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

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To study the impact of purse-seine fishing on deliberately released sardine, two laboratory experiments were performed to explore the effect of net confinement for 10, 20, 40, and 60 min at 18 and 23°C. A third experiment considered two levels of fish density while confined for 20 and 40 min at 16°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 to the probability of dying (mean values of 16.3 and 2% for dead fish and survivors, respectively), and fin erosion was a long-term stress response observed in both dead and surviving fish. Time of confinement was an important stressor, with survival rates decreasing significantly with increasing periods in the net, and temperature having an additional negative effect. Density effects were less conclusive, but there was some indication that survival correlated with biological condition (heavier fish were more likely to survive). It seems that delayed mortality after release can be substantial, although death is not certain 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*, stress reactions, stressors.

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Introduction

The fate of released or slipped pelagic fish at the end of purse-seine fishing operations has been poorly studied. Although the issue has been considered for fisheries targeting mackerel (*Scomber scombrus*; Lockwood *et al*., 1983), herring (*Clupea harengus*; ICES, 2000), and sardine (*Sardinops sagax*, Mitchell *et al*., 2002; *Sardina pilchardus*, Stratoudakis and Marçalo, 2002), mortality rates are difficult to estimate because immediate mortality is generally low and the consequences to surviving escapees are difficult to evaluate (Mitchell *et al*., 2002; 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 the final stages of the operation before slipping (Pawson and Lockwood, 1980; Lockwood *et al*., 1983; Misund and Beltestad, 1995; Mitchell *et al*., 2002). However, all 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 the principles controlling collateral fishing-related mortality (Davis and Olla, 2001; Davis, 2002) because death may be delayed until some time after capture and 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 with delayed mortality resulting from capture (Davis, 2005). However, most studies of delayed fishing mortality have focused on the effects of towed fishing gears or recreational fisheries. Such activities lead to considerable changes in environmental conditions, such as air exposure or the crossing of steep thermoclines (Sangster *et al*., 1996; Suuronen *et al*., 1996; Broadhurst *et al*., 2006; Gingerich *et al*., 2007; Suski *et al*., 2007), and are therefore easier to isolate and to simulate the main stressor associated with fishing. In contrast, purse-seining is selective and targets schooling fish that are not so easy to maintain and manipulate in captivity, and the catch does not leave the water, leading to a general perception that such 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.*, 2008b) to use, for the first time, fully acclimated, unstressed fish in controlled experiments where final stages of the purse-seining operation 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) sardine, and the short- and long-term physiological and physical reactions described. Fish were observed for up to 10 d after simulated fishing, though a subsample of fish was sacrificed immediately before and after the net confinement to evaluate the level of physiological stress reaction compared with field observations from the Portuguese commercial purse-seine fishery for sardine (Marçalo *et al.*, 2006).

Material and methods

Live sardine were captured by commercial purse-seiners operating off western Algarve in southern Portugal, following the methods described by Marçalo *et al.* (2008b). The sardine used for Experiments 1 and 2 were captured in February 2007 at night and in relatively rough sea conditions (wave height $\sim$1.5–2 m), and those for Experiment 3 were captured in April 2007 in daylight (the net was set around sunrise) and in good sea conditions (wave height $\sim$0–0.5 m). Survival rates of the sardine after 2 weeks in captivity were $\sim$40 and $>$80% for fish captured in February and April, respectively. Fish were allowed to acclimate for a period of at least 2 weeks before any experiment, based on previous evidence that capture- and transfer-related mortality practically ceases within a week and that physiological equilibrium after fishing and transport stress is re-established by the second week (Marçalo *et al.*, 2008b).

Experimental set-up

Fishing simulation experiments were performed at the Aquaculture Research Station of IPIMAR in Olhão between February and June 2007 using a single rectangular 10-m^3 tank subdivided into four compartments of equal volume by removable lateral-framed panels (Figure 1a; Marçalo *et al.*, 2008b). All compartments were supplied with seawater (at ambient temperature) with an open-system water circulation and variable water flow, aerated using airstone diffusers placed at the centre of each compartment. As a conventional purse-seine cannot be set in captivity, a netting device was designed and constructed with the same mesh (18 mm) used by commercial purse-seiners operating off the Portuguese coast (Figure 1b), to allow simulation of the commercial dry-up time (Marçalo *et al.*, 2006). The fishing device was made with enough height and width to allow encirclement of the fish shoal present in each compartment. Two plastic tubes holding the net allowed the device to be placed in the back end of each compartment. Each tube was held by a technician who was responsible for sliding the tube against the lateral compartment wall and slowly pushing the whole 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, then pushed from the outside, allowing the net to be lifted and the fish confined in a purse.

The fish destined for Experiment 1 were immediately introduced into each compartment of the experimental tank after arrival at the station, and the remaining fish were held at a stocking tank located nearby. For Experiment 2, a new batch of sardine was transferred from the stocking tank, subdivided equally per compartment, and allowed to acclimate for 2 weeks. The reduced availability of fish led to there being fewer fish per compartment in this experiment (Table 1). The absence of controlled water temperature in the experimental tank led to small daily variations between Experiments 1 and 2 ($\pm$2°C). The sardine used in Experiment 3 and obtained from the second fishing trip (April 2007) were also introduced immediately into the experimental tank, but the acclimation period was longer (1.5 months), so allowing for the seasonal increase in water temperature ($>$5°C) to provide sufficient contrast to test the environmental effect.

