MONASH University

Reproductive and energetic consequences of fishing capture on chondrichthyan species

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Abstract

Chondrichthyans are one of the most threatened groups of aquatic vertebrates, with fishing activity being the most severe and frequent threat. More than immediate and delayed mortality, when animals are discarded after capture, fishing-capture stress can cause various long-term, sub-lethal consequences impacting energy allocation, behaviour, growth, immune function and reproduction. Despite the severe impacts that these alterations can have on population viability, few studies have investigated this issue in chondrichthyans, limiting the assessment of the full extent of fishing capture consequences. The overall aim of my research was to increase the knowledge on the long-term energetic and reproductive consequences of fishing-capture stress.

To investigate the effect of fishing capture on the energy allocation of animals, I estimated aerobic metabolic rate (MR), as a proxy for the energy consumption, by measuring oxygen uptake rates (MO_2) and compared values measured in minimally stressed animals with those recorded immediately after the same animals had experienced a fishing capture simulation. Pregnant *Trygnorrhina dumerilii* experienced trawling capture and air exposure, while *Callorhinchus milii*, *Mustelus antarcticus* and *Heterodontus portusjacksoni* were exposed to stress related to gillnet capture.

After capture, increased MRs were recorded in *H. portusjacksoni*, while the other species manifested decreased MRs and in *C. milii*, MRs were still lower than unstressed values seven days after initial fishing capture, indicating an incomplete recovery. Most likely, animals respond to fishing-capture stress increasing energy use to fuel homeostasis restoration or the flight response, but when the stress is too severe or prolonged, animals exhaust the available energy reserves and a metabolic decline is triggered to preserve remaining energy for activities essential to sustain life.

The two opposite metabolic responses likely result from the different sensitivity of the species and the different severity of the stress elicited by the two fishing-capture conditions simulated.

Reviewing the literature available on reproductive consequences caused by anthropogenic stressors in teleost and chondrichthyan species, I highlighted important research gaps, including the lack of data relative to consequences caused by fishing-capture stress in chondrichthyans. To begin to address these gaps, for *T. dumerilii*, the energy consequences of capture stress were compared to the energy cost of near-term pregnancy to verify whether this activity was affected by fishing-capture. Trawling simulation decreased MR to values similar to those estimated for females post-partum, suggesting a reduced energy investment in the maintenance of pregnancy.

Further, I researched potential alterations occurring in *T. dumerilii* neonates when mothers were exposed to trawling-capture stress during late pregnancy. Compared to control neonates, neonates born from stressed mothers had higher granulocyte to lymphocyte ratio (indicating a stressed state), lower body mass and smaller yolk-sac volume at birth, slower growth, lowered boldness and, after a simulated predator attack, swam for shorter distances and altered their burying response.

These results suggest that animals surviving after capture suffer from significant sub-lethal effects. The observed energy alterations likely affect energy allocation to important biological activities, impairing them. Indeed, the reduced amount of energy invested in pregnancy maintenance likely impairs embryonic development. Further, the incomplete MR recovery suggests serious consequences originating from the prolonged depression of activities essential for life. The consequences of the neonatal alterations need to be studied in a natural environment, but these first data suggest a possible reduction in neonates' survival rate impacting recruitment and population abundance. Given the severe population consequences that potentially originate from the observed energetic and reproductive alterations, these data might be useful to support

informed decisions on the development of effective management strategies to ensure the conservation of these threatened populations.

Thesis Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer reviewed journals (Journal of Experimental Marine Biology and Ecology), one manuscript that has been accepted for publication in a peer reviewer journal (Frontiers in Marine Science) and three manuscripts that are being revised for resubmission to scientific journals at the time of thesis submission.

The core theme of the thesis is *to increase the knowledge on the long-term consequences of fishing capture in chondrichthyans, with particular focus on alterations in energy allocation and reproduction*. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Monash University School of Biological Sciences under the supervision of Associate Professor Richard D. Reina and Dr Terence I. Walker of Monash University.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Chapter 5 is a narrative published in *Journal of Experimental Marine Biology and Ecology*. I and Juan Manuel Molina contributed equally (40%) in the conception, design, ethics, permits, data collection, data analysis and manuscript preparation. Terence Walker and Richard Reina collaborated equally (10%) in supervision and manuscript editing.

In the case of chapters 2, 3, 4 and 6, my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
2	A review of reproductive consequences of fishing-capture stress in chondrichthyans: insights from teleost research	Being revised for resubmission to Fish and Fisheries	80%. Concept, literature search, and manuscript preparation	1) Terence Walker 10% Supervision and manuscript editing 2) Richard Reina 10% Supervision and manuscript editing	*No*
3	Influence of reproductive state and fishing-capture stress on the metabolic rate of a viviparous chondrichthyan	Being revised for resubmission to Conservation Physiology	80% Conception, design, ethics, permits, data collection, data analysis, manuscript preparation	1) Terence Walker 10% Supervision and manuscript editing 2) Richard Reina 10% Supervision and manuscript editing	No
4	The effect of fishing capture stress on the metabolic rate and swimming activity of a holocephalan	Being revised for resubmission to Physiological and Biochemical Zoology	80% Conception, design, ethics, permits, data collection, data analysis, manuscript preparation	1) Terence Walker 10% Supervision and manuscript editing 2) Richard Reina 10% Supervision and manuscript editing	No
6	Prolonged impairment of neonate fitness following maternal exposure to fishing-capture stress during late pregnancy in a chondrichthyan species	Accepted for publication in Frontiers of Marine Science	80% Conception, design, ethics, permits, data collection, data analysis, manuscript preparation	1) Terence Walker 10% Supervision and manuscript editing 2) Richard Reina 10% Supervision and manuscript editing	No

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Licia Finotto

Student signature: **Date:** 24/09/2020

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Richard David Reina

Main Supervisor signature: **Date:** 24/09/2020

Acknowledgements

I still remember my thought after attending the first lecture of my master degree: I sure love sharks, but I will never become a physiologist, let alone work on respirometry. And here I am, quite a few years later, with a PhD thesis that focuses on physiology and respirometry, and I have to admit, I enjoyed (almost) every moment. This has been possible only thanks to the advice and guidance that my two supervisors gave me. Richard, there has been many hitches to my project, from having no fish to having no place where to house the fish, but you have always been positive, helpful and supporting. You always allowed me to pursue my own interests during my PhD, giving me large freedom, up to the point of trusting me in buying some very expensive fishes. All this immensely helped me become an independent researcher. Terry, a legend in fishery and shark science, your endless wisdom and enthusiasm for all new discoveries greatly motivated my curiosity. You always had time to discuss best fishing gears or experiment settings and your invaluable feedbacks always made me look at things from different angles. And also, thanks for checking on animals in Queenscliff, it allowed me to have some days off during the sometimes longer than expected experiments.

A huge thank you has to go to all those people without whom my project would not have been possible.

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Thanks to Juan who introduced me to the magic world of respirometry. I will cherish carrying a huge respirometer around campus and the puzzled looks of people, the great moments spent on the boat chatting with Maurice and the endless hours spent waiting for sharks to breathe. The time outside work was also great, and I have to thank you and Emilia for having looked after me and my poor balance at the Foo Fighters concert.

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made so many sacrifices, from having to listen to me rambling on sharks and lessen known sea creatures, up to the point of 'allowing' your only child to move to Australia, 14373 km away from Home, because you understood that this was what I needed to grow as a researcher, but even more as a person. I missed you (except for the snoring); I am pretty sure you missed me too and I can only imagine what you went through to allow me follow my dreams. I have not told you this often, but I would not be the person I am if it was not for your help, scoldings, unconditional support, love and sacrifice and for this, I immensely thank you! I love you.

Chapter 1. GENERAL INTRODUCTION

1.1. CHONDRICHTHYANS AND FISHERIES

Chondrichthyans (sharks, rays and chimeras) are one of the most threatened groups of vertebrates (Dulvy et al., 2014), with 28.6% of the known species classed in one of the three highest categories of extinction risk adopted for the Red List by the International Union for the Conservation of Nature (IUCN, 2020). Many chondrichthyan populations have undergone severe declines in abundance in the recent decades (Baum et al., 2003; Dulvy et al., 2008; Dulvy et al., 2014), including the 99% decline observed between 1950 and 1990 for the oceanic whitetip shark (*Carcharhinus longimanus*) in the Gulf of Mexico (Baum and Myers, 2004) and the 75% decline recorded over 15 years for some large shark species in the North-East Atlantic (Baum et al., 2003). The globally critically endangered school shark (*Galeorhinus galeus*) is estimated to have declined 88% in its six genetically separate populations over a period of three generations (79 years; Walker et al., 2020). Also, local extinction is recorded for the blue skate (*Dipturus batis*) and the white skate (*Rostroraja alba*) in UK waters (Ellis et al., 2005). The high vulnerability of this group of species is the result of shared life-history traits, such as long-lived, late sexual maturity, long reproductive cycles, low fecundity and long embryonic development (Camhi et al., 1998; Dulvy et al., 2008), resulting in a relatively low reproductive rate and low capacity for recovery after population depletion (Camhi et al., 1998; Dulvy et al., 2008).

Threats to chondrichthyan populations include habitat degradation, pollution and climate change (Dulvy et al., 2008; Halpern et al., 2008; Dulvy et al., 2014). However, pressures associated with fisheries exploitation are considered the most common and severe (Pauly et al., 2005; Dulvy et al., 2008; Halpern et al., 2008; Ferretti et al., 2010; Dulvy et al., 2014). It has been estimated that ~100 million chondrichthyans are caught annually in fisheries around the world (Worm et al.,

2013) and overexploitation is a main cause of the steep decline observed in some populations (Pauly et al., 2005; Dulvy et al., 2008; Halpern et al., 2008; Ferretti et al., 2010; Dulvy et al., 2014). However, few fisheries directly target chondrichthyans, while the major proportion of animals, up to around 50% globally (Stevens et al., 2000), is accidentally caught in fishing operations targeting other species and is termed bycatch. Due to low economic value or existing management measures, the majority of bycatch animals are released back into their environment after capture. Discarding is a concerning problem, not only because reliable estimates on the amount of animals affected are scarce but also because few data are available on the post-release fate of the discarded animals (Ferretti et al., 2010; Molina and Cooke, 2012).

Due to injuries and stress suffered during capture procedures, some of the discarded animals die, immediately or soon after release (14 days; Frick et al., 2010a, b; Heard et al., 2014; Martins et al., 2018). Large variability exists between species and fishing gears (Skomal, 2007; Mandelman and Skomal, 2009; Dapp et al., 2015), but with the immediate mortality rate as high as 91% in longline-caught scalloped hammerhead shark (*Sphyrna lewini*; Morgan and Burgess, 2007) and delayed mortality as high as 63% in gillnet-caught whiskery shark (*Furgaleus macki*; Braccini et al., 2012), mortality is the most severe outcome of fishing capture and the largest impact on populations. However, for animals that do not die due to fishing capture, survival does not come without a cost. The physiological and energetic alterations associated with the fishing-capture stress response have long-term, sub-lethal effects that impair several biological activities (Skomal and Mandelman, 2012) and need to be thoroughly understood.

1.2. CONSEQUENCES OF FISHING-CAPTURE STRESS

The stress response is a mechanism highly conserved among vertebrates (Wendelaar-Bonga, 1997) that allows maintaining or restoring homeostasis in the presence of a stressful event, thereby increasing survival chances (Barton and Iwama, 1991; Wendelaar-Bonga, 1997; Skomal

and Mandelman, 2012). It is generally subdivided into three phases, the first being the *primary response,* which starts with the perception of the stress by the nervous system that triggers a hormonal cascade terminating in the release of the stress hormones. Among other functions, stress hormones stimulate the mobilization of the energy reserves needed to fuel the 'fight or flight response' or to maintain and restore homeostasis during the *secondary response* (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012). Stress hormones can also directly act at the levels of specific biological activities or organs (Wendelaar-Bonga, 1997; Sapolsky et al., 2000; Bobe and Labbé, 2010; Li and Leatherland, 2012) and influence gene expression patterns (Sapolsky et al., 2000). When stress is prolonged or severe, these physiological and energetic alterations culminate in *tertiary responses*, which are long-term effects observed at the organism and population levels and include impairments to reproduction, energy allocation, behaviour, immune function and growth (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012).

A substantial number of studies investigated the short-term physiological, immunological and haematological effects of fishing-capture stress in chondrichthyans (Frick et al., 2010a, b; Van Rijn and Reina, 2010; Skomal and Mandelman, 2012; Gallagher et al., 2014; Dapp et al., 2016; Guida et al., 2016a). The results of these studies indicate wide variation related to diverse species sensitivity (Mandelman and Skomal, 2009; Frick et al., 2010a; 2010b; Gallagher et al., 2014) and severity of the stress elicited by different fishing gears (Frick et al., 2010a; Dapp et al., 2015; Martins et al., 2018). Species' sensitivity to stress is heavily influenced by their respiratory mode, with species capable of buccal pumping being less sensitive than obligate ram ventilator species that need to constantly swim for gill oxygenation (Dapp et al., 2015). Restraining fishing gears, such as gillnets and trawl nets, that hamper swimming, are the ones triggering the most severe consequences, especially for ram ventilating species (Dapp et al., 2015). Other characteristics of the fishing operations, including capture duration (Frick et al., 2010b; Heard et al., 2014), air

exposure occurrence and duration (Frick et al., 2010b; Heard et al., 2014), environmental variables such as temperature (Guida et al., 2016b) and handling techniques, can all influence the stress load associated with capture and the severity of the consequent alterations.

Long-term consequences of fishing-capture stress have the potential to impact fitness and recruitment (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012), thereby reducing the already low ability of chondrichthyan populations to recover from depletion. Nevertheless, few data exist on tertiary responses for chondrichthyan species and urgent research is needed. The occurrence of post-capture mortality and the severity of the short-term, sub-lethal consequences differ largely between different chondrichthyan species (Mandelman and Skomal, 2009; Frick et al., 2010a; 2010b; Gallagher et al., 2014), and, similarly, the long-term stress responses are likely characterized by a large variability. Moreover, evaluating stress consequences in wild animals is complicated by a number of uncontrollable parameters that can affect and hinder the interpretation of the responses, including stress elicited by the sampling procedures and health status and previous history of the animal (Johnstone et al., 2012; Johnstone et al., 2017). Therefore, only by studying a variety of fishing-capture stress indices and responses, including reproductive and energetic consequences, in a diverse range of species and fishing-capture circumstances, will it be possible to understand the complex issue of fishing-capture consequences and draw general conclusions. These fundamental data will help evaluate the full extent of fishing-capture effects and develop risk assessment and population dynamic models (Chin et al., 2010; Wheeler et al., 2020; Walker et al., in review) used to assess fishery sustainability and design effective management measures to improve the conservation status of depleted populations (Punt and Walker, 1998; Pribac et al., 2005; Walker, 2005a, b).

1.3. ENERGETIC CONSEQUENCES

The stress response, while being necessary to increase survival chances in case of stressful events (Schreck et al., 2001), is also energetically expensive (Schreck and Li, 1991; Mc Ewen and Wingfield, 2003; Schreck, 2010). The amount of energy animals have available for different biological activities is largely fixed (Ware, 1982; Calow, 1985), therefore the energy needed to sustain the stress response is inevitably diverted from the investment in these activities, with potential consequent impairments (Calow, 1985; Mc Ewen and Wingfield, 2003; Schreck, 2010). Measuring energy alterations and changes in energy allocation emerging after animals experience stress is fundamental to gain a better understanding of the full impact of particular stressors. Any alteration in energy allocation to important biological activities associated with fitness, such as growth, swimming, reproduction or immune function, should be primarily researched. A common method used to investigate energy use is estimating an animal's aerobic metabolic rate (MR) by measuring its oxygen uptake rate. According to the amount of activity performed by the animals, different types of MRs can be estimated. Standard MR (SMR) represents the amount of energy used by fasted animals to sustain essential maintenance and life-supporting activities when no movement other than self-righting is performed. Routine MR (RMR) is the energetic expenditure of a fasted animal performing an unspecified amount of movement, while maximum MR (MRM) is the maximum amount of energy consumption an animal can sustain before reaching exhaustion (Chabot et al., 2016).

Metabolic rate estimates have been largely used to investigate energetic alterations caused by anthropogenic stress in teleost fishes (Chan and Woo, 1978; Dalela et al., 1980; Barton and Schreck, 1987; Morgan and Iwama, 1996; Davis and Schreck, 1997; Herskin, 1999; Sloman et al., 2000; Palanivelua et al., 2005; Collins, 2019). Physical stress can severely impact energy consumption in rainbow trout (*Oncorhynchus mykiss*) by causing a 120% increase in baseline levels

(Barton and Schreck, 1987). Fishing-capture stress, involving struggling, handling and air exposure, triggers a similar large increase in energy use (Brick and Cech, 2002; Clark et al., 2012; Cooke et al., 2014), that ultimately can cause measurable reductions in growth and fecundity, up to 100% and 62%, respectively (Watson et al., 2020). Energetic studies in chondrichthyans have mainly investigated baseline energy consumption, temperature influence and the cost associated with swimming activity (Lowe, 2001; Neer et al., 2006; Whitney et al., 2016; Di Santo et al., 2017). Very few studies researched the consequences of stress, focusing primarily on the effects of environmental stressors, including oxygen levels, salinity and climate change related variables such as a decrease in seawater pH and increase in temperature (Sepulveda et al., 2007; Di Santo and Bennett, 2011; Guffey and Goss, 2014; Di Santo and Kenaley, 2016; Morash et al., 2016; Tunnah et al., 2016). However, the use of MR estimates to investigate energetic alterations caused by fishing-capture stress seems promising (Bouyoucos et al., 2019), as highlighted by the only two studies investigating this issue. In lemon sharks (*Negaprion brevirostris*), 1 h of longline simulation caused an 8% increase in RMR (Bouyoucos et al., 2017) and in blacktip reef sharks (*Carcharhinus melanopterus*), 3 min of gillnet capture and 1 min of air exposure caused a 120% increase in SMR (Bouyoucos et al., 2018). These results confirm the severe impact that fishing capture can have on animals' energy use but also highlight the large difference that characterizes responses of different species and those elicited by different fishing-capture procedures.

The consequences for the biological activities impacted by the stress-induced energetic alterations will likely be more severe the longer these alterations persist (Davis and Schreck, 1997). Therefore, the recovery time needed for metabolic rates to return to minimally-stressed values needs investigation to understand better the extent of the energetic consequences overall. In chondrichthyans, following fishing-capture stress, the metabolic recovery time ranges from 3 to 20 h (Bouyoucos et al., 2017; Bouyoucos et al., 2018). However, given that some of the reported

values are estimates rather than direct measurements, and because of the potentially high variation in recovery time, the process of recovery needs evaluating in more species.

The energetic consequences of stressful events are usually compared to aerobic scope, an estimate of the energy available that animals can allocate to different activities (Clark et al., 2013). Aerobic scope is calculated as the difference between MMR and SMR, although its use might be misleading. Animals can sustain MMR only for brief periods before exhaustion (Brett and Blackburn, 1978; Scarabello et al., 1991; Reidy et al., 1995; Bouyoucos et al., 2017) and during normal activities, their MR is largely below MMR (Norin and Clark, 2016). So it follows that comparison of the stress-induced energetic alterations to this maximal energy consumption might underestimate the full consequences. A better evaluation should be obtained by relating the energetic alteration caused by fishing-capture stress to the cost of important biological activities. Hindering this comparison is the fact that, other than for swimming and digestion (Parsons, 1990; Sims and Davies, 1994; Di Santo and Kenaley, 2016), estimates of energetic cost for other activities are lacking (Chabot et al., 2016).

Activities associated with reproduction, such as mating behavior, parental care (Hinch and Collins, 1991; Gillooly and Baylis, 1999), gamete production (Parker, 1972) and, in viviparous species, pregnancy (Carrier et al., 2004; Hamlett et al., 2005; Trinnie et al., 2012), are among the most energetically-demanding and are likely to be severely impacted by capture-induced alterations in energy allocation. Given the essential role of unimpaired reproduction to building population abundance, the need to understand the extent of potential impairments to this activity is clear. The cost of pregnancy, as measured in the teleost sailfin molly *Poecilia latipinna* (Timmerman and Chapman, 2003) and Korean rockfish *Sebastes schlegelii* (Boehlert et al., 1991), is unknown for chondrichthyans, let alone reports of alterations in energy allocation to this activity

following stress. Thus, the lack of information on energy allocation, particularly in response to fishing stress, highlights the urgent need for further research.

1.4. REPRODUCTIVE CONSEQUENCES

Impacts on reproduction following a fishing-capture event originate from a direct action of stress hormones on organs and biological processes involved in reproduction, including genetic expression and the production of reproductive hormones (Wendelaar-Bonga, 1997; Sapolsky et al., 2000; Bobe and Labbé, 2010; Li and Leatherland, 2012). Moreover, the impact also depends on the alteration in the amount of energy remaining for allocation to reproductive activities after investment in sustaining the stress response (Calow, 1985; Wendelaar-Bonga, 1997; Sapolsky et al., 2000; Mc Ewen and Wingfield, 2003; Schreck, 2010). In teleosts, various reproductive consequences have been observed in relation to events that trigger the response of the stress axis, both in stressed adults and in offspring of stressed parents (intergenerational consequences). Adult impairments include alterations in reproductive hormone concentrations (Carragher et al., 1989; Cleary and Pankhurst, 2000), mating behavior (Morgan et al., 1999; O'connor et al., 2009), gonadal mass (Goos and Consten, 2002; Sopinka et al., 2014) and gamete size (Campbell et al., 1992, 1994; Mileva et al., 2011), number (Mileva et al., 2011; Shourbela et al., 2016) and quality (Bobe and Labbé, 2010; Sousa et al., 2015). In oviparous species, further effects include egg fertilization rate (Campbell et al., 1994; Eriksen et al., 2013), embryo hatching rate (Okanlawon, 2010; Eriksen et al., 2013) and timing of hatching (Gagliano and Mccormick, 2009). In offspring, stress experienced by the parents can impact size at hatch (Eriksen et al., 2013; Capelle et al., 2017), growth rate (Eriksen et al., 2013; Nesan and Vijayan, 2016), occurrence of malformations (Eriksen et al., 2013; Shourbela et al., 2016), amount of energy reserves (Gagliano and Mccormick, 2009; Eriksen et al., 2013), stress response (Colson et al., 2015; Nesan and Vijayan, 2016), swimming performance (Sopinka et al., 2014; Sopinka et al., 2016), behaviour (Eaton et al., 2015;

Best et al., 2017), learning ability (Sloman, 2010; Eaton et al., 2015), reproductive output (Magierecka, 2019) and survival (Soso et al., 2008; Shourbela et al., 2016). The effects of stress may manifest also in the second generation of offspring, i.e., those produced by the offspring of stressed parents (transgenerational effects; Magierecka, 2019). The reproductive consequences of fishing-capture stress are marginally researched, but the effects observed resembles those caused by other anthropogenic stressors, including decreased reproductive hormone concentrations (Cleary and Pankhurst, 2000; Bayunova et al., 2002), delayed gonadal development (Rowland, 1988), atresia (Cleary and Pankhurst, 2000; Hall et al., 2009), decreased follicle diameter (Rowland, 1988), decreased semen osmolarity and pre-activated sperm motility (Allyn et al., 2001), altered parental care (Cooke et al., 2002), increased occurrence of offspring malformations (Morgan et al., 1999) and decreased offspring size (Ostrand et al., 2004).

Very few studies have investigated reproductive impairments caused by fishing-capture stress in chondrichthyans; nevertheless, the little information available depicts a concerning situation. In many viviparous species, fishing capture can cause the abortion of eggs or non-viable embryos, or the premature parturition of viable, partially or completely developed neonates (Adams et al., 2018). Abortion and premature parturition, given the low survival chances of premature neonates (Charvet-Almeida et al., 2005; Campbell et al., 2018), are the most severe outcomes, causing the complete failure of the reproductive event (Adams et al., 2018; Wosnick et al., 2019). However, even when neonates survive, fishing capture still affects size at birth and induces a persistent alteration of the immune response in neonates (Guida et al., 2017), indicating a state of stress (Van Rijn and Reina, 2010) and potential survival impairments. Strong similarities exist between teleost and chondrichthyan stress response mechanisms, secondary stress responses and energetic consequences (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012), and it is likely that reproduction is also similarly affected, with many aspects potentially impacted by fishing-

capture stress but not yet investigated in chondrichthyans. Further investigation of reproductive impairments in chondrichthyans is essential not only because of the poor conservation status of many populations (Dulvy et al., 2014; IUCN, 2020) but also because of their prolonged reproductive cycles (Hamlett et al., 2005). In many species, the reproductive cycle lasts 2–3 years, meaning that between the production of two successive litters of offspring 2 or 3 years pass (Walker, 2005b, 2007; Rochowski et al., 2015), and, during this time, females invest large amount of energy in oocyte and histotroph production and/or in sustaining the long pregnancy (Hamlett et al., 2005; Trinnie et al., 2012). Any reproductive impairment will impact the large energy investment that has been allocated to reproduction during past years and many years may have to pass before animals can reproduce again. This, in association with the intrinsically low reproductive rate of many chondrichthyan species (Camhi et al., 1998; Dulvy et al., 2008), suggests that population recovery will be further severely hindered by any reproductive impairments. Therefore, it is essential to understand better the reproductive consequences caused by fishing-capture stress to identify potential species that most urgently need management and to implement effective conservation measures.

1.5. RESEARCH AIM AND OBJECTIVES

The main aim of this thesis is to improve our understanding of the sub-lethal consequences of fishing-capture stress in discarded chondrichthyans, focusing particularly on long-term, tertiary responses. More accurate information will provide a rational basis for the development of sound management measures improving the conservation status of this taxon. To achieve this aim, I focused on three main objectives.

1) Identify the potential long-term reproductive consequences caused by fishing-capture stress.

2) Quantify the changes in energy expenditure caused by fishing-capture stress.

3) Determine the variability of the energetic responses that follow fishing-capture stress.

Thesis data chapters (2–6) contribute to the aim of the thesis as described in Figure 1.1 and briefly outlined below.

Objective 1 is addressed in Chapters 2, 3 and 6. Chapter 2 reviews the reproductive impairments observed in teleosts and chondrichthyans exposed to anthropogenic stressors, including fishing-capture stress. Chapter 3 compares the energetic alterations potentially caused by fishing capture with the energetic cost of near-term pregnancy estimated in a viviparous chondrichthyan species to understand whether capture-induced energetic alterations could compromise reproduction and, if so, to what extent. Metabolic rates estimated through respirometry and oxygen uptake rate measurements serve as a proxy for aerobic energy use. Chapter 6 investigates the alterations observed in neonates following exposure of their mothers to fishing capture simulation during pregnancy to understand whether maternal stress impacts neonatal fitness.

Objective 2, addressed in Chapters 3, 4 and 5, is to compare the energy expenditure of minimally stressed animals with values estimated immediately after exposing the same animals to fishing-capture simulation. In Chapter 4 metabolic rates are also estimated seven days after fishing-capture simulation and compared to values estimated immediately after capture simulation and in minimally stressed animals to investigate metabolic recovery time.

Objective 3, addressed in Chapters 3, 4 and 5, is to compare the energetic responses observed in several species, of diverse taxonomic groups and sensitivity to fishing-capture stress, subjected to capture by different fishing gears.

1.6. THESIS STRUCTURE

This thesis consists of seven chapters, including a general introduction (Chapter 1 - this Chapter), five data chapters (Chapters 2–6) and a general discussion (Chapter 7).

Chapter 1 provides a brief overview of the current knowledge on the effects of fishing-capture stress in chondrichthyans, highlighting important research gaps, especially for energetic and reproductive consequences, that need addressing. This Chapter also outlines the aim and objectives of the thesis and its structure.

Chapter 2 is a comprehensive review of reproductive impairments observed in both teleost and chondrichthyan fishes following exposure to anthropogenic stressors. Based on the strong similarity of responses observed in teleosts and chondrichthyans and on reproductive traits peculiar to chondrichthyans, suggestions are made on reproductive traits potentially impacted in chondrichthyans and what consequences might result.

Chapter 3 investigates the energetic alteration caused by simulated trawling and air exposure stress in pregnant southern fiddler ray (*Trygonorrhina dumerilii*). The energetic expenditure of near-term pregnancy was calculated by comparing metabolic rates estimated in pregnant females with those estimated soon after parturition. Fishing-induced energetic alterations were evaluated in the context of the energy requirements of pregnancy.

