3; 1 x 4; 1 x 5; 2 x 3; 2 x 4; 2 x 5; 3 x 4; 3 x 5; 4 x 5
Treatment x day	12	10724,2	893,7	0,86	0,587	
Nearest - neighbor ANOVA						
Treatment	4	378,707	94,677	97,238	<0,001	** ** ** ** * ** ** ** **
Day	4	265,174	66,293	68,087	<0,001	1 x 2; 1 x 3; 1 x 4; 1 x 5; 2 x 3; 2 x 4; 2 x 5; 3 x 4; 3 x 5; 4 x 5
Treatment x day	16	197,642	12,353	12,69	<0,001	
Swimming speed ANOVA						
Treatment	4	3560,058	890,014	9,53	<0,001	** ** ** *
Day	4	8640,717	2160,18	23,14	<0,001	1 x 2; 1 x 3; 1 x 4; 1 x 5; 2 x 3; 2 x 4; 2 x 5; 3 x 4; 3 x 5; 4 x 5
Treatment x day	16	3292,949	205,809	2,21	0,004	

[a] Treatment: 1, control; 2, 20 low – experiment 1; 3, 20 low – experiment 2; 4, 20 low - experiment 3; 5 – 40 high - experiment 3.
Significant groups are underlined: *, <0,05; **, <0,001

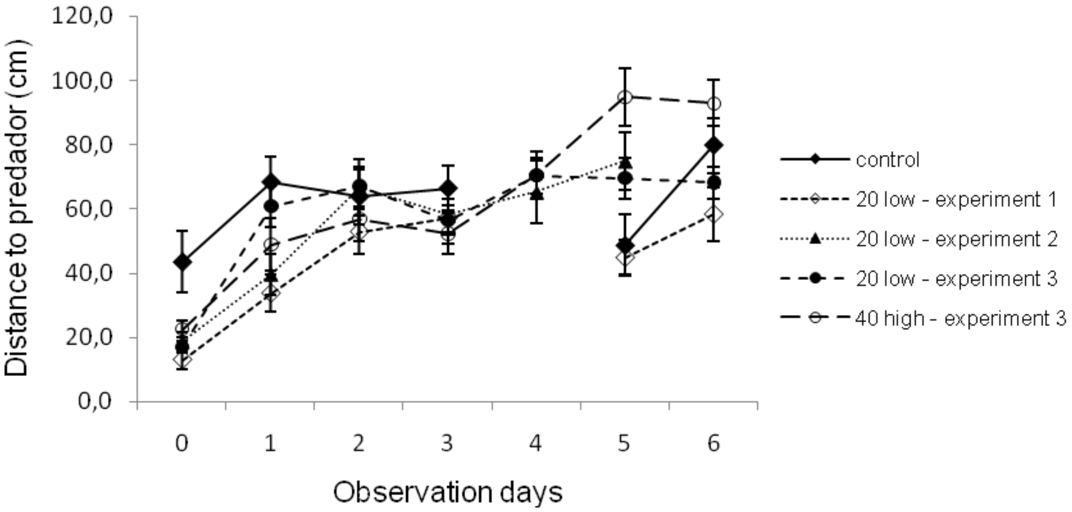

Figure 5.2 – Mean sardine distance to predator (± SE) for all treatments after simulated purse seine fishing. Sardines exposed to sea bass at day 0.

Nearest neighbor patterns were altered with the presence of predators in opposite directions for control or fished sardines (Figure 5.3). While control fish showed an immediate increase in group cohesiveness, revealed by the decrease of nearest neighbor distances to about half of what was observed prior to exposure to the predators (day -1) up to day 1, with fish gradually relaxing and maintaining constant distances until the end of the observation period, sardines in all fished tanks immediately dispersed after suffering the fishing impact, maintaining this behavior even in the presence of the predator for many hours during day 1 and even day 2, gradually adjusting to form tighter schools in the following days. ANOVA results revealed a significant treatment effect (F = 97.24, df = 4, P < 0.001), with multiple comparisons giving highly significant differences between most treatments (Tukey, p < 0.001), except between treatments 4 and 5 (experiment 3). Significant differences from all fished groups when compared with the control further support the effect of the fishing stressor, here represented by the time and density applied. Differences within mild severity groups indicate an experimental effect for this parameter that could be related with the interference of unmeasured effects of experimental variables such as sample size (n) or water temperature. Experiment 3 while testing two severity groups, it maintains variables such as sample size (n) and water temperature equal, thus we consider that the non significance between treatments 4 and 5 can indicate that there are no differences between levels of severity.