Counting mistakes on the day of the arrival of the sardine from sea (Experiments 1 and 3), or sardine jumping from one compartment to another, caused some deviations from the initial number of fish per compartment for all experiments and treatments (Table 1). Also, for all treatments during each experiment, some sardine escaped under the simulation device (Table 1). The

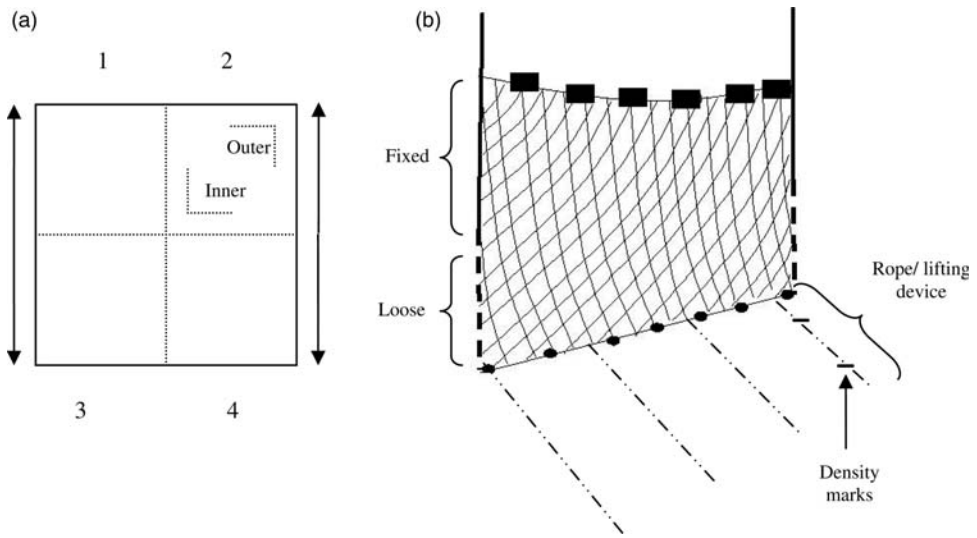

Figure 1. Diagram showing (a) the 10-m^3 subdivided tank, arrows representing the direction of operation, and (b) the netting device.

Table 1. Summary information of field and captivity operations to provide the sardine used in the three experiments.

Experiment	Date of capture	Date of experiment	Acclimation in experimental tank (d)	Observation (d)	Temperature (°C)	Factor	Treatment	n_{total}	$n_{confined}$	Mean length (cm)	Mean weight (g)	Mean condition factor
1	2 February 2007	19 February 2007	17	10	17.7	Time; low temperature	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	2 February 2007	14 March 2007	13	9	15.6	Time; density	1–20 min/low density	63	55	18.7 ± 1.8	53.9 ± 21.4	7.8 ± 1.6
							2–20 min/ high density	54	49			
							3–40 min/low density	67	61			
							4–40 min/ high density	63	48			
3	19 April 2007	5 June 2007	47	10	23.0	Time; high temperature	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			

Biological data are presented as mean ± s.e.

following sections refer in turn to each of the three experiments performed. For readability purposes, the factors tested are referred to as "time", "density", and "temperature".

Experiments

Experiment 1: the effect of simulated fishing duration at low temperature

The sardine were confined in a net for 10, 20, 40, and 60 min, based on final net-hauling times (where the purse is completely bunted to reduce the water volume and to increase the fish density sufficiently for pumping onboard) observed during commercial purse-seine operations (Marçalo *et al.*, 2006). Each compartment was treated individually, and 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 (i.e. allowed to swim freely over the "headrope" 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 (an approximate visual estimate). On the day of the experiment (day 0), before netting (pre-stress) and before slipping (post-stress), six fish were randomly sampled for blood (caught by a hand net while swimming freely—pre-stress; caught by a handnet from inside the purse—post-stress) and sacrificed for further biological analysis (more details are provided by Marçalo *et al.*, 2008a). Fish mortalities were recorded daily for 10 d. The average water temperature was 18°C.

Experiment 2: the effect of simulated fishing duration and density

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

Experiment 3: the effect of simulated fishing duration at high temperature

All the methodologies were performed according to that described for Experiment 1, but just five sardine were sampled randomly for pre- and post-stress analysis, and acclimation was longer, to investigate the environmental effect (the average water temperature was 23°C).