Chapter 4 investigates the energetic consequences of simulated gillnet capture in elephant fish (*Callorhinchus milii*). These energetic alterations were evaluated in the context of changes in swimming activity measured after fishing capture simulation. The recovery time of the energetic alterations was determined.

Chapter 5 is a published paper (Molina et al., 2020) designed and executed as a collaboration. In this chapter, energetic alterations caused by gillnet capture were investigated in two species characterized by different ecological traits and sensitivity to fishing-capture stress, the sedentary,

resilient Port Jackson shark (*Heterodontus portusjacksoni*) and the active swimming, more sensitive gummy shark (*Mustelus antarcticus*).

Chapter 6 investigates reproductive consequences observed in *T. dumerilii* neonates when mothers were exposed to fishing-capture stress during pregnancy. Specifically, neonate growth rate, yolk sac volume at birth, yolk sac consumption rate, swimming performance and burying and boldness behaviours were investigated.

Chapter 7 is a general discussion integrating the results of all data chapters. The implications of these findings for fishery sustainability and management measures are also discussed. Research limitations and knowledge gaps that still need investigation are addressed.

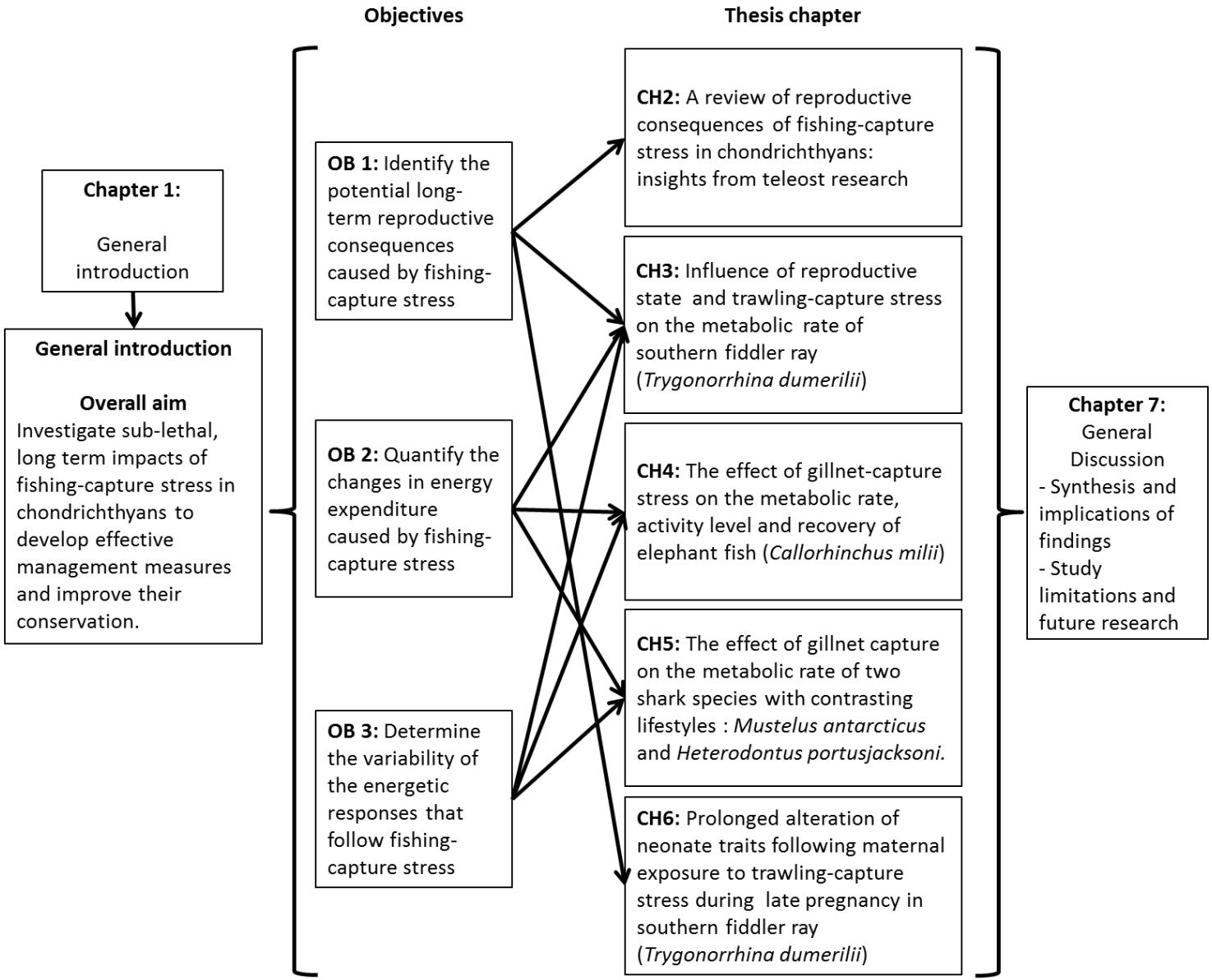

Figure 1.1. Outline of thesis structure indicating which objectives are addressed in each chapter.

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Chapter Two

A review of reproductive consequences of fishing-capture stress in chondrichthyans: insights from teleost research

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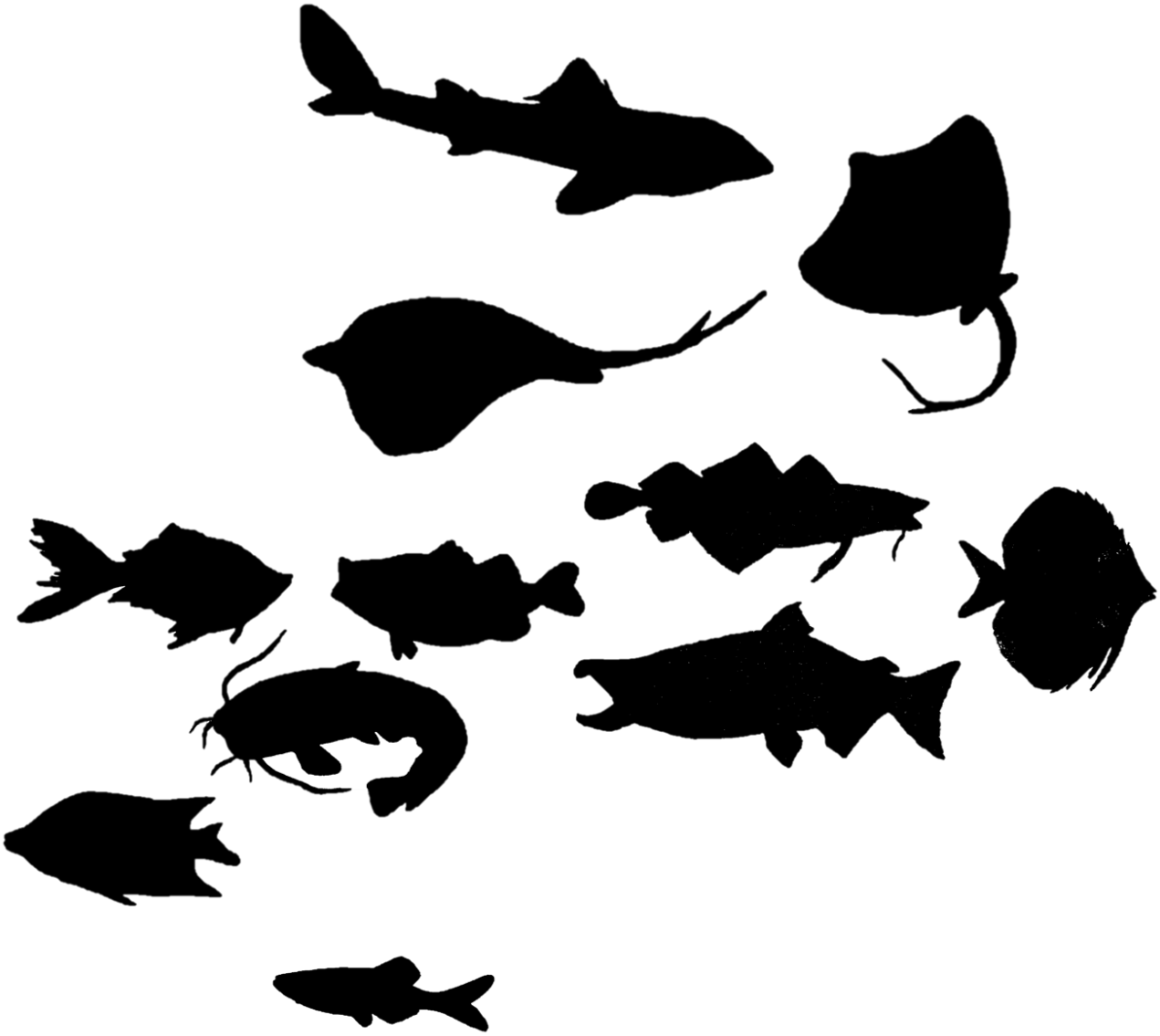

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ABSTRACT

Stress is a constant presence in the life of fishes, and the stress response elicited, despite being essential, can cause long-term alteration to various biological processes. Given reproduction's critical importance, reproductive impairments need to be thoroughly studied, especially in the context of the steep decline in abundance occurring in many fish populations in general, and chondrichthyan populations in particular. Fishes experience a wide variety of stressors, both natural and anthropogenic, but currently fishing capture is one of the most severe. In this review, we briefly synthesise the current knowledge of the reproductive effects of anthropogenic stressors in teleost fishes, and then focus on consequences of fishing-capture stress and chondrichthyan responses. Stress impairs many aspects of reproduction both in adults and offspring, from reproductive hormone levels to gamete quality and from size at birth to offspring survival. Data on the effects of fishing-capture stress and on the consequences in chondrichthyans are limited, and, despite differences, several similarities emerge with teleost responses. Based on these data and on resemblances between teleost and chondrichthyan physiological, immunological and energetic responses to stress, we speculate on possible chondrichthyan reproductive traits that are likely impacted by stress. We also propose potential measures that could lessen the severity of the reproductive effects of fishing-capture stress and highlight traits and knowledge gaps that need investigation. Better understanding will help determine real costs and sustainability of fisheries practices and thereby contribute to develop more effective conservation measures.

2.1. INTRODUCTION

Natural stress is a constant presence in the life of animals, which have evolved mechanisms to cope with natural variation and avoid major damage (Boonstra, 2013). The stress response is the fundamental process that allows an organism to overcome a threat through promoting physiological, biochemical, immunological and behavioural alterations, such as the production and release of response molecules (stress hormones) and changes in locomotion, respiration, hydromineral regulation and tissue repair. The purpose of such changes is to support activities that are necessary to cope with stress, to maintain or restore homeostasis, and to survive (Wendelaar-Bonga, 1997; Schreck et al., 2001). Despite being an adaptive response, chronic or severe stressors may overcome the ability of the organism to compensate, and the stress response, with the associated increased stress hormone concentrations and energy expenditure, becomes detrimental to the animal (Schreck and Li, 1991; Wendelaar-Bonga, 1997; Mc Ewen and Wingfield, 2003; Schreck, 2010; Watson et al., 2020). More and more often, animals now face anthropogenic stressors (Halpern et al., 2008; Sih et al., 2011), which are human-induced, rapid environmental changes outside animals' evolutionary experience and include climate change, habitat degradation, fishing capture and pollution (Sih et al., 2011; Boonstra, 2013). Anthropogenic stressors represent severe threats with potentially profound consequences for biological processes, animal fitness and species conservation Indeed, these stressors can reduce growth and food assimilation capacity, induce immune suppression, and cause reproductive impairment (Wendelaar-Bonga, 1997).

Many fish populations have undergone a steep decline in abundance, leading to poor conservation status (Worm et al., 2009; Froese et al., 2012, 2013; Pauly and Zeller, 2016; FAO, 2018). Chondrichthyans (sharks, rays, skates and chimaeras) are one of the most threatened taxonomic groups (Dulvy et al., 2014) with 28.6% of the known species classed as threatened

(IUCN, 2020). The vulnerability of this fish group originates from their shared life-history traits of late maturity, long life span, long embryonic development and production of few offspring compared to teleost fishes and resulting in low reproductive rate and capacity to recover from population depletion (Camhi et al., 1998; Dulvy et al., 2008). Anthropogenic stressors, including those associated with fishing, pollution, habitat degradation and climate change, are the main threats to fish populations (Dulvy et al., 2008; Halpern et al., 2008; Dulvy et al., 2014; Walker et al., in review). Currently, fishing capture is by far the most frequent and detrimental event that wild fishes might experience (Pauly et al., 2005) and bycatch (i.e., discarded, non-target species) is an important problem, especially for chondrichthyans (Ferretti et al., 2010; Molina and Cooke, 2012). Due to management requirements or low economic values, an annual estimated 9.1 million tons of marine animals (Pérez Roda et al., 2019), mainly fishes (Zeller et al., 2018), are discarded into the ocean after capture. Many of these are already dead or die soon after being discarded, but some will ultimately survive (Dapp et al., 2015). Animals that survive must cope with the stressors induced by fishing capture, including constraint and injury caused by fishing gear, exposure to air, handling and changes of ambient temperature and pressure. Understanding the longer-term consequences of capture stress is essential to evaluate the full impact of fisheries and reproductive consequences deserve particular attention, given that any impairment to this critical process undermines population and species conservation (Schreck et al., 2001; Sopinka et al., 2016a), particularly for already depleted groups such as chondrichthyans.

This review aims to synthesise, for both teleost and chondrichthyan fishes, the existing research on reproductive impairments resulting from a stress response elicited by anthropogenic stressors. For teleosts, comprehensive reviews have treated the reproductive consequences caused by stress (Schreck et al., 2001; Milla et al., 2009; Leatherland et al., 2010; Schreck, 2010; Li and Leatherland, 2012; Pankhurst, 2016; Sopinka et al., 2016a) so we briefly summarize these

reponses and include recently described alterations in offspring behaviour and reproductive output, and transgenerational effects. Our primary focus is on the effects of fishing-capture stress, largely excluded from most of the previous reviews, and on responses of chondrichthyans in particular. A recent review has summarized the known reproductive consequences of anthropogenic stressors for chondrichthyans, including fishing capture (Wheeler et al., 2020). On top of the information provided by these authors, we include some references of the effect of fishing-capture stress on reproductive hormone concentrations and ovulation and we discuss the broader repercussions of the fishing-induced consequences on animal fitness and reproductive success. Our review also includes unpublished results for a chondrichthyan species (Chapter 6) that confirm the similarity of stress-induced reproductive impairments between teleost and chondrichthyan fishes. Moreover, acknowledging the little information available for chondrichthyans, utilizing data obtained from teleost studies, we predict other potential reproductive impairments that can occur due to fishing-capture stress in chondrichthyan species. Based on the reviewed consequences of different fishing-capture conditions, we also suggest measures that might reduce the occurrence and severity of the reproductive effects. Finally, we highlight other gaps in knowledge that need addressing as a step towards developing more effective population assessment models and improving the conservation status of fish populations.

2.2. THE STRESS RESPONSE AND MECHANISMS OF REPRODUCTIVE IMPAIRMENT

A stress response is the set of sensory perceptions, hormonal cascades and physiological, metabolic and behavioural changes arising in an animal after a stressful event and is necessary to restore homeostasis and survive. The stress response in fishes comprises three phases (Barton and Iwama, 1991; Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012). During the *primary response*, the stressful event is perceived by the hypothalamus and the hypothalamic-

sympathetic-chromaffin cell axis and the hypothalamus-pituitary-interrenal axis (HPI) are activated, stimulating the biosynthesis and secretion of stress hormones, catecholamines and glucocorticoids. Cortisol (Wendelaar-Bonga, 1997) and 1α-hydroxycorticosterone (Anderson, 2012; Skomal and Mandelman, 2012; Ruiz-Jarabo et al., 2019) are the main glucocorticoids in teleosts and chondrichthyans, respectively. Among other functions, in teleosts, stress hormones are known to enhance blood oxygen transport and stimulate the mobilization of energy substrates through the release of glucose from glycogen reserves, the enhancement of free fatty acid metabolism and the inhibition of protein synthesis (Wendelaar-Bonga, 1997). These are aspects of the *secondary response* observed both in teleosts and chondrichthyans and are needed to fuel the increased level of activity and energy demand required to restore homeostasis and perform the 'fight or flight response' (Watson et al., 2020). Physiological and biochemical alterations culminate in *tertiary responses*, which are long-term consequences occurring at the whole organism and population levels, including changes in feeding, behaviour, growth, reproduction and immune competence (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012; Watson et al., 2020).

Reproductive consequences likely result from both a direct action of stress hormones and an indirect consequence of the stress-induced energy shortage (Wendelaar-Bonga, 1997; Sapolsky et al., 2000; Watson et al., 2020). In teleosts, basal glucocorticoid levels are necessary to regulate several biological processes, both in adults (Ayson, 1989; Wendelaar-Bonga, 1997; Milla et al., 2009) and in embryos (Nesan et al., 2012; Nesan and Vijayan, 2013, 2016). However, the high glucocorticoid levels resulting after severe stress responses are deleterious. Glucocorticoids can directly affect specific reproductive pathways, organs or transcriptional patterns. This includes acting at the gonadal level, modifying the patterns of the hypothalamus-pituitary-gonadal axis, the primary regulator of reproduction in fishes (Pankhurst, 2008; Awruch, 2013; Pankhurst, 2016), and influencing the synthesis of mRNA and proteins fundamental during reproductive events and

gonadal and gamete development (Bobe and Labbé, 2010; Li and Leatherland, 2012). Additionally, stressed females produce eggs with higher cortisol concentrations (Stratholt et al., 1997; Eriksen et al., 2011; Kleppe et al., 2013; Faught et al., 2016) that can affect embryo traits by disrupting gene expression during the first developmental phases (Sapolsky et al., 2000; Hillegass et al., 2008; Li et al., 2010, 2011, 2012; Nesan and Vijayan, 2012, 2016). An indirect effect originates from the energetic trade-offs created with the emergence of the need to cope with stress. The amount of energy available for the functioning of all biological activities is largely fixed (Ware, 1982; Calow, 1985) and the energy mobilized to support the stress response (Schreck and Li, 1991; Wendelaar-Bonga, 1997; Mc Ewen and Wingfield, 2003; Schreck, 2010; Bouyoucos et al., 2017; Bouyoucos et al., 2018; Molina et al., 2020) is necessarily diverted from other processes (Calow, 1985; Wendelaar-Bonga, 1997; Mc Ewen and Wingfield, 2003; Schreck, 2010) with potential impairments to those activities, including reproduction. The energy depletion might be exacerbated by glucocorticoid-induced lack of appetite, reduced food assimilation capacity and reduced immune competency (Wendelaar-Bonga, 1997; Watson et al., 2020).

2.3. REPRODUCTIVE IMPAIRMENTS IN TELEOST FISHES

Despite the severe effects and high occurrence of pollution and climate change, reproductive impairments caused by these stressors are not included in our review because they also originate from a direct, specific action of the given pollutant or environmental variable on particular organs or processes involved in reproduction (Pandey, 2000; Pankhurst and Munday, 2011; Söffker and Tyler, 2012; Ullah and Zorriehzahra, 2015; Wheeler et al., 2020). Our review describes only reproductive impairments resulting from the elicitation of the stress response itself. Various stressors have been investigated, directly applied or simulated with administration of cortisol to the parents, through food, injection or implant, or treating eggs with cortisol, through injection or bath. Some stress might not be sufficiently intense to alter the indicators investigated (Wingfield

and Sapolsky, 2003; Watson et al., 2020) and reproductive responses also differ according to stress timing during the reproductive cycle (Contreras-Sánchez et al., 1998), stress type, intensity, frequency, duration, and the associated potential for habituation (Schreck et al., 2001). Mimicking stress through cortisol administration does not comprise the entirety of the stress response that begins with the perception of the stress and involves hormones other than cortisol (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012). Similarly, treating the eggs with cortisol cannot simulate the alterations to gamete quality that stress causes independently from the allocation of cortisol to the eggs (refer to section 2.3.1). Altogether, these differences in methodologies further contribute to the variability in the observed responses. Differences exist also between species due to various reproductive modes (Wingfield and Sapolsky, 2003; Pankhurst, 2016) and sensitivity to stress, between population, whether sourced from the wild or held in captivity (Cleary and Pankhurst, 2000; Johnstone et al., 2012; Sopinka et al., 2015b) and between individuals, due to animals' previous stress history, health (Wingfield and Sapolsky, 2003; French et al., 2007; Johnstone et al., 2012), size, life stage (Watson et al., 2020), sex, age and social status (Schreck et al., 2001; Sopinka et al., 2015b). Nevertheless, the perception and response to stress are somewhat stereotypic (Wendelaar-Bonga, 1997; Pankhurst, 2016) and, with caution, generalisations can be made.

2.3.1. Parental effects

'Parental effects' comprises the reproductive consequences observed in stressed adults, from gonadal development to gamete quality. One of the most commonly reported consequences is the decline in plasma levels of the main reproductive hormones: 17β-estradiol (E_2), progesterone (P_4) and testosterone (T) in females and testosterone and 11ketosterone (11KT) in males (Pickering et al., 1987; Carragher and Sumpter, 1990; Melotti et al., 1992; Foo and Lam, 1993b, a; Pankhurst and Dedual, 1994; Jardine et al., 1996; Clearwater and Pankhurst, 1997; Coward et al., 1998;

Haddy and Pankhurst, 1999; Pottinger et al., 1999; Cleary and Pankhurst, 2000; Bayunova et al., 2002; Kleppe et al., 2013) and, in some species, gonadotropins (hormones that stimulate the synthesis and secretion of the other reproductive hormones; Carragher et al., 1989; Choi et al., 2017). These alterations can occurr as soon as 2 h after stress exposure (Pottinger and Carrick, 2000) and be as pronounced as a 50% decrease in T and a 35% decrease in E_2 in male and female brown trout (*Salmo trutta*; Carragher et al., 1989). Most aspects of reproduction, from gonadal development (Miura et al., 1991; Cavaco et al., 1998), maturation of oocytes, (Selman and Wallace, 1989) and maintenance of follicular integrity (Janz and Van Der Kraak, 1997; Wood and Van Der Kraak, 2002), to reproductive behaviour (Wingfield et al., 1990; Magee et al., 2006; Milla et al., 2009) and timing of reproductive activities (Wallace, 1985; Miura et al., 1991; Cavaco et al., 1998; Milla et al., 2009), are regulated by a complex mix of interacting reproductive hormones (Borg, 1994; Nagahama et al., 1995; Schulz et al., 2010). Therefore, the stress-induced decline in reproductive hormone concentrations might be among the causes of other observed reproductive impairments.

Stress alters courtship in Atlantic cod (*Gadus morhua*) despite not affecting reproductive output in terms of spawning frequency, number of eggs produced and fertilization or hatching rate (Morgan et al., 1999). Moreover, cortisol exposure causes the masculinization of behaviour in female mosquito fish that attempt copulation with untreated females (*Gambusia affinis*; Knapp et al., 2011). Reproductive behaviours are essential components of successful reproduction and further research studying the population consequences of these alterations is needed.

Having invested energy in a stress response, an animal can either invest its remaining energy to complete the current reproductive seasonal cycle or save it for a later event (Schreck et al., 2001). Reproductive cessation after stress is observed in some oviparous teleosts (Mileva et al., 2011), occasionally with almost all stressed females undergoing complete reabsorption of the yolk

from the oocytes of the follicles before ovulation, termed atresia (Rowland, 1988). Anecdotal observations suggest that stress also causes parturition in some viviparous poecilid species (Schreck et al., 2001).

When reproduction is to occur, given the large amount of energy needed for gamete production (Parker, 1972), the trade-off that normally exists between number and size of gametes (Roff, 1992) can be intensified by the stress-induced energy shortage. The lower amount of energy available after a stressful event would prevent females from producing yolk (i.e., embryos' nutritional reserves) for the same, optimal number of oocytes (Watson et al., 2020) and promote partial atresia, through reabsorption of yolk from some of the oocytes (Coward et al., 1998; Cleary and Pankhurst, 2000), to recover previously allocated energy reserves for eventual redistribution to the other follicles. Indeed, a reduction in plasma vitellogenin levels, the main component of yolk, is reported after stress (Carragher et al., 1989; Foo and Lam, 1993a; Sopinka et al., 2014). Consequently, both a reduction in oocyte size and a reduction in oocyte number (fecundity) are observed. Females stressed late during oocyte maturation, once the number of oocytes is established, produce smaller oocytes but exhibit unaltered fecundity (Campbell et al., 1992, 1994; Contreras-Sánchez et al., 1998). Conversely, when stress is applied early, females produce fewer oocytes of unaltered size (Soso et al., 2008; Sopinka et al., 2014). Severe stressors early in oogenesis may cause alteration in both oocyte size and number due to their significant impact on energy resources (Mileva et al., 2011; Shourbela et al., 2016). Similarly, a highly stressful event, triggering atresia, can cause a reduction in gamete number even during late vitellogenesis (Cleary and Pankhurst, 2000). As a consequence of atresia and the production of smaller and/or fewer oocytes, a decrease in ovary mass is also reported in stressed females (De Montalembert et al., 1978; Rowland, 1988; Carragher et al., 1989; Foo and Lam, 1993a; Clearwater and Pankhurst, 1997; Cleary and Pankhurst, 2000; Hall et al., 2009; Sopinka et al., 2014). Testis development and

spermatogenesis require a lower amount of energy (Parker, 1972); nevertheless, stress-induced reduction in the number of sperm produced in rainbow trout (*Oncorhynchus mykiss*; Campbell et al., 1992) and negative effects on testicular mass are reported (Carragher et al., 1989; Goos and Consten, 2002). The decrease in reproductive hormone concentrations also contributes to gonadal impairments (Carragher et al., 1989; Clearwater and Pankhurst, 1997; Coward et al., 1998; Cleary and Pankhurst, 2000; Goos and Consten, 2002) Both gamete number and size are important determinants of male and female reproductive success (Parker, 1972; Roff, 1992) and offspring survival (Miller et al., 1988; Leggett and Deblois, 1994; Sogard, 1997) and the population consequences of these stress-induced alterations might be severe.

A stressful event can also affect gamete quality, causing DNA damage in developing follicles (Sousa et al., 2015) and altering the nature and quantity, usually reducing it, of the fundamental substances that females transfer to the egg, such as components of the innate immune system, mRNAs, proteins, vitamins and hormones (Li and Leatherland, 2012; Sousa et al., 2015). Stressed mothers can also transfer detrimental compounds to the oocytes, including excessive cortisol (Stratholt et al., 1997; McCormick, 1998; Andersson et al., 2011; Kleppe et al., 2013; Faught et al., 2016) and other biologically active compounds that can influence gene expression altering embryonic development. Moreover, in *O. mykiss* males, confinement stress causes a 90% decrease in sperm motility (Bobe and Labbé, 2010). All these traits contribute to gamete quality and the stress-induced alterations might impact gametes' ability to fertilize or to be fertilized, and impede the development of normal offspring (Bobe and Labbé, 2010). Accordingly, in some species, stress causes a reduction in oocyte fertilization (Campbell et al., 1994; Akar, 2011; Eriksen et al., 2013; Shourbela et al., 2016) and hatching rates (up to 20% decrease in *O. mykiss*; Campbell et al., 1992; Contreras-Sánchez et al., 1998; Gagliano and Mccormick, 2009; Li et al., 2010; Okanlawon, 2010; Eriksen et al., 2013; Shourbela et al., 2016).

In stressed females, both premature and delayed ovulation are reported. *Oncorhynchus mykiss* stressed towards the end of follicular maturation, close to ovulation, ovulate earlier (Contreras-Sánchez et al., 1998), potentially to reduce the cost of oocyte maintenance when energy needs to be invested to cope with stress (Contreras-Sánchez et al., 1998). Conversely, *O. mykiss* stressed at the beginning of vitellogenesis or throughout the maturation period, delay ovulation (Campbell et al., 1992; Foo and Lam, 1993a) because the stress-induced limitations in energy reduce the allocation of energy in follicular development, delaying the process and the subsequent ovulation (Foo and Lam, 1993a; Contreras-Sánchez et al., 1998). Data on males are scarce, but delays in spermatogenesis and gonadal development are reported in the common carp (*Cyprinus carpio*; Goos and Consten, 2002). These alterations might affect reproductive success given that the timing of all reproductive processes is finely regulated to ensure that embryos and offspring encounter suitable conditions for their survival and development (Cushing, 1975; Lowerre-Barbieri et al., 2011). Similarly, the fact that in a cichlid species (*Neolamprologus pulcher*) stressed females take significantly longer to spawn a second time (Mileva et al., 2011) might reduce the number of spawning events and eggs produced during the reproductive season, impacting total reproductive output (Lowerre-Barbieri et al., 2011).