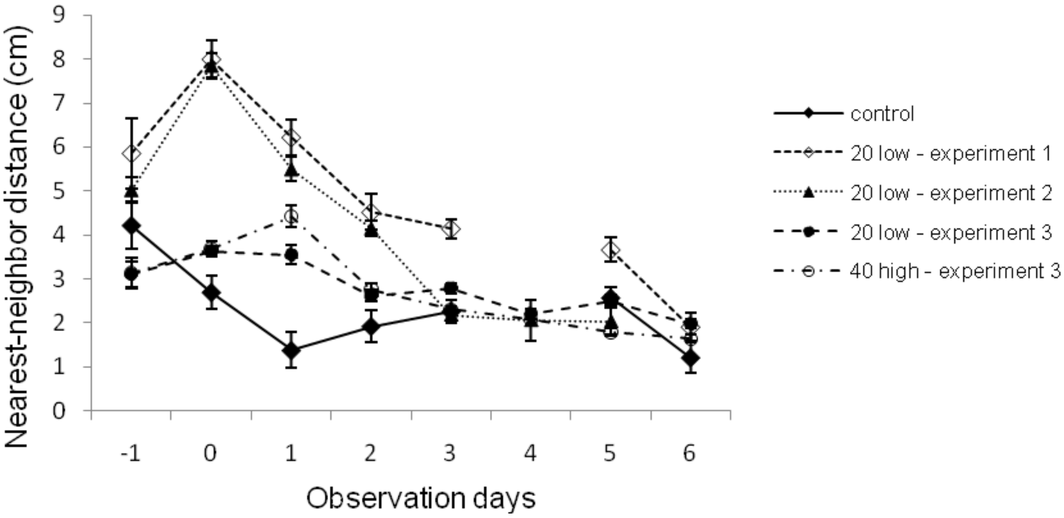

Figure 5.3 – Mean nearest-neighbor distances (± SE) for sardine exposed to sea bass predators for all treatments after simulated purse seine fishing. Day -1 refers to mean data before sea bass exposure; sardines exposed to sea bass at day 0.

Sardine swimming speed was relatively low for all treatments prior to any fishing event or exposure to predators (day -1; Figure 5.4), with sardines swimming around the tank in a loose school. After fishing simulation and exposure to predators, sardines in the control had a fivefold significant increase in swimming speed, which was maintained for at least 48 hours, while fished tanks had slower speeds for the same period. Very fast movements of the control group were common, where the school always tried to move away from the predators as they approached, maintaining maximum predator – prey distances, while sardines in fished treatments were visually more dispersed and rarely engaged in sprints. A significant treatment effect (ANOVA; *F* = 9.53, df = 4, $p < 0.001$) was only observed for all fished treatments when compared with the control (Tukey; $p < 0.001$). As previously stated for nearest neighbor distances, significant differences between all fished groups and the control show the significant effect of the fishing stressor (time and density), while non significant multi comparison results between treatments 4 and 5 of experiment 3 may support once more the absence of difference between levels of severity.