Laboratory sampling

Fish collected for blood sampling were retrieved from each compartment with a handnet and sedated with 2-phenoxyethanol (300 ppm). Once the 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 the fish were immediately sacrificed by severing the spinal cord behind the head. The blood was immediately refrigerated and the haematocrit values determined within 24 h by initially homogenizing at room temperature, then placing the blood sample into capillary tubes and centrifuging for 5 min at 2380 *g* in an autocrit centrifuge (Hettich EBA 20). Although blood cells become anoxic and swell if the 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 h is a common routine for analysis of blood in veterinary laboratories, including inhibiting other mechanisms (e.g. glucose consumption by blood cells; Marçalo *et al.*, 2006). After haematocrit analysis, the remaining blood samples were centrifuged at 609 *g* for 10 min, then the plasma was stored at -20°C for subsequent analysis of cortisol at the laboratory of the Lisbon Faculty of Veterinary Medicine, following the 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, and at the end of the observation period (day 9 or 10), all survivors were collected from each compartment with a handnet, sedated, and checked 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), and other standard biological parameters (sex, macroscopic maturity state, fat index, gonad mass) were also recorded (for details on sardine biological sampling, see Silva *et al.*, 2006). Fish condition was estimated from its condition factor ($F_C = 1000\, M_T/L_T^3$). Assessment of scale loss was performed by adapting the method of Main and Sangster (1990), and evaluation of the extent of caudal fin erosion followed the fin condition factor method presented in Latremouille (2003). Both these methods are described in detail by Marçalo *et al.* (2008b).

Statistical analysis

Exploratory data analysis for the fish sampled at day 0 (comparisons pre- and post-fishing, and the effect of fishing on cortisol and haematocrit) revealed some abnormally high values of cortisol. Because of the small sample sizes, removing these data by considering them to be outliers would be inappropriate, so a $\log(x + 1)$ transformation was used to stabilize the variances. To evaluate the overall effect of fishing on cortisol, haematocrit, 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 because 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 Experiment 2 to evaluate the effect of density and fishing time on cortisol and haematocrit. Terms not statistically significant were removed, and corresponding simpler models were analysed.

For the analysis of survival data, we conducted an exploratory analysis via Kaplan–Meier (KM) survival estimators (Kaplan and Meier, 1958), then implemented a Cox proportional hazards regression model (CPH; Cox, 1972). The KM survival estimators allow visual comparison of survival over time as a function of the different treatments and are presented as a function of both experiment and fishing time (the latter to visualize the temperature effect). To preclude the need for any parametric assumptions, a non-parametric bootstrap procedure (see Efron and Tibshirani, 1993, for detail of the bootstrap) was implemented, re-sampling fish within experiments and treatments, and obtaining 95% confidence intervals for the KM survival estimates. Interpretation of the CPH model is analogous to a regression model, but in this case the parameters relate to a higher or a 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 analyses (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 as well as for those which were alive at the end of the observation period, using similar *t*-tests to those described above. Although we tried to model these physical parameters as a function of the different treatments, results were not consistent, so are not presented. In general and for simplicity, we refer to statistical significant results for tests with *p*-values of <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".

Results

In all, 969 sardine (365 in Experiment 1; 247 in Experiment 2; 357 in Experiment 3) were used for the three experiments. Uncontrolled fish escapes were observed during the fishing operations, ranging (per tank compartment) from 13 to 24%, 8 to 24%, and 21 to 32%, respectively, for Experiments 1–3. There was no variation in sardine mean length across experiments (mean length 18.7 cm) or in mean weight for the first two experiments (mean weight 54.0 g). However, a considerable percentage ($\sim$30%) of fish from Experiments 1 and 2 (the first wild stock) had an uncommonly high condition factor for the season (spawning season), whereas the sardine used in Experiment 3 (the second wild stock) had a slightly lower and less variable weight, leading to a lower mean condition factor for the fish used in that experiment (Table 1).

Physiological patterns

Both physiological parameters, log-transformed cortisol and haematocrit (Figure 2), showed significant changes immediately after simulated fishing, and the direction of change agreed 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^{-1} ($n = 68$) and a median post-stress value of 7.1 μg dl^{-1} ($n = 68$). However, although median concentrations pre-fishing were only slightly lower than the mean value observed at the onset of fishing at sea (2 μg dl^{-1}; 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^{-1}; Marçalo *et al.*, 2006). Reliable haematocrit data were not obtained for three 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 35.9%; Marçalo *et al.*, 2006) were not attained, indicating that simulated fishing was sufficient to elicit a significant stress reaction, but its