An extreme consequence of exposure of females to high concentrations of cortisol is the development of male external morphological characteristics (Japanese rice fish *Oryzias latipes* and *G. affinis*; Grillitsch et al., 2009; Knapp et al., 2011). Further studies must investigate potential masculinization effects of stress-increased cortisol concentrations, whether they involve gonadal development and to what extent mating and reproductive success are impaired.

2.3.2. Intergenerational effects

'Intergenerational effects' include alterations occurring in offspring of stressed parents after fertilization, from the embryonic stage to adulthood. When mentioning 'stressed offspring' in this

review, we refer to offspring (i.e., embryos, neonates or larvae) hatched from cortisol-treated eggs or produced by parents that experienced stress.

The timing of embryonic development and hatching is highly regulated (Cushing, 1975; Lowerre-Barbieri et al., 2011) and any alteration potentially compromises offspring survival. Few studies examine stress-induced alteration in hatching time (Stratholt et al., 1997; McCormick and Nechaev, 2002; Gagliano and McCormick, 2009; Sloman, 2010; Magierecka, 2019) and anticipated hatching has been observed in damselfish (*Pomacentrus amboinensis*; Gagliano and McCormick, 2009) and three-spined stickleback stressed larvae (*Gasterosteus aculeatus*; Magierecka, 2019). Accordingly, an increased cell proliferation rate is recorded in stressed embryos (McCormick and Nechaev, 2002; Li et al., 2012), possibly affecting the complex process of embryogenesis (Li et al., 2012).

The realization of the fine-tuned pattern of gene expression during embryogenesis is crucial for organs and tissues development, but is altered in oocytes produced by stressed females (Faught et al., 2016) by the increased cortisol concentration acting as a transcriptional regulator (Sapolsky et al., 2000), with up to hundreds of genes either up- or down-regulated (Kleppe et al., 2013). In *O. mykiss*, immune-related genes are initially up-regulated and later down-regulated, resulting in impairment of the immune system functionality (Li et al., 2011). Both enhanced and depressed expression of growth-related genes is observed in the same species (Li et al., 2010, 2012) and is likely associated with increased embryonic cell proliferation rate (Li et al., 2012) and late offspring growth rate (Li et al., 2010). In zebrafish (*Danio rerio*), the expression of genes related to brain development is affected, conditioning neurogenesis in areas of the brain associated with behavioural control and HPI axis response and consequently causing altered boldness and risk-taking behaviour in offspring (Best et al., 2017). Similarly, the expression of key genes for HPI axis functioning is down-regulated and associated with alteration in larval stress

responses (Nesan and Vijayan, 2016). Alterations in gene expression are occasionally associated with morphological abnormalities in the hatchlings (Hillegass et al., 2008), as severe as heart malformations and functionality disruptions when the expression of key genes for heart morphogenesis and contraction is suppressed (Nesan and Vijayan, 2012).

Parental stress increases the occurrence of malformations in the offspring (Aiyelari et al., 2007; Okanlawon, 2010; Eriksen et al., 2013; Shourbela et al., 2016), ranging from minor absence of symmetry (Eriksen et al., 2008; Gagliano and McCormick, 2009) to conspicuous skeletal (Eriksen et al., 2007; Hillegass et al., 2008; Nesan and Vijayan, 2016) and cardiac malformations (Nesan and Vijayan, 2012). Generally, more than impairing the functioning of the impacted organs and tissues (Nesan and Vijayan, 2012), malformations are associated with decreased fitness, growth (Andrades et al., 1996), survival rates (Eriksen et al., 2007), resistance to oxidative stress (Barahona-Fernandes, 1982), swimming performance (Helling et al., 2003), predator avoidance (Bergstrom and Reimchen, 2003) and auditory and dispersal abilities (Gagliano et al., 2008), highlighting the possible severe consequences of the stress-induced increase in malformation incidence. An extreme alteration is the increase in the proportion of males when Argentinian silverside (*Odontesthes bonariensis;* Hattori et al., 2009) and *O. mykiss* larvae (Van Den Hurk and Van Oordt, 1985) are treated with cortisol. Specific research should investigate whether the increased concentrations of cortisol transferred in the eggs by stressed mothers can influence offspring sex determination.

Offspring size at birth and later in life correlates with fitness and survival because small individuals are typically disadvantaged (Miller et al., 1988; Leggett and Deblois, 1994; Sogard, 1997) due to locomotor drawbacks associated with their size that reduce prey-capture and predator-avoidance abilities (Blaxter, 1986; Bailey and Houde, 1989). Similarly, growth rate influences survival because faster growth reflects a more rapid passage through the most

vulnerable life stages when body size is small (Meekan and Fortier, 1996; Bergenius et al., 2002; Wilson and Meekan, 2002). In some species, parental stress reduce offspring size (Mccormick, 1998, 1999; Ostrand et al., 2004; Capelle et al., 2017; Magierecka, 2019), persisting up to 7 months after birth in *S. salar* (Eriksen et al., 2006, 2013). Parental stress do not alter (Contreras-Sánchez et al., 1998; Eriksen et al., 2006; Magierecka, 2019) or even increases larval growth rates (Eriksen et al., 2006; Li et al., 2010; Eriksen et al., 2013; Nesan and Vijayan, 2016). However, excessively fast growth is associated with oxidative damage (Alonso-Alvarez et al., 2007) and shortened telomeres (Pauliny et al., 2015), potentially leading to faster ageing and decreased survival (Ricklefs, 2006; Metcalfe and Alonso-Alvarez, 2010).

At birth, stressed larvae have fewer endogenous reserves (yolk and oil globules; McCormick, 1998, 1999; Eriksen et al., 2006; Gagliano and McCormick, 2009; Eriksen et al., 2013) which represent an essential source of sustenance used for tissue growth and maintenance during the early periods after birth. Smaller reserves might reduce growth rates (Rothschild, 1986) and the starvation period that offspring can endure (Miller et al., 1988; Leggett and Deblois, 1994; Eriksen et al., 2006), reducing survival. However, in other species, no difference is recorded (Stratholt et al., 1997). In stressed *S. salar*, stressed offspring show a reduced yolk-sac consumption rate in the first weeks after hatching, possibly in response to a lower initial amount of resources and the need to retain them for as long as possible (Eriksen et al., 2006).

Studies variously report stress-induced decreased (Eriksen et al., 2007; Soso et al., 2008; Li et al., 2010; Okanlawon, 2010; Shourbela et al., 2016), unchanged (Campbell et al., 1992, 1994; Contreras-Sánchez et al., 1998; Eriksen et al., 2006; Sloman, 2010; Li et al., 2012; Sopinka et al., 2014; Magierecka, 2019) or increased offspring survival (Gagliano and McCormick, 2009; Capelle et al., 2017), even in the same species. Survival is usually determined under protected laboratory conditions, with abundant food and absence of predation and competition, both of which

profoundly influence survival, and experiments typically end within a month after birth. These observations might explain why unaltered or even increased survival is observed.

Parental stress in several ways affects offspring performance. In stressed *O. mykiss* offspring, an initial increase and a later decrease in the concentration and activity of some elements of the innate immune system are observed, suggesting an immunosuppressive effect (Li et al., 2011). However, despite the potential for a stress-mediated immune compromise, impaired infection resistance has not yet been detected (Contreras-Sánchez et al., 1998).

Stressed offspring manifested both decreased (Auperin and Geslin, 2008; Colson et al., 2015) and increased (Sloman, 2010; Nesan and Vijayan, 2016) basal cortisol levels, indicating modifications of the HPI axis functioning. Both alterations are likely deleterious, given the crucial role of cortisol in regulating biological processes (Ayson, 1989; Wendelaar-Bonga, 1997; Milla et al., 2009; Nesan et al., 2012; Nesan and Vijayan, 2013, 2016) and the energy expenditure associated with a potential continuous state of HPI axis activation (Wendelaar-Bonga, 1997). Additionally, in *O. mykiss*, *G. aculeatus* and *D. rerio*, parental stress suppresses the increase in cortisol concentration that should occur when offspring are challenged with a stressful event (Auperin and Geslin, 2008; Colson et al., 2015; Nesan and Vijayan, 2016; Magierecka, 2019). Failing to sufficiently increase cortisol production and elicit a stress response may reduce fitness, given these responses are essential in maintaining homeostasis (Nesan and Vijayan, 2016). Similarly, when challenged with a stress, *D. rerio* larvae hatched from stressed parents manifest a reduced increase in the magnitude of the rise in heart rate (Nesan and Vijayan, 2012). These observations indicate a general reduction in cardiac performance and confirm the impaired ability to respond to stress through the cardiovascular adjustments necessary to sustain the fight-or-flight response (Wendelaar-Bonga, 1997). Higher basal heart rates in stressed embryos (McCormick and Nechaev, 2002) and lower heart rates in stressed larvae (Nesan and Vijayan, 2012) are also recorded. These

dysfunctions are particularly concerning because they are recorded in offspring that do not manifest heart malformations. Larvae that developed severe cardiac malformations following parental stress manifest even more severe consequences, such as extremely irregular heartbeats, suggesting a complete disruption of heart functions (Nesan and Vijayan, 2012).

Offspring of stressed *S. trutta* have higher metabolic rates (Sloman, 2010), and while this might confer an advantage in acquiring a dominant position (McCarthy, 2001), it is also associated with higher energy usage (Glazier, 2015) and faster depletion of the energy reserves (Gagliano and McCormick, 2009). An increased rate of unsuccessful feeding attempts is observed in stressed offspring, indicating a reduced feeding ability that might potentially further reduce energy availability (Eriksen et al., 2011). In addition, stressed offspring of some salmonids species manifest altered burst swimming rates (Sopinka et al., 2014; Sopinka et al., 2016b), and increased swimming rates, being a hyperactive response, may cause a quicker depletion of the energy stores (Sopinka et al., 2014). Burst swimming is used to quickly escape when attacked (Sopinka et al., 2016b) and the observed less efficient performance might also impair offspring ability to escape predators.

Cortisol treatment of eggs does not affect offspring oxidative stress or the concentration of antioxidant compounds in sockeye salmon (*Oncorhynchus nerka*). However, maternal cortisol levels are negatively correlated with offspring antioxidant capacity (Taylor et al., 2016), suggesting that maternal factors, other than the sole stress-induced increase in egg cortisol concentration, may contribute to impair the offspring antioxidant system and therefore result in damage to cells and the whole organism (Metcalfe and Alonso-Alvarez, 2010).

A stress experienced by the parents can also affect offspring behaviour, and enhanced aggressiveness towards conspecifics and mirror images is usually reported in salmonids and guppy (*Poecilia reticulata*; Sloman, 2010; Eriksen et al., 2011; Eaton et al., 2015; Sopinka et al., 2015a). In

one case, aggressiveness was lower in stressed than unstressed offspring (Burton et al., 2011). Higher aggressiveness improves competitiveness and acquisition of resources and dominance, consequently increasing fitness (Sloman, 2010; Eriksen et al., 2011; Sopinka et al., 2015a). However, aggressiveness is energetically demanding (Castro et al., 2006; Ros et al., 2006) and increases the chances of being detected by predators (Martel and Dill, 1993; Fuiman and Magurran, 1994; Archard and Braithwaite, 2003), potentially reducing survival in stressed offspring. Stressed offspring have enhanced behavioural reactivity (Colson et al., 2015) towards sudden stimuli and novel environments, manifesting more pronounced 'flight' (i.e. increase in activity level; Espmark et al., 2008; Eriksen et al., 2013; Colson et al., 2015; Best et al., 2017) and 'freezing responses' (Eriksen et al., 2011). A rise in activity causes increases in energy expenditure (Colson et al., 2015) and predation risk (Martel and Dill, 1993; Fuiman and Magurran, 1994; Archard and Braithwaite, 2003) and the freezing response, being associated with fear and stress, activates the HPI axis and its deleterious consequences. The flight and the freezing responses are more intense in stressed offspring, and their associated negative aspects are likely more severe in these offspring than in those hatched from non-stressed parents. In stressed offspring, boldness is either unaltered (*G. aculeatus*; Magierecka, 2019) or increased (Sopinka et al., 2015a; Best et al., 2017). Increased boldness leads to exploratory behaviours that facilitate finding food but also expose animals to an increased risk of predator encounter (Martel and Dill, 1993; Fuiman and Magurran, 1994; Archard and Braithwaite, 2003). Learning ability and memory are key for orientation, foraging, social interaction, predator avoidance and reproduction (Kelley and Magurran, 2003; Odling-Smee and Braithwaite, 2003; Braithwaite, 2005). In some species, parental stress does not alter these abilities (Cortez Ghio et al., 2016) but in others, it causes memory deficits (Sloman, 2010; Eaton et al., 2015).

Only one study conducted on *G. aculeatus* investigated the effect of parental stress on the reproductive output of the offspring. Offspring of stressed parents produced fewer but heavier eggs (Magierecka, 2019), seemingly investing more on the quality rather than the quantity of the eggs. However, when egg predation risk is among the main sources of mortality, a reduction in the number of eggs produced, despite their higher quality, will likely impact recruitment.

2.3.3. Transgenerational effect

Stressors that in addition to inducing a stress response also directly influence reproductive processes or organs, such as temperature and pH alterations associated with climate change (Pandey, 2000; Pankhurst and Munday, 2011; Söffker and Tyler, 2012; Ullah and Zorriehzahra, 2015; Wheeler et al., 2020), are known to cause transgenerational effects in teleost fishes (Munday. 2014; Donelson et al., 2017; Servili et al., 2020). However, data on stressors that only act through the triggering of the stress response are scarce, and research on *G. aculeatus* is the only report. Transgenerational effects are those observed in the offspring (F2 generation) produced by the offspring (F1 generation) of stressed parents (F0 generation). While hatching rate of F2 eggs are not affected by stress experienced by F0 females, stressed F2 fry have larger size despite the unaltered developmental time, indicating a faster embryonic growth (Magierecka, 2019). Faster growth can be advantageous (Meekan and Fortier, 1996; Bergenius et al., 2002; Wilson and Meekan, 2002), but can also result in faster ageing and decreased survival (Ricklefs, 2006; Metcalfe and Alonso-Alvarez, 2010). Because this is the only study investigating transgenerational effects of anthropogenic stressors, these results cannot be extrapolated to other fish species.

2.4. REPRODUCTIVE IMPAIRMENTS OF FISHING-CAPTURE STRESS

Despite fishing currently being one of the main threats for wild fish populations (Dulvy et al., 2003; Pauly et al., 2005; Dulvy et al., 2014), the reproductive consequences of fishing-capture

stress have been only marginally investigated. Many of the most researched stressors, such as chasing, crowding, handling and air exposure, closely reflect conditions experienced during fishing capture procedures, suggesting strong similarities in the responses elicited.

Accordingly, fishing-capture stress decreases reproductive hormone concentrations (Melotti et al., 1992; Jardine et al., 1996; Clearwater and Pankhurst, 1997; Haddy and Pankhurst, 1999; Cleary and Pankhurst, 2000; Bayunova et al., 2002), delays gonadal development (Rowland, 1988), causes atresia (Rowland, 1988; Clearwater and Pankhurst, 1997; Cleary and Pankhurst, 2000; Hall et al., 2009), inhibits follicle growth (Rowland, 1988) and decreases female fecundity, up to a 62% reduction in the number of follicles in European sea bass (*Dicentrachus labrax*) exposed to angling (Watson et al., 2020). In Murray cod (*Maccullochella peelii*;) the onset of ovarian impairments is rapid and partial atresia occurs within 48 h from gillnet capture (Rowland, 1988). Fishing capture also decreases white bass (*Morone chrysops*) semen osmolarity, which in turn causes pre-activated sperm motility. As motility of spermatozoa is brief, pre-activated sperm once ejaculated, might not be able to reach and fertilize the eggs, thereby reducing male reproductive success (Allyn et al., 2001).

Parental care in male largemouth bass (*Micropterus salmoides*) and smallmouth bass (*Micropterus dolomieu*) are affected by fishing-capture stress. Angling procedures cause a reduction in males' reproductive success (O'connor et al., 2009) with more severe consequences as fishing intensity increases (Philipp et al., 1997). These consequences likely originate from the reduction in the energy budget, immune competence (O'connor et al., 2009) and aggressiveness of males defending the nest (Philipp et al., 1997) that are caused by the fishing-capture stress. However, explanations not related to the elicitation of the stress response, such as a higher degree of nest abandonment due to the increased egg predation during the absence of males, cannot be excluded (Philipp et al., 1997).

Fishing-capture stress experienced by parents can also affect offspring traits, including increasing the occurrence of offspring malformations, to the point that all offspring of stressed *G. morhua* present some sort of deformity (Morgan et al., 1999). Despite no observed effect on offspring survival (Morgan et al., 1999), the increased occurrence of malformations might reduce survival of those offspring produced by mothers that experienced fishing-capture stress, later in life (Morgan et al., 1999; Eriksen et al., 2007). Moreover, parents that experienced fishing-capture stress produce offspring with decreased size compared to unstressed offspring (Ostrand et al., 2004).

The stress-induced reproductive impairments observed both in parents and offspring might have severe consequences on reproductive success and population recruitment (Schreck et al., 2001; Sopinka et al., 2016a). However, while severe fishing-capture conditions, such as long periods of capture by angling or longlining, cause a reduction in reproductive hormone levels (Clearwater and Pankhurst, 1997; Cleary and Pankhurst, 2000) and fecundity (Watson et al., 2020) and an increase in malformation occurrence (Morgan et al., 1999), milder fishing operation, such as short angling capture periods, have no consequences for gonadal development (Lowerre-Barbieri et al., 2003; Hall et al., 2009, 2017), egg hatching success (Booth et al., 1995), egg viability or development (Pettit, 1977). Therefore, modifications of fishing practices aimed at reducing the severity of the stress elicited might represent viable options to reduce the negative population consequences originating from reproductive impairments.

2.5. REPRODUCTIVE IMPAIRMENTS IN CHONDRICHTHYAN FISHES

For chondrichthyans, very few studies report the reproductive consequences of anthropogenic stress, in general, and fishing-capture stress, in particular (Wheeler et al., 2020), and only four have purposely investigated these impairments. The most severe consequence is that, in elasmobranchs (sharks, skates and rays), fishing capture induces parturition in at least 93

viviparous species, occurring on average in 24% of the captured pregnant females. Depending on the stage of gestation when fishing capture is experienced, females can abort eggs or non-viable neonates, or give birth to partially or completely developed neonates, i.e., premature parturition (Adams et al., 2018). In some species, including shortnose guitarfish (*Zapteryx brevirostris*; Wosnick et al., 2019) and *Potamotrygon amandae* (de Sousa Rangel et al., 2020), all pregnant females aborted their pups when captured. Some species, such as the Kapala stingaree (*Urolophus kapalensis*), are so sensitive that only 20 minutes of trawling causes capture-induced parturition (Campbell et al., 2018). Scarce information exists on the survival of premature neonates, impeding the drawing of any general conclusion. Charvet-Almeida et al. (2005) mention that premature neonates of several freshwater stingrays rarely survive, regardless of the developmental stage and Campbell et al. (2018) report that neonates died soon after capture-induced parturition. Overall, it seems likely that the survival rate will be low for all but almost fully-developed neonates. If neonates survive, no information is available on their fitness or performance, such as feeding or swimming ability. Even more concerning, of the identified 93 elasmobranch species, nine are classed on the IUCN Red List for species extinction risk as endangered or critically endangered (Adams et al., 2018). Moreover, the opportunistic nature of the observations on fishing vessels and the remark that capture-induced abortion or parturition can be delayed for several hours after capture (Wosnick et al., 2019; de Sousa Rangel et al., 2020; LF personal observations) suggest that the phenomenon may commonly go unnoticed and is underestimated.

When premature parturition does not occur soon after fishing capture (up to 28 h; Wosnick et al., 2019), parturition duration does not seem to be impacted by the stress. Indeed, pregnant southern fiddler rays (*Trygonorrhina. dumerilii*) exposed to trawling simulation gave birth within the reported parturition period and in the same time frame as unstressed females (Guida et al., 2017), even when stressed close to parturition date (average of 24 days before; Chapter 6).

However, trawling-capture stress alters the timing of other reproductive processes. Indeed, trawling stress induces premature ovulation in small-spotted catshark (*Scyliorhinus canicula*) within 3 h after capture (Dodd and Duggan, 1982), suggesting that oocytes are ovulated before the conclusion of vitellogenesis, thereby reducing the amount of resources for embryonic development. Moreover, egg-laying has been observed upon handling of the Port Jackson shark (*Heterodontus portusjacksoni*; Adams et al., 2018) and soon after trawl capture and transport of *S. canicula* (LF personal observation). Similarly, sometimes oviparous females dissected after capture bear only one egg case and since in species with short single oviparity females typically lay two egg cases at a time (Musick and Ellis, 2005), it has been suggested that the other egg case was prematurely laid due to fishing-capture stress, termed capture-induced oviposition (Kyne et al., 2011; Bustamante et al., 2013; Rigby, 2015). The egg case might have also been laid shortly before capture and stronger evidence is needed to confirm the existence of capture-induced oviposition. Nevertheless, and despite possibly seeming a lesser consequence compared to capture-induced abortion and parturition, given that eggs are self-sustaining, capture-induced oviposition might be of considerable importance for species with multiple oviparity and sustained single oviparity. In these species, eggs are laid after an extended period retained inside the mothers' reproductive tract, meaning that a substantial part of the embryonic development occurs in that protected and regulated environment (Nakaya, 1975; Nakaya et al., 2020). The occurrence of capture-induced oviposition will reduce the time the eggs spend in the maternal environment and might cause disruptions to embryonic development and increase predation (Nakaya et al., 2020). During the time spent in the uterus, the egg case undergoes sclerotization (Koob and Cox, 1993), which is essential for the development of its antibacterial and antifouling properties (Thomason et al., 1996; Hamlett et al., 1998). Shorter retention in the uterus might affect these characteristics, exposing embryos to higher infection rates and affecting osmoregulation and oxygenation if the

surface of the egg case becomes heavily colonized. After selecting a substrate with suitable environmental conditions and low predator presence (Trujillo et al., 2019), most species secure the eggs in crevices (Powter and Gladstone, 2008), buried (Boisvert et al., 2015) or attached to hard substrate (Smith and Griffiths, 1997). Eggs spontaneously oviposited during the capture event or found on the vessel and discarded to the sea might not encounter appropriate environmental conditions. The consequences are unknown, but development failure is a possible outcome if eggs must maintain a specific orientation for embryogenesis, like in elephant fish, *Callorhinchus milii* (Boisvert et al., 2015).

Fishing-capture stress also negatively affects parental condition, reducing body mass in females (Guida et al., 2017) and increasing mortality rate in pregnant females (Wosnick et al., 2019) and in reproductively active males (Prado et al., 2018). Capture stress does not influence reproductive hormone levels in *T. dumerilii* (Guida et al., 2017) but P_4 levels in male bonnethead shark (*Sphyrna tiburo*) decrease following intense fishing-capture stress (Manire and Rasmussen, 1997). The difference likely originates from the diverse sensitivity to fishing capture, with *T. dumerilii* being particularly resilient (Manire et al., 2001; Martins, 2017).

Fishing-capture stress can alter offspring characteristics. Neonates born from *T. dumerilii* stressed during pregnancy show 11.9% and 27% decrease in total length and body mass compared to unstressed neonates (Guida et al., 2017) and body mass is reduced even when the stress is applied closer to parturition (Chapter 6). In the same species, stressed neonates manifest an increased ratio of granulocytes to lymphocytes (Guida et al., 2017; Chapter 6), an immune alteration typically elicited by stressors (Van Rijn and Reina, 2010), suggesting that parental exposure to fishing capture induces a state of chronic stress in the offspring. The alteration is potentially long-lasting, originating after maternal stress and persisting until birth, at least 40 days later (Guida et al., 2017), and, given the energy requirement of mounting and maintaining an

immune response (French et al., 2007), can compromise other biological activities (Wendelaar-Bonga, 1997).

Even stressors occurring near parturition (within an average of 24 days before), when embryonic development is close to completion, can have severe impacts. Exposure of pregnant *T. dumerilii* to trawling simulation reduces the volume of the residual yolk sac in neonates at birth, despite stress being applied after the conclusion of vitellogenesis and could have not impacted the initial allocation of yolk to the oocytes (Chapter 6). A potential explanation is the fact that stressed neonates have to invest more energy in other biological processes, such as the immune response (French et al., 2007). Monitoring growth and survival of these neonates housed in captivity for the first month after birth did not indicate a difference in survival between stressed and unstressed neonates but showed a reduced growth increment in total length for stressed neonates (Chapter 6) that, as for teleost, might increase the predation risk (Meekan and Fortier, 1996; Bergenius et al., 2002; Wilson and Meekan, 2002). The reduced growth was observed despite both groups of neonates consuming the residual internal yolk sac reserves at the same rate (% decrease in remaining yolk sac; Chapter 6), potentially because, in stressed neonates, part of the energy obtained from yolk sac metabolism has to be diverted from the allocation for growth to sustain the prolonged elicited immune response (French et al., 2007).

After a simulated predator attack during a swimming test, *T. dumerilii* neonates born from mothers exposed to trawling simulation burst-swim for shorter distances (Chapter 6). The inability of neonates to distance themselves enough from predators, remaining in their field of view, might increase the risk of being individuated and predated. In the same study, neonate boldness was tested both in an undisturbed situation and after a simulated predator attack. In the undisturbed situation, stressed *T. dumerilii* neonates delay their first movement and spend a smaller amount of time swimming and exploring the surrounding area, indicating decreased boldness, potentially

reducing their ability to find food and compete (Archard and Braithwaite, 2003). The higher boldness of unstressed neonates, being associated with higher visibility, might increase predation risk (Martel and Dill, 1993; Fuiman and Magurran, 1994; Archard and Braithwaite, 2003). However, when simulating a predator attack, the difference in boldness disappeared while both stressed and unstressed neonates showed marked reduction in boldness.

2.6. INSIGHTS ON CHONDRICHTHYAN IMPAIRMENTS BASED ON TELEOST RESEARCH

There are differences between teleost and chondrichthyan stress responses, particularly in the use of different primary energy substrates (carbohydrates in teleost and lipid in chondrichthyan), the difference in the main stress hormones and the inconclusive evidence for the glucocorticoid action of 1-α-OH in chondrichthyans (Ruiz-Jarabo et al., 2019). Indeed, in small spotted catshark (*Scyliorhinus canicula*), despite after air exposure an increase in 1-α-OH concentration has been observed in association with enhanced glycolysis and gluconeogenesis, a direct, causal relationship between 1-α-OH increase and the induction of glycolysis and gluconeogenesis has yet to be definitely proven (Ruiz-Jarabo et al., 2019). Nevertheless, the main physiological, energetic and immunological consequences of stress are comparable. Moreover, also the stress-induced alterations in the few reproductive traits investigated in chondrichthyans are similar to effects observed in teleost fishes (refer to sections 2.3 and 2.5). Indeed in chondrichthyans, as in teleosts (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012), stress, and specifically fishing-capture stress, causes immediate or delayed mortality (Frick et al., 2010b; Dapp et al., 2017), physiological disruption, such as increases in stress hormone levels, haematocrit and lactate and glucose concentrations (Frick et al., 2009; Mandelman and Skomal, 2009; Skomal and Mandelman, 2012; Martins, 2017; Martins et al., 2018; Ruiz-Jarabo et al., 2019), alteration in the cellular energetic charge (Guida et al., 2016a), immunological impairments (Van Rijn and Reina, 2010; Heard et al., 2014; Guida et al., 2017), behavioural alteration (Holts and

Bedford, 1993; Klimley et al., 2002; Nakano et al., 2003; Danylchuk et al., 2014; Guida et al., 2016b; Martins, 2017; Raoult et al., 2019) Chapter 4) and energetic disruption, including increased (Bouyoucos et al., 2017, 2018; Molina et al., 2020) and decreased energy use (Molina et al., 2020; Chapter 3 and 4). Therefore, given the absence of sufficient specific research on chondrichthyans, we draw upon what is known of teleost fishes responses and chondrichthyan life history to speculate on possible reproductive consequences of fishing-capture stress in chondrichthyans.

