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RESEARCH ARTICLE

# Temperature-dependent metabolic consequences of food deprivation in the European sardine

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## ABSTRACT

Aquatic ecosystems can exhibit seasonal variation in resource availability and animals have evolved to cope with the associated caloric restriction. During winter in the NW Mediterranean Sea, the European sardine *Sardina pilchardus* naturally experiences caloric restriction owing to a decrease in the diversity and quantity of plankton. However, ongoing global warming has had deleterious effects on plankton communities such that food shortages may occur throughout the year, especially under warm conditions in the summer. We investigated the interactive effects of temperature and food availability on sardine metabolism by continuously monitoring whole-animal respiration of groups of control (fed) and food-deprived sardines over a 60-day experiment in winter (12°C) or summer (20°C) conditions under natural photoperiod. In addition, we measured mitochondrial respiration of red muscle fibres, biometric variables and energy reserves of individuals sampled at 30 and 60 days. This revealed that winter food deprivation elicits energy saving mechanisms at whole animal and cellular levels by maintaining a low metabolism to preserve energy reserves, allowing high levels of survival. By contrast, despite energy saving mechanisms at the mitochondrial level, whole animal metabolic rate was high during food deprivation in summer, causing increased consumption of energy reserves at the muscular level and high mortality after 60 days. Furthermore, a 5-day re-feeding did not improve survival, and mortalities continued, suggesting that long-term food deprivation at high temperatures causes profound stress in sardines that potentially impairs nutrient absorption.

**KEY WORDS: Bioenergetics, Red muscle, Mitochondria, Respirometry, Global warming, Small pelagic fish**

## INTRODUCTION

Small pelagic fish are ecologically essential because of their central position in the food web (Cury et al., 2000), and are economically important as a fishery resource for humans (Pikitch et al., 2014). In the Mediterranean Sea, the European sardine, *Sardina pilchardus*, is the most emblematic small pelagic fish species owing to its abundance and the associated large fishery industry. Sardines feed on a variety of prey, from small phytoplankton to large zooplankton (Costalago and Palomera, 2014; Rumolo et al., 2016). In this temperate zone, the composition of plankton communities varies seasonally, dominated by small primary producers in winter and by large phytoplankton and zooplankton species in summer (Romagnan et al., 2015). Moreover, in winter, plankton productivity is limited, with low plankton biomass in surface waters (Auger et al., 2014). Consequently, in winter, sardines are likely to endure periods of natural caloric restriction, which could be associated with periods of obligatory food deprivation. The Mediterranean Sea is oligotrophic and anthropogenic climate change may exacerbate this (Irwin and Oliver, 2009) by decreasing primary production (Auger et al., 2014) and plankton diversity (Boyce et al., 2010) or by changing the phenology of plankton growth (Hordoir and Meier, 2012), especially in summer (Adrian et al., 2006; Lewandowska et al., 2014; Suikkanen et al., 2013). This would then lead to an increased likelihood of caloric restriction for sardines because of a decrease in the size of their prey. Small plankton species are probably less energetic than larger species (Zarubin et al., 2014) and, in sardines, small plankton also cause a shift in foraging mode from prey capture to filter-feeding (Costalago et al., 2015; Garrido et al., 2007). In captive sardines, we have shown that this foraging shift triggers a decline in body condition and growth, in association with the development of mechanisms of energy sparing at the level of mitochondria (Thoral et al., 2021b). Negative effects of global warming on plankton community biomass and diversity could cause sardines to face caloric restriction throughout the year, with profound consequences for their energy balance. This situation should create an energy imbalance between increased metabolic demands of planktivorous fish (owing to higher temperatures) and decreased plankton resources (Hays et al., 2005), which could in turn compromise fish's survival. These modifications of plankton communities may be a major explanatory factor for the disastrously reduced sardine populations in the Gulf of Lions in the NW Mediterranean Sea (Saraux et al., 2019; Van Beveren et al., 2014).

Food deprivation in a warm environment should represent a major energetic challenge for ectothermic organisms such as fishes (Auer et al., 2016), because temperature will raise their metabolism and energy demands (Schulte, 2015). Mitochondrial metabolism, which produces the majority of cellular energy (ATP), increases with temperature. Warming could lead to higher energy wastage from increased proton leakage (i.e. LEAK respiration) and/or to a decrease in mitochondrial efficiency for ATP synthesis (Pichaud et al., 2019; Pörtner et al., 1999; Roussel and Voituron, 2020; Thoral et al., 2021b). On the other hand, food deprivation elicits a decrease in LEAK respiration in fish, which is associated with an increase in mitochondrial coupling efficiency (Salin et al., 2018). If we assume

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that high temperature would impede the decrease in LEAK respiration during food deprivation, which is mandatory to reduce energy expenditure and promote survival, then an interaction between hot temperature and food deprivation could lead to harmful physiological consequences for fishes, including the sardine.

We investigated the combined physiological effects of food scarcity and seasonal temperature, by measuring the consequences of food deprivation for 30 or 60 days in sardines at 12°C (winter temperature) and 20°C (summer temperature) for individual survival and for aerobic metabolism at the whole-animal and cellular level. To do so, we measured whole-animal metabolic rate as rate of oxygen uptake as well as mitochondrial bioenergetics in red muscle, a highly aerobic tissue in fishes (Rome et al., 1988). Protein and lipid levels were also assessed in white muscle to estimate whole-animal energy reserves. In addition, extended food deprivation could eventually impair the capacity to digest and assimilate food (Zaldúa and Naya, 2014). Therefore, sardines were re-fed after 30 and 60 days of food deprivation, while monitoring mortality rates as a proxy of recovery from starvation.