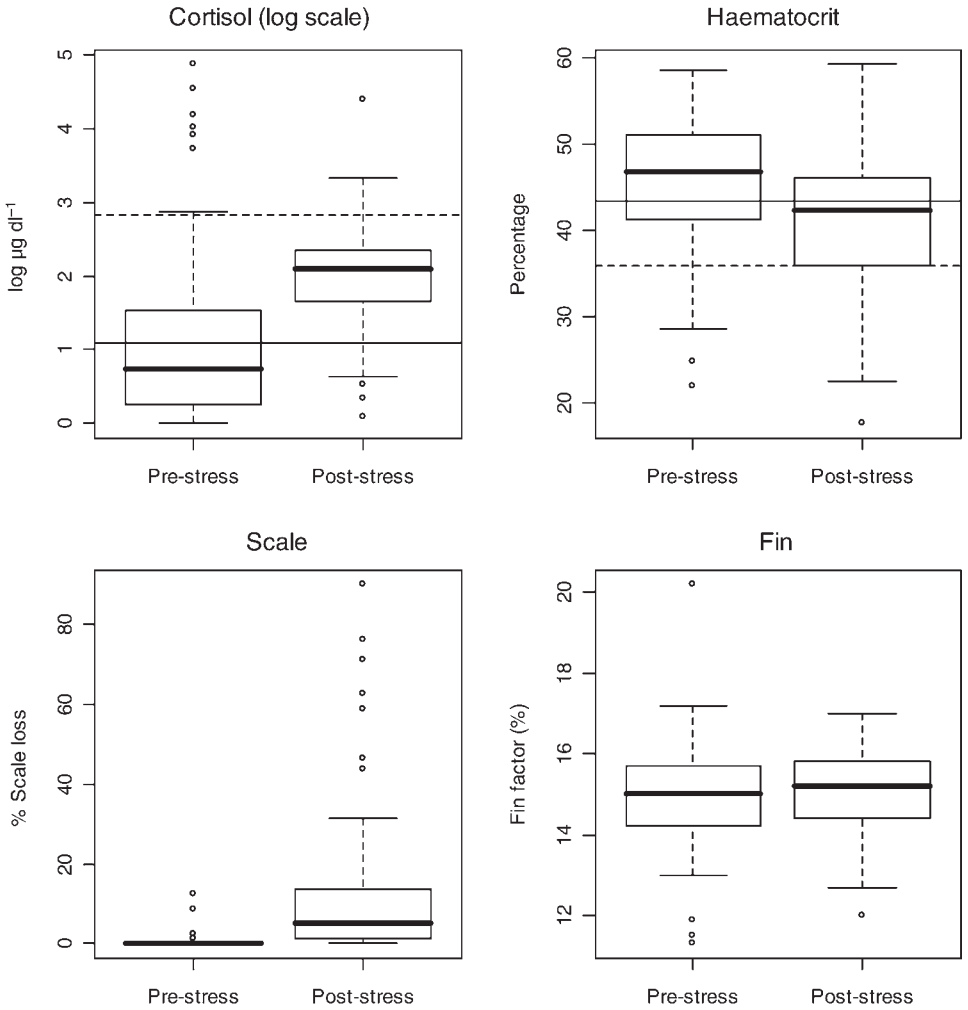

Figure 2. Boxplots of physiological parameters (cortisol and haematocrit, top row) and physical parameters (scale loss and fin factor, bottom row) as a function of pre- or post-stress condition, pooled across experiments and tanks (treatments). The box stretches from the 25th to the 75th percentile. The line across the box represents the median, and 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).

impact was possibly less acute than that of a commercial fishing operation.

Stressor effects on physiology

Simulated fishing duration and temperature

The ANOVA analysis (Table 2) used to test the effect of operational (time and density) and environmental (temperature) factors showed that for the data on simulated fishing at low and high temperatures (Experiments 1 and 3, respectively) combined, simulated fishing significantly increased cortisol ($F = 9.75$, $p < 0.001$), but had no effect on haematocrit ($F = 2.01$, $p = 0.13$), whereas 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).

Simulated fishing duration and density

The ANOVA also revealed that compared with a low density of sardine, a high density of sardine in the net led to significantly higher haematocrit ($F = 8.54$, $p = 0.008$) and no apparent effect on cortisol ($F = 2.31$, $p = 0.14$), whereas simulated fishing 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).

Physical injury

Simulated fishing led to a statistically significant increase in scale loss ($p < 0.001$), from means of 1 and 5% scale loss for pre- and post-stress, respectively (Figure 2). No significant differences were observed for the fin factor (mean of 15.0% for both pre- and post-stress fish; $p = 0.99$). As for the physiological parameters, there are no reference values available to compare the results of physical injury sustained in captivity with those of live capture. Overall, dead fish show a significantly higher scale loss than surviving fish at the end of the experiments ($p < 0.001$; Figure 3). Survivors have minimal scale loss (mean 2%), close to the scale loss observed for fish sampled before simulated fishing (mean 1%, although with some extreme cases of up to 13% loss). The differences observed in scale loss (mean 13% for fish sampled post-stress, 27% for dead fish, and 2% for survivors) indicate

Table 2. ANOVA values for operational and environmental variables affecting physiological parameters in pre- and post-fishing conditions.

Physiological parameter	Experiment	Comparison	Mean square	Value of *F*-statistic	d.f.	*p*-value
Cortisol	1 and 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 and 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