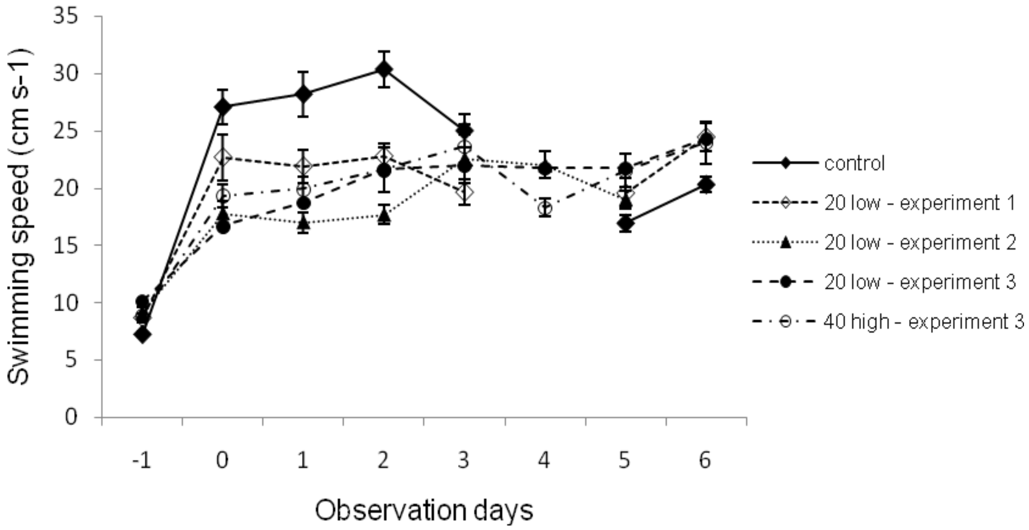

Figure 5.4 – Mean swimming speed (± SE) for sardine exposed to sea bass predators for all treatments after simulated purse seine fishing. Day -1 refers to mean data before sea bass exposure; sardines exposed to sea bass at day 0.

5.5 – Discussion

The present study demonstrates that simulated purse seine fishing and slipping in captivity using acclimated sardines can provide useful information on sardine behavior reactions facing predators after deliberate release or escape from commercial fishing operations. The results provided evidence that fished sardines suffer immediate and long term (up to 3 days) behavioral impairments, when compared with control fish (fishing not simulated). For the three parameters analyzed, fished sardines had significantly lower swimming speed, increased predator approach and nearest neighbor distance, were initially disoriented, more lethargic and formed less cohesive schools than control fish. Although results concerning deaths due to predation are not conclusive, the behavior impairments observed suggest a strong likelihood of increased vulnerability to predators for several hours or even days after deliberate release (“slipping”) from purse seines.

Acclimated sardines were subjected to 4 treatments simulating the final stages of purse seine fishing operations, where the chosen fishing time and bunting intensity (density) intended to compare the effect of a mild and severe stress (20 minute fishing at low density and 40 minute at high density respectively) on the behaviour of escapees. Initial intentions were to maintain experimental variables such as prey sample size (n) or water temperature alike along the different experiments. However, for the sample size, some adjustments were made after high fishing mortality rates were observed on the severe stress treatment in experiment 2, leading to its elimination from the analysis, (since the reduced number of survivors invalidated the video analysis). Subsequently, sample size was reduced to about half in the mild and severe treatments of experiment 3, while water temperature changes were uncontrolled because they were season dependent. Also the stressors applied in this study were deliberately scaled down in order to reduce fishing mortality and obtain behavioural measurements of recovering fish, following Ryer (2002), who suggested that survivors should be considered the important subject from the ecological and population dynamics point of view, in contrast with injured fish that will die in direct consequence of stress. Nonetheless, despite of variations in experimental variables, some of them controlled (sample size) or uncontrolled (water temperature), which did not allow consistent experimental replication, conclusions on the relative importance of some factors that may influence sardine behaviour after simulated fishing in captivity can be drawn.

Behavior responses of sardines after simulated fishing were significantly different compared with control fish. It was immediately observed that control fish were more alert, constantly moving away from the predator with fast movements in dense coherent schools, while fished sardines allowed sea bass to approach at shorter distances, had slower swimming performance and were widely dispersed for a long period of time (general view shown in Figure 5.5).

Figure 5.5 – Image showing sardine behavior aspects in the control tank (top) and in a fished tank (bottom)

A significant fishing effect was evident in all the parameters analyzed: increased predator approach, increased nearest-neighbor distances (decreased schooling) and decreased swimming speed. This occurred for fish in fished treatments regardless of whether the stressors were mild (less time and low density inside the net) or severe (more time and high density inside the net), with results suggesting the same impact for both levels of severity. Thus, regardless of stressor intensity, impairments that can compromise sardine behavior and turn them more vulnerable to predation were observed, which goes in line with previous findings in laboratory experimentation testing other species and fisheries (Mesa, 1994; Mesa et al. 1994; Ryer, 2002).

There was an evident experimental effect in the results obtained, especially for nearest neighbor distances, where results of experiment 3 deviate clearly from experiments 1 and 2. The causes for these deviations are not clear, possibly due to confounding effects from differences in sample size or water temperature - the latter known to be an important stressor affecting negatively fish survival rates after fishing events and consequently behavior patterns (Davis, 2002). However, most importantly, the pattern in nearest neighbor is similar for fished fish in all experiments, with all fished fish dispersing after fishing for both levels of severity, in contrast with the aggregation behavior of control fish. Finally, for all parameters, recovery periods for fished sardines could be retrieved from the main differences from the control. For instance, achieving increased distances from the predator occur within a short period of time (1 day), while for nearest-neighbor and swimming speed, recovery was only attained within 2-3 days.