## MATERIALS AND METHODS

### Fish and experimental design

Sardines [*Sardina pilchardus* (Walbaum 1792)] were captured off Sète (France) by a purse seine in spring and acclimated at the Ifremer station of Palavas-Les-Flots for 1 month following the procedures detailed in Queiros et al. (2019) to undergo bacteriological and viral analyses and to wean fish from live prey to commercial aquaculture feed. Fish were maintained in an outdoor 4.5 $m^3$ tank for 3 months and then in an indoor 3 $m^3$ tank for an additional 4 months, tanks were supplied with water directly pumped from the sea and filtered through sand filters. All experimental protocols complied with French and EU legislation regarding animal experimentation and were approved by the regional ethics committee for animal experimentation no. 36 (Languedoc-Roussillon) (project #10622).

Water temperatures were not controlled except to maintain a minimum of 10°C in winter and maximum of 25°C in summer. The photoperiod was adjusted weekly to follow the natural cycle. Sardines were fed twice a day with commercial pellets, rations were adjusted according to prevailing temperature in order to maintain a body condition close to 1, based on previous experiments and monthly biometries (daily feeding rates of 0.3% and 0.6% of fish mass at 12°C and 20°C, respectively). To allow for individual identification, a Tiny-tag (Biolog-id, Bernay, France, 0.03 g i.e. <0.5% of sardine lowest body mass) was implanted in the dorsal muscle for individual identification, under anaesthesia (140 ppm benzocaine); all sardines survived the procedure and rapidly recovered normal behaviour.

At experimental onset, 304 sardines were distributed into eight experimental 90 liter tanks (38 sardines per tank), to obtain a homogenous size and mass distribution among tanks (body mass=14.17±2.53 g; body length=120.52±5.44 mm; means±s.d.). Prior to transfer, sardines were anaesthetized and total body length and mass were recorded (to the nearest 0.1 mm and 0.01 g, respectively).

This transfer was followed by 10 days of acclimation to the new tanks before the food deprivation experiment started. Sardines were divided in four treatments (see Fig. 1), with two replicates per treatment: (i) control sardines fed twice a day at 12°C (FED12); (ii) control sardines fed twice a day at 20°C (FED20); (iii) sardines food deprived at 12°C (FAS12); and (iv) sardines food deprived at 20°C (FAS20). The chosen temperatures were reached after gradual daily increase (+ ∼2°C $day^{-1}$) from 13°C to 20°C for FED20 and FAS20, or decrease from 13°C to 12°C for FED12 and FAS12

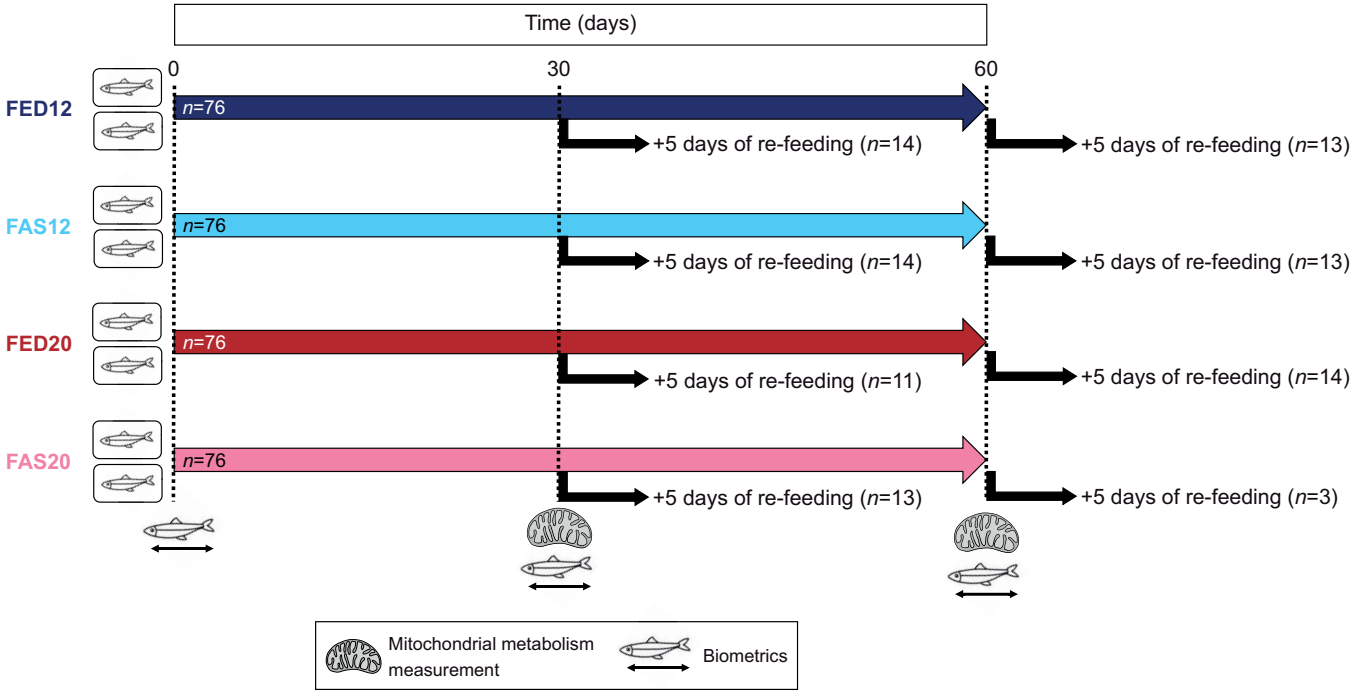


**Fig. 1. Experimental protocol with four treatments in duplicate.** Control sardines were fed twice a day at 12°C (FED12), or at 20°C (FED20), another group of sardines were food-deprived at 12°C (FAS12) or 20°C (FAS20). Some fish from food-deprived and control groups were re-fed for 5 days after 30 or 60 days of food deprivation. Other fish were removed from their respective tanks at 30 or 60 days to measure the mitochondrial metabolism in red muscle samples. $\dot{M}_{O_2}$ was measured between 0 and 60 days. Biometrics were performed at 0, 30 and 60 days and mitochondrial metabolism measurements were done after 30 and 60 days.