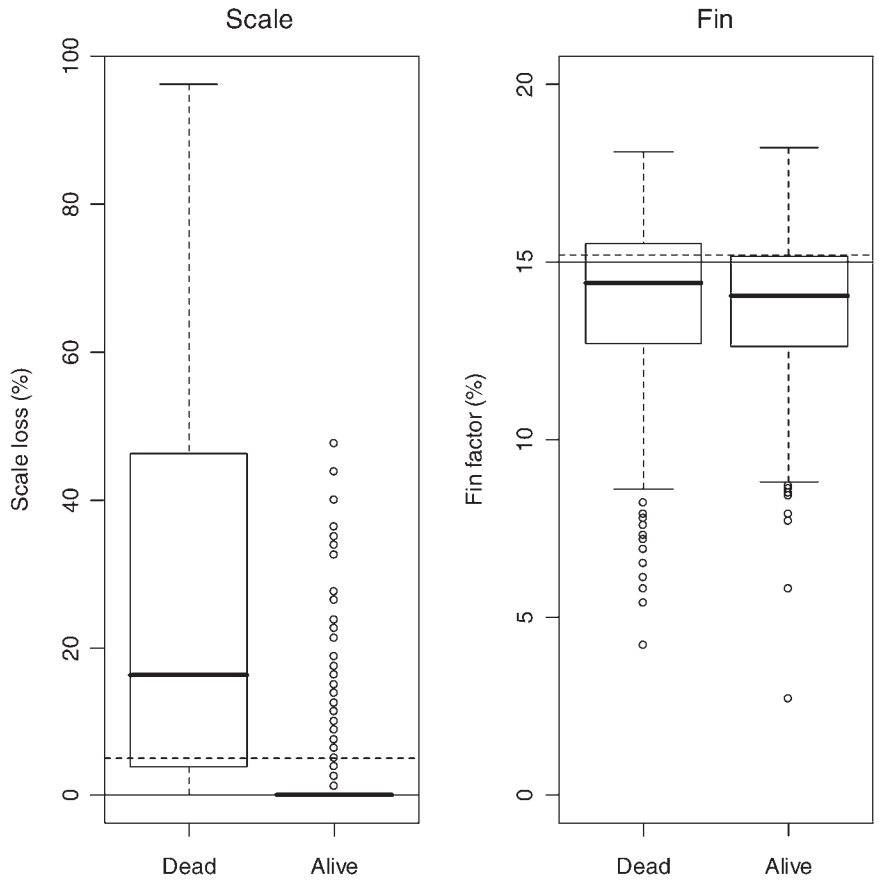

Figure 3. Boxplots of physical parameters of dead and surviving fish at termination day, pooled across experiments and tanks (treatments). The box stretches from the 25th to the 75th percentile. The line across the box represents the median, and the ends of the vertical line indicate the minimum and maximum data values. Individual points are considered outliers. Also shown are median observations for pre-stress (solid line) and immediate post-stress (dashed line).

that such loss is associated with an increased probability of dying after capture, agreeing with the observations presented in Marçalo *et al*. (2008b). The effects on the fin factor were not significant between dead fish and survivors (mean 13.6% for dead fish, 13.7% for survivors; $p = 0.68$). However, the fin factors for dead fish and survivors are smaller than the values measured on day 0 (mean 15% for both pre- and post-sampled fish), indicating a delayed fishing effect. Gradual post-capture fin erosion in dead fish and fish sampled alive after 2 weeks in captivity was also observed by Marçalo *et al*. (2008b), mainly from day 1 on.

Survival

Sardine survival in each experiment as a function of observation days is shown separately for each treatment in Figure 4. For all experiments and treatments, most mortality was within the first 5 d, followed by low daily rates of mortality in the remaining

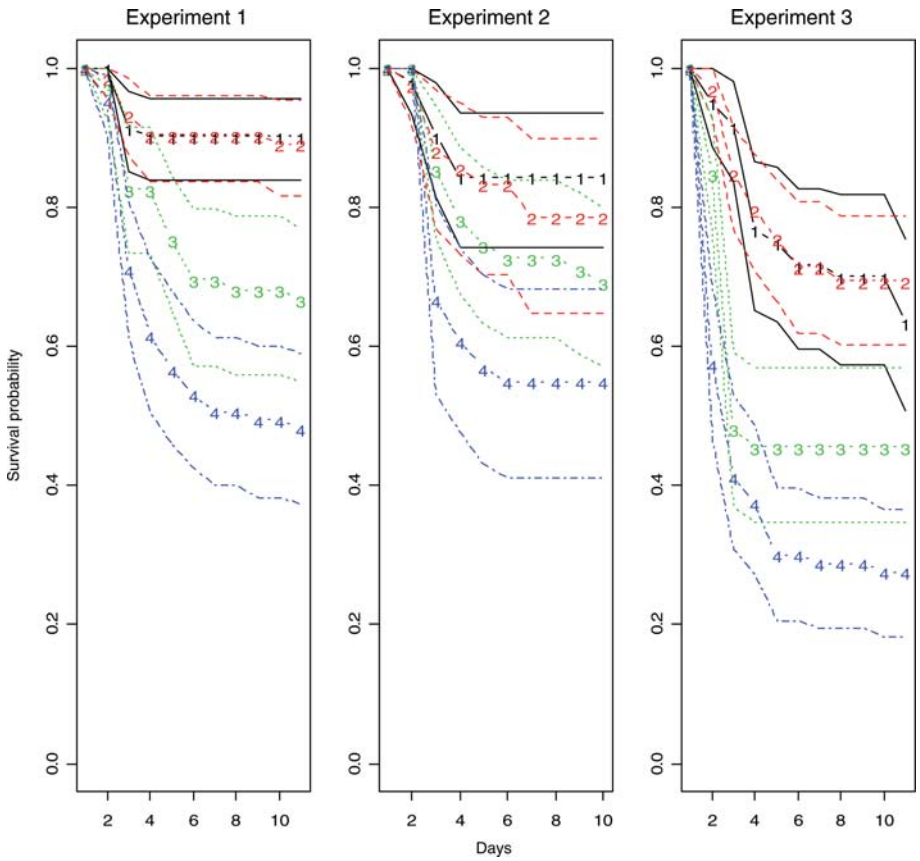

Figure 4. Treatment survival (KM survival estimators) as a function of observation day for each experiment. The solid line for 1 and dashed lines for 2–4 represent 95% confidence intervals for the survival curves obtained by a non-parametric bootstrap. Numbers refer to treatments in each experiment (more detail is provided in Table 1).