As in teleosts, fishing-capture stress and the associated altered energy allocation (Bouyoucos et al., 2017, 2018; Molina et al., 2020) and reproductive hormone levels (Manire and Rasmussen, 1997) experienced during vitellogenesis might alter maternal allocation to the developing follicles. This would likely result in the transfer of increased amount of stress hormones and altered amounts of energy resources and other compounds such as hormones, mRNA and proteins, possibly impairing embryonic gene expression and development and offspring performance. If stress hormones are transferred to the oocyte, given the extended embryonic development of up to three years for some viviparous species such as the greeneye spurdog shark (*Squalus chloroculus;* Rochowski et al., 2015) and one year for some oviparous species such as *H. portusjacksoni* (Rodda and Seymour, 2008), embryos will be exposed to their effects for many months, certainly longer than teleosts, with potentially more significant consequences.

Chondrichthyan reproductive modes are subdivided according to the type of maternal energy provisioning to the embryos (Hamlett et al., 2005). In lecithotrophic species (oviparous and some viviparous species), mothers provide the embryo only with yolk sac reserves, while in matrotrophic species (viviparous species only), females supply additional nutrition (Dulvy and Reynolds, 1997; Hamlett et al., 2005). The intergenerational effects reported for *T. dumerilii* are concerning because they are observed in a lecithotrophic species (Marshall et al., 2007) even though the embryos' sole nutritional supply, the yolk sac, was not impacted because the stress

occurred after vitellogenesis was concluded and the yolk sac was formed (Guida et al., 2017; Chapter 6). This observation indicates that other mechanisms besides a reduced energy supply, including embryonic exposure to maternal stress hormones, are involved in causing the reproductive impairments. Moreover, in matrotrophic species, the stress-induced energetic constraints (Bouyoucos et al., 2017, 2018; Molina et al., 2020), affecting maternal ability to provide additional nutrients, will likely cause more severe effects. Alterations in nutrient supply are likely to last during the maternal metabolic recovery period (up to 7 days in *C. milii*; Chapter 4) potentially causing a significant impact for fast-developing embryos *in utero* (Marshall et al., 2007).

Viviparous species are possibly more sensitive to maternal stress because of the close physiological connection between mother and embryos and the additional supply of nutrients involved in some species. Nevertheless, oviparous species may also be impacted. Along with the allocation of higher stress hormone concentrations in the yolk, if a stress occurs after completion of vitellogenesis but before egg encapsulation in the egg case (Carrier et al., 2004), stress hormones might be deposited in the jelly surrounding the oocyte in the egg case, as observed in birds (Rubolini et al., 2005), and therefore impact embryos during their long development (Serena, 2005; Rodda and Seymour, 2008).

Some chondrichthyans have extremely prolonged follicle development (ovarian cycle), i.e, 3 years in *S. chloroculus* (Rochowski et al., 2015) and in school shark (*Galeorhinus galeus*; Walker, 2005b). Therefore, the probability of an animal experiencing fishing-capture stress at some point during the reproductive cycle and thereby suffer reproductive impairment is high and particularly concerning for *G. galeus*, whose global status has recently been classed as critically endangered (Walker et al., 2020). Moreover, it is common for some species, such as spiny dogfish (*Squalus acanthias*), gummy shark (*Mustelus antarcticus*), *G. galeus* and *T. dumerilii*, to undergo

vitellogenesis and gestation concurrently (Stenberg, 2005; Walker, 2005b; Marshall et al., 2007; Walker, 2007) and so any stress suffered potentially affects two offspring generations, impacting both the pregnancy cycle, and the current developing embryos, and the ovarian cycle and the future embryos that will develop from the currently maturing oocytes. Unlike teleosts, many oviparous chondrichthyans reproduce throughout the year or have only a very brief resting period (Capapé et al., 2008) and potentially all mature animals captured are in a reproductively active state and at risk of reproductive consequences if captured and released. Another consequence of reproduction occurring over very long cycles is that any stress-induced reproductive impairments has the potential to severely impact population abundance because one or more years must pass before a chondrichthyan can complete a subsequent reproductive cycle and contribute to the recovery of the population.

Sharks and rays have complex reproductive behaviours and courtship. Specific body movements, such as body arching, following and paired swimming in nurse sharks (*Ginglymostoma cirratum*), are performed by females to signal their receptivity and by males to attract female choice. Mating behaviours also include aggression displays and bites towards females and between males competing for hierarchy and females (Pratt and Carrier, 2005). Social aggression is regulated by the production of gonadal hormones (Tricas et al., 2000) and the 'following' behaviour of males is suggested to be induced by females releasing pheromones (Demski, 1990). Given the important role of reproductive hormones in regulating these behaviours, the alterations in their levels observed after a fishing-capture stress (Manire and Rasmussen, 1997) might alter reproductive behaviour and reduce the probability of a successful mating.

2.7. RESEARCH PRIORITIES FOR CHONDRICHTHYANS

Given that fishing-capture stress seems to have a high potential to cause severe consequences for reproduction in chondrichthyans and given the poor conservation status of many of these species, priority should be given to further researching the consequences of fishing-capture stress on reproduction. Additional research is also needed because the only four studies purposely investigating fishing-induced reproductive impairments refer to three ray species (Guida et al., 2017; Wosnick et al., 2019; de Sousa Rangel et al., 2020; Chapter 6), two belonging to the same batoid family, the Trygonorrhinidae, sharing the same reproductive mode of lecithotrophic viviparity (Marshall et al., 2007; Wosnick et al., 2019) and being quite resilient to fishing-capture stress (Martins, 2017; Wosnick et al., 2019). Moreover, in two studies, animals were subjected to similar fishing capture simulation (Guida et al., 2017; Chapter 6).

Different species (Frick et al., 2010a, 2010b), even within the same taxonomic family (Carcharhinidae; Mandelman and Skomal, 2009), exhibit wide variation in sensitivity to stress, primarily related to respiratory mode. Species able to buccal pump and extract oxygen when constrained are more able to withstand the stress, whereas obligate ram ventilating species are more sensitive (Dapp et al., 2015, 2016). Differences in responses might also be related to fishing gear types, with fishing gear like trawl nets and gillnets that restrict animal movement and oxygen uptake being particularly stressful (Dapp et al., 2015).

Given this variability in stress responses (Mandelman and Skomal, 2009; Dapp et al., 2015) and the likely different impact of stress on the reproduction of species characterized by different reproductive modes (refer to section 2.6), one of the first priorities should be expanding the investigation to species belonging to diverse taxonomic groups, with different reproductive modes and sensitivity. Besides occasional observations on fishing-induced ovulation and oviposition, no data are available for oviparous chondrichthyans despite the responses observed in teleosts

indicating that oviparous species can be highly impacted. Investigating the effect of fishing-capture stress experienced during vitellogenesis or the egg-laying season, such as the transfer of higher stress hormone concentrations to the eggs or stress-induced oviposition, should be another focus. Wild animals are exposed to several stressors simultaneously (Orr et al., 2020) and the effect of fishing-capture stress should be studied in association with habitat degradation, pollution and the environmental changes foreseen as a consequence of climate change, all proven to severely impact chondrichthyan reproduction (Wheeler et al., 2020). This is particularly important for oviparous species, because developing embryos in their egg case cannot move away from areas affected by these stressors (Wheeler et al., 2020). Moreover, in several oviparous chondrichthyans, predicted seawater temperature and pH for the end of the century are known to impact embryonic development and hatchling phenotype, such as survival, metabolic rate and feeding ability (Di Santo, 2016; Rosa et al., 2017; Di Santo, 2019). Understanding potential interacting effects of fishing-capture stress with other stressors on reproduction will help better assess the vulnerability of chondrichthyan species to anthropogenic stressors through an ecological vulnerability risk assessment (Chin et al., 2010; Wheeler et al., 2020; Walker et al., in review). This information is necessary to improve the predictions obtained from stock assessment and population dynamics models, in order to assess whether fishery exploitation and bycatch are sustainable or if management measures need to be implemented (Punt and Walker, 1998; Pribac et al., 2005; Walker, 2005b, a).

2.8. MANAGEMENT PRIORITIES FOR CHONDRICHTHYANS

Despite the paucity of data, the poor conservation status of many chondrichthyans and the high occurrence of bycatch (Dulvy et al., 2014) suggest that a precautionary approach should be adopted to reduce the occurrence and severity of potential reproductive impairments. Among the severe threats to these populations, fishery capture is both the most severe and easiest to

manage. Due to the high synchronicity of the reproductive cycle in many species (Walker, 2005b; Marshall et al., 2007; Walker, 2007), reducing anthropogenic impact on known pupping and egg-laying grounds in periods of the year coinciding with the most sensitive processes of the reproductive cycle, such as near-term pregnancy or oviposition (de Sousa Rangel et al., 2020), would be one of the best solutions. This measure might not be achievable, however, if these areas are also important fishing grounds. In the viviparous *P. amandae*, the biggest females, carrying the highest number of embryos, are most susceptible to abortion and premature parturition after fishing capture (de Sousa Rangel et al., 2020). Therefore, protecting the larger and more fecund animals (uterine fecundity is usually positively correlated with female size; (Marshall et al., 2007; Walker, 2007; de Sousa Rangel et al., 2020)), by implementing the use of size-selective fishing gears, would likely reduce the occurrence of capture-induced premature parturition and abortion, potentially without reducing the catch. However, despite potentially reducing the occurrence of capture-induced parturition, these actions would not protect animals during other important reproductive processes, such as vitellogenesis. In teleosts, the severity of the reproductive consequences relates to the severity of the fishing-capture stress (refer to section 2.4) and reducing the severity of fishery-induced reproductive impairments in chondrichthyans might be possible by attenuating the magnitude of the stress experienced by animals. There is a growing body of research highlighting that reducing capture duration, air exposure and handling and implementing fishing best practices decreases the measurable stress suffered by chondrichthyans (Frick et al., 2010a, 2010b; AFMA, 2014; Heard et al., 2014; Campbell et al., 2018; Martins et al., 2018). These can be the first, feasible and effective management measures adopted to prevent reproductive impairments, while waiting for specific data to reveal the sensitivity of chondrichthyan reproduction and highlighting species that most urgently need management attention.

2.9. CONCLUSIONS

Most of the stress-related research in fishes focuses on lethal effects and overlooks long-term consequences such reproductive impairments. However, whilst some variability exists, it is unquestionable that stress severely impacts reproduction in fishes, potentially affecting population recruitment and sustainability. Many knowledge gaps persist and work remains to be done to properly understand this phenomenon. In addition to the relative paucity of studies on chondrichthyans, research focusing on transgenerational effects or offspring behavioural, performance and reproductive alterations is also lacking. Present investigations are limited to fecundity, offspring size and short-term survival and exclude other possible impacts on traits, such as swimming and feeding ability that also contribute to offspring fitness. Data on teleost viviparous species and males are scarce, but research shows that the male contribution to reproduction is affected with consequences for offspring survival (Campbell et al., 1994; Philipp et al., 1997; Allyn et al., 2001) and, in viviparous species, embryos spend a long time inside the body of the mother, possibly being chronically exposed to maternal stress hormones. The little data available suggest severe impairments in these circumstances and highlight the need for further research.

Given the variability in the observed responses, it is paramount to investigate reproductive impairments, particularly in sensitive and endangered species. In this context, research on chondrichthyans must be prioritized given their critical conservation status (Dulvy et al., 2008, 2014) and the severity of the reproductive impairments so far observed (Guida et al., 2017; Adams et al., 2018; Wosnick et al., 2019; de Sousa Rangel et al., 2020; Chapter 6). Working with the most endangered and sensitive chondrichthyan species might not be feasible; however, this should not stop research advancement. Data obtained from proxy species, that are easily sourced in large numbers and housed in captivity, such as *S. canicula* (Ruiz-Jarabo et al., 2019) and *T. dumerilii*

(Guida et al., 2017; Chapter 3 and 6), will provide important, preliminary data that could be extrapolated to other species helping develop effective conservation measures. Indeed, only by understanding the actual consequences of different anthropogenic stressors, and their interaction, on reproduction, recruitment and population abundance will it be possible to design more informed fisheries management and conservation measures that might help reverse the trend of decline of these species (Watson et al., 2020).

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Chapter Three

Influence of reproductive state and fishing-capture stress on the metabolic rate of a viviparous chondrichthyan

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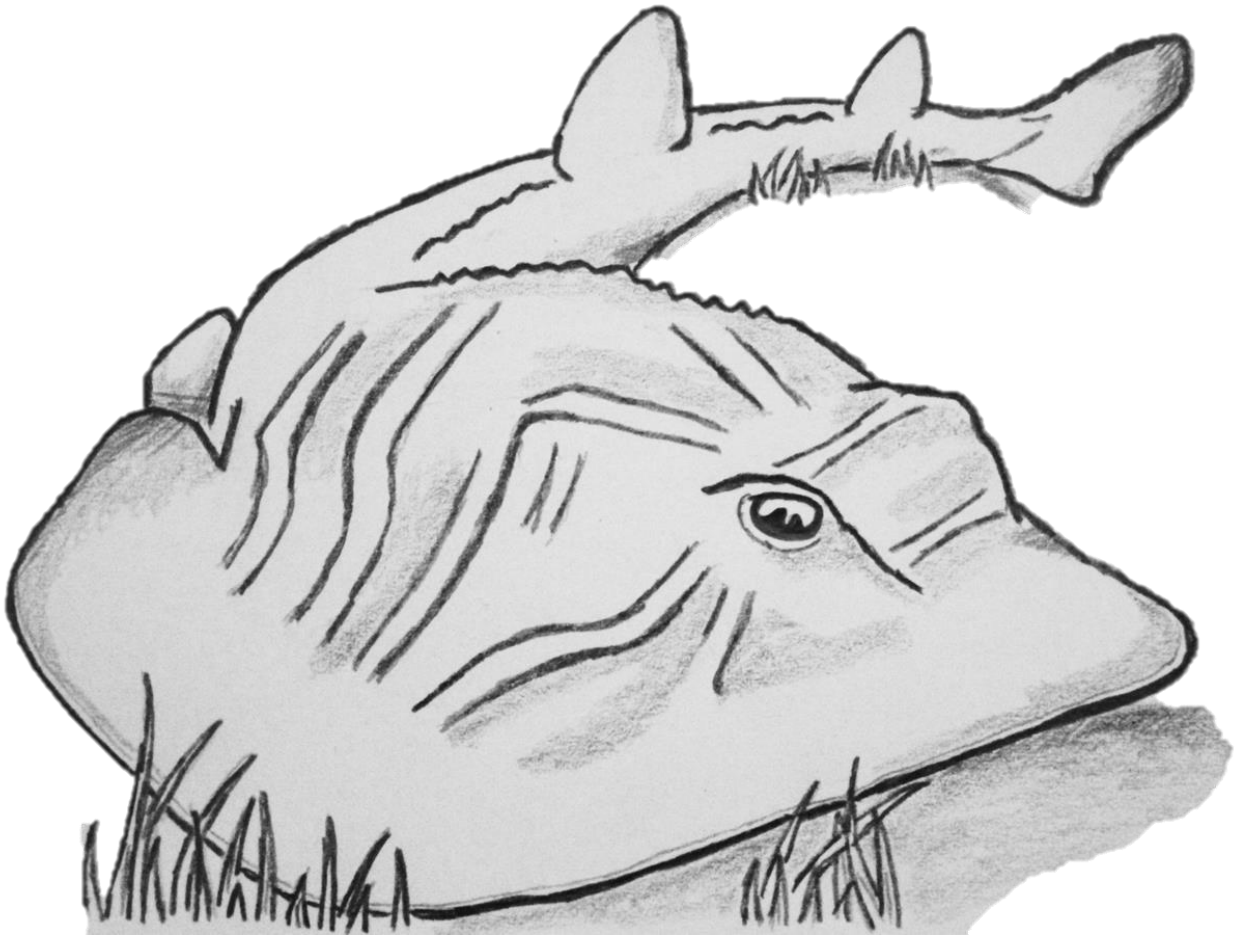

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Drawing by Licia Finotto

ABSTRACT

The effects of fishing capture extend beyond immediate or delayed death. In animals released back in the environment (discard), the stress response elicited by fishing-capture is necessary to restore homeostasis and increase survival, but is also energetically costly. This energy is diverted from other biological activities, possibly impairing them. Elasmobranchs are among the most threatened vertebrate groups, and estimating capture-induced energetic alterations and comparing them to the cost of important biological activities is necessary to assess implications for fishery sustainability. We estimated alterations in aerobic metabolic rates (MRs, a proxy for energy usage) in response to trawling simulation and air exposure and pregnancy cost in southern fiddler rays (*Trygonorrhina dumerilii*), a commonly discarded species. Metabolic rates were estimated twice in pregnant females, before (Unstressed MR) and immediately after trawling-capture simulation and air exposure (After-capture MR), and once after females gave birth (Post-partum MR). After trawling simulation, MO_2 (80.93 ± 3.87 mg O_2 kg^{-1} h^{-1}) was 32.1% lower than MO_2 measured in pregnant unstressed females (119.17 ± 5.77 mg O_2 kg^{-1} h^{-1}). This decrease in energy expenditure seems to be triggered by an initial, excessive energetic investment in the stress response, and aimed at shutting down non-essential activities, thus redirecting energy to processes essential for survival. The cost of near-term pregnancy was a 74.7 ± 39.9% increase in MO_2 compared to values recorded in females after parturition. Capture simulation decreased MRs to values lower than those estimated post parturition, suggesting a capture-induced reduction in energy allocation to pregnancy sustenance, potentially resulting in severe reproductive impairments. These data, applied in population models and ecological risk assessments, may support developing effective conservation measurements.

3.1. INTRODUCTION

Chondrichthyan populations (sharks, rays and chimeras) face high risk of extinction and fisheries exploitation represents the main threat, with most of the animals captured as bycatch (unwanted capture) and commonly released back in the environment (Stevens et al., 2000; Dulvy et al., 2014; Zeller et al., 2018; IUCN, 2020). Discarded animals are released to an unknown fate and this complicates understanding the full consequences of fisheries capture (Molina et al., 2012). The effects of fishing-capture stress extend beyond immediate death and in teleost fishes, discarded animals undergo physiological (Wendelaar-Bonga, 1997), behavioural (Morgan et al., 1999; Sopinka et al., 2015), immunological, growth (Wendelaar-Bonga, 1997), energetic (Barton et al., 1987) and reproductive alterations (Schreck et al., 2001; Sopinka et al., 2016). Given the difficulties of working with large, long-lived animals, studies on chondrichthyans mainly focus on short-term effects of fishing capture, such as physiological blood alterations and immediate and delayed mortality (Frick et al*.,* 2010a, b; Van Rijn et al., 2010; Frick et al*.,* 2012; Gallagher et al., 2014). Few studies have investigated long-term consequences.

A stress response is necessary for an animal to maintain or restore homeostasis and to survive fishing capture (Schreck et al., 2001), but is an energetically-demanding process (Mc Ewen et al., 2003; Schreck, 2010). The amount of energy available for all biological activities has a fixed limit (Ware, 1982; Calow, 1985), so the energy that animals expend to cope with stress becomes unavailable for other activities, potentially impairing them (Calow, 1985; Mc Ewen and Wingfield, 2003; Schreck, 2010). In teleost fishes, metabolic rate (MR) estimates have been used to evaluate the alterations in energy allocation following exposure to stress, showing that even minor physical disturbances can cause a 121% increase in energy use compared to baseline levels (Barton and Schreck, 1987), and that the energy impairment following angling capture can cause losses of up to 62% in yearly fecundity in European sea bass (*Dicentrachus labrax*; Watson et al., 2020). MR

studies of chondrichthyans are mainly restricted to the evaluation of baseline values or of alterations caused by changing environmental conditions, such as water temperature, oxygen concentration and salinity (Di Santo et al., 2011, 2016; Morash et al., 2016; Tunnah et al., 2016; Whitney et al., 2016) and only a few have investigated the energetic responses to fishing-capture stress, observing contrary responses in different species (Bouyoucos et al., 2017, 2018; Molina et al., 2020). Chondrichthyans vary in their physiological response and sensitivity to fishing capture (Mandelman et al., 2009; Frick et al., 2010a, b; Dapp et al., 2015; Bouyoucos et al., 2018) and more research estimating MR alterations in response to capture is needed to better evaluate the impacts on bycatch species and thereby improve fisheries management and conservation measures for this threatened group of fishes. Moreover, investigating stress consequences in free-living vertebrates is complex and requires a broad approach encompassing various physiological and ecological measures (Johnstone et al., 2012) if reliable interpretations are to be drawn. The approach requires research on a range of species and a focus on largely unstudied stress proxies, such as metabolic alterations.

Stress-induced alterations in energy allocation are usually compared to the aerobic scope, which is the maximum amount of energy that an animal has available to perform all its biological activities (Clark et al., 2013). Animals can maintain this maximal energetic expenditure usually for only a short period before becoming exhausted (Brett and Blackburn, 1978; Reidy et al., 1995; Bouyoucos et al., 2017) and, during spontaneous activity, MR is usually well below (Norin et al., 2016). Therefore, relating the cost of stress to this maximal MR value could underestimate the consequences of the energetic disruption. Instead, an ecological perspective on the magnitude of the energetic alteration caused by fishing-capture stress should be obtained by comparing this change with the amount of energy needed to sustain important biological activities, such as growth, gamete production, pregnancy or immune response, rather than with the aerobic scope.

Unfortunately, estimates of the energy cost of these activities, except for swimming and digestion, are scarce, further complicating the evaluation of the long-term effects of stress (Chabot *et al.*, 2016). Reproduction, and particularly pregnancy, are among the most important and most energetically demanding activities (Parker, 1972; Carrier et al., 2004; Hamlett et al., 2005; Trinnie et al., 2012) and more studies evaluating the impact of fishing-capture stress on these activities are needed. Even more so, given the high occurrence (in up to 24% of all pregnant females captured) of capture induced abortion and premature parturition observed in 93 viviparous elasmobranch species (Adams et al., 2018).

In this study, we estimated aerobic MRs, recording oxygen uptake rates (MO_2) using respirometry measurements, to investigate the impact of simulated trawling capture and air exposure (Frick et al., 2010b) on an endemic southern Australian chondrichthyan, the southern fiddler ray (*Trygonorrhina dumerilli*). *Trygonorrhina dumerilli* is a viviparous species, commonly caught by professional and recreational fishers but almost always released because of its low economic value and not esteemed for consumption (Walker et al., 2007; Last et al., 2009). Despite being able to survive severe fishing-capture stress (Guida et al., 2017; Martins, 2017), reproductive impairments and neonatal consequences occurred in *T. dumerilii* when pregnant females experienced simulated trawl capture and air exposure (Guida et al., 2017). Pregnant females occupy areas that are commonly exploited by commercial fisheries (Marshall et al., 2007), so we further investigated the energetic response to capture stress of *T. dumerilii* during this critical phase of their life history. By comparing MRs of females in a pregnant reproductive state with their MRs estimated when in a post-partum state, we calculated the energetic cost of late-term pregnancy and then compared it with the energy response to the fishing-capture stress, therefore, assessing its energetic impact in the context of this important biological activity.

3.2. MATERIALS AND METHODS

3.2.1. Animal collection and husbandry

Between March and April 2018, 14 pregnant *T. dumerilii* females were hand-collected while snorkeling in Swan Bay, Victoria, Australia. Pregnancy was determined by the presence of embryos in the uteri using ultrasound scanning (L6.2 linear transducer probe at 8–5 MHz, Ibex Pro Portable Ultrasound, E. I. Medical Imaging, USA). Upon collection, up to three animals (20 kg combined mass) were placed in an onboard 200-L holding tank, with a continuous flow of ambient seawater provided until arrival (< 2 h) to the housing facility (Victorian Fisheries Authority, Queenscliff, Victoria, Australia). Animals' body mass to the nearest 0.1 kg (BM), total length to the nearest 0.5 cm (TL) and volume to the nearest 0.1 L (Vol) were measured. The volume was calculated by immersing the animal in a tub (2.0 × 1.0 × 0.7 m; L × W × H) containing a known volume of water and measuring the volume of water displaced. Females were tagged with different colour combinations of cable ties. Animals were randomly assigned to either the Trawl Group (n=7) or the Control Group (n=7) and transferred to two separated 9,000-L circular housing tanks (maximum biomass of 45 kg each). Tanks were located outside, under a covered structure and supplied with continuously flowing ambient seawater and aeration. Animals were exposed to natural photoperiod and fed sardines and prawns twice a week (~10% of their total body mass per week). Water temperature in the tanks could not be controlled but approached values recorded in the adjacent Swan bay (± 1 °C) and followed the seasonal variation (no difference between housing tanks; mean ± standard error (SE): 17.2 ± 0.2 °C, range: 14.8–19.2 °C). After parturition, female BM and Vol were measured again. Animals were maintained in captivity until a maximum of 4 days after parturition and released back in Swan Bay after a veterinary health-check. All experiments were performed under Monash University Animal Ethics Protocol BSCI/2016/37, Parks Victoria

research permit n° 10008588, Victorian Fisheries Authority general permit RP1286 and complied with current Australian law.

3.2.2. Experimental design

The schematic of the experimental design is described in Figure 3.1. Females were randomly divided in two treatment groups. Females in the 'Trawl' group underwent trawling simulation and air exposure during late-pregnancy (see below), while females in the 'Control' group were not exposed to capture stress.

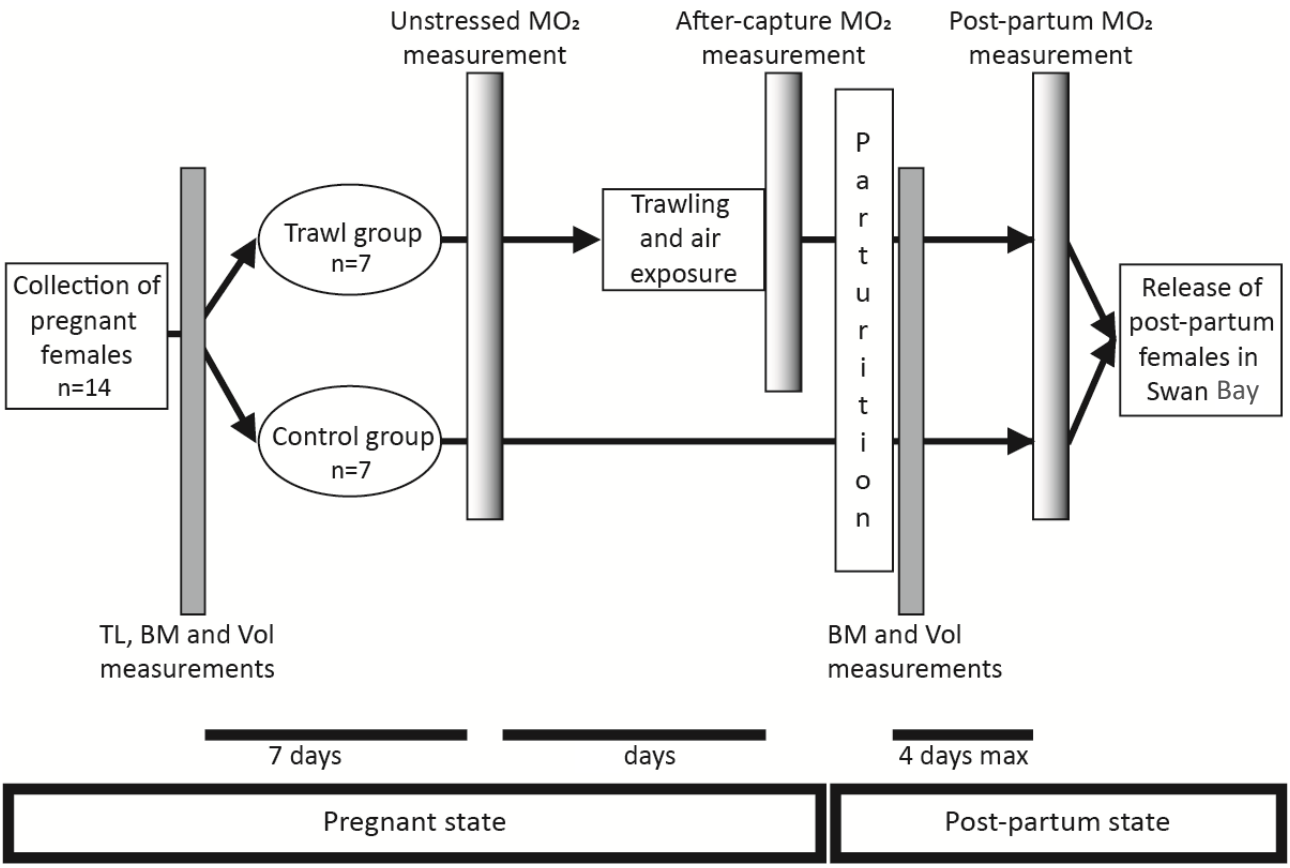

Figure 3.1. Schematic of the experimental design. Squares represent procedures that *Trygonorrhina dumerilii* females experienced. Ellipses represent the two different treatment groups in which pregnant females were randomly divided (Trawl and Control). Black arrows indicate the passage from one procedure/measurement to the next, separately for each treatment group. Vertical grey bars indicate the moments when total length (TL), body mass (BM) and volume (Vol) of the females were measured. The vertical white line indicates when parturition occurred. Vertical blurred bars indicate when MO_2s were measured. If the bar intercepts only one of the arrows, the measurement was performed only on that treatment group. Horizontal black segments represent the number of days passing between different procedures/measurements (not in scale). Horizontal black bars indicate when females were considered in a pregnant or post-partum state. n indicates numerosity.