groups (−0.5°C day$^{-1}$). These temperatures of 12 and 20°C were chosen because they occur in the Mediterranean Sea in winter and summer, respectively (Feuilloley et al., 2020; Teulier et al., 2019; Thoral et al., 2021a). More information about sardine holding can be found in the supplementary Materials and Methods. Biometrics were recorded at day 0, 30 and 60 by weighing each individual. The relative change in body mass per day between 0 and 30 days of experiment and between 30 and 60 days of experiment was calculated as follows:

$$\%\ \text{Change in body mass} = \left(\frac{\Delta\text{Body mass}}{\text{Initial body mass}\times\Delta t}\right)\times 100. \quad (1)$$

All tanks were checked twice daily for mortalities. To assess the potential of food-deprived sardines to restart feeding after 30 and 60 days, 5 to 8 fish per tank (11–14 fish per treatment) were transferred to a new tank, where they were fed for 5 days; transfer of fed groups controlled for any effects of handling. Given the high mortality in the FAS20 group, only 3 sardines were re-fed in this group after 60 days of food deprivation. The experimental treatment and assays extended from 25 January to 10 April.

### *In vivo* oxygen consumption

*In vivo* group metabolism was measured as oxygen consumption by the sardines in their custom-designed holding tanks, using automated stop–flow respirometry (Steffensen, 1989) modified for open tanks (McKenzie et al., 2007, 2012), exactly as described in Queiros et al. (2021). Briefly, this system alternated periods in a cycle comprising 30 min when the tanks were aerated and received a water supply versus 30 min when water supply and aeration were stopped, and the fish consumed the oxygen in the water. Water $O_2$ saturation never fell below 80% during the 30 min of closed respirometry and was rapidly restored when the tanks received a flow of aerated water. Water was mixed constantly in the tanks so there was a linear decline in water oxygen concentration owing to consumption by the sardines. This was recorded continuously by oxygen probes (Firesting O2, PyroScience, Germany) and associated software (PyroScience Oxygen Logger). Oxygen consumption ($\dot{M}_{O_2}$) was then calculated using a custom script in R software, to transform oxygen saturation (in %) into oxygen concentration, based upon established values of oxygen solubility as a function of temperature and salinity (measured twice a day in each tank). The slopes of decreasing oxygen concentration over time were estimated through a linear model using an automated R script. The first and last minute of measurement were removed before estimating the slopes, and only slopes with $R^2$>0.8 were retained. Measurements collected during fish handling, feeding or any intervention on the tanks were removed. Thus, only routine respiration was considered, including spontaneous activity and the postprandial period. The $\dot{M}_{O_2}$ was calculated in mg $O_2$ h$^{-1}$ g$^{-1}$ of fish, from the decline in water $O_2$ concentration and considering the total volume of water and the total biomass of the fish (McKenzie et al., 2007, 2012). The changes in the total biomass of each tank were taken into account in $\dot{M}_{O_2}$ calculations. The cyclical measures were then summed up into daily median rates of oxygen uptake. Because of the data distribution, the number of cycles measured per day was not homogenous (owing to interventions in tanks etc.) and the number of fish decreased over the experiment, we therefore used the medians of respiration only for days when more than 5 cycles were measured and more than 12 fish were present. This threshold was chosen because when the number of individuals was lower than 12, the biomass was too low to provide consistent slopes with $R^2$>0.8.

### Dissection and red muscle mitochondrial respiration

After 30 and 60 days of food deprivation±2 days, 7 to 8 sardines were removed from each tank, sacrificed by anaesthetic overdose (1000 ppm benzocaine), weighed (body mass is shown in Table 2) and rapidly dissected to measure muscle composition and mitochondrial bioenergetics. These measurements were also done at day 0 and data are presented in Table S1.

A small sample of red muscle (5.67±1.20 mg) was taken on the midline of the flank, weighed and rapidly placed into ice-cold buffer MiR05 (0.5 mmol l$^{-1}$ EGTA, 3 mmol l$^{-1}$ $MgCl_2$, 60 mmol l$^{-1}$ K-lactobionate, 20 mmol l$^{-1}$ taurine, 10 mmol l$^{-1}$ $KH_2PO_4$, 20 mmol l$^{-1}$ HEPES, 110 mmol l$^{-1}$ sucrose, 1 g L$^{-1}$ free fatty acid bovine serum albumin, pH=7.1 at 30°C, Kuznetsov et al., 2008), for immediate use in mitochondrial respiration measurements. Fish were then dissected to assess sex, maturity stage (97% of fish were mature) and total mass of red muscle and white muscle. White muscle samples were stored at −80°C for subsequent analyses. Gonad mass and proportion are reported in Table S2.