Sardine delayed mortality rates caused by fishing were proportional to the severity of the stressor, according to prior observations on the species using similar fishing simulations (A. Marçalo, unpublished data), ranging from 3 to 7 % and 43 to 70 % for the mild stress and severe treatments respectively, and contrasting with the control tank which presented negligible mortality (1.1 %). Sample size had no effect in mild stressor treatments, but an effect was observed in the severe stressor treatments, with smaller sample size associated with a 25 % reduction in fishing mortality. Some mortality variations observed for all mild stressor treatments may be related to factors other than time or density, namely variations in water temperature, sardine acclimation to the experimental tank or unobserved predator interactions and captivity effects (physical interaction with the tank). Pronounced differences among survival rates for the different levels of severity applied are not comparable and do not correlate with the levels of behaviour impairment observed for each respective treatment.

Many studies, focusing mainly on trawl gear effects on demersal species, have shown that stress may impair fish behavior and response to predators. Further, some of these studies have demonstrated predator-prey interactions with a wide variety of species (Ryer, 2002, 2004; Ryer et al. 2004; Davis, 2005), tested under different light conditions (Olla et al. 1997; Ryer and Olla, 1998*a*), as well as predator interference with feeding habits (Ryer and Olla, 1998*b*). The focus has been on the sub-lethal effects of impairments in the escapees and their ecological meaning (impact on schooling ability, orientation, reflex responses, migration, feeding or reproduction; Davis, 2005), rather than directly on lethal predation levels, as the latter depends mostly on predation intensity in the fishing vicinity and is difficult to quantify. Our results indicate that sardines subjected to different levels of stress through simulated purse seine fishing and despite considerable differences in their susceptibility to direct stress-induced mortality, appear equally susceptible to suffering sub-lethal effects from behavioral impairments, therefore increasing their vulnerability to predation.

The present study presents a new approach for measuring behavioral impairments that small pelagics may suffer when deliberately released from purse seine nets through slipping. Experimental conditions may have been limited in terms of consistent replicates for each stressor severity, but results clearly elucidate for the first time behavior impairment patterns for simulated slipped sardines. The results indicate that added to the delayed mortality directly resulting from the interaction of fishing stressors that lead to physical injury and physiological impairment (which are proportional to the severity of the stressor), unobserved mortality of individuals that survive fishing but become behaviorally impaired and are predated in the following hours or days is highly likely. However, this study and results are only the launch to complement the detection of unobserved delayed mortality in the purse seine fishery, since the study of interactions with other factors that affect survival, namely physiological stress levels, degree of physical injury, temperature or biological (e.g. fish size), to name just a few, were absent. It also shows that quantification of direct or indirect unobserved delayed mortality in small pelagics escaping purse seine nets is difficult as it depends on many factors/conditions that occur during the fishing event (Marçalo et al. 2008, A. Marçalo, unpublished data). Thus, there is an urgent need to improve methods to monitor the fate of fish escaping or deliberately released from purse seine nets, not only focusing on physiological stress responses and physical injury, but also behavior impairment and subsequent predation.

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CHAPTER

Conclusions

Post fishing delayed mortality has been studied for some métiers and fish species, especially demersal (Broadhurst et al. 2006). The exact mechanisms leading to mortality after discarding are not completely clarified and depend on the highly variable operational, environmental or biological factors that characterize each case study (Davis 2002). In the absence of any work specifically dedicated to the impact of the main factors or stressors acting during purse seine fisheries on slipped (slipping being the term used to describe the discarding of fish directly from the net during purse seine activities) fish, this thesis provides new information on the survival, stress, physical and behavior responses of sardine *Sardina pilchardus*, the most important pelagic resource targeted by purse seine fisheries along the Portuguese coast. In **chapter 2**, short term evolution of sardine stress responses are described and likely stressors identified during operations at sea. Maintenance of the species in captivity described in **chapter 3** brings an insight to the post capture mortality, and the physiological and physical impact evolution. Laboratory experiments in **chapter 4** allow the simulation of the last phases of purse seining (the most stressful period during capture) and slipping, in order to explore the effect of operational and environmental stressors. Fishing duration and water temperature are important stressors contributing to delayed mortality. Physiological variables do not predict or correlate with observed mortality, while physical damage (especially scale loss) is an important predictor of the probability of dying. Finally, in **chapter 5**, behavioral responses of slipped sardines when facing predators are investigated to account for any impairment suffered during fishing that could contribute to unobserved mortality after slipping. The current chapter summarizes the major findings of this research, with the aim to better understand the factors/stressors acting during purse seine fishing operations and sardine responses that may affect their survival after slipping, and propose lines of future research and action.