Animals in the Trawl group individually underwent three separate trials to measure MO_2 (hereafter referred to as MO_2-class). The first two, Unstressed and After-capture MO_2 classes, were measured in females in a pregnant state, and the last, Post-partum MO_2 class, was measured in females after they gave birth. The Unstressed MO_2-class was measured in pregnant females at

least seven days after initial capture to allow recovery and habituation to the housing conditions (Guida et al., 2017) and represented the routine aerobic energy requirements of minimally stressed animals, but included the energetic cost of pregnancy. Five days were allowed to pass after the Unstressed MO_2 trial to allow recovery from minor stressors potentially involved in the respirometry trial (Martins et al., 2017). Then, the After-capture MO_2-class was measured immediately following capture simulation (see below). This MO_2-class estimated the energetic response to fishing-capture stress of animals in a pregnant state. Finally, the Post-partum trial occurred within four days of parturition. Females in the Control group experienced two trials to evaluate their Unstressed and Post-partum MO_2 classes and no respirometry measurements corresponding to the After-capture MO_2-class took place. Trawl females experienced confinement in the respirometry chamber an additional time compared to Control females, and this might have influenced their stress load and successive measurements. However, spending up to 3 h in a respirometry chamber did not cause physiological alterations associated with stress in blacktip reef sharks (*Carcharhinus melanopterus;* Bouyoucos *et al.*, 2018) and it is unlikely that the additional respirometry confinement biased the measurements. We adopted a repeated measures design for determining MO_2 of each animal for the separate MO_2 trials to allow control for the high inter-individual variability in MR evident from other studies (Molina *et al.*, 2020). Food processing and digestion can alter the energetic load of sedentary species (Sims *et al.*, 1994), so to remove this influence, MO_2s were measured in animals in a post-absorptive state at least 48h after the last feeding event.

3.2.3. Respirometry trial

MO_2 trials were performed using a rectangular plastic tank as the respirometry chamber (120cm x 60cm x 45cm, 324 L volume; 5 mm thickness; Figure 3.2), with an air/water tight plastic lid. The tank was equipped with a water inlet and outlet connected to an external water pump

used to mix the water inside the respirometry chamber, without creating a current strong enough to disturb the animals and force them to swim against it (Clark et al., 2013). An oxygen sensor meter (Hach IntelliCAL™ LDO101 Field Luminescent/Optical Dissolved Oxygen sensor meter; ±0.1 mg/L accuracy) was placed close to the water outlet and used to measure changes in dissolved oxygen concentration (DO) and temperature inside the chamber during MO_2 trials.

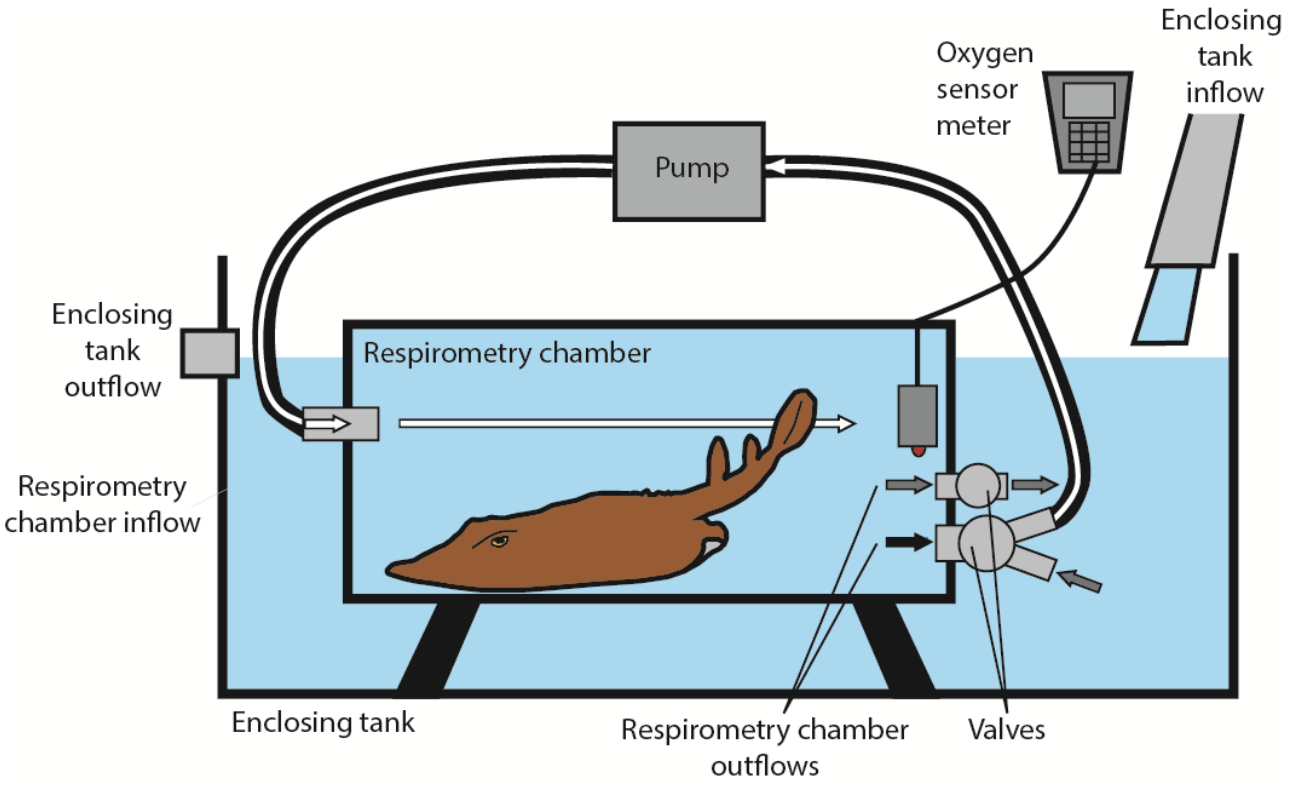

Figure 3.2. Schematic representation of the respirometry chamber. Grey arrows indicate water flow during the flushing phase, black arrows indicate water flow during the measurement phase and white arrows indicate common water flow during both phases. The closure or opening of the specific valve allowed the transition from the measurement to the flushing phase (adapted from Molina *et al.*, 2020).

The respirometry chamber was placed within a 5,000-L tank and females were individually netted and hand-transferred from their housing tank to the respirometry chamber at the time of MO_2 trials. Netting, handling and transfer of the animals took <2 min. Once an animal was placed in the respirometry chamber, it was allowed to settle till it began to rest on the bottom of the

chamber (<2 min), at which time the chamber was sealed and the MO_2 trial began. Values of DO were recorded at 30-sec intervals during eight measurement cycles, each of 15 min duration. Successive measurement cycles were separated by a 15-min period during which time the chamber was flushed with oxygenated seawater from the enclosing 5,000-L tank. At the end of these flushing periods, the inlet to the pump was manually changed again to resume water circulation within the sealed respirometry chamber for another measurement cycle. To avoid animals suffering from oxygen deprivation, DO was monitored continuously and never allowed to fall lower than 80% saturation. During all procedures, animal well-being was periodically monitored by visual inspection of its behaviour through the transparent lid. No animals showed signs of visible suffering or stress. Continuous recording of animals' swimming activity within the respirometry chamber was not possible, and only non-standardized, sporadic observations were performed. It emerged that, apart from the initial agitation after the introduction in the respirometry chamber and some sporadic periods of movement (usually lasting <1 min), animals spent most of the time resting on the bottom. Therefore, as proposed for other sedentary species (Di Santo and Bennett, 2011), the estimated MRs can be classified as Resting RMRs (RRMR), which are defined as the MRs of animals performing an unspecified, low amount of activity (Chabot *et al.*, 2016).

At the end of the 8th measurement cycle (after ~4 h) the lid of the respirometry chamber was removed and the animal hand-transferred back to the housing tank. The respirometry chamber was emptied, washed and refilled with seawater between MO_2 trials of different animals. If trials were performed in different days, the chamber was left empty and allowed to dry to reduce bacterial proliferation. A blank trial was performed to account for background respiration inside the respirometry chamber. The chamber was sealed and DO recorded during 6 cycles of 15 min duration as in a normal measurement trial, without an animal inside. Background respiration was

calculated and subtracted from all other measurements during MO_2 calculation (see Data analysis). To account for circadian rhythm, all respirometry trials occurred during daylight, always between 8 am and 4 pm.

3.2.4. Trawling simulation and air exposure

To simulate trawling capture, females in the Trawl group were dip-netted from the housing tank and individually placed inside a cod-end (monofilament net bag with stretched mesh diameter of 102 mm and bag length of 110 cm) mounted on a circular support hanging from a metal bar suspended over the 19,000-L experimental tank. In the centre of the tank, a rotating wood panel (2.0 × 1.0 m: W × H) generated a water current of ~0.6 m/s in front of the stationary cod-end, simulating the condition of towing a net through the water during trawling operations (Frick et al., 2010b). Each female was towed individually for 7 h. At the end of the trawling simulation, the female was retrieved from the cod-end and placed for 30-min air exposure in an empty tub (2.0 × 1.0 × 0.7 m; L × W × H) outdoors in a shaded area. This trawling simulation represents a best-case scenario compared to commercial conditions as it does not include the additional stress derived from the presence of other animals or objects in the cod-end causing pressure, injuries, crowding, packing and hypoxia. Only one respirometry chamber was accessible, thus only one female could be towed at each time and to partially offset this unavoidable bias, the duration in the cod-end was 7 h (Frick et al., 2010b). At the end of the air exposure period, the female was immediately hand-transferred to the respirometry chamber and MR of these stressed animals was measured as described earlier. Females in the Control group experienced the same dip-netting, handling and transfer (to a different 5,000-L tank) procedures but neither experienced towing and air exposure nor had their MO_2 measured at this time. The experimental tank was supplied with constant seawater flow so that the whole mass of water was replaced between two

different trawl simulations (occurring in successive days), reducing the possibility that stress-related chemical clues influenced the responses of animals treated subsequently.

3.2.5. Post-Partum MR

Parturition was confirmed by the presence of neonates in the housing tanks. The identification of the female(s) that gave birth was made first by counting the number of pups. *Trygonorrhina dumerilii* litter size ranges from two to five pups (Marshall et al., 2007), so the presence of four or more pups indicated that potentially two females gave birth the same night. Identification of individual mothers was made by visual inspection of females' body appearance, because near-term pregnancy is characterized by evident bulges of the dorsal side of a female's body, which disappears immediately after parturition (de Sousa Rangel et al., 2020). Parturition was then confirmed by ultrasound scanning, with those females suspected to have given birth being dip-netted and drawn near to the side of the housing tank where, while submerged and lightly restrained, they were checked with the ultrasound. The protocol allowed assigning each female to specific parturition events.

The number of embryos born from each female was recorded (Fecundity) after every parturition event. On one occasion, two females gave birth the same night. An estimated number of embryos was available for each female from ultrasound scanning performed at initial capture. Additionally, a preliminary experiment highlighted that neonate total length at birth was positively correlated with maternal size and that siblings have similar coloration patterns (Chapter 6). Based on these three variables, all neonates were assigned to the respective mother.

In some cases, parturition occurred on days when the respirometry chamber was in use, in which case the Post-partum MO_2 trial was delayed until the respirometry chamber was available, but always performed within 4 days after parturition.

3.2.6 *Respirometry data processing*

For each of the eight measurement cycles of a MO_2 trial for a female, the values of compensated dissolved oxygen concentrations (DO, $mgO_2\ L^{-1}$) recorded were plotted against time and a linear regression slope was calculated. R^2 values of the calculated slopes were all higher than 0.85. Measurements recorded during the blank trial were processed similarly and the slopes calculated for the 8 cycles were averaged to obtain a single value for background respirometry that was subtracted from the slope calculated for each of the eight measurement cycles when calculating MO_2 (see below). MO_2 protocols recommend allowing an adequate time (at least 2 h and up to 24 h) for the animal to recover from the mild handling/air exposure stress and to acclimate to the novel environment represented by the respirometry chamber (Clark et al., 2013). One of the main aims of this study was to investigate the effect of fishing-capture simulation on MO_2 and, as the process of recovery begins soon after the stress ceases (Reidy et al., 1995; Cooke et al., 2014), After-capture MO_2 needed to be measured immediately after the simulation and no habituation period could be introduced. For comparison purposes, Unstressed and Post-partum MO_2s were also measured immediately after the animal was placed in the respirometry chamber. To account for the potential increase in MO_2 caused by the initial handling/air exposure stress, a repeated measures ANCOVA was used separately for each animal and MO_2 trial to detect whether the interaction between time, as a continuous variable, and measurement cycle, as a categorical variable, had any effect on DOs. This allowed determining whether the slopes of DO decline calculated for the eight sequential measurement cycles differed significantly from one another and, if so, only data obtained after the values of the slope stabilized were used to calculate the MO_2 and included in the following statistical models (see Molina et al., 2020 for example). MO_2 was calculated using the equation:

$$MO_2 = (O - B) \times Vol \times BM^{-1}$$

where MO_2 is the mass-specific oxygen uptake rate measured as $mgO_2\ h^{-1}\ kg^{-1}$, O is the regression slope previously calculated and expressed as $mgO_2\ L^{-1}\ h^{-1}$, B is the background respirometry calculated for each MR trial expressed as $mgO_2\ L^{-1}\ h^{-1}$, Vol is the volume of water in L contained in the respirometry chamber, calculated as the difference between the volume of the chamber and the volume of the animal, and BM is the wet body mass of the animal in kg.

As mentioned, water temperature could not be controlled and followed seasonal variation. The IntelliCAL probe automatically corrected the DO values measured according to temperature. However, given that MO_2 is strongly influenced by temperature (Schmidt-Nielsen, 1997), calculated MO_2s were temperature-corrected to 17°C, the average temperature recorded during the experimental period, using $Q_{10} = 2.3$ (Neer et al. 2006; Whitney et al. 2016).

3.2.7. Data analysis

3.2.7.1. Morphological traits

The normality of total length, body mass and volume datasets was assessed using the Shapiro-Wilk test, and homoscedasticity was verified using Levene's test. A t-test was used to investigate any differences in total body length at capture between females of the Trawl and Control Groups. Linear mixed-effects models (LME) were used to examine the influence of the interaction between Group (Control and Trawl) and State (Pregnant and Post-Partum) on female body mass and volume. The ID of the females was included as a random effect (BodyMass ~ Group * State + (1|ID); Volume ~ Group * State + (1|ID)). This and the following models were validated evaluating the homoscedasticity of the generated residuals. Significance of the fixed effects was evaluated using a Wald test and potential differences between means were investigated using a Holm-Šídák post hoc test.

3.2.7.2. Relationship between MO_2 and BM

The normality of the error structure of the MO_2 data sets was assessed with a Quantile-Comparison Plot and a kurtosis and skewness test. Separately for Unstressed and Post-partum MO_2 values, the relationship between body mass and MO_2s was investigated, after log transformation of the data, using an LME that included body mass as a fixed effect and female ID as a random effect. Because body mass did not influence MO_2 values (see results), it was excluded from further analysis.

3.2.7.3. Influence of MO_2-class and Group on MO_2 values

To investigate which effect had a significant influence on MO_2, an LME, including the interaction between Group (Control and Trawl) and MO_2-class (Unstressed, After-capture and Post-partum) as a fixed effect and the ID of the animal as a random effect, was used (MO_2 ~ Group * MO_2-class + (1|ID)). Presented results are derived from the most parsimonious model, obtained from the full model through stepwise backward elimination of non-significant terms. Initially, Tank ID (Control or Trawl female housing tank) and the number of embryos carried by each female (Fecundity) were included as additional random factor in the model. However, the inclusion of these factors did not improve the model (confirmed by higher Akaike (AIC) and Bayesian information criterion values (BIC)) and these random factors were therefore excluded from the final model.

3.2.7.4. Pregnancy cost

For each female, the cost of pregnancy was calculated by subtracting Post-partum MO_2 values from Unstressed values and dividing the result by the Post-partum MO_2 to obtain the proportional increase in energy needed to sustain pregnancy. A linear model was used to analyse the influence of Fecundity on the cost of pregnancy. Prior to any analysis, the values of proportional cost of

pregnancy were transformed as the square root; for clarity, the results are presented as percentage.

Results are reported as mean ± SE. All tests used a significance level of $\alpha \leq 0.05$. Data were processed using R statistical software (R Core team 2019).

3.3. RESULTS

Despite being confirmed in an advanced state of pregnancy, one Control female never gave birth. From ultrasound scanning, no mouth movements associated with gill oxygenation was observed in the embryos, suggesting that the embryos were likely non-viable. The Unstressed MO_2 measured from this female is comparable to values observed in the other females and was included in the analysis, but the Post-partum MO_2 trial was not performed. The other females gave birth within the parturition period reported in the literature (Marshall et al., 2017), between 20th of April and 24th of May 2018, an average of 25.7 ± 4.9 (6–39) and 22.8 ± 4.8 (8–43) days after Unstressed MO_2 measurement for Trawl and Control females, respectively.

3.3.1. Morphological traits

Morphological data are reported in Table 3.1. There was no significant difference in total length, body mass or volume between the Trawl group and the Control group (Total length: $F_{1, 12} = 0.61$, $p = 0.55$; body mass: $F_{1, 12} = 0.15$, $p = 0.69$; volume: $F_{1, 12} = 0.67$, $p = 0.41$). Pregnant versus post-partum state had a significant effect such that both body mass ($F_{1, 11} = 37.89$, $p < 0.0001$) and volume ($F_{1, 11} = 17.03$, $p < 0.0001$) were significantly lower for the females when measured post-partum than when pregnant.

Table 3.1. Total length (mean ± S. E., range and number of animals used (n)), body mass and volume of female *Trygonorrhina dumerilii* in the Control Group and Trawl Groups (Group) in Pregnant and Post-Partum State. For each variable separately, different superscript letters indicate statistical differences ($p < 0.05$) between Group and Condition.

Dependent variable	**Group**	**Pregnant state**	**Post-Partum state**
Total length (cm)	Control	95.8 ± 1.8 (88.0–103.5) (n=7) [a]	
	Trawl	93.4 ± 3.8 (78.0–105.0) (n=7) [a]	
Body mass (kg)	Control	6.0 ± 0.3 (5.2–7.8) (n=7) [a]	5.2 ± 0.3 (4.5–6.6) (n=6) [b]
	Trawl	5.8 ± 0.5 (3.8–7.2) (n=7) [a]	5.1 ± 0.5 (3.2–6.0) (n=6) [b]
Volume (L)	Control	4.8 ± 0.2 (4.5–5.8) (n=7) [a]	4.3 ± 0.2 (3.8–5.3) (n=6) [b]
	Trawl	4.6 ± 0.4 (3.2–5.8) (n=7) [a]	3.9 ± 0.3 (2.8–4.8) (n=6) [b]

3.3.2. Relationship between MO_2 and BM

The relationship between body mass and MO_2, both Unstressed and Post-partum values, was not significant (Unstressed MR $F_{1,12} = 0.32$, $p = 0.57$; Post-Partum $F_{1,11} = 0.003$, $p = 0.87$).

3.3.3. Influence of MO_2-class and Group on MO_2 values

The effect of Group (Control or Trawl) on MO_2 was not significant ($F_{1,151} = 0.95$, $p = 0.33$), while both MO_2-class (Unstressed, After-capture and Post-partum; $F_{2,151} = 80.53$, $p < 0.0001$) and the interaction between Group and MO_2-class ($F_{2,151} = 9.12$, $p = 0.002$) had a significant effect on MO_2. Both trawling stress and parturition caused a significant decrease in MO_2 values compared to Unstressed MR (Control: 118.75 ± 7.85 $mgO_2\ kg^{-1}\ h^{-1}$; Trawl: 119.17 ± 5.77 $mgO_2\ kg^{-1}\ h^{-1}$; Control Unstressed – Control Post-partum and Trawl Unstressed – Trawl After-capture: $p < 0.0001$; Trawl Unstressed – Trawl Post-partum: $p = 0.03$) and, in the Trawl group, trawling stress caused a significantly greater decrease in MO_2 compared to the one observed after parturition (Trawl After-

capture MO_2 = 80.93 ± 3.87 mgO_2 kg^{-1} h^{-1}: Trawl Post-partum MO_2 = 99.07 ± 4.95 mgO_2 kg^{-1} h^{-1}; p = 0.02). On the other hand, the Post-partum MO_2 measured in the Control group (69.14 ± 7.30 mgO_2 kg^{-1} h^{-1}) did not differ from the After-capture MO_2 measured in the Trawl group (p = 0.97). In the Trawl group, trawling simulation caused a 32.1% decrease in MO_2 compared to Unstressed MO_2. The significance of the interaction between Group and MO_2-class, indicates that parturition had a different effect on the two treatment groups, causing a significantly greater MO_2 decrease in the Control (41.8%) compared to the Trawl Group (16.9%; Figure 3.3).

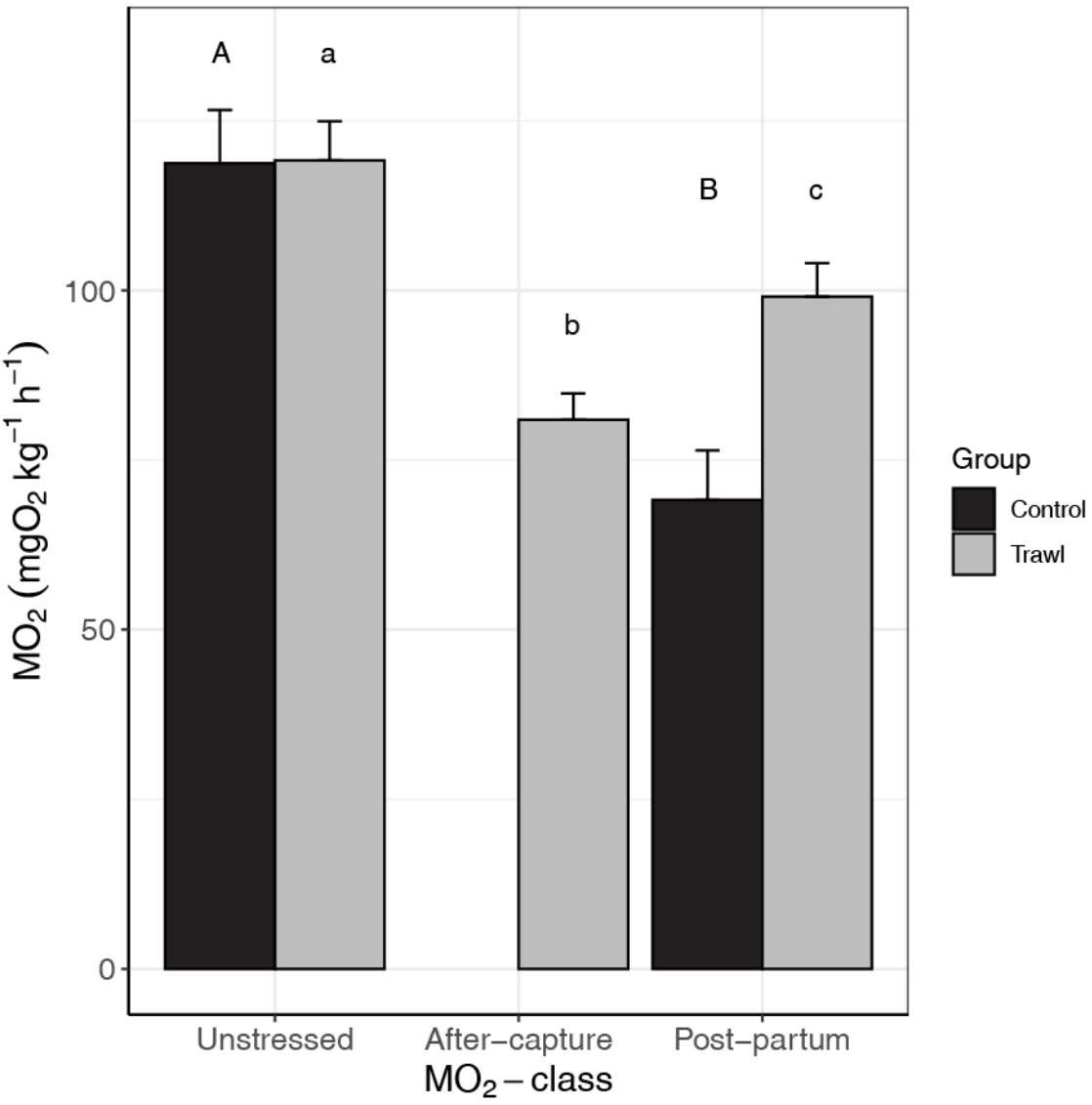

Figure 3.3. MO_2 values (mgO_2 kg^{-1} h^{-1}; mean values ± SE) for the different treatment groups (Control, black boxes, and Trawl, grey boxes) and MO_2-classes (Unstressed (n=14), Stressed (n=7) and Post-Partum (n=13)). Different uppercase and lowercase letters indicate statistically significant differences ($p < 0.05$) between different MO_2-classes within the Control and Trawl group, respectively. No significant difference was found between Trawl and Control group in either the Unstressed or the Post-partum MO_2-class.

3.3.4. Pregnancy cost

One Trawl female showed an increased Post-partum MO_2 compared to Unstressed values, consequently the proportional cost of pregnancy was negative. This female gave birth to a

stillborn neonate in an advanced state of decomposition and, similarly to what was observed in the female that never gave birth, no mouth movement was detected in the embryo while *in utero*, and this likely explains the negative cost of pregnancy recorded. This female was excluded from further analyses.

The cost of pregnancy represented a 74.7 ± 39.9% increase above the Post-partum MO_2. The number of embryos carried by each female did not influence the proportional cost of pregnancy ($F_{1,10} = 0.018$, $p = 0.89$).

3.4. DISCUSSION

Investigating the energetic alterations caused by fishing-capture stress is an important area of research, but at present data for chondrichthyans are scarce. The lack of estimates of the energetic cost of important biological activities (Chabot et al., 2016) hinders determining the full extent of any metabolic alteration caused by stress. To partially address this knowledge gap, we aimed at investigating potential metabolic consequences of simulated trawling and air exposure on pregnant *Trygonorrhina dumerilii*. We also aimed at evaluating the cost of pregnancy for the first time in a chondrichthyan species. Surprisingly, results highlighted that the fishing-capture simulation did not increase MO_2 but significantly decreased it. Moreover, fishing-capture exposure reduced MO_2 to values lower than those observed in females post-parturition, suggesting that fishing-capture might impair pregnancy maintenance with possible consequences for reproduction and neonates. The cost of late-term pregnancy is a 74.7 ± 39.9% increase in energy expenditure compared to post-partum condition, and is similar to values observed in teleosts (Boehlert et al., 1991; Timmerman and Chapman, 2003).

The stress response and the re-establishment of homeostasis following fisheries capture are energetically demanding processes and after simulated trawl we expected to measure an increase in MO_2, as observed in other species (Bouyoucos et al., 2017, 2018; Molina et al., 2020). Instead, in

T. dumerilii, fishing capture caused a 32.1% decrease in RRMR. Animals' swimming activity levels during measurements were not collected and the observed MO_2 decline potentially originated from a decrease in this activity. Some chondrichthyans use resting as a strategy to cope with fishing-capture stress (Guida et al., 2016b) thereby reducing the amount of energy invested in movement and reallocating it to the stress response. While this might be the case for *T. dumerilii* too, during both Unstressed and After-capture MO_2 trials, animals spent almost all the time resting and the difference in swimming activity level between these trials was unlikely large enough to explain the observed MO_2 decrease. Moreover, gummy sharks (*Mustelus antarcticus*) and elephant fish (*Callorhinchus milii*), similarly showing decreased MO_2 after simulated fishing-capture, showed a decrease also in their resting MO_2, measured in animals only performing self-righting movements. These observations seem to indicate that the metabolic decrease is not, or at least not only, caused by a reduction in the swimming activity and that other physiological mechanisms must be involved. Habituation to the respirometry chamber the second time animals were placed in it, and the consequent lower stress load might have influenced the measurement of After-capture MO_2. However, *C. milii* does not show habituation to the respirometry chamber (Chapter 4) and spending time in a respirometry chamber does not represent a stressful event in itself for *C. melanopterus* (Bouyoucos et al., 2018). This indicates that the observed MO_2 decrease was unlikely to have been caused by our measurement methods. The lack of habituation, while somewhat hindering the possibility to consider the measured MO_2 as baseline values (see supplementary materials, Appendix I), do not invalidate the reliability of the results on the energetic effect of fishing-capture stress and different reproductive conditions. Indeed, all measurements were performed in the same conditions (lack of habituation) and potential associated higher stress condition.