Respiration of red muscle fibres was measured using Oxygraph-2 K high resolution respirometers (Oroboros® Instruments - WGT Austria), with measurements carried out at the relevant acclimation temperature of the fish. Muscle samples were transferred to respirometer chambers containing 2 ml MiR05, and mitochondrial function was measured at the acclimation temperature, with a substrate-uncoupler-inhibitor titration protocol adapted from Teulier et al., 2019. Briefly, glutamate (10 mmol l$^{-1}$), malate (2.5 mmol l$^{-1}$), ADP (1 mmol l$^{-1}$) and succinate (5 mmol l$^{-1}$) were sequentially added to obtain the phosphorylation respiration rate (i.e. OXPHOS respiration). Oligomycin (1.25 µg ml$^{-1}$) was then added to inhibit ATP synthase to obtain the basal non-phosphorylating respiration rate (i.e. LEAK respiration). Antimycin A (22.5 µmol l$^{-1}$), a complex III inhibitor, was then added in excess to fully inhibit the mitochondrial respiration in order to measure any residual non-mitochondrial respiration. All measured respiration rates were then corrected for this residual respiration. Integrity of the outer mitochondrial membrane was checked with the addition of cytochrome *c* (10 µmol l$^{-1}$) by verifying an absence of stimulation of the respiration after this injection (Teulier et al., 2019). As the mean effect of the cytochrome was the same for all study groups, no data were removed from the analyses. All measured mitochondrial variables were normalized to body mass for statistical analyses.

### Protein and lipid assays

Proteins and lipids were assayed on white muscle samples after being frozen at −80°C, with the total amount of lipid and protein expressed in mg g$^{-1}$ wet fish. As described in Brosset et al. (2015), lipids were extracted from ∼0.15 g white muscle using a solvent mixture (chloroform:methanol 2:1 v/v) (Folch et al., 1957). Extracts were then spotted on SIII chromarods (Iatron Laboratories) to separate lipid classes (aliphatic hydrocarbons, sterol and wax esters, ketones, triacylglycerols, free fatty acids, free fatty alcohols, free sterols, diacylglycerols, acetone mobile polar lipids and phospholipids) in three successive developing baths in solvents of increased polarity. Chromatograms were then analysed using the Peak Simple software and lipid classes quantified using a standard calibration curve obtained for each lipid class. All experiments were done by the same person, with one replicate of each sample. The proportion of free fatty acids

(FFAs) was checked, and all samples containing more than 10% FFAs were removed from analyses (out of a total of 172 assays, 8 assays with ∼12% FFAs were removed; Brosset et al., 2015) as this may be a sign of sample degradation during storage. All lipid classes were then summed to obtain total lipids.

As detailed in Queiros et al. (2019) for protein assays, ∼10 mg of muscle samples (10.25±0.54 mg) were lyophilized and ground with a ball mill (MM400, Retsch GmbH, Germany) then immersed in a 1.5 ml solution containing 10% SDS (Sigma Aldrich, France), 1.5% protease inhibitor cocktail (cOmplet, Sigma Aldrich, France), milliQ water solution (lysis solution adapted from Campus et al., 2010) and subjected to four cycles of 15 min in an ultrasonic bath (300 Ultrasonik, Ney Company, USA) alternating with 3 min vortex. Extracts were clarified for 10 min at 3000 ***g*** at 4°C and protein content quantified by the BCA method (Pierce, Thermo Fisher Scientific, France). All samples were measured in duplicate, and intra- and inter-plate protein variations were 3.1% and 5.0%, respectively.

### Statistical analysis

Effects of food deprivation, temperature and time were evaluated for all physiological and morphometric characteristics by linear-models (LMs) or linear mixed-models (LMMs) to account for dependency of the data when there was more than one measure per individual (i.e. for analyses of the relative change in body mass). Temperature (12°C or 20°C), treatment (control or food deprivation), time (30 or 60 days) as well as their triple interaction and the three double interactions were included in models as fixed effects. The model was then simplified according to a stepwise procedure by removing non-significant interactions and fixed effects (only when not involved on any remaining interactions). Groups were then compared through *post hoc* tests, using the emmeans or glht function, when any of the interactions were significant ($P<0.050$). Normality of residuals was tested using a Shapiro–Wilk test. When normality assumptions were not met, data were transformed using a monotonous positive transformation: the Box Cox transformation. Normality was then re-tested on residuals and the model run with the transformed data. When results from analyses on transformed data were similar to those obtained on raw data, results on raw data were kept for clarity purposes.

Survival analyses were performed based on the distribution of survival times using the Cox proportional-hazards regression model (Lin and Wei, 1989) to assess the effect of temperature and food deprivation on fish survival over 60 days. Data from re-feeding are presented as % survival over the 5 day period, with no statistical analyses owing to small sample sizes. After pooling the respiration data between 0 and 30 days and between 30 and 60 days, we compared dependence of metabolic rates on temperature, treatment and periods, and their interactions, with linear mixed-models and *post hoc* analyses. The level of significance was $P<0.050$. Values are presented as means ±s.e.m. Statistical analyses were performed in R v. 4.0.3 using the packages lme4 (https://CRAN.R-project.org/package=lme4), lsmeans (https://CRAN.R-project.org/package=lsmeans) and multcomp (https://CRAN.R-project.org/package=multcomp).

## RESULTS

All model results are presented in Table S3.

### Survival rate

As seen in Fig. 2, both high temperature (odds ratio=6.035; $CI_{95}$=[2.519–14.460]) and food deprivation (OR=3.344, $CI_{95}$=[1.619–6.907]) had a detrimental effect on survival, which was significantly lower after 60 days of food deprivation at 20°C (44%) compared with the other groups (FED20, 86%; FAS12, 94%; FED12, 98%). Although we did not perform statistical analyses because of small sample sizes, it appears that re-feeding sardines after a period of 30 or 60 days of food deprivation did not improve survival (Table 1). Rather, food-deprived sardines transferred to another tank for re-feeding appeared to have even lower survival rates than the sardines remaining without food in their tank, an effect that did not occur in control fish and might have been exacerbated at high temperature.