The ultimate goal will be to contribute for a better knowledge of the most important direct and indirect mechanisms affecting survival/mortality of sardine escapees and to make suggestions to the fishing sector in order to enhance the probability of sardine survival after slipping.

Results obtained in chapter 2 demonstrate that commercial purse seining induces significant stress responses in sardines. Physiological parameters such as cortisol, glucose, haematocrit, haemoglobin, ionic balance and ATP catabolites were modeled as a function of time in the net, and biological (sex, reproductive state and condition) and operational variables (catch, light level and phase of fishing operation). Time spent in the net has the most important effect on all parameters, whereas biological and the other operational factors have a minor impact. Mean values of some physiological parameters at the end of the fishing operation are similar to peak stress values and show patterns reported in literature for other teleost species (Barton and Iwana 1991, Wandelaar Bonga 1997), whereas patterns of other parameters such as haematocrit and haemoglobin are not so in line with the most frequent observations for other species. Nonetheless, these short term stress reactions only showed the physiological evolution during capture. To further investigate the impact of purse seining on the survival or mortality of slipped sardines, laboratory experimentation was carried out and assumed to be the most reliable and practical way to explore links between magnitude of the stressor, short term and long term stress reactions (using the values obtained in the field for the species as a reference and guarantee that the simulated fishing impact is realistic) and subsequent survival. In chapters 3 and 4 it is revealed that physiological parameters are important measures for predicting stress fishing impacts, although also as previously stated in related works for demersal fisheries (Davis et al. 2001; Davis 2002), they do not always correlate with observed mortality when compared with other variables such as physical damage, and ultimately other sources that may contribute to unobserved mortality such as behavior impairments (chapter 5). Also, stressors studied in chapter 2, such as the fact that time spent in the net (considered one of the most important stressors, in detriment of others such as catch load/density) were further explored in chapter 4. In chapter 4, simulations showed that mortality correlates positively with increased time in the net, while density effects are less conclusive. Also, while in chapter 2 it was referred that environmental factors such as water thermoclines are less restrictive in purse seine fisheries in comparison with demersal fishing, in chapter 4 it was demonstrated that sea surface temperature is an important factor leading to decreased survival of escapees. Subsequently, it is expected that during warmer months, especially in the Portuguese Southern region (that experiences frequent daily sea surface water temperatures above 20 º C) delayed mortality of slipped fish may be higher, as the most stressful stages of the fishing operation take place at the water surface (further developed in the next paragraphs).