Several potential causes can explain the decrease in MO_2we observed. Animals trapped in a trawl cod-end may rely on anaerobic metabolism to survive and function, but by not consuming oxygen, their energy use is not measurable through respirometry. While in the cod-end, animals performed sporadic, brief (< 2 min) bouts of active swimming, which engages white muscles tissue powered by anaerobic metabolism (Svendsen et al., 2010). Similarly, air exposure and the associated low oxygen level, could have triggered the anaerobic metabolism (Burggren et al., 1980; van den Thillart et al., 1994). Accordingly, *T. dumerilii* subjected to a similar trawl simulation showed increased levels of plasma lactate as an end product of anaerobic glycolysis, confirming that some anaerobic metabolism is involved in response to fishing capture (Martins, 2017). However, given the accumulation of toxic wastes, animals can sustain anaerobic metabolism only for short periods (Chabot et al., 2016) and it seems unlikely that *T. dumerilii* relied primarily on it for the entire capture duration. Moreover, immediately following anaerobic work and air exposure, oxygen uptake steeply increases to rebuild the depleted oxygen stores (Svendsen et al., 2010), a response that is opposite to what we recorded in this study as even MO_2 measured immediately after the animals were placed in the respiromtry chamber following capture simulation were significantly lower than MO_2 measured in unstressed animals. Therefore, it is improbable that an increase in anaerobic metabolism is the main explanation for the observed metabolic decline.

A rapid depletion of the energetic resources and the consequent metabolic decline might be another cause of the observed metabolic alterations. A similar response, the metabolic depression, is commonly observed in teleosts exposed to hypoxia/anoxia, temperature changes and drought (Hand, 1996), and in epaulette sharks (*Hemiscyllium ocellatum*) facing severe hypoxia (Routley et al., 2002), where it is suggested to be initiated by the buildup of adenosine, an end product of ATP use (Renshaw et al., 2002). Once the concentration reaches a threshold signalling

depleted cellular energetic reserves, adenosine acts as an inhibitory neuromodulator, shutting down all non-essential biological activities to save energy for vital processes and maintain high ATP levels in the brain, avoiding apoptosis and brain damage (Renshaw et al., 2002). In *M. antarcticus*, fishing-capture simulation causes the exhaustion of ATP reserves in liver and muscle, while brain and heart ATP levels are preserved (Guida et al., 2016a), suggesting that the response to this stress is similar to the response to hypoxia. During trawling stress, animals likely invest more energy in the stress response to restore homeostasis at first, with a concomitant increase in MO_2 that goes unnoticed because occurring while *T. dumerilii* females were still in the net. Afterwards, given the high severity and/or prolonged duration of the stress and the limited ability to enhance the release of glucose from hepatic glycogen stores (Routley et al., 2002), animals exhaust all the available plasma glucose (Frick et al., 2010a; Martins et al., 2018). The concomitant adenosine accumulation might then trigger a reduction in aerobic metabolic demand. In other animal taxa, metabolic depression is induced by large decreases in extracellular pH (Guppy et al., 1999). Plasma pH decreases in chondrichthyans experiencing fishing capture (Skomal et al., 2012; Bouyoucos et al., 2018) and this might be another possible mechanism of the metabolic decline observed in *T. dumerilii*.

In other chondrichthyans, MO_2 increased after fishing-capture stress. Whether MO_2 increases or decreases possibly depends on a species' intrinsic sensitivity to the stressors associated with the fishing-capture simulated and the intensity of those stressors. Increased oxygen consumption occurs either in resilient species (e.g., Port Jackson sharks *Heterodontus portusjacksoni*; Molina et al., 2020) or following mild fishing simulation (Bouyoucos et al., 2017, 2018). These circumstances likely lead to minor stressful and allostatic states that could be overcome by moderately increasing the energy allocation to the stress response, without exhausting the energy resources and incurring in a large pH decrease or adenosine build-up triggering metabolic decline. Conversely,

MO_2 of sensitive species, after an initial increase, decreases to preserve the remaining energetic resources, potentially due to the extreme homeostatic alteration resulting from the fishing simulation and the inability to offset it (Molina et al., 2020; Chapter 4). Although *T. dumerilii* is a resilient species (Guida et al., 2017; Martins, 2017), exposure of females to highly stressful fishing practices (Dapp et al., 2015), such as trawl and air exposure, was likely sufficient to trigger metabolic depression. Moreover, our study animals being pregnant might have enhanced the sensitivity of *T. dumerilii* (Wosnick et al., 2019), contributing to the observed response. Further studies investigating the effect of a similar capture simulation in *T. dumerilii* males and/or non-pregnant females may help understand whether the severe alteration of the metabolic response observed in this study is a result of the pregnant condition or of the highly stressful trawling simulation.

Pregnancy cost has a large influence on baseline energetic expenditure, representing a 74.7% increase over post-partum RRMR value. The large cost of pregnancy originates from the need to maintain a suitable uterine environment, ensure oxygen delivery to the embryos and remove toxic metabolic wastes produced by them. This translates into increased maternal cardiac output to enhance blood circulation and increased oxygen uptake to meet embryonic demands, with the associated increase in branchial pump work and cost of ionic and osmotic regulation as more blood passes through the gills (Webb and Brett, 1972; Thibault and Schultz 1978; Hamlett et al., 2005). Additionally, increased energy is needed to move a heavier and bulkier body due to higher water drag and fatigue (Makiguchi et al., 2017). Cost of pregnancy increases with advancing gestational stages because initially most of the tissues constituting the embryo (yolk and water) are inert, but as development progresses, embryos consume more oxygen and produce more metabolites (Boehlert et al., 1991; Timmerman and Chapman, 2003), increasing the metabolic demand on the mother. The values we obtained from *T. dumerilii* were measured close to

parturition (about 3 weeks before) and likely represented the greatest increases to MR during pregnancy experienced in that species. *Trygonorrhina dumerilii* is a lecithotrophic species with no histotroph (Marshall et al., 2007) and the only nutrients mothers supply to the embryos are allocated in the yolk sac (Hamlett et al., 2005). We expect that the cost of pregnancy would be substantially higher in matrotrophic species, in which females provide embryos with other nutrients beside yolk (Hamlett et al., 2005). Similarly, the cost of pregnancy is expected to increase as the number of embryos *in utero* increases (Boehlert et al., 1991). However, in our study, no significant relationship between cost of pregnancy and fecundity was observed. This unexpected observation is likely the result of the fact that the range of fecundity investigated was limited (2–4 embryos, only one female carrying 4 embryos), the sample size was small and the variability in females' previous condition was likely large, an unavoidable bias of working with animals sourced from the wild (Johnstone et al. 2012). Further research with a larger number of animals is needed to better investigate changes in the energy cost of pregnancy in relation to females' fecundity.

Fishing-capture stress reduced MO_2 to values lower than MO_2 measured after parturition, meaning that pregnant females experiencing trawling stress had energy expenditure lower than females in a post-partum condition. The largest component of the increased oxygen consumption during pregnancy appears to be consumed by the developing embryos (80.9%; Boehlert et al., 1991). Therefore, the reduction in maternal oxygen uptake we observed following fishing-capture stress might impact embryonic respiration. Moreover, a reduced investment in pregnancy maintenance might reduce the quality of the uterine environment due to the accumulation of toxic metabolites produced by the embryos that the mother cannot clear (Webb and Brett, 1972; Thibault and Schultz 1978; Hamlett et al., 2005). We did not investigate recovery, but after a fishing-capture stress, RMR took 3–20 h to decrease back to resting values (Bouyoucos et al., 2017, 2018). The metabolic decline persisted for up to 24 h after osmotic stress (Morash et al., 2016;

Tunnah et al., 2016) and, in the holocephalan *C. milii*, up to 7 days after fishing-capture stress (Chapter 4). Given the rapid development of *T. dumerilii* embryos (Marshall et al., 2007), maternal energetic disruptions, even when short-lasting, potentially severely impact embryonic development and, along with other physiological alterations, likely contribute to neonatal impairments observed when female *T. dumerilii* are exposed to trawling simulation during pregnancy (Guida *et al.*, 2017; Chapter 6). The suggested reduced energy allocation to pregnancy maintenance and the associated inability of trawled females to maintain an optimal uterine environment, might be among the causes of the observed reduction in neonatal size (Guida *et al.*, 2017, Finotto *et al.*, unpublished) and yolk sac volume at birth (Finotto *et al.*, 2021). In these circumstances, neonates might have to increase energy expenditure to lessen the consequences of this sub-optimal environment, ultimately consuming their energy store, the yolk sac, faster and leaving lower resources to sustain growth. Similarly, an increase in uterine concentration of toxic metabolites could be one of the causes of the stressed condition observed in neonates at birth (Guida *et al.*, 2017, Finotto *et al.*, 2021).

The fishing-capture treatment towing the animals individually for 7 h is slightly longer than typical commercial durations of fisheries in the general area of our study, which range from 4–6 h (Frick et al., 2010). However, the treatment excludes a variety of other highly variable stressors such as compression, injury, and associated hypoxia from the presence of other animals and debris in the cod-end, and environmental changes in temperature and pressure. Similarly, the conditions encountered after the end of the capture simulation are mild, with an absence of predators and occurring in an optimal environment with abundant food. All this considered, and despite longer tow durations, our capture simulation was unlikely to have overstressed the animals, rather we think it represented a best-case scenario compared to commercial procedures (Frick et al., 2010b; Guida et al., 2017; Martins, 2017). More importantly, despite being a mild to

moderate trawl treatment, this capture simulation measurably decreased pregnant females MO_2, indicating the need to investigate the effects of the more extreme conditions occurring during commercial trawl operations.

Despite the resilience of *T. dumerilii*, fishing-capture stress has important energetic and reproductive impacts not previously considered, and mitigation actions need to be implemented. Indeed, the energetic impairments observed in pregnant females might be contributing to causing neonatal consequences, which can affect neonatal survival and, consequently, population abundance (Chapter 6) with potential ecosystem-level impacts. The reduction of tow and air exposure durations and better handling practices (AFMA, 2014), by reducing the stress load, will potentially avoid the triggering of the metabolic decline and the resultant reproductive impairments. Actions to reduce the likelihood of encountering and/or capturing pregnant females in areas where they are in high abundance would also be beneficial, particularly during January–April when embryonic growth is rapid (Marshall et al., 2007). Although *T. dumerilii* extinction risk status is least concern (IUCN, 2020), our results provide important information in support of improved protection of more sensitive and threatened chondrichthyans, such as the closely related shortnose guitarfish (*Zapteryx brevirostris*) and spotted shovelnose ray (*Aptychotrema timorensis*; IUCN, 2020). The inclusion of these data in population models and risk assessments will be fundamental to assess the full consequences of fishing-capture events and to develop sound management measures aimed at improving the conservation status of these threatened populations (Watson et al., 2020).

3.5. CONCLUSIONS

The results of our study provide new information and insights on the stress response of a chondrichthyan species to fishing-capture stress. The observed metabolic decrease in *T. dumerilii* in response to fishing-capture stress supports the results recorded in other species (Molina et al.,

2020; Chapter 4) and, equalling the decrease measured after parturition, points to possible reproductive and intergenerational consequences caused by this energetic disruption. We also report the first estimates of the energetic cost of pregnancy for any chondrichthyan species, providing baseline data necessary to identify potential long-term effects of MO_2 alterations occurring after fishing-capture stress.

Metabolic impairment caused by fishing capture and its recovery needs to be investigated in a larger number of chondrichthyans and associated with blood and tissue analysis aimed at identifying the mechanisms of the metabolic depression. Further researching the effects of metabolic depression on reproductive success is needed, given the importance of this process for the maintenance of sustainable populations. The understanding of these reproductive consequences will benefit from a more accurate evaluation of pregnancy cost, integrating the energetic cost of the whole gestation period (Boehlert et al., 1991) and investigating species characterized by different reproductive modes.

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Chapter Four

The effect of fishing-capture stress on the metabolic rate and swimming activity of a holocephalan

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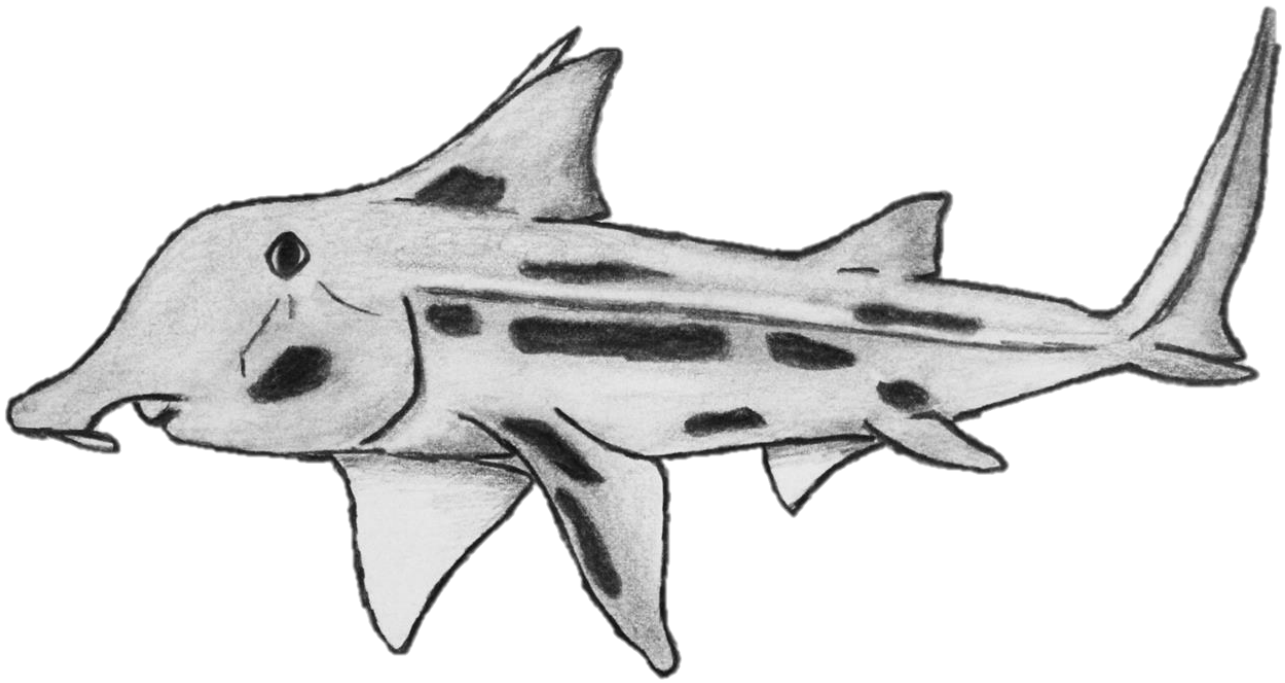

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Drawing by Licia Finotto

ABSTRACT

Overfishing, capture mortality and consequences following release of surviving animals represent severe threats to chondrichthyans. Although holocephalans, or chimeras, are common bycatch and discarded species, other than mortality, little is known of fishing-capture stress impacts. The stress response elicited after fishing capture is essential to increase survival chances but is also energetically demanding and affects the amount of energy available for other biological activities, with potential impairments. We measured the effect of 30-min simulated gillnet capture on metabolic rates (MR), a proxy for energy use estimated through oxygen uptake rates (MO_2), metabolic recovery pattern and swimming activity of elephant fish (*Callorhinchus milii*). Immediately after simulated capture, Active and Inactive MO_2 were 27.5% and 43.1% lower than unstressed values. This metabolic decline is likely an adaptation for reducing the energy allocated to non-essential activities, thus redirecting it to the stress response and processes essential for immediate survival. Supporting this suggestion, animals spent 38.5% less time moving after gillnet capture compared to unstressed conditions, probably as a result of a reduction in the amount of energy allocated to swimming. After 7-days, Active MO_2 and swimming activity returned to unstressed values, while Inactive MO_2 was still 15.0% lower. Even though metabolic decline may enhance survival chances, the associated decrease in swimming activity might increase predation occurrence and slow the physiological recovery after a fishing event. Moreover, some of the activities involved in Inactive MO_2 are fundamental for life maintenance and its prolonged depression might have long-term repercussions for life sustenance and health as a consequence of capture.

4.1. INTRODUCTION

Holocephalans, or chimaeras, are an order of mainly deep-sea chondrichthyans for which biological and physiological data are fragmentary (Didier et al. 2012, 2014; IUCN 2020). Most of the species are not presently classed at risk of extinction (IUCN 2020), but holocephalans are increasingly being captured in commercial fishing operations as fishing grounds expand to deeper regions (Didier et al. 2012; Watson and Morato 2013). The growing retention of these animals and the fishing-capture stress experienced by animals discarded because of low market values, bag limits, and other management measures increasingly threaten holocephalan populations globally (Stevens et al. 2000; Simpfendorfer and Kyne 2009). Moreover, in the waters of southern Australia, of 10 holocephalan species with low resilience to fishing stressors, 40% are presently highly vulnerable to stock depletion, despite fisheries management actions halving fishing effort and closing all waters of depth greater than 700m to most fishing (Walker et al. in review).

For elasmobranchs (sharks and rays), the effects of fishing-capture stress extend beyond death, to include physiological (Frick et al. 2009, 2010b), behavioural (Sundström and Gruber 2002; Danylchuk et al. 2014; Bouyoucos et al. 2017), immunological (Van Rijn and Reina 2010; Skomal and Mandelman 2012), energetic (Bouyoucos et al. 2017, 2018; Molina et al. 2020; Chapter 3) and reproductive alterations (Guida et al. 2017; Adams et al. 2018; Wosnick et al. 2019; Chapter 6). Only one study has investigated the physiological consequences of fishing-capture stress in a holocephalan, highlighting the high sensitivity of the species studied (Martins et al. 2018). Therefore, given that holocephalans have low reproductive rates and consequently reduced capacity to recover from fishing exploitation (Bell 2012; Didier et al. 2012; Finucci et al. 2017), the increasing influence of fishing-capture stress and the high occurrence of bycatch (Walker and Gason 2007; Burch et al. 2018) call for further research on the sub-lethal effects of fishing. Understanding stress consequences in free-living vertebrates requires investigating a suite of

physiological and ecological measures (Johnstone et al. 2012), so it is important to increase our knowledge through targeted research on this data-poor taxonomic group.

After any stress, an energetically-costly stress response is elicited to restore homeostasis and to enhance survival (Schreck et al. 2001). The energy used to fuel this response (Schreck and Li 1991; Mc Ewen and Wingfield 2003; Schreck 2010) is subtracted from the fixed amount available to sustain other biological activities, potentially impairing them (Ware 1982; Calow 1985; Mc Ewen and Wingfield 2003; Schreck 2010). Metabolic rate (MR) estimates have been used to investigate the energy costs of fishing-capture stress in teleost fishes (Clark et al. 2012; Cooke et al. 2014), and more recently in sharks and rays (Bouyoucos et al. 2017, 2018; Molina et al. 2020; Chapter 3) and might similarly be informative for holocephalans. Metabolic rates are typically estimated by measuring oxygen uptake (MO_2) of an animal in different states of activity, with each MR type associated with different behaviours and ecological significance. Standard metabolic rate (SMR) is the amount of energy used for organism maintenance by an animal at rest, with no voluntary muscular movements, while routine metabolic rate (RMR) is measured when animals perform an unspecified amount of activity, and maximum metabolic rate (MMR) is the maximum energetic capacity that an animal can sustain before exhaustion (Chabot et al. 2016). During normal activities, animals rarely approach MRM, while RMR and SMR represent the main components of energy expenditure (Norin and Clark 2016). Measuring changes in MR types allows investigating how animals adjust energy allocation after a stressful event and focusing on SMR and RMR will improve our understanding on the consequences that these alterations have on activities associated with animal welfare and survival, including homeostasis, osmoregulation, organismal maintenance and swimming (Chabot et al. 2016). As important as it is to investigate the short-term energy changes, it is equally important to describe the metabolic recovery pattern to evaluate the extent of the stress consequences. Indeed, the longer the metabolic alterations

persist, the worse the consequences might be (Davis and Schreck 1997). Nevertheless, only one study has directly measured metabolic recovery after fishing-capture stress (Bouyoucos et al. 2017) and, given the large variability in responses to stress characteristic of chondrichthyans, further research is needed.

Changes in animal behaviours following a stressful capture event, including predator avoidance, shoaling, use of refuges, vigilance, equilibrium, and most importantly, swimming performance and speed, can increase the predation risk to discarded animals (Ryer 2004; Raby et al. 2014). In chondrichthyans, fishing capture alters diving behaviour (Holts and Bedford 1993; Klimley et al. 2002; Nakano et al. 2003), swimming speed (Holland et al. 1993; Sundström and Gruber 2002; Gurshin and Szedlmayer 2004; Raoult et al. 2019), the pattern of movement (Martins 2017) and reflex and swimming abilities (Danylchuk et al. 2014). Investigating potential swimming impairments is important, even more so in the context of a concomitant reduction in the energy budget available to sustain movement. We do not know how stress affects swimming performance in holocephalans, and further research is needed, particularly considering their migratory and shoaling habits (Didier et al. 2012).

The elephant fish, *Callorhinchus milii*, is an Australian holocephalan captured both in commercial and recreational fisheries in southern Australia and New Zealand (Walker and Gason 2007; Braccini et al. 2009) and due to bag limits and low commercial value, a large number of animals is discarded after capture (~58% of the total catch in 2014; Burch et al. 2018). The species experiences a high rate of delayed mortality following discarding (Braccini et al. 2012) and severe physiological alterations as a consequence of the stress experienced (Martins et al. 2018). In this paper, we investigated the energetic consequences of gillnet capture simulation on female *C. milii* by estimating MR alterations through the respirometry method and measuring MO_2. We measured both Active MO_2, a proxy for RMR, using data recorded while animals were swimming,

and Inactive MO_2, a proxy for SMR, using data collected while animals were resting. Any potential immediate alterations in the energetic expenditure and the metabolic recovery period were examined comparing the MO_2 of females measured before, immediately after and seven days after simulated gillnet capture. Simultaneous with MO_2 measurements, we recorded the amount of time the animals spent swimming or resting to detect any changes in swimming time.

4.2. MATERIALS AND METHODS

4.2.1. Animal collection and husbandry

In April and July 2019, female *C. milii* (n=7) were collected from Barwon estuary and Corner Inlet (Victoria, Australia) using hook and line or purse seine. Animals were held onboard the fishing vessel in a 200-L holding tank with a continual flow-through of ambient seawater and transported to the housing facility (Victorian Fisheries Authority, Queenscliff, Victoria, Australia) in a transport tank constantly supplied with pure oxygen. Collection and transport took 2–5 h and a maximum of 3 animals (7 kg biomass) was held together at any time. At the facility, the body mass of each animal was measured to the nearest 0.1 kg before transferring it to a 19,000-L circular housing tank (maximum biomass of 11 kg) placed outside under a covered structure and supplied with continuously flowing seawater and aeration. The colour pattern of each animal allowed identification without the use of tags. Animals were exposed to natural photoperiod and ambient water temperature and fed sardines and shellfish twice a week (~10% of their total body mass weekly). Water temperature in the tanks could not be controlled but approached values recorded in the adjacent Swan bay (± 1°C) and followed the seasonal variation (no difference between housing tanks; mean ± SE: 14.3 ± 0.4 °C, range: 13.1–17.1 °C). Captivity lasted for a maximum of 30 days and, after veterinary health check, animals were released into the adjacent Swan Bay. The first females collected were laying eggs, but they had ceased before the end of the 10-day habituation period. All experiments were performed under Monash University Animal Ethics

Protocol BSCI/2018/07, animals were collected under Victorian Fisheries Authority general permit n° RP1364 and all work complied with current Australian law.

4.2.2. Experimental design

The schematic of the experimental design is described in Figure 4.1. For each female separately, the oxygen uptake rate (MO_2) was measured during three separate trials, hereafter referred to as MO_2-class. The Unstressed MO_2-class was measured in minimally stressed animals at least ten days after initial capture to allow recovery and habituation to the housing conditions. Then the After-capture MO_2 class measurement occurred after a further seven days with the measurements taken immediately following simulated gillnet capture (see below). The Recovery MO_2 class was measured seven days after the capture simulation. We adopted a repeated measures design for determining MO_2 of the different MO_2-classes to allow control for the high inter-individual variability in MO_2 evident from other studies (Molina et al., 2020). To reduce the influence of food processing and digestion on the energy demand of animals (Chabot et al. 2016), MO_2s were measured in animals at least 24h after the last feeding event.

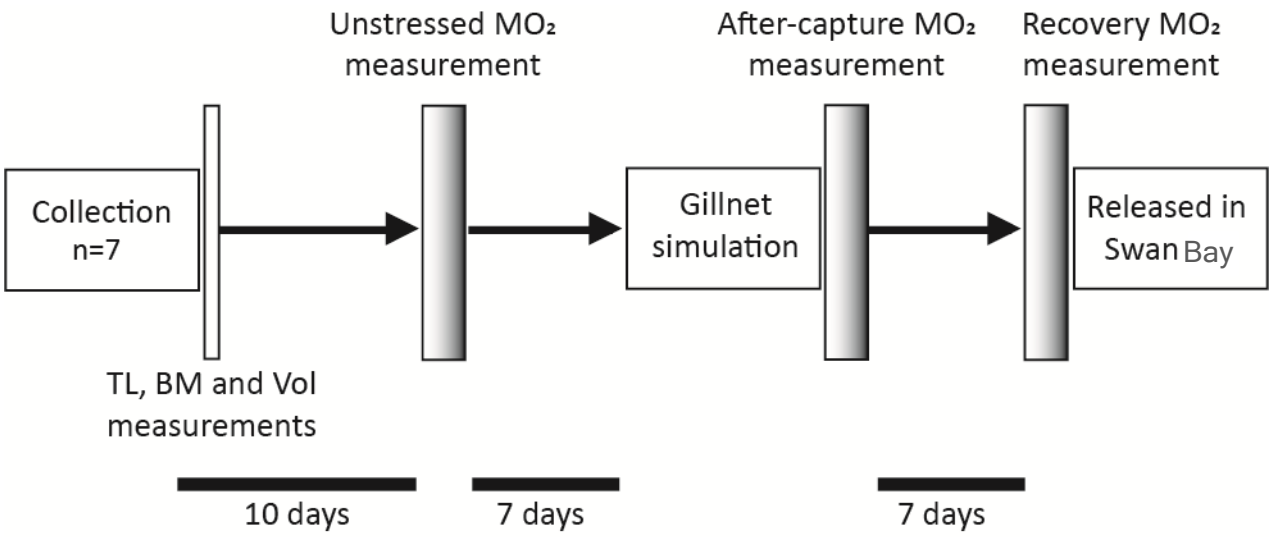

Figure 4.1. Schematic of the experimental design. Squares represent procedures that *Callorhinchus milii* females experienced. Black arrows indicate the passage from one procedure/measurement to the next. The vertical white bar indicates when female total length (TL), body mass (BM) and volume (Vol) were measured. Vertical blurred bars indicate when different MO_2-classes were measured. Horizontal black segments represent the number of days passing between different procedures/measurements (not in scale). n indicates numerosity.

4.2.3. Respirometry trial

Oxygen uptake rate trials were performed using a circular plastic respirometry chamber (105 cm diameter x 34 cm height, 295-L volume; 5 mm thickness; Figure 4.2) in which animals were able to swim. The chamber was sealed using a double layer of thick plastic sheets laid down on the water surface and tightly secured around the rim of the tank using strips of foam and several large plastic clamps (Barnett et al. 2016). This method prevented the formation of an air space above the surface of the water. The tank was equipped with a water inlet and outlet connected to an external water pump that maintained even mixing of the water inside the respirometry chamber without creating a current strong enough to disturb the animals and cause them to swim against it (Clark et al. 2013). An oxygen sensor meter (Hach Intellical™ LDO101 Field Luminescent/Optical dissolved Oxygen sensor meter of ±0.1 mg/L accuracy), placed close to the water outlet, was used

to detect dissolved oxygen concentration (DO) and temperature inside the chamber during MO_2 measurements.