### Metabolic rates

The metabolic rates of sardines in their tanks are shown in Fig. 3. Regardless of the period, metabolism at 20°C was higher than at 12°C, both in fed ($P<0.001$) and food-deprived fish ($P<0.001$). An effect of food deprivation was only visible at 20°C between 30 and 60 days, when FAS20 metabolic rate was lower than FED20 ($P<0.001$). Finally, the respiration of FED20, FAS12 and FAS20 increased over the two periods (FED20: $P<0.001$; FAS12: $P=0.065$; FAS20: $P<0.001$).

### Biometric characteristics

Regardless of the time period, food deprivation significantly decreased body mass at both temperatures ($P<0.001$; Table S3), with total and daily mass loss significantly higher in FAS20 fish compared with FAS12 fish ($P<0.001$, Table 2 and Fig. 4).

Similarly, food deprivation decreased absolute red muscle mass ($P<0.001$) but not the ratio of red muscle mass to body mass (Table 2). Absolute and relative red muscle mass were also lower overall at 20°C than at 12°C, regardless of nutritional status or time period ($P<0.001$, Table 2).

White muscle absolute and relative mass were also negatively affected by food deprivation and temperature (Table S3), both being lower at 20°C compared with 12°C after 30 days of food deprivation ($P<0.05$ and $P=0.063$, respectively; Table 2). These variables were also lower in fed fish at 20°C after 60 days compared with 30 days ($P<0.05$ and $P<0.05$, respectively).

The proportion of proteins in white muscle was negatively affected by food deprivation ($P<0.001$) and decreased overall at 20°C compared with 12°C ($P<0.01$, Table S3). It was also lower after

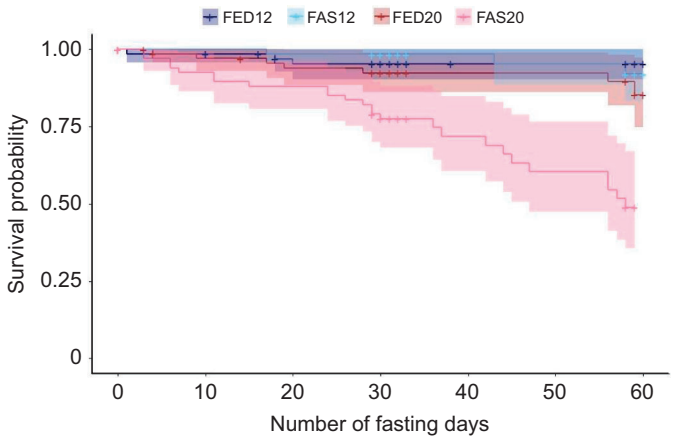


**Fig. 2. Survival of fed and food-deprived sardines at 12°C and 20°C during the 60 days of experiment.** All groups are represented, with the fed fish at 12°C (FED12, dark blue) and 20°C (FED20, red) and the food-deprived fish at 12°C (FAS12, pale blue) and 20°C (FAS20, pink). The crosses represent individuals that were taken for mitochondrial measurements, as well as those placed on the re-feeding regimen, i.e. for which survival was not measured until the end of the experiment.

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**Table 1. Survival rate of sardines when re-fed after food deprivation at 12°C or 20°C**

| | Fed | | Food-deprived | |
|---|---|---|---|---|
| | 12°C | 20°C | 12°C | 20°C |
| 30–35 days | 100% (31/31) | 100% (34/34) | 100% (31/31) | 92% (25/26) |
| Re-fed fish 30–35 days | 100% (14/14) | 79% (9/11) | 82% (12/14) | 59% (8/12) |
| Re-fed fish 60–65 days | 100% (13/13) | 93% (13/14) | 69% (9/13) | 33% (1/3) |

The number of surviving fish out of the total number is shown in brackets.

60 days compared with 30 days ($P$<0.05). However, the proportion of total lipids exhibited a more complex pattern: it was lower at 20°C compared with 12°C after 30 days of food deprivation ($P$<0.001) but increased in fish at 20°C after 60 days of food deprivation compared with 30 days ($P$<0.05).

### Mitochondrial respiration

The LEAK respiration was significantly affected by temperature (Table S3), being higher at 20°C than 12°C, especially in fed fish after 60 days ($P$<0.05, Fig. 5). Food deprivation also affected the LEAK respiration at 20°C after 60 days, as it was significantly lower in FAS20 compared with FED20 fish ($P$<0.05), and when compared with FAS20 fish after 30 days ($P$<0.05).

The OXPHOS respiration was mainly affected by time (Table S3), as it decreased with time in fed fish at 12°C ($P$<0.05), and in food-deprived fish, regardless of the temperature ($P$<0.01). A significant interaction between treatment and time also exists (Table S3), with OXPHOS respiration being significantly lower at 20°C in food-deprived fish after 60 days ($P$<0.05; Fig. 5).