period in captivity, confirming prior observations made after commercial fishing (Marçalo *et al.*, 2008b). Also, in all experiments, simulated fishing 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 min in the net) and significantly less survival for longer simulated fishing duration (40 or 60 min, when survival could decrease to 30% after 10 d). For simulated fishing at low and high temperature (Experiments 1 and 3, respectively), there was no statistically significant difference in survival between 10 and 20 min duration, whereas mortality after 60 min was higher than after 40 min, though statistically not significant for the sample sizes and levels of variation observed. Similarly, for simulated fishing duration and density (Experiment 2), the density in the net had no effect at shorter duration (20 min), but mortality was higher for the denser bunting for longer duration, although again the difference was not statistically significant. Finally, the survival curves plotted separately for each simulated fishing duration across experiments (Figure 5) show a significant temperature effect on survival that is consistent across durations; survival at 23°C is ∼20% less than that at lower temperatures (16 or 18°C), at any level of simulated fishing duration.

Survival was modelled as a function of simulated fishing duration (net confinement), water temperature, fish density, and fish weight (Table 3), to explore the relative influence of these factors. The results of the analysis confirm that increasing simulated fishing time and water temperature increase the risk of death, but also that high density contributes to an increased risk (although statistically not significant, likely because of the small sample size used). There is also evidence that heavier (larger and more robust) fish have increased survival probabilities (Figure 6). Surviving fish at the termination of the experiment were significantly heavier ($p < 0.001$) than fish that died [mean weight of dead fish 40.9 g ($n = 297$) and of survivors 51.9 g ($n = 536$)]. This conclusion is further supported by analysing the condition factor over time (all fish pooled across treatments and experiments; results not presented), which shows that the sardine sacrificed at day 0 (a random sample, $n = 136$) had significantly higher condition factors than those that died during their first day in captivity ($n = 131$, $p < 0.001$), suggesting that physically more fit sardine are less likely to die.

Discussion

The results of this study have demonstrated that using acclimated sardine in purse-seine fishing simulations can permit an assessment of the impact of operational, environmental, and biological variables on the survival and physiological and physical conditions of slipped pelagic fish. Off Portugal, commercial purse-seining operations before bunting (e.g. school pursuit, net shooting, and hauling) constitute a stressful event that can exceed 1.5 h (Marçalo *et al.*, 2006). In these laboratory experiments, only the final part of the operations, when the net is fully bunted, was simulated because previous studies have indicated that this is when most of the stress and physical damage arise through increased