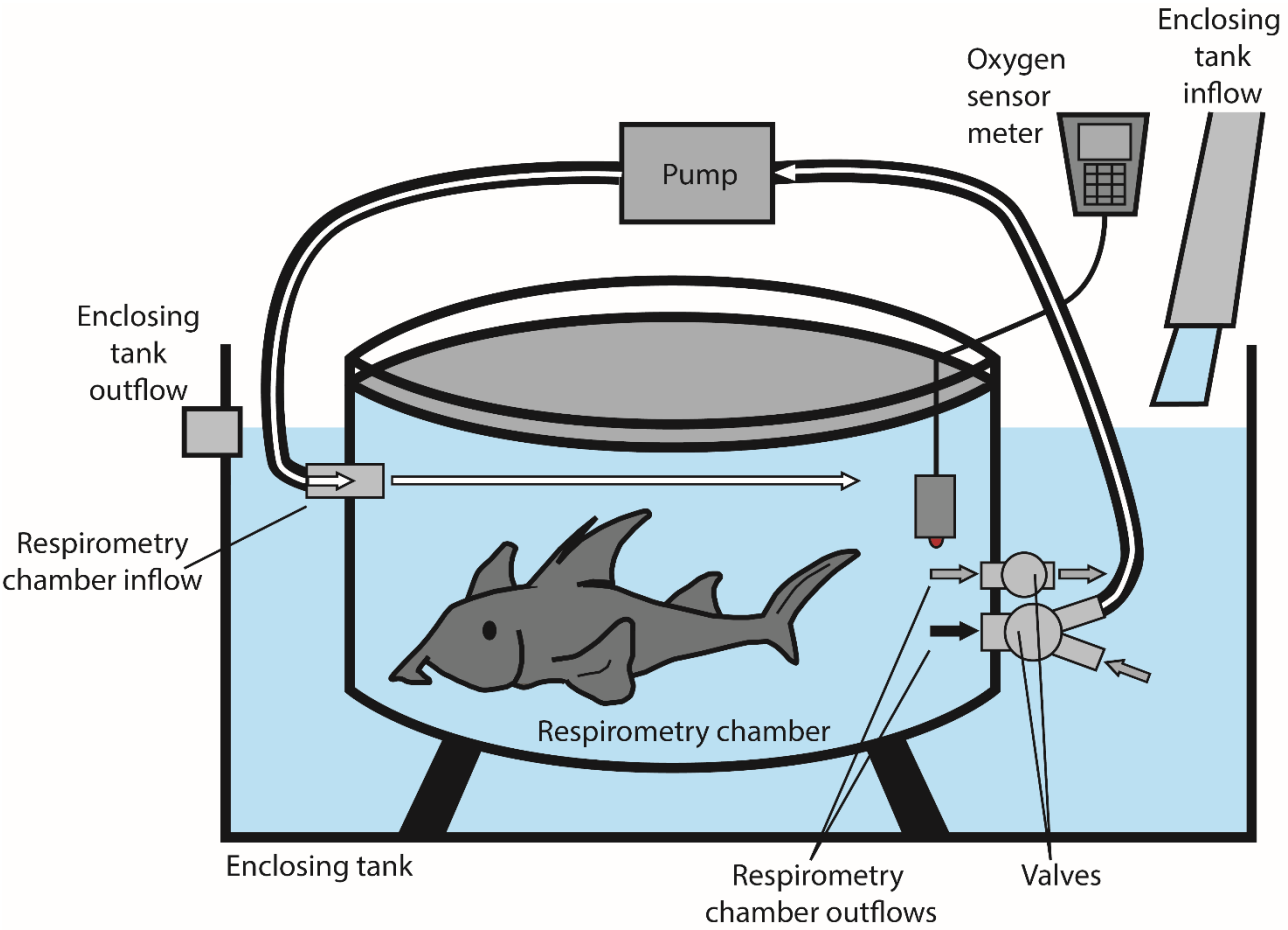

Figure 4.2. Schematic representation of the respirometry chamber. Grey arrows indicate water flow during the flushing phase, black arrows indicate water flow during the phase measurement of oxygen depletion and white arrows indicate common water flow during both phases. The closure or opening of the specific valve allowed the transition from the measurement to the flushing phase (adapted from Molina et al., 2020 with permission).

The respirometry chamber was placed in a 5,000-L tank and the females were individually netted and hand-transferred from the housing tank to the respirometry chamber (< 2 min) at the time of each trial. After placing a female in the respirometry chamber, it was allowed to settle to its cruising swimming pattern (< 2 min), then the surface of the chamber was sealed with plastic

sheets and the MO_2 trial began. Values of DO ($mgO_2\ L^{-1}$) were recorded at 30-sec intervals during eight measurement cycles, each of 15-min duration. Each measurement cycle was separated by a 15-min period during which the respirometry chamber was flushed with oxygenated seawater obtained from the enclosing 5,000-L tank. At the end of the flushing period, the inlet to the pump was changed to resume water recirculation within the respirometry chamber for the next measurement cycle. To avoid animals potentially suffering from oxygen deprivation, DO was monitored continuously and never allowed to fall lower than 80% saturation before reflushing the tank. During all procedures, animal well-being was monitored by visual inspection of its activity level through the transparent plastic sheets. No animal showed visible signs of distress. After the end of the eighth cycle, the plastic sheets were removed, and the animal was transferred back to the housing tank. A blank trial was performed at the beginning and end of each MR trial to detect background respiration inside the respirometry chamber. The chamber was sealed and DO monitored for 15 min as during a normal measurement cycle, without an animal inside. Background respiration was calculated and subtracted from all other measurements before MO_2 calculation (see Data analysis). The respirometry chamber was emptied, washed and refilled with seawater between each animal. If trials were performed in different days, the chamber was left empty and allowed to dry to reduce bacterial proliferation. To account for circadian rhythm, all respirometry trials occurred during daylight, always between 8 am and 4 pm.

4.2.4. Gillnet simulation

Gillnet capture was simulated seven days after the Unstressed MO_2 trial to allow animal recovery from minor stressors potentially involved in the procedure. A 1.5 m long x 1.5 m deep panel of monofilament gillnet was placed vertically on the bottom of a 5,000-L experimental tank, attached to a wooden frame mounted on top of the tank. The size of the mesh varied according to the size of the animal (100 mm, 130 mm or 180 mm between opposite knots of stretched

diamond-shaped mesh). Each female was individually dip netted, transferred to the experimental tank and placed in a pocket of the gillnet created by drawing the footrope to below the surface (Frick et al. 2009). Each animal became enmeshed by its own movement, but the degree of enmeshment was controlled to avoid reduced ventilation and injury of the gills from constraint of the operculum (Bell 2012). Our simulated gillnet capture probably represented a milder stress than typical commercial fishing operations in which movement of captured animals can cause severe entanglement and operculum obstruction (Dapp et al. 2017). In our study, enmeshment was controlled to ensure standardization of the fishing-capture stress conditions in all females and to avoid the death of any of the animals, the likelihood of which was investigated in a previous study (Martins et al. 2018).

Gillnet capture simulation lasted for 30 min, then the female was disentangled from the net and hand-transferred to the respirometry chamber located in an adjacent tank to measure After-capture MO_2 as described above. The disentangling and transfer process took less than 2 min and air exposure was shorter than 30 sec. The experimental tank was supplied with a constant seawater flow so that the whole volume of water was replaced between two successive gillnet simulations, reducing the possibility of stress-related chemical clues influencing the responses of animals treated subsequently.

4.2.5. Recovery MR

The animals were kept in their holding tank for seven days to allow recovery from the simulated gillnet capture before measuring Recovery MO_2 as described above. The recovery period length was chosen in accordance with the results of a previous study indicating a measurable physiological recovery of *C. milii* within three days after fishing-capture simulation (Martins et al. 2018).

4.2.6. Swimming time

During each of the eight measurement cycles of a MO_2 trial, the time the animal spent actively swimming or resting on the bottom of the respirometry chamber, equalling the full duration of 15 min, was recorded. Animals were considered 'at rest' when lying on the bottom of the tank and the only activities performed were sporadic movements of the pectoral fins for self-righting. The start of swimming was defined by energetic movements of the pectoral fins causing the animals to move at least one body-length away from the starting point. In all cases, animals swam at least a full circumference of the tank before stopping. The end of swimming was determined by the cessation of pectoral fin movements and the return of the animals to the bottom of the tank. Swimming speed could not be reliably estimated, so these data were not recorded. However, soon after being placed in the respirometry chamber, all females settled to a constant cruising speed which was maintained for the whole duration of the swimming time during the different measurement cycles.

4.2.7 *Respirometry data processing*

For each of the eight measurement cycles of a MO_2 trial, the values of dissolved oxygen concentrations (DO) were categorized according to the animal's swimming activity. From these data we calculated Active and Inactive MO_2, referred to as 'MO_2-type' hereafter. The data recorded while the animal was swimming were used to determine Active MO_2, while data measured when it was at rest were used to determine Inactive MO_2. Because MO_2 takes some time to decrease and to stabilize at its Inactive MO_2 after switching from swimming to resting (Piiper et al. 1977), only periods during which an animal rested continuously for at least 5 min were included in Inactive MO_2 calculations and DOs recorded during resting periods shorter than 5 min were excluded from MO_2 analysis. When two swimming periods were separated by a period of rest (or vice versa), data were rearranged and aggregated to obtain a single swimming period

and a single resting period (Figure 4.3). In this way, for each of the eight measurement cycles, only a maximum of one series of DO measurements for swimming and one series of DO measurements for resting were obtained and used to calculate RMR and SMR types.

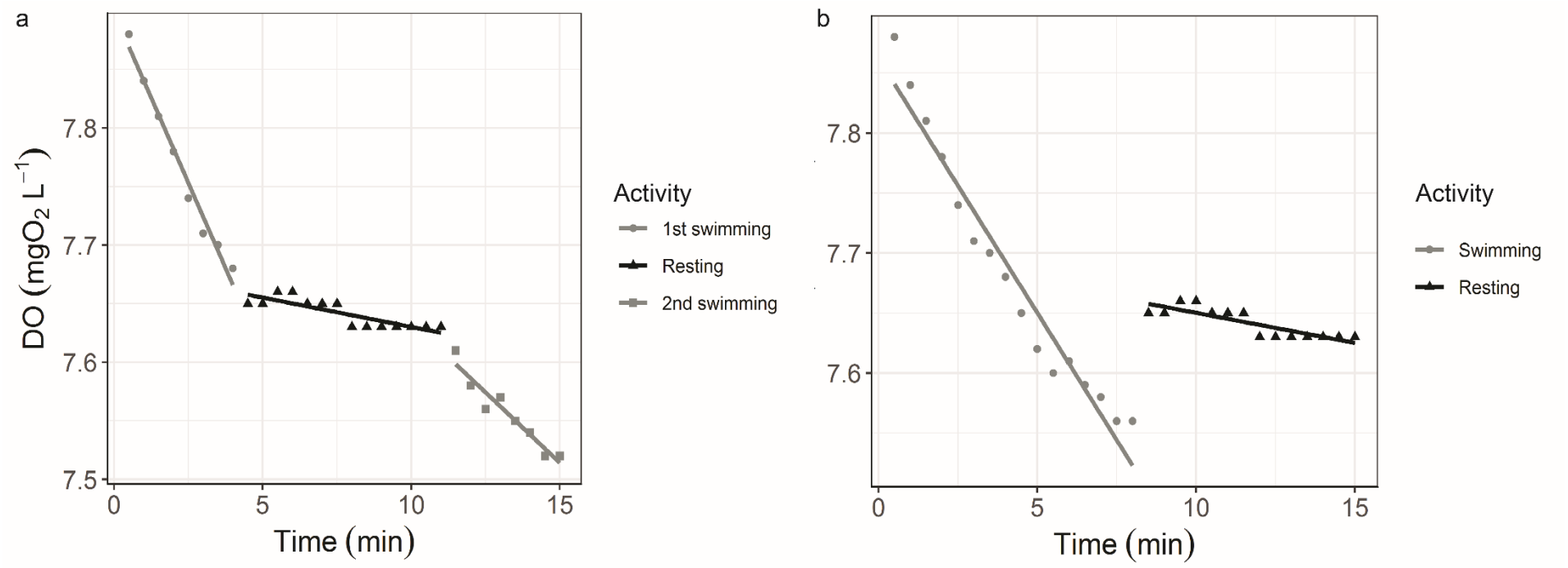

Figure 4.3. Example of rearrangement of dissolved oxygen data (DO; mg L^{-1}) according to an animal's swimming activity during one of the eight measurement cycles comprising a MR trial. (a) Original DOs plotted against time in the respirometry chamber (dots, triangles and square). A separate line is fitted by linear regression for each successive period of swimming or resting activity. (b) Rearranged data: the two period of activity, previously separated by a period of resting, has been aggregated to obtain a continuous series of DOs. Two regression lines were fitted to the single resting and swimming periods.

Separately for the swimming and resting series of data and for the eight measurement cycles, DOs were plotted over time and the slope of the decrease in DO was calculated by regression analysis. R^2 values of the calculated slopes were all higher than 0.85. Data recorded during the two blank trials were processed similarly and the values of the slopes obtained were averaged to obtain a value for the background respiration that was subtracted from the previously calculated slopes during MO_2 calculation (see below). The introduction of a habituation period (2–24 h) before measuring MR is recommended to allow animals to recover from the mild handling and/or air exposure and to adjust to the novel environment of the respirometry chamber (Clark et al. 2013). However, the main aim of our study was to investigate the effect of fishing-capture simulation on MO_2 and, because the process of recovery from stress begins as soon as the stress ceases (Reidy et al. 1995; Brick and Cech 2002; Cooke et al. 2014), we needed to measure After-capture MO_2 immediately after fishing-capture simulation, so no habituation period was used. For comparison purposes, Unstressed and Recovery MO_2s were also measured immediately after the animal was placed in the respirometry chamber. To account for the potential increase in MO_2 caused by the initial handling/air exposure stress, separately for each MO_2 trial and for Active and Inactive MO_2 data, an ANCOVA, including DO as the dependent variable and the interaction between time and measurement cycle as an independent factor, was used to test whether the slopes calculated for the eight different measurement cycles differed significantly from one another. If slopes differed, only data obtained after the slopes stabilized were used for calculating MO_2s and included in the statistical models (see Molina et al. 2020 for example). MO_2 was calculated using the equation:

$$MO_2 = (O - B) \times Vol \times BM^{-1} \quad (1)$$

where MO_2 is the mass-specific metabolic rate expressed as $mgO_2\ h^{-1}\ kg^{-1}$, O is the regression slope previously calculated and expressed as $mgO_2\ L^{-1}\ h^{-1}$, B is the background respiration

regression slope calculated for each MO_2 trial expressed as $mgO_2\ L^{-1}\ h^{-1}$, Vol is the volume of water in L contained in the respirometry chamber and BM is the body mass of the animal in kg.

As mentioned, water temperature could not be controlled and followed seasonal variation. The IntelliCAL probe automatically corrects measured DO values according to temperature. However, given the strong influence that temperature has on MO_2 (Schmidt-Nielsen, 1997), calculated MO_2s were temperature-corrected to 14°C, the average temperature recorded during the experimental period, using $Q_{10} = 2.3$ (Neer et al. 2006; Whitney et al. 2016).

4.2.8. Data analysis

4.2.8.1. Body mass - MO_2 *relationship*

The normality of the error structure of the datasets was assessed using Quantile-Comparison Plots, Shapiro-Wilk, kurtosis and skewness tests. The relationship between body mass and Unstressed MO_2 was investigated, separately for Active and Inactive MO_2 and after log-transformation of the data, using GLMEs including body mass as a fixed effect and female ID as a random effect (MO_2 ~ BM + (1|ID)). Because body mass did not influence MO_2 values (see results), it was excluded from further analyses.

4.2.8.2. MO_2-class and MO_2-type effects on MO_2 values

To investigate differences in MO_2 values, a GLME including the interaction between MO_2-class (Unstressed, After-capture and Recovery) and MO_2-type (Inactive MO_2 and Active MO_2) as a fixed effect and female ID as a random effect was used (MO_2 ~ MO_2-class * MO_2-type + (1|ID)). Models were validated evaluating the homoscedasticity of the generated residuals. Significance of the fixed effects was evaluated using a Wald test and a Holm-Šídák post hoc test was used for comparison of means.

4.2.8.3. Energetic cost of animal activity

For both Unstressed MO_2s and After-capture MO_2s, the energetic cost of animal activity (CA_P), which is the percentage increase in energy use needed to switch between resting and active states (Whitney et al., 2016), was calculated. Using Active and Inactive MO_2 values obtained from the previous model, CA_P was calculated as:

$$CA_P = (\text{Active } MO_2 - \text{Inactive } MO_2)/\text{Inactive } MO_2 * 100 \quad (2)$$

4.2.8.4. Animal swimming time

For each MO_2 trial separately, the proportion of time the animal spent swimming during each of the eight measurement cycles was calculated by dividing the total time spent swimming (min) by the duration of the measurement cycle (15 min). Before analysis, values obtained from the eight cycles were transformed as the arcsin of the square root, but the results are presented as percentages for clarity. Differences in the transformed data were tested using a LME, including MR-class as a fixed effect and animal ID as a random effect (SwimmingTime ~ MO_2-class + (1|ID)).

Data were processed using R statistical software (R Core team 2019). Results are reported as mean ± standard error and all tests used a significance level of $\alpha \leq 0.05$.

4.3. RESULTS

4.3.1. Body mass - MO_2 relationship

For both Inactive MO_2 ($F_{1,5} = 0.02$, $p = 0.88$) and Active MO_2 ($F_{1,5} = 0.005$, $p = 0.95$), there was no significant relationship between Unstressed MR and body mass.

4.3.2. MO_2-class and MO_2-type effects on MO_2 values

The class of MO_2 measurement (Unstressed, After-capture or Recovery; $F_{2,159} = 377.21$, $p < 0.0001$), MO_2 -type (Inactive MO_2 or Active MO_2, $F_{1,159} = 1494.40$, $p < 0.0001$) and their interaction ($F_{2,159} = 16.48$, $p = 0.0003$) all had a significant effect on MO_2. Within each MO_2-class, Inactive MO_2

was significantly lower than Active MO_2 ($p < 0.0001$). For Inactive MO_2, all MO_2-classes significantly differed from one another (Unstressed – Recovery, Unstressed – After-capture and Recovery – After-capture: $p< 0.0001$; Unstressed = 59.41 ± 5.94 $mgO_2\ h^{-1}\ kg^{-1}$; After-capture = 33.78 ± 4.42 $mgO_2\ h^{-1}\ kg^{-1}$; Recovery = 50.49 ± 3.57 $mgO_2\ h^{-1}\ kg^{-1}$). For Active MO_2, Unstressed (116.97 ± 3.99 $mgO_2\ h^{-1}\ kg^{-1}$) and Recovery MO_2s (111.84 ± 5.36 $mgO_2\ h^{-1}\ kg^{-1}$) did not differ ($p = 0.72$) but were both significantly higher than After-capture MO_2 (81.05 ± 5.55 $mgO_2\ h^{-1}\ kg^{-1}$; $p < 0.0001$; Figure 4.3). Relative to Unstressed values, gillnet capture caused a 27.5% and a 43.1% decrease in Active and Inactive MO_2, respectively. Recovery Inactive MO_2 was 15.0% lower than Unstressed Inactive MO_2 and 49.6% higher than After-capture Inactive MO_2.

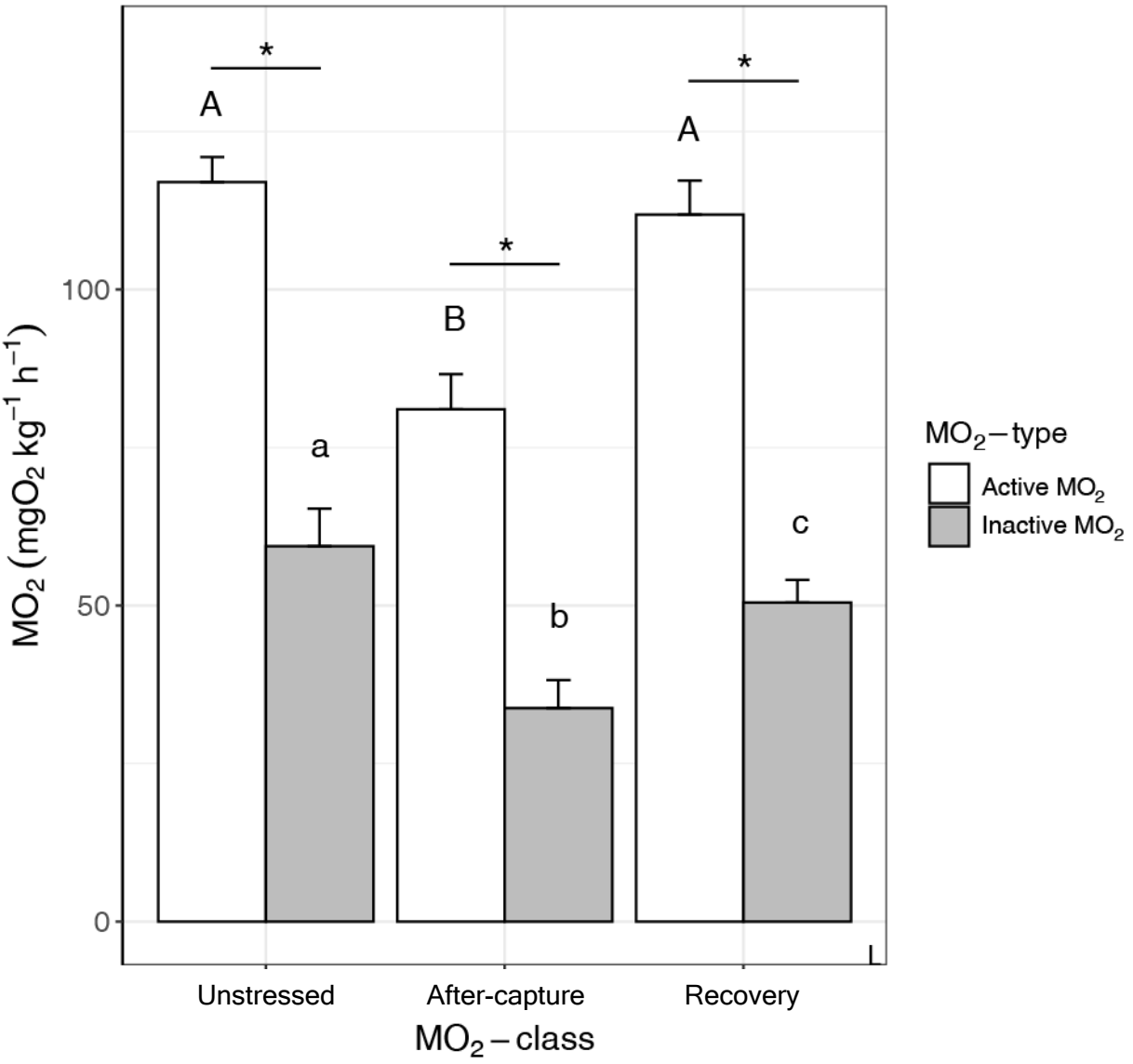

Figure 4.4. Mean ± SE ($mgO_2\ kg^{-1}\ h^{-1}$) of Active MO_2 (white boxes) and Inactive MO_2 (grey boxes) for each MO_2-class: Unstressed (n=7), After-capture (n=7) and Recovery (n=7). *indicates statistically significant differences ($p < 0.05$) between Active and Inactive MO_2 within the same MO_2-class. Different uppercase and lowercase letters indicate statistically significant differences between different MO_2-classes within Active and Inactive MO_2, respectively.

4.3.3. Energetic cost of animal activity

The cost of Activity (CA_P) represented a 96.9% and 139.9% increase compared to Unstressed and After-capture Inactive MO_2 values, respectively.

4.3.4. Level of animal swimming time

The percentage of time that animals spent swimming significantly differ between MO_2-classes ($F_{2,141}$ = 20.78, p<0.0001). Time spent swimming in the Unstressed (62.9 %) and Recovery measurements (52.8 %) did not differ (p = 0.06), but they were both higher than After-capture values (38.7 %; Unstressed – After-capture: p <0.0001; Recovery – After-capture: p = 0.02). Gillnet capture caused a 38.5% decrease in swimming time compared to Unstressed values.

4.4. DISCUSSION

Metabolic alterations caused by fishing-capture stress in chondrichthyans are an important research area, especially given the observed species-specific responses (Bouyoucos et al. 2017, 2018; Molina et al. 2020) and reproductive implications (Chapter 3). For the first time for a holocephalan, we aimed at investigating possible capture-related metabolic alterations and the recovery pattern. Results showed that MO_2 measured while animals were both swimming (Active MO_2) and resting (Inactive MO_2) were significantly reduced by a 30-min gillnet simulation. After seven days from capture stress, Active MO_2 increased back to minimally stressed values, while recovery for Inactive MO_2 was only partial.

Given the energy expenditure associated with the fishing-stress response observed in teleost fishes (Clark et al. 2012; Cooke et al. 2014), an increase in MO_2 might be expected as a result of fishing-capture treatment also in chondrichthyans. Nevertheless, in elasmobranchs (sharks and rays), a fishing-capture stress both increases (Bouyoucos et al. 2017; Bouyoucos et al. 2018) and decreases MO_2 (sharks: Molina et al. 2020; rays: Chapter 3). In our study, 30 min of gillnet capture simulation caused a 27.5% and a 43.1% decrease in Active MO_2 and Inactive MO_2, respectively. There are several possible explanations for this result. It is likely that an initial increase in energy expenditure and aerobic metabolism, aimed at restoring homeostasis, does occur soon after animals are placed in the gillnet. However, as capture is prolonged, the recovery process, and the

associated decrease in MO_2 (Bouyoucos et al. 2017; Bouyoucos et al. 2018), might start while animals are still entangled in the net, and therefore might have gone unnoticed as in our experiment we started measuring MO_2 only at the conclusion of the gillnet simulation. Supporting this hypothesis, after an initial period of struggling when placed in the net (<10 min), animals settled on the bottom of the tank without any further energetic movement, potentially allowing for MO_2 recovery to start. However, given that recovery usually takes several hours (Bouyoucos et al. 2017; Bouyoucos et al. 2018) and capture simulation lasted only 30 min, the constant decline in MO_2 with time characteristic of a recovery process should have been recorded during After-capture MO_2 measurements, but it was not, as animals' MO_2 stabilized within the first hour in the respirometry chamber. Moreover, all MO_2s measured at the end of the gillnet simulation are significantly lower than values measured in minimally stressed animals, indicating that, as well as recovery, other mechanisms are involved.

A triggering of a metabolic decline caused by the initial excessive energy consumption can be another cause of the decreased MO_2, somewhat similar to the responses observed in teleosts exposed to hypoxia/anoxia, temperature changes and drought (Hand, 1996), and in epaulette sharks (*Hemiscyllium ocellatum*) facing severe hypoxia (Mulvey and Renshaw 2000; Routley et al. 2002). As a consequence of this large energy use, the likely build-up of adenosine, a product of ATP degradation, acts as a neuronal inhibitor intended to preserve energy reserves in the heart and the brain (Guida et al. 2016a) and maintain biological processes fundamental for survival, while suppressing unnecessary activities (Renshaw et al. 2002). In other instances, MO_2increased after fishing-capture without any sign of metabolic decline potentially because, either due to the species being resilient to stress (Molina et al. 2020) or the stress imposed being mild (Bouyoucos et al. 2017, 2018), the initial increased energy investment in the stress response was sufficient to restore homeostasis. The decrease observed in *C. milii* fits this explanation because this species is

sensitive to stress (Martins et al. 2018) and, even though the fishing-capture simulation was relatively mild (Dapp et al. 2015), the associated stress was probably enough to severely disrupt *C. milii* homeostasis and cause the consequential large energy use triggering metabolic decline. Monitoring the activity level revealed a significant reduction in the time the animals spent swimming after capture simulation (38.5% decrease) that might also explain the decline in MO_2. Nevertheless, Inactive MO_2 also declined, indicating that the reduction in the activity level is only one of the possible causes and that other physiological mechanisms must contribute. After-capture MO_2 was measured after animals experienced the respirometry chamber first during the Unstressed trial, so habituation to the respirometry chamber might have occurred, and the associated lower stress experienced, compared to the first MO_2measurements, might also explain the decrease in MO_2measured after capture simulation. However, the return of Active MO_2 to Unstressed values after the recovery period, when the animals experienced the respirometry measurement for the third time, supports the absence of habituation and the validity of the measured decrease in MO_2.