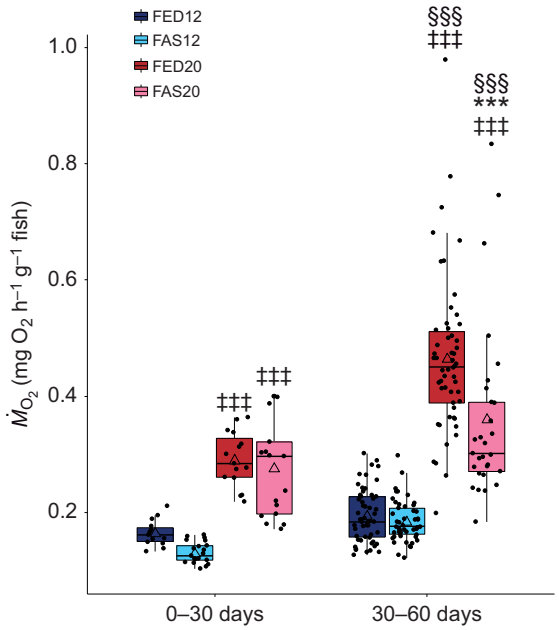


**Fig. 3. Median respiration rates of sardines between 0 and 30 days and between 30 and 60 days, measured by tank respirometry.** Statistics are given according to *post hoc* comparisons from the retained model (here the full model: Respiration rates~Treatment×Temperature×Time). ‡Significant effect of temperature at same period of time and same treatment (‡‡‡$P$<0.001). *Significant effect of food deprivation at same time and temperature (***$P$<0.001). §Significant effect of time for same group (§§§$P$<0.001). The triangle represents the mean of each group. Bar is median; box indicates 1st and 3rd quartiles; whiskers show the $\dot{M}_{O_2}$ cycles measured and retained for analysis within each group at each measurement interval.

## DISCUSSION

The results demonstrate that the effects of food deprivation on metabolism were exacerbated at high temperature in sardines, with a more rapid use of energy reserves and a greater depression of mitochondrial metabolism. This was indicative of entry into a critical phase of food deprivation, as revealed by the quicker and larger decrease in body and muscle mass and higher mortality at warm temperatures. The physiological consequences of food deprivation are separated into three sequential phases: phase I, a short period of metabolic adjustment during which protein utilization decreases; phase II, a fasting period characterized by the development of many energy-saving mechanisms during which lipids are the main energy fuel and protein utilization remains at a steady low level; phase III, a critical phase during which amino acids are oxidized, protein utilization increases and the survival of the individual becomes compromised (Bar, 2014). Entry into the critical phase seems to have occurred between 30 and 60 days of food deprivation at summer temperatures, since mortality was increased in warm food-deprived individuals. These negative impacts of food deprivation must be attributed, to some degree, to the higher metabolic rates at warm temperature. As the survival of sardines was not impacted by food deprivation at low temperature, winter conditions would seem to allow implementation of energy-saving mechanisms that endure over time.

**Table 2. Characteristics of fish at time of euthanasia**

| | 12°C | | | | 20°C | | | |
|---|---|---|---|---|---|---|---|---|
| | 30 days | | 60 days | | 30 days | | 60 days | |
| | Fed | Food-deprived | Fed | Food-deprived | Fed | Food-deprived | Fed | Food-deprived |
| *N* | 10–14 | 16–17 | 16–18 | 15–16 | 12–13 | 11–13 | 16 | 13–14 |
| Sex ratio (M/F) | 9/5 | 10/6 | 11/7 | 10/6 | 5/7 | 5/7 | 6/10 | 8/5 |
| Body mass (g) | 13.00±0.48 | 12.21±0.49 | 13.20±0.56 | 11.58±0.4 | 13.72±0.66 | 10.08±0.67 | 12.23±0.46 | 9.85±0.43 |
| Red muscle mass (g) | 1.24±0.08 | 1.14±0.08*** | 1.27±0.08 | 1.15±0.10*** | **1.09±0.06** | **0.79±0.08***** | **1.06±0.07** | **0.77±0.08***** |
| Red muscle (%) | 9.52±0.40 | 9.18±0.37 | 9.61±0.41 | 9.84±0.54 | **8.05±0.34** | **7.75±0.56** | **8.59±0.42** | **7.61±0.46** |
| White muscle mass (g) | 2.41±0.12 | 2.25±0.13 | 2.60±0.19 | 1.94±0.10 | 2.81±0.23 | 1.54±0.19‡ | 2.07±0.17§ | 1.37±0.12 |
| White muscle (%) | 18.73±0.85 | 18.35±0.75 | 19.47±0.91 | 16.65±0.53 | 20.23±0.89 | 14.99±1.21 | 16.68±0.93§ | 13.68±0.86 |
| Protein (% in white muscle) | 11.19±0.97 | 9.89±0.57*** | 10.96±0.55§ | 7.79±0.87***,§ | **10.07±0.95** | **8.03±0.71***** | **9.46±0.65**§ | **6.41±0.67***,§ |
| Lipid (% in white muscle) | 18.50±0.17 | 23.91±0.22 | 17.48±0.17 | 26.16±0.36 | 20.78±0.25 | 12.10±0.12‡‡‡ | 16.64±0.22 | 19.37±0.21§ |

Data shown are means±s.e.m. ‡Significant effect of temperature at same period of time and for same treatment (‡$P$<0.05;‡‡‡$P$<0.001). Bold indicates a global effect of temperature ($P$<0.001 for red muscle and $P$<0.01 for protein). *Global effect of food deprivation (*$P$<0.05; **$P$<0.01; ***$P$<0.001). §Significant effect of time (§$P$<0.05).