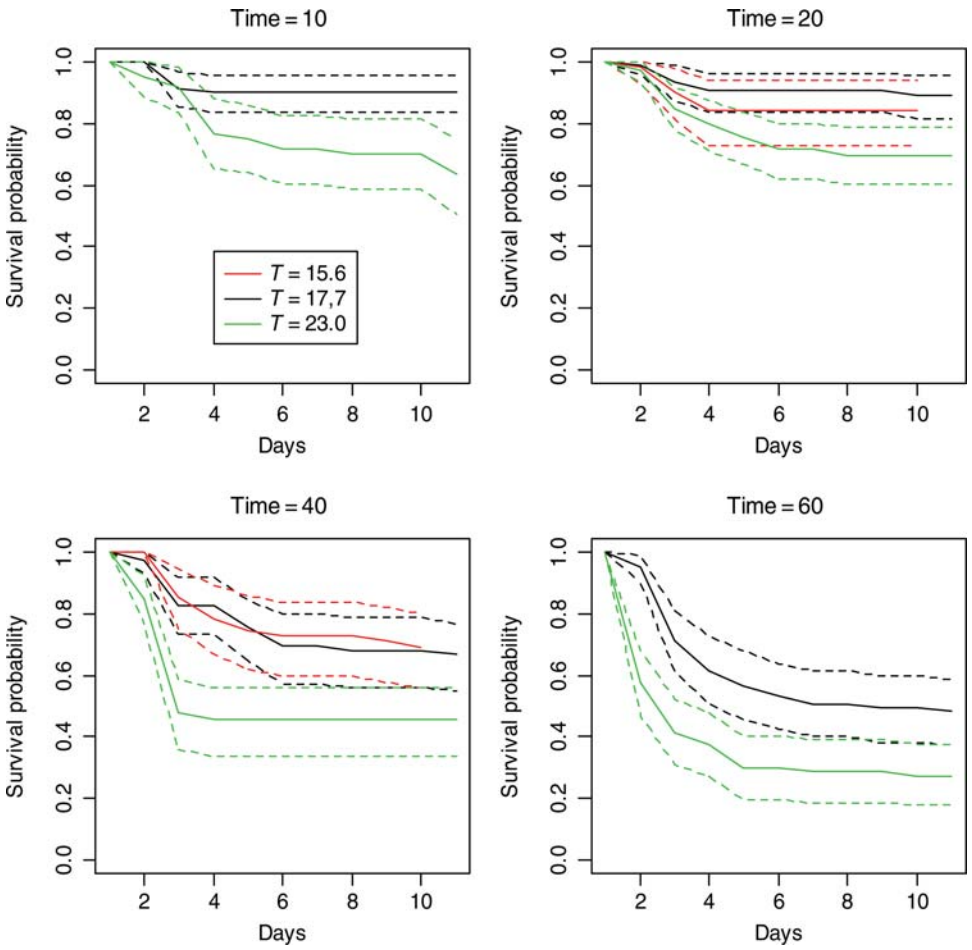

Figure 5. Experiment survival (KM survival estimators) as a function of observation day for each fishing time treatment level (in min), showing a temperature effect. Dashed lines represent the 95% confidence intervals for the survival curves obtained by a non-parametric bootstrap.

Table 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.

Factor	Coef (s.e.)	Exp(coef)[a] (95% *CI*)	*p*-value
Time (20 min)	−0.068 (0.229)	0.934 (0.596, 1.464)	0.77
Time (40 min)	1.012 (0.215)	2.750 (1.804, 4.192)	$<10^{-5}$
Time (60 min)	1.460 (0.206)	4.304 (2.873, 6.448)	$<10^{-5}$
Temperature (17.7°C)	−0.407 (0.244)	0.666 (0.412, 1.075)	$<10^{-5}$
Temperature (23.0°C)	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
Total weight	−0.061 (0.006)	0.941 (0.931, 0.951)	$<10^{-5}$

[a]A value of Exp(coef) of 1 indicates the absence of a change in a hazard attributable to a given variable, a value >1 means a larger risk, and a value <1 a lower risk (or protection factor) associated with the corresponding variable.

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 sardine before 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 to captivity. The levels of cortisol increased significantly immediately after simulated fishing, but did not attain the concentration observed at the end of purse-seine 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 attributable 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 was in line with the patterns observed previously for the species in the wild (Figure 2), in which a significant increase in cortisol and a decrease in haematocrit during commercial fishing have been reported (Marçalo *et al.*, 2006). Cortisol rise is a common primary response of fish subjected to stressful conditions, and haematocrit increases in most teleosts to facilitate oxygen transportation and to reduce cardiac costs (Wood *et al.*, 1983; Barton

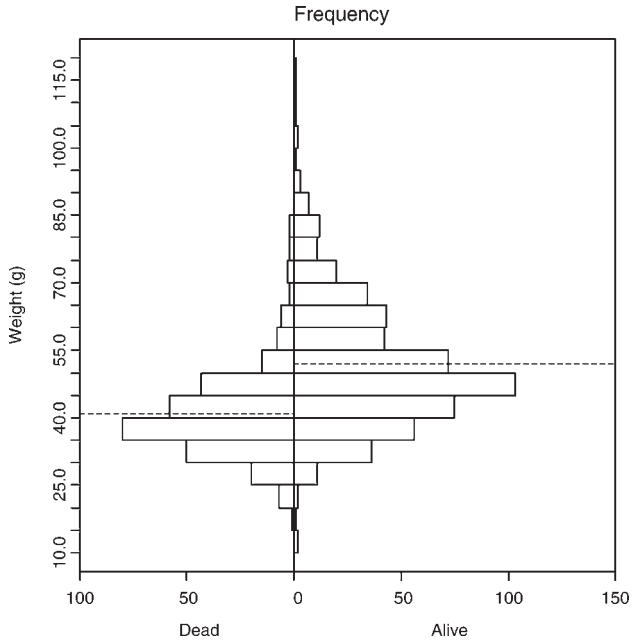

Figure 6. Weight frequency distributions of dead and surviving fish at the termination of the experiment. The horizontal dashed lines represent the mean weight.

and Iwama, 1991; Bonga, 1997). Such a response has been noted for several species, notably small pelagic fish such as Atlantic mackerel (*S. scombrus*; Swift, 1982; Boutilier *et al.*, 1984). The contradictory observed decrease in haematocrit in sardine (as shown by Marçalo *et al.*, 2006, 2008b) may be similar to observations by Bourke *et al.* (1987) of skipjack tuna (*Katsuwonus pelamis*) that suffered increased blood volume and osmolarity in the first hours post-capture. For sardine, we can suggest at this stage that the main cause of a haematocrit decrease is related to the potential for fluid (water) to shift between tissues and the blood (here, the osmotic shift of water out of the muscle intracellular compartment into the extracellular fluids/blood plasma compartment), which is caused by increased permeability of the gills under enhanced stress (Wood *et al.*, 1983; Wood, 1991). Further, the mechanism can be the result of sodium and chloride entering from the external seawater faster than they can be pumped out, raising plasma osmotic pressure, and creating in turn an internal osmotic imbalance. This then draws water out of the muscle intracellular compartment into the extracellular fluids/blood plasma compartment and blood plasma, so diluting the red blood cells and decreasing the haematocrit.