A reduction in the activity level during (Guida et al. 2016b) and after fishing capture (Danylchuk et al. 2014; Raoult et al. 2019) has also been observed in other species and is likely an adaptation for reducing the energy spent to sustain swimming, thereby redirecting it to fuel the stress response and increase survival chances. This is particularly true for *C. milii* in the context of the triggered metabolic decline, when animals, after exhausting almost all their energy resources (Manire et al. 2001; Frick et al. 2010a; Martins et al. 2018), have to primarily direct the remaining energy to sustain essential processes, to the detriment of swimming (Mulvey and Renshaw 2000; Routley et al. 2002). Although it saves energy, reduced mobility could increase predation risk (Ryer 2004; Raby et al. 2014), especially in southern Australian waters that are home to several large predatory shark species (mako *Isurus oxyrinchus*, white *Carcharodon carcharias* and broadnose

sevengill shark *Notorynchus cepedianus*; Last and Stevens 2009) that might scavenge on discarded animals. The reluctance of *C. milii* to swim post-stress, potentially originating from the increase in the cost of activity compared to the Unstressed condition (44.4% increase), might translate to a lower responsiveness to approaching predators or other dangers, further reducing the ability to escape these threats (Ryer 2004; Raby et al. 2014). *Callorhinchus milii* is a social species that undertakes migration in shoals (Last and Stevens 2009; Bell 2012; Didier et al. 2012) and reduced swimming performance might prevent discarded animals from keeping pace with the rest of the shoal, leaving them isolated with potentially impaired survival and reproductive success (Pitcher and Parrish 1993). Swimming and ram ventilation, enhancing gas exchange (Clark and Seymour 2006; Brooks et al. 2011), seem to hasten the recovery after stress (Milligan et al. 2000; Farrell et al. 2001). Indeed, some shark species increase swimming activity during capture, potentially switching to ram-ventilation to overcome the low efficiency of buccal pumping and to mitigate the severity of the physiological alterations (Bouyoucos et al. 2017); instead, the decreased activity level of *C. milii* might slow their recovery. However, in the context of a capture-induced metabolic decline, reduced swimming activity and maintenance of vital processes seem to be prioritized over the need for adequate ram ventilation and rapid recovery (Molina et al. 2020), despite potentially leaving animals exposed to predatory attack and social impairment. Swimming alterations are particularly noteworthy, given that they persist for at least 4 h after fishing stress (the duration of the MO_2 trial).

Investigating metabolic recovery is important for estimating the overall energetic cost of stressors (Davis and Schreck 1997). Recovery from a fishing-induced increase in MO_2 took 5.4±2.5 h in *N. brevirostris* (Bouyoucos et al. 2017) and an estimated 8.4±5.8 h in blacktip reef sharks (*Carcharhinus melanopterus*, Bouyoucos et al. 2018). In contrast, MR was still lower than baseline 24 h after osmotically-induced stress (Morash et al. 2016; Tunnah et al. 2016). In *C. milii*, Active

MO_2 and swimming activity returned to Unstressed values within seven days from fishing-capture simulation, but Inactive MO_2 recovery was only partial. Given that some components of the Inactive MO_2 represents the energy used to fuel activities involved in life sustenance, the prolonged reduction in Inactive MO_2 might cause severe long-term repercussions on the organism, potentially compromising survival (Chabot et al. 2016). Animals prioritizing the recovery of Active MO_2 and of swimming activity over Inactive MO_2, despite the large amount of energy that has to be devoted to supporting movement and the assumption that some processes contributing to Inactive MO_2 remain suppressed, seems to indicate further the importance of swimming activity for this species. The decline in Active MO_2 appears to be primarily driven by the reduction in the activity level, given that it recovers as animals return to Unstressed swimming patterns. In contrast, the decline in Inactive MO_2 is likely caused by alterations in physiological processes that do not completely recover within the monitored period. After a similar fishing-capture stress, *C. milii* blood physiological variables returned to minimally stressed values within 2–3 days (Martins et al. 2018). However, Inactive MO_2 alterations may be caused by variables commonly not monitored in stress studies, such as cellular energy charge (Guida et al. 2016a). In *T. dumerilii*, immunological variables remain altered up to 28 days after stress (Guida et al. 2017), suggesting that, while secondary responses (blood physiological variables) usually recover in a short time (24–72 h; Frick et al. 2009, 2010b; Martins 2017), whole-organism tertiary responses, including altered immune function and energy use, are longer-lasting.

Compared with other chondrichthyans, *C. milii* is quite sensitive to stress (Braccini et al. 2012; Martins et al. 2018). Although reducing gillnet soaking times seems to increase survival (Martins et al. 2018), the energetic alterations and the changes to swimming activity we observed suggest consequences such as a higher predation occurrence that might reduce survival in nature even when physiological impairments *per se* would not have been lethal. Further, all animals in our

study were highly responsive to touch and struggled to free themselves during their removal from the gillnet, and in field studies using behavioural and reflex indices for condition assessment, this level of responsiveness would likely be seen as an indication of an animal in good condition (Manire et al. 2001; Braccini et al. 2012). Such an assessment would potentially cause an underestimation of the post-capture mortality (Raoult et al. 2019) if the altered swimming activity we observed is associated with increased predation rate in nature. Although the extinction risk status of *C. milii* is 'least concern' (IUCN, 2020), there is mounting evidence for the need to reduce fishing impacts. Additional to the past decline in the abundance of the Bass Strait stock (Punt et al. 2004) and the occurrence of discarding by recreational fishers using rod and reel (up to 20%; Braccini et al. 2012), growing anecdotal reports indicate dwindling numbers in inshore waters. Furthermore, a recent risk analysis shows that the species is highly vulnerable to further population decline at present levels of fishing combined with current high levels of global carbon emissions and consequent effects of climate change, and moderately vulnerable to low to medium emissions scenarios (Walker et al. in review). It is true that of different gear types, gillnet elicited the greatest stress response in *C. milii* (Martins et al. 2018), but field hook and line are likely to cause similar consequences as the one elicited by our gillnet capture simulated in a protected environment, especially when animals' handling is prolonged and executed without proper caution (Didier et al. 2012; Martins et al. 2018). Reductions in fishing time and air exposure and the adoption of better handling practices (AFMA 2014) will reduce the stress imposed and might avoid triggering metabolic decline, or at least reduce the extent of the energetic alteration, consequently promoting post-release recovery. Management measures will be especially beneficial in known nursery areas and where predator abundance is high. Given the paucity of data (Didier et al. 2012), our results contribute fundamental information for the management of

other holocephalans, including the two congeneric species, the Southern American elephant fish *C. callorynchus* and the Cape elephant fish *C. capensis*.

4.5. CONCLUSIONS

Investigating the metabolic responses to fishing capture of a holocephalan species provided new information on the stress response of *C. milii* and confirmed the relatively high sensitivity of the species and the similarity of the energetic response with other chondrichthyan species (Molina et al. 2020; Chapter 3). The metabolic recovery pattern suggests that the energetic impairments are long-lasting and, with the associated reduction in activity levels, might increase the risk of predation and reduce the survival of animals post-release.

Supporting metabolic measurements with blood and tissue analysis will help identify the mechanisms causing the metabolic depression and the causes of the incomplete recovery of Inactive SMR. Understanding the consequences of the prolonged alteration of biological processes involved in organismal maintenance (Inactive SMR) should be investigated along with field tracking studies to confirm reduction in swimming activity and to verify potential associated increases in predation occurrence.

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Chapter Five

The effect of gillnet capture on the metabolic rate of two shark species with contrasting lifestyles

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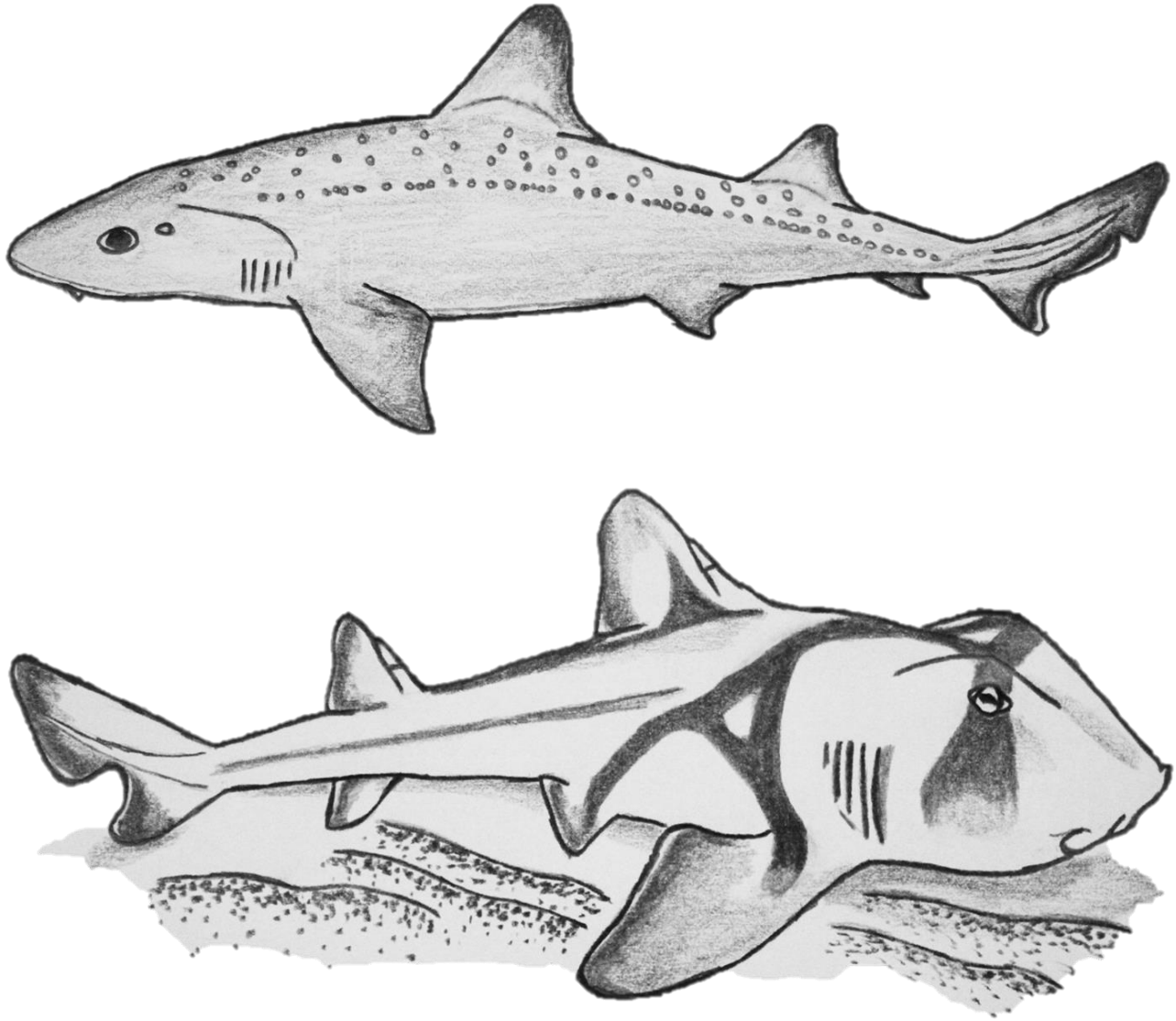

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The effect of gillnet capture on the metabolic rate of two shark species with contrasting lifestyles

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A B S T R A C T

In this study we investigated the metabolic consequences of simulated gillnet capture in species with contrasting life-history characteristics, the gummy shark, *Mustelus antarcticus* and the Port Jackson shark, *Heterdontus portusjacksoni*. Pre-stress standard and routine metabolic rates (MR) of individuals of these species were measured and compared to the MR obtained after simulated gillnet capture. Standard MRs of *M. antarcticus* and *H. portusjacksoni* were 168.5 ± 61.9 mgO_2 $kg^{-0.67}$ h^{-1} and 144.1 ± 20.4 mgO_2 $kg^{-0.67}$ h^{-1}, respectively. Routine MRs of *M. antarcticus* and *H. portusjacksoni* were 202.6 ± 63.5 mgO_2 $kg^{-0.67}$ h^{-1} and 166.4 ± 11.2 mgO_2 $kg^{-0.67}$ h^{-1}, respectively. The simulated gillnet-capture treatment decreased the MR of *M. antarcticus* by 59.3% and increased that of *H. portusjacksoni* by 21.6%. Our results confirm that *M. antarcticus* is highly sensitive to gillnet capture, exhibiting significant variability in MR among individuals and also a high delayed mortality rate. We interpret the decrease in MR as a compensatory mechanism for reducing oxygen consumption to cope with the stress imposed by capture. Mortality in this species was associated with higher pre-stress MR, and lower post-stress MR. In contrast, *H. portusjacksoni* showed mild increases in MR. Their resilience to capture stress most likely relies on effective buccal pumping and lower basal MR. A mixed-effects model approach permitted us to identify the main sources of variation in the MR measured, which were the individual differences, treatment effects, species variations and delayed mortality. The comparative approach employed in this work allowed us to understand and provide reliable estimates of the effects of fishing on the MRs of these two species.

1. Introduction

Fishery by-catch is a threat to the stocks of chondrichthyan species worldwide (Stevens et al., 2000). Indeed, about50% of the mass of sharks caught in fisheries globally are discarded (Oliver et al., 2015). Sharks caught as by-catch (non-target and not retained) or of size below the legal minimum length for target and byproduct species are returned to the water after experiencing interactions with fishing gear as well as air exposure and handling. These stressors can have lethal outcomes or detectable short- and long-term sub-lethal effects (Molina and Cooke, 2012; Guida et al., 2017; Van Rijn and Reina, 2010; Frick et al., 2012). Research on the adverse effects of fishing on chondrichthyans in recent years (e.g., Bouyoucos et al., 2017; Frick et al., 2009, 2010a, 2012; Gallagher et al., 2014; Guida et al., 2017; Barragán-Méndez et al., 2019) indicates that these fishes exhibit high variation in their tolerance to current fishing practices. For example, a given fishing-gear configuration might have high impact on one species while having a low impact on another (Dapp et al., 2016a, 2016b; Frick et al., 2010b). Explaining the differences between species in their responses to fishery-related stressors requires a comparative approach that addresses the underlying biological mechanisms associated with the stress response.

The physiology of chondrichthyan species is understudied compared to teleost species of high commercial value (Dowd et al., 2006; Clark and Seymour, 2006; Sepulveda et al., 2007; Clark et al., 2013). One useful area of study of chondrichthyan physiology is the determination of energy use through measurement of metabolic rates (MR) (reviewed by Carlson et al., 2004 and Bernal et al., 2012). According to the level of activity, the MR of an animal can be categorized as standard (SMR), routine (RMR) or maximum MR (MMR) (for an extended discussion on terminology, see Chabot et al., 2016). SMR represents the energy expended in maintaining homeostasis, which in fish includes breathing and self-righting movements (Chabot et al., 2016). RMR represents normal activity associated with locomotion, prey seeking, digestion and active feeding (Dowd et al., 2006; Di Santo and Bennett, 2011), while MMR is the maximum energy output an individual animal of a species can produce, which in fish is typically estimated using exhaustive exercise experiments (Cutts et al., 2002; Roche et al., 2013; Di Santo et al., 2016). The study of MR allows scientific measurement of how an animal alters energy expenditure in response to a controlled stressful event (Kühnhold et al., 2017; Johnstone et al., 2012).

The physiological response to a stressful event in an animal involves the energetically demanding process of allostasis (Schreck et al., 2001). The energy required has to be diverted from limited reserves available for the functioning of other biological processes, such as growth, reproduction and immunocompetence (Ware, 1982; Calow, 1985; Schreck, 2010; Schreck and Li, 1991; McEwen and Wingfield, 2003),

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leading to possible impairment in these processes. Several of the most significant sub-lethal effects from fishing-capture stressors include physiological and behavioural alterations (Morgan and Crim, 1999), change in energy allocation (Barton and Schreck, 1987), haematological and immunological disruptions (Van Rijn and Reina, 2010; Skomal and Mandelman, 2012), and movement and reproductive disruption (Clearwater and Pankhurst, 1997; Ostrand et al., 2004; Guida et al., 2017). As such, measurements of MR in response to fishing capture could be used to estimate the amount of energy allocated to the stress response, and improve our understanding of the consequences of fishing capture for chondrichthyan species.

In this study, we explore interspecific differences in physiological responses to stress associated with fishing capture by comparing respirometric measurements of metabolic rate of the gummy shark *Mustelus antarcticus* and the Port Jackson shark *Heterodontus portusjacksoni*. *Mustelus antarcticus* is an active demersal species, endemic to southern Australia and sensitive to capture, with longer capture times leading to delayed mortality (Frick et al., 2010a, 2010b, 2012). Although targeted by both commercial and recreational fisheries, captured animals exceeding catch quota or below legal minimum length are released (Walker, 1999). In contrast, *H. portusjacksoni* is a common by-catch species that is mostly released alive (Walker et al., 2005). This species, also endemic to southern Australia, is epibenthic, oxygenates its gills primarily by buccal pumping and shows a comparatively high tolerance to capture stress (Frick et al., 2009, 2010a, 2010b). Because *M. antarcticus* and *H. portusjacksoni* have different sensitivity to stress, a comparative study of their metabolic responses to capture provides a better understanding of the fate of released sharks.

2. Materials and methods

2.1. Animal collection and husbandry

Animals for experimentation were collected (Victorian Fisheries Authority Permit RP1306) off the south coasts of French and Phillip islands in the state of Victoria, Australia (Fig. 1). *Mustelus antarcticus* and *H. portusjacksoni* were collected on a small commercially-operated vessel using a demersal longline with 200 baited hooks, with a soak time of 2–3 h during November and December 2017. Onboard the fishing vessel, the animals were immediately placed in a temporary holding tank containing flowing ambient seawater. Each animal was tagged, measured for total length and body mass, and each female underwent ultrasound examination in the water to determine whether it contained eggs or embryos in utero. Pregnant females were excluded from this study given that the energetic cost of pregnancy could introduce unwanted variation to the experiment. Upon return to port, the animals were transferred into 4000-L transport tanks mounted on a truck, containing seawater aerated with pure oxygen. A maximum of 6 animals were transported in a single tank, accounting for a total biomass of up to 37.8 kg. Transportation took approximately 2 h, where on arrival at the housing facilities, animals were transferred by dip net into two 20,000-L holding tanks containing seawater recirculated through a biofilter, a protein skimmer and temperature regulators. The average biomass in the two tanks was 33.4 kg. The animals were fed a diet of fish and prawns twice a week (~5% of their total body mass per week) and care was taken to ensure all animals in the tank ingested their weekly ration. Animals were allowed to recover from the stress caused by the initial collection and transportation and to acclimate to the housing conditions for at least 7 days before the trials started (Frick et al., 2009). The animals appeared to be in good condition and displayed normal behaviour in the holding tank. Individual *M. antarcticus* swam slowly most of the time, and interacted with each other, at times resting on the bottom of the tank. Individual *H. portusjacksoni* mostly rested on and moved along the bottom of the tank for extended periods, actively moving up when food was offered, and then swimming for a few minutes before returning to the bottom. During this period, the sharks experienced a natural photoperiod and the water temperature was controlled at 16 ± 2 °C, which is within the temperature range they would experience in the wild at that time of year. Water quality variables were monitored daily by measuring temperature, salinity, pH, nitrate and nitrite concentration to ensure optimal housing conditions (Conte, 2004).

2.2. Respirometry

The MR measurements were made for each animal using a respirometry array (Fig. 2). We performed an initial determination of MR baseline levels (i.e., control treatment) and a subsequent determination of MR immediately following simulated gillnet fishing capture (i.e., gillnet fishing treatment).

The experimental animals consisted of 13 *M. antarcticus* of total length (TL) mean ± SE (range) of 970 ± 200 (720–1365) mm and

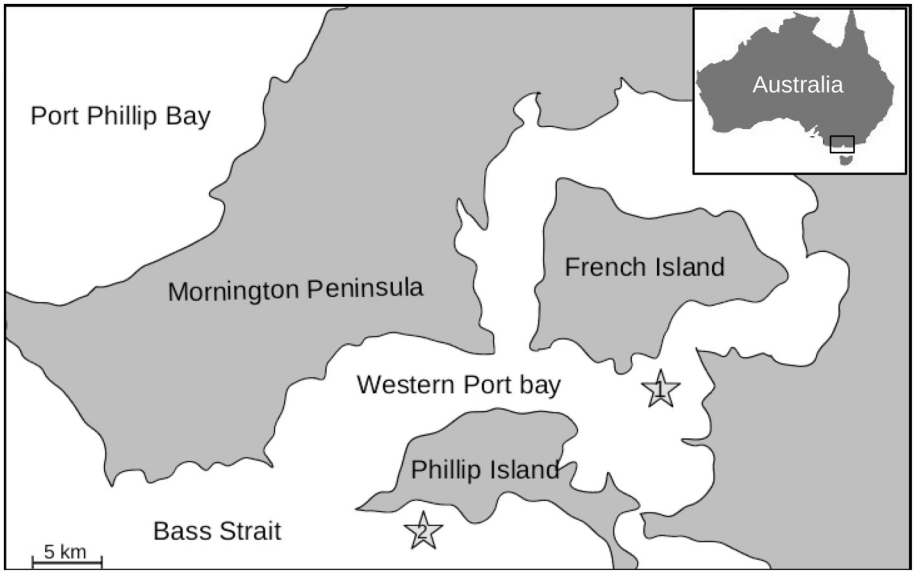

Fig. 1. Collection sites for *Mustelus antarcticus* and *Heterodontus portusjacksoni* in Victoria, Australia, during November–December 2017. The stars show the positions of the sites. Site 1: 38°36′25.9″S, 145°30′21.8″E; Site 2: 38°28′41.8″S, 145°04′40.7″E).

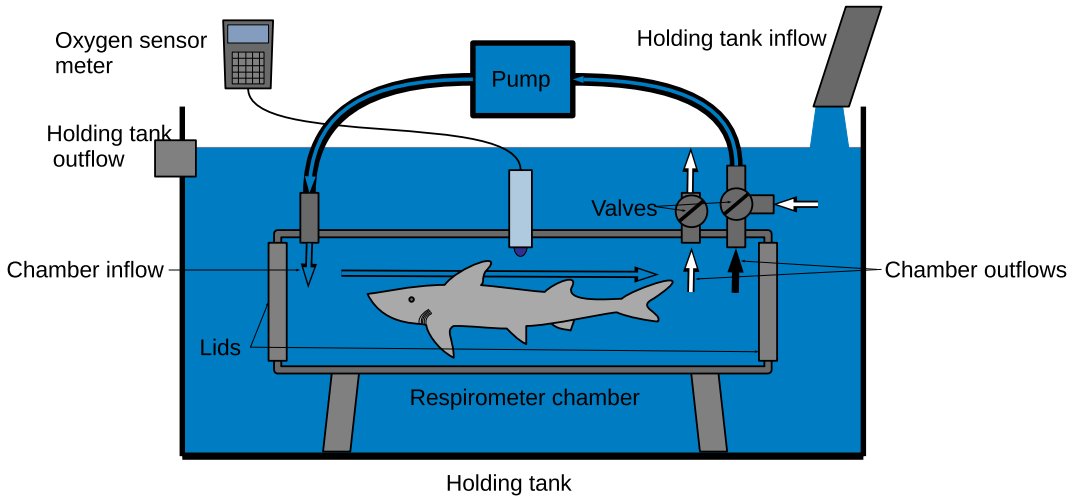

Fig. 2. Respirometry array placed in the holding tank. White arrows indicate water flow during the replenishment phase, black arrows indicate water flow during the measurement phase and blue arrows indicate common water flow during both phases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

total body mass (M) mean ± SE (range) of 4.35 ± 3.16 (0.9–10.6) kg and 5*H. portusjacksoni* of TL 610 ± 100 (520–780) mm and M of 1.90 ± 1.39 (1.0–4.3) kg. Given that the energy spent in digesting food in sedentary species can greatly increase MR (Clark et al., 2013), animals were not fed for 24 h prior to the respirometry trials (based on the digestion times estimated for *Scyliorhinus canicula* by Sims et al. (1996)) to ensure a postabsorptive state (Chabot et al., 2016).

The respirometry array consisted of a cylindrical PVC tube (~10 mm thickness) respirometer chamber, 400 mm in diameter and 2000 mm in length (a volume of 251.2 L), with watertight lids on each end, inlets and outlets of the water pump, and a Hach Intellical™ LDO101 Field Luminescent/Optical Dissolved Oxygen sensor meter (± 0.1 mg L^{-1} accuracy). The respirometry array was placed and submerged in one of the holding tanks 24 h prior to the trial, and left there to prevent adding stress to the animals, as additional stress would result from handling and air exposure necessary for the transfer between tanks. Leaving the array in the holding tank also ensured that the animals became accustomed to the setup (Chabot et al., 2016; Clark et al., 2013). At the beginning of each trial, air pockets were eliminated by simultaneously rotating the chamber and flushing it with water.

For each trial, one animal was led into the respirometer chamber, which was then sealed for a series of respirometry cycles, where each cycle consisted of an oxygen measurement phase and a replenishment phase. The measurement phase was conducted with the chamber sealed, allowing the animal to consume the oxygen in the chamber. During this phase, the pump circulated the water in a closed circuit, taking water from the rear and returning it to the front of the respirometer chamber at a rate of 32 L min^{-1}. The placement of the inlets and outlets ensured an even mix, in order to minimize the measuring error (Clark et al., 2013), and minimum drag associated with water flow. The replenishment phase consisted of flushing the chamber with oxygenated water by changing the pump inflow to take fresh seawater from the holding tank. The duration of the measurement phase was 10 min, but was reduced if the animal consumed oxygen too rapidly, in order to prevent the dissolved oxygen concentration (DO) falling below 80% saturation. The replenishment phase was also 10 min, or the period required to reach the initial DO saturation (which never exceeded 20 min). The periods of these phases are in accordance with those presented in the literature for obtaining maximum accuracy in DO measurements (Clark et al., 2013).

Dissolved oxygen was measured continuously during the measurement phase, and was logged in the sensor meter as average DO per minute. Water temperature was also measured at the same intervals using the temperature sensor integrated in the DO sensor meter (± 0.1 °C accuracy). During the replenishment phase, the DO was monitored for animal welfare reasons, but not recorded. Activity and welfare were monitored with a submersible camera placed inside the chamber, near the water pump inlet. Between 4 and 8 cycles were run for each animal, with some animals having less than 8 cycles because they needed to be taken out of the respirometer chamber earlier after exhibiting signs of acute stress responses, or becoming stuck sideways while moving inside the respirometer chamber.

2.3. Baseline MR

Each animal underwent a baseline respirometry trial (i.e., control treatment) to determine its SMR and RMR. Certain species exhibit clearly defined rest and active periods, in which SMR and RMR can be measured conveniently (Clark et al., 2013; Chabot et al., 2016; Sims et al., 2006). For *M. antarcticus* and *H. portusjacksoni*, prior to starting the respirometry trials, a 24-h period of observation of the animals´ activity indicated no particular rest period, with the sharks alternately swimming and resting at different times. As such, we determined the SMR and RMR based on the monitored activity recorded by the camera inside the respirometer chamber (Fig. 3). Activity was treated as a binary response variable where the animal was classed as being at rest or active. An animal was at rest when on the bottom of the chamber and only performing breathing and other minor movements to maintain posture, and active when the animal was swimming inside the chamber. The swimming pattern of the animals was generally haphazard; they swam towards the front of the chamber, turned, then swam towards the back of the chamber and repeated this pattern one or more times before resting on the bottom of the chamber again. Sometimes the turning occurred before reaching either end of the chamber, or the turning movement was the only movement performed. Cycles during which the animals were active more than 50% of the time were used for estimating RMR, whereas cycles during which the animals were at rest for more than 50% of the time were used for estimating SMR.

After the procedure, the chamber was opened and the animal released back to the holding tank, where it remained with the other animals, undisturbed for at least 30 days before the gillnet capture simulation described below. All surviving animals returned to their normal behaviour in the holding tank the next day after this procedure.

A blank respirometry test was performed to account for bacterial respiration inside the respirometer chamber. The chamber was sealed without an animal inside, and DO was monitored for one hour. We did