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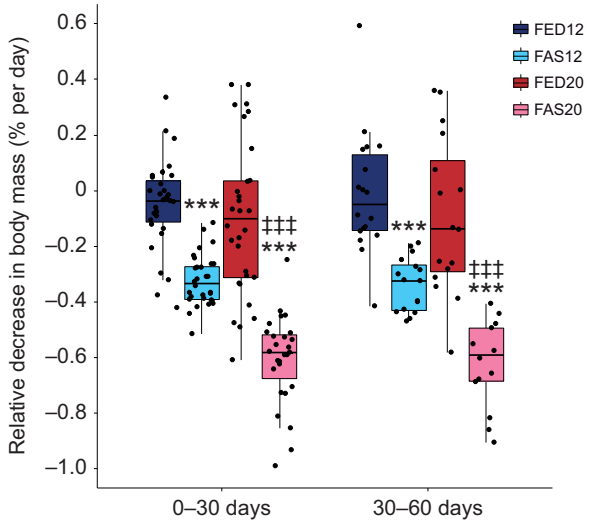


**Fig. 4. Relative change in body mass between 0 and 30 days, and between 30 and 60 days in sardines.** All groups are represented, with the fed fish at 12°C (FED12, dark blue) and 20°C (FED20, red) and the food-deprived fish at 12°C (FAS12, pale blue) and 20°C (FAS20, pink). Statistics are given according to *post hoc* comparisons from the retained model (Change in body mass~Treatment×Temperature) after backward selection from the full model. ‡Significant effect of temperature at same period and for same treatment (‡‡‡$P$<0.001). *Significant effect of food deprivation (***$P$<0.001). Bar is median; box indicates 1st and 3rd quartiles, whiskers show the individuals in each group at each measurement interval.

### Interaction between temperature and food deprivation

As expected, fish deprived of food at high temperature faced a major energetic paradox, with the need to save energy to survive but in a warm environment that increases energy expenditure. For instance, food deprivation leads to a decrease in metabolic rate in fishes, as a consequence of physiological and behavioural energy saving strategies (Cook et al., 2000; McKenzie et al., 2014), while an increase in temperature leads to an increase in metabolism (Schulte et al., 2011). In our study, despite a decrease in respiration after 60 days of food deprivation, the metabolic rate of food-deprived fish at 20°C remained higher than that measured at 12°C in fed or food-deprived fish. Moreover, as observed in Queiros et al. (2021), the respiration of sardines food deprived at 20°C actually increased after 50 days of food deprivation, which may indicate an entry into the critical fasting phase (Bar, 2014; Queiros et al., 2021). At a cellular level, the combination of food deprivation and warmth for 60 days elicited energy-saving mechanisms characterized by a decrease in mitochondrial maintenance costs (i.e. lower LEAK respiration) and in oxidative capacity (i.e. lower OXPHOS respiration). This combination of decreases in mitochondrial maintenance cost (LEAK respiration) and power (OXPHOS respiration) occurs during the critical phase of food deprivation in birds (Bourguignon et al., 2017; Monternier et al., 2017).

The mobilization of different energetic reserves depends on the food deprivation phase (Bar, 2014). Carbohydrates are mobilized first from liver and muscle, followed by lipids and then proteins after several weeks, leading to a decrease in tissue and body mass (Blasco et al., 1992). In our study, two months of food deprivation caused a decrease in protein in white muscle, linked to a decrease in muscle mass (Table 2), which resulted in a loss of body mass that was exacerbated at 20°C. However, we observed no change in lipid proportions in white muscle at 12°C, and a decrease after 30 days of food deprivation at 20°C but followed by an increase after 60 days. While this increase in lipid proportion after 60 days seems surprising, it might be related to the fact that it is relative to fish mass. Indeed, if lipids are no longer the main energy substrate during the critical phase of food deprivation (Bar, 2014), the absolute quantity should remain relatively stable while fish mass decreases due to the massive use of proteins, resulting in an increase