Post-fishing stress levels in sardine as measured by cortisol and haematocrit were not proportional to the magnitude of the stressors (operational and environmental factors tested in the experiments). The sardine exposed to simulated fishing at low and high temperatures (Experiments 1 and 3, respectively) did experience a duration effect for cortisol but not for haematocrit, and there was no temperature effect on any of the physiological variables. In terms of simulated fishing and density (Experiment 2), high density had a significant effect on haematocrit but not cortisol, whereas fishing duration had no effect on either variables in this experiment. The lack of correspondence within physiological stress levels and stressor intensity, taking into account the observed levels of mortality, indicates that physiological stress induced by net-confinement, density, and elevated water temperature were not useful predictors of potential delayed mortality, indicating just a stress reaction. As stated by Davis *et al.* (2001), caution should be taken when using physiological variables to evaluate stress that ultimately results in mortality. Nevertheless, failure to induce the post-stress levels in the laboratory similar to those attained for the same parameters in the field (here mostly attributable to the absence of full simulation of field conditions or absence of predators), or a 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 behaviour, reproductive, and immune system effects), should be investigated further to understand delayed mortality.

In all experiments, delayed mortality mainly took place in the first 3–4 days post-fishing, a pattern similar to that described in previous studies on the species during capture, transport, and preliminary observation in captivity (Olmedo *et al.*, 1990; Mitchell *et al.*, 2002; Marçalo *et al.*, 2008b). 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 rather 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 their time of retention inside the net was increased in the three experiments. Some mortality was observed even for a very short holding duration (10 min), but most deaths followed holding times of 40 or 60 min. Before the fishing simulations, the sardine in the tank compartments were mortality-free for several days, so 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 proposed as the main causes of stress build-up and physical damage, so 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 greater fish density.

Fishing simulations at 23°C reduced survival rates by ~20% for all treatments when compared with experiments performed at 16 or 18°C. Higher water temperatures lead to physiological changes such as increase in cardiac outputs and a decline in fish performance and are 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°C 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 temperature. Such temperatures may be experienced during some fishing events in the southern regions of the sardine's range, i.e. during summer off southern Portugal, in the Mediterranean Sea, or off the coast of northwest Africa (Coombs *et al.*, 2006; Ganias *et al.*, 2007).

The CPH model indicated that heavier (physically fitter and in better condition) fish had a lesser probability of dying after fishing. This is in line with the notion that smaller fish tire faster and are more susceptible to injury 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 a higher condition factor) are less likely to die may also be considered a factor that confounded the results of Experiment 3. Although higher water temperature is suggested as the most probable cause of decreasing survival rates, we cannot eliminate the theory that lower condition factor (favoured by decreased weight) of the fish used in this experiment may have led to less survival. Despite this, earlier observations during capture, transport, and maintenance of the species in captivity indicated that water temperature during operations was among the most important variables influencing survival rates (Marçalo *et al.*, 2008b), whereas other laboratory observations indicated that sardine acclimation at lower water temperature reduced post-capture immediate and delayed mortality (AM, unpublished data).

The comparison of the physical condition (scale loss and fin erosion) between fish before and after fishing (Figure 2) indicated that simulated fishing significantly increased scale loss, a process expected because of the abrasion suffered by fish during the final stages of purse-seining. The limited scale loss of survivors (mean 2%) at the end of the observation period compared with the extensive scale loss of fish that died after slipping (mean 27%) demonstrated that this physical parameter is closely associated with the probability of dying after release. Further, the importance of and trends in scale loss after capture have been discussed previously during experiments on the introduction of the species in captivity (Marçalo *et al.*, 2008b), where the observed reduction in scale loss with time in captivity indicated that fish with greater scale loss died earlier than those with limited loss. Looking into the daily evolution of the fin factor pooled across all fish (pre- and post-stress, dead and survivors; data not shown), we observed that fin erosion decreased up to day 6 and then increased, but never reached the values observed at simulated pre-fishing. This pattern was also observed by Marçalo *et al.* (2008b) for both dead and surviving fish, with dead fish having less fin erosion at all times. Therefore, trends in fin erosion showed that this parameter is a long-term stress response only revealed days after the fishing stressor, and most likely related to immunity suppression and caused by bacterial infection (Latremouille, 2003).

Our results have shown that a longer duration of the final purse-seine operation (when the net is completely bunted) and fishing at higher sea surface temperatures reduce the probability of survival of the fish slipped. Although the primary physiological stress reactions did not seem to correlate with the probability of survival, future work should focus on tertiary stress reactions (such as decreased resistance to disease) or indirect stress effects (such as behavioural impairment) to explore their importance in delayed mortality. As an example, ongoing work on predator–prey interactions after slipping revealed impairments related to sardine shoal formation, swimming speed, and predator–prey distances that may increase vulnerability to predation and be an additional source of unobserved mortality after deliberate release from purse-seines (Marçalo *et al.*, 2008a). Physical injuries, especially scale loss, were among the most important causes of death, so modifications to commercial fishing practice that may reduce abrasion could lead to a higher probability of survival for slipped fish.

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