Whereas field studies have been used to study the development of fishing gear modifications that may reduce discard mortality and are the key in trying to assess and describe the main operational or other factors/stressors that will have an impact on delayed mortality of escapees, the need to complement the field work with laboratory experimentation has proved to be essential in order to accomplish the goal of predicting delayed mortality after discards and to study the links between all possible reasons that lead to death (physical, physiological, biological or general behavioral; Davis 2002). The study conducted in Chapter 3 aimed to optimize conditions of capture, transport and acclimation of sardines to laboratory conditions in order to perform fishing simulations with the species (Chapter 4 and 5). This goal was accomplished, with the trials also providing preliminary data on the survival, physical impact and physiological status of sardines after capture. Not neglecting the fact that added to the capture process itself, sea and land transportation were an added source of stress and physical damage (although the impact is diminished by the absence of the final fish offloading to the seiner), the “hypothetical” survival pattern of sardines that escape purse seine nets was evaluated for the first time. Survival rates varied considerably depending on an array of factors during the capture and transportation process at sea and in land and husbandry conditions and their interaction. Most deaths for all trials occurred in the first 5 days, stabilizing thereafter and being negligible or absent within 2 weeks. Among the factors affecting survival in captivity, environmental stressors seemed to be important to explore, as demonstrated by the inverse correlation of survival and increased sea water temperature during capture, or thermal shock during introduction in captivity. Short term physiological parameters studied, showed peak values in the beginning of the capture and acclimation process, gradually reestablishing and showing inverse temporal trends from those observed during the commercial capture event described in chapter 2. This not only proved the reestablishment of the short term physiological status, but also the good maintenance condition that allowed the recovery from the stress of capture and transportation. Nonetheless, long term stress impacts were revealed with the temporary inhibition of the reproductive system, demonstrated by the almost immediate significant decrease of gonadossomatic index (GSI). Physical status of captured sardines was evaluated, where patterns of scale loss or fin erosion were compared between live and dead fish. Scale loss turned out to be an important predictor of survival or mortality probability, where those fish with higher percentage of scale loss die earlier. For all trials analyzed, surviving fish have minor scale loss (< 5 %) compared with significantly higher and highly variable scale loss of dead sardines, which is further supported with the results observed for physical injury in Chapter 4. Similarly, fin erosion was another important parameter reflecting not only another aspect of physical damage but also immuno-deficiency. Results demonstrated that the fin erosion is significantly higher in dead fish, although it is consistently observed for both dead and live fish. The fact that the fin erosion is intensified not immediately after capture but from the second day onwards is a reflection of the delayed effect of the stress response (long term response), also observed in Chapter 4.

As stated previously, laboratory experimentation is an important tool to support fish delayed mortality studies. Although it can be criticized for lack of realism, laboratory studies allow an approach in testing reasons that trigger the stress response that subsequently lead to mortality that could not be possible in the field, for many reasons including higher costs (Davis 2002; Davis and Ryer 2009). To date only a few studies have been dedicated to purse seine fisheries and the fate of pelagic escapees (Pawson and Lockwood 1980; Lockwood et al. 1983; Misund and Beltestad 1995, 2000; Mitchell et al. 2002), and none fully used laboratory acclimated fish and/or fishing simulations. In the work presented in chapter 4, the effect of important factors/stressors observed during live capture (chapter 2) or during capture, transportation and introduction of the species in captivity (chapter 3), namely operational (time of capture and density) and environmental (sea surface water temperature), could be closely explored through fishing simulations. Different stressor severity levels were simulated and links with physical and physiological responses could be also considered in this work. Further, taking advantage from previous evidence demonstrated during live capture observations (Chapters 2 and 3), it was concluded that the final part of the fishing process is the most stressful, since this is the period when the net is completely bunted and fish suffer increased stress and manipulation that occur during the fish offloading process. Therefore, fishing simulations were adapted to conditions that better suited the intentions and were the most practical and easy to perform, where only the encircling and bunting of the net was performed. The work presented in chapter 5 aimed to study behavior responses of sardines that may be affected by the impact of fishing, and that may lead to unobserved mortality due to increased vulnerability to predation. According to related works, behavior impairment is an ecologically significant measure that contributes to fishing collateral delayed mortality, where finding ways to measure its impact on the survival of escapees is essential and only possible so far with laboratory experiments (Davis 2005; Ryer and Olla 1998a, 1998b; Ryer 2002; Ryer et al. 2008). Thus, as in chapter 4, laboratorial experimentation was once more developed to allow closer observations which would have been difficult in the wild, particularly because of the difficulty in performing predator-prey interaction studies in the wild, which depend mostly on the proximity and availability of predators at the time of fish discarding (here at the time of slipping).