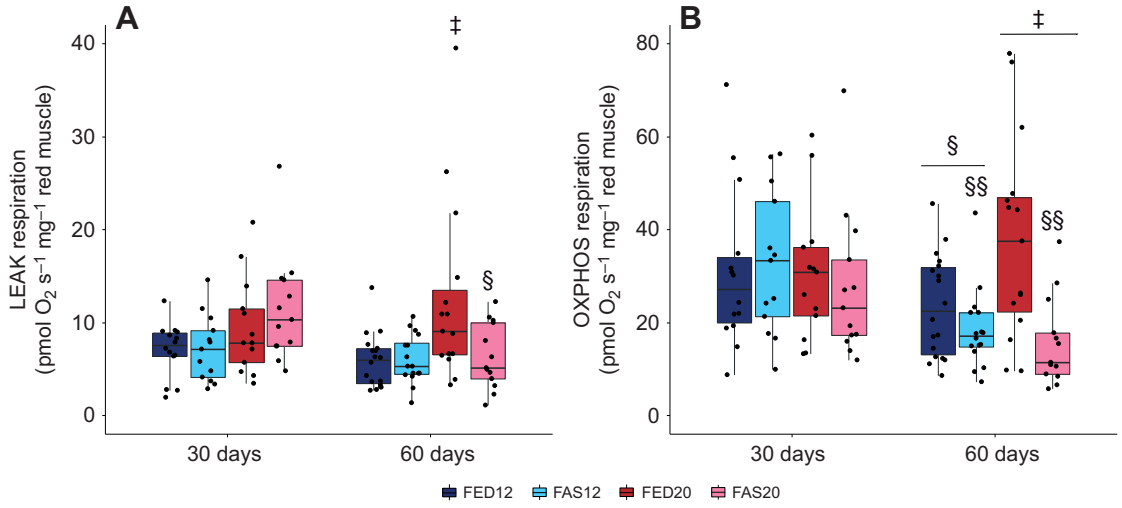


**Fig. 5. LEAK (A) and OXPHOS (B) respirations of sardines depending on the treatment and period.** The respiration is expressed in pmol $O_2$. $s^{-1}$. $mg^{-1}$ of red muscle and has been measured at the acclimation temperature of the fish. All groups are represented, with the fed fish at 12°C (FED12, dark blue) and 20°C (FED20, red) and the food-deprived fish at 12°C (FAS12, pael blue) and 20°C (FAS20, pink) after 30 and 60 days of the experiment. Statistics are given according to *post hoc* comparisons from the retained model after backward selection from the full model [LEAK respiration~Treatment×Temperature×Time; OXPHOS respiration~(Treatment×Time)+(Temperature×Time)]. ‡Significant effect of temperature at same period of time and same treatment (‡$P$<0.05). In B, ‡ indicates an overall effect of temperature, regardless of treatment. §Significant effect of time (§$P$<0.05; §§$P$<0.01). In B, § indicates a significant effect of time for same temperature and the presence of this sign with a horizontal bar indicates an overall effect of time, regardless of temperature. Bar is median; box indicates 1st and 3rd quartiles, whiskers show the individuals in each group at each measurement interval.

in lipid proportion, as muscle is the main lipid store in sardines (Venugopal and Shahidi, 1996).

In summary, the use of energy reserves, in association with cellular energy saving, was sufficient to allow food-deprived individuals to survive at 12°C but not at 20°C. Consequently, the combination of warming and food deprivation leads to a major decline in body mass (Chatzifotis et al., 2018) and survival rate.

### Ineffective re-feeding at high temperatures

It is interesting that mortality in fish deprived of food at 20°C could not be avoided by a subsequent 5 days of re-feeding. This might appear surprising as many studies have shown beneficial effects of re-feeding after long-term food deprivation. In Atlantic salmon *Salmo salar*, 10 days of refeeding are sufficient to regenerate tissue mass and enzyme activities after 50 days of food deprivation at 9°C (Van Dijk et al., 2005), additionally suggesting that cold temperatures could be beneficial for survival in food-deprived fish. Moreover, after 3 weeks of food deprivation, 1 week of re-feeding leads to compensatory growth and more efficient use of food by roach (*Rutilus rutilus*; Van Dijk et al., 2002). On the contrary, our observations suggest that 60 days of food deprivation might be too long for a full recovery of sardines after re-feeding. Perhaps, long-term food deprivation can lead to morphological changes in the digestive tract (Zaldúa and Naya, 2014), which could result in poor absorption of nutrients, making re-feeding ineffective. In addition, as mortality rate increased in food-deprived fish at 12°C after re-feeding, notably after 60 days of food deprivation, the stress caused by handling could have been too great for a positive effect of re-feeding (Olsen et al., 2002). However, it has been shown in various fish species that several weeks of food deprivation do not affect resistance to an acute stress, like a handling disturbance (Davis and Gaylord, 2011; Jørgensen et al., 2002; Mørkøre et al., 2008). Nevertheless, as no effect of handling was observed on fish from control treatments at 12 or 20°C, this suggests that food-deprived sardines might become more sensitive to additional stress (Vijayan and Moon, 1992), a phenomenon that could play an important role in the wild where multiple additional stressors exist (predation, pollution, pathogens etc.).

### Adaptation to food deprivation in winter

In our study, food deprivation in cool winter conditions did not seem to be especially problematic for sardines. Respiration of food-deprived fish was equivalent to, or lower than, fed fish at the same temperature, as observed in the ectothermic amphibian *Bufo marinus* after 9 days of food deprivation at 10°C (Trzcionka et al., 2008). In the same study, a decrease in LEAK respiration in response to food deprivation combined with cold exposure was observed in *B. marinus* liver but not muscle. In our study, mitochondrial respiration was maintained after 30 days and only slightly decreased after 60 days for OXPHOS respiration in red muscle. Moreover, multiple physiological variables, such as lipid content and the relative mass of red and white muscle, did not change over the 60 days of food deprivation (Table 2), as has been observed for liver substrates in *R. rutilus* after 21 days of food deprivation at 4°C (Van Dijk et al., 2005). As resources are naturally scarce during winter, sardines may have adapted to a lack of food in the cold season.

### Concluding remarks

In sardines, long-term winter food deprivation did not have adverse physiological consequences, eliciting energy-saving mechanisms that endured over time. However, food deprivation in summer was clearly very problematic; mitochondrial adjustments for cellular energy saving were not sufficient to compensate a high metabolic rate, leading to declines in body mass and survival after 60 days of food deprivation. Furthermore, 5 days of re-feeding did not improve survival of the sardines, especially at 20°C, suggesting that long-term food deprivation in a warm environment may cause irreversible deterioration of the ability to assimilate nutrients. This could have potentially catastrophic consequences if it occurred in wild populations, especially considering that summer food availability may be more variable in the future.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: C.S., L.T.; Methodology: D.J.M., C.S., L.T.; Validation: E.T., D.R., C.S.; Formal analysis: E.T., C.S.; Investigation: E.T., E.G., G.D., Q.Q., D.J.M., J.-H.B., L.M., L.T.; Resources: D.R., D.J.M., C.S., L.T.; Writing - original draft: E.T.; Writing - review & editing: E.T., D.R., E.G., G.D., Q.Q., D.J.M., J.-H.B., L.M., C.S., L.T.; Visualization: E.T., C.S.; Supervision: C.S., L.T.; Project administration: C.S., L.T.; Funding acquisition: C.S., L.T.

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#### Data availability

Data are available from Figshare: 10.6084/m9.figshare.21909759.v2.

#### ECR Spotlight

This article has an associated ECR Spotlight interview with Elisa Thoral.

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