Results from the simulations performed in chapter 4 demonstrated that survival rates correlate inversely with time inside the net, increased sea surface water temperature has an added negative effect and that density effects are not as clear, and finally biological effects (fish condition) seem to have an important contribution to survival (heavier fish were more likely to survive). Also, although simulations in chapter 4 had a smaller physiological impact when compared with results presented in chapter 2 at the end of commercial fishing operations (maybe due to the absence of a complete operational activity), blood physiology proved once more to be essential only to show the stress impact of fishing, but did not correlate with mortality rates observed, as also suggested in other related works (Davis et al. 2001; Davis 2002). Thus, physiological direct (short term) responses are not to be considered one of the main reasons leading to post fishing mortality of sardine or to be used solely in delayed mortality studies. Physical damage, especially scale loss, is an important cause of death, where results observed in chapter 3 were confirmed in the laboratory (chapter 4) and it is noted once more that fish with higher chances to survive after escape have minor scale loss (0-5 %). Longer term physical responses such as fin erosion, which is the reflection of immune suppression and increased bacterial action was also established as a symptom affecting all fish as a result of stress, although with an increased magnitude in debilitated fish or fish that have more chances to die as observed in chapter 3. Also, unknown consequences for the species can arise from the temporary cessation of reproductive activity found with the study conducted in chapter 3, and considered an important stress response, although spontaneous spawning in captivity was observed within 2 months. In chapter 5, another aspect of indirect responses was observed, namely behavior responses that affect survivors after release from fishing nets. Here, behavior impairments at the level of predator approach, nearest-neighbor distance or swimming performance (speed) could be determined for sardines after capture and released (slipped). When compared to a control, post capture surviving sardines allowed closer approaches of the predator, had increased nearest neighbor distances, which could also reflect wider schools, and also presented decreased swimming speeds. Although mortality due to predation was not conclusive, behavior impairments indicate how slipped sardine escapees have increased vulnerability to predation, which may lead to unobserved and unaccounted mortality.

Overall, it is important to emphasize the fact that for all slipped escapees, whether or not they will survive from a combination of short term/direct responses (specially physical-scale loss), they will also suffer long term/indirect stress responses, here demonstrated by suppression of the reproductive and immune systems and behavior impairments, which are detrimental to survival and will affect the species at the biological and ecological level.

Final remarks

This thesis had two main objectives: the first concerning the determination of factors/stressors that contributed to mortality of slipped sardines after purse seine fishing operations and the study of responses of the fishing impact at the physiological, physical and behavior level; secondly and foremost the tentative quantification of mortality that results from slipping. The studies carried out led to detailed information on the main stressors occurring during fishing, especially time inside the net during the final stages of the operations. With the fishing simulations in chapter 4, it is demonstrated that if time inside the net during fish offloading exceeds 10-20 minutes, survival rates of escapees decrease significantly. These survival rates can be reduced by up to 20 % with increased sea water temperatures. Sardines with better condition factor have improved chances to survive and physical responses such as scale loss are among the most important direct cause of death. Long term stress and behavior responses are added sources of delayed mortality.

Concerning the second aim of the thesis, this work can be taken as an important step to confirm and account for mortality that occurs after slipping and that is neglected and not quantified for stock assessment studies. However, generalized quantification of mortality resulting from slipping is concluded to be ambitious at this stage, since arrays of different confounding factors at the level of the season, capture and catch affect every single purse seining trip. Thus, the presented studies are the initial step to account for slipping mortality due to purse seining, but further investigation in this area is suggested, mainly with more replication of the experimental work or looking forward to test other important operational factors (e.g. light conditions in which fishing operations occur), or mixture of other factors (e.g. biological versus environmental, namely testing the effect of condition factor at different sea water temperatures). Nonetheless, from the results presented, some suggestions for the fishing sector can be drawn to improve fishing methods in order to minimize mortality of slipped fish or reduce slipping. Thus, for the Northern coast where daily quotas are applied and sardine is the main target and landed species, skippers should always estimate as early as possible the catch load (experienced skippers commonly do a visual estimate with good precision rates) during hauling and prior to reaching complete bunting of the net, followed by the release of any excess catch (this not only favors maneuvers with decreasing hauling time, as also decreases resulting stress to the fish, improving chances of survival of the fish allowed to escape), either by lowering the head rope or using selectivity devices such as panels with increased mesh sizes as described in Gonçalves et al. (2008). Further, any excess catches should be shared with boats that have not fished and are located in the vicinity, or always brought onboard and markets found to place that fish (even if sold as feed to aquaculture systems). In the Southern/Algarve regions where catches are not as abundant and are composed mostly of mixed species, the same methodology applies and greater care should be taken during Summer months where sea surface temperatures are frequently above 20 ºC. Finally, for all areas, fishers should be advised to not bunt the net very tightly in order to decrease the risk of dramatic abrasion and subsequently increased scale loss. Thus, as fishing methods can be modified to reduce slipping of excessive or unwanted catches and related delayed mortality, observations in the field lead to the conclusion that these modifications are possible as they are already practiced occasionally in certain locations. However, modifications to fishing, where new ideas are not usually easy to implement and take some sensitive effort to apply, should be discussed within established working groups composed of fishers, industry representatives and researchers in order to reach agreements and if possible law enforcement.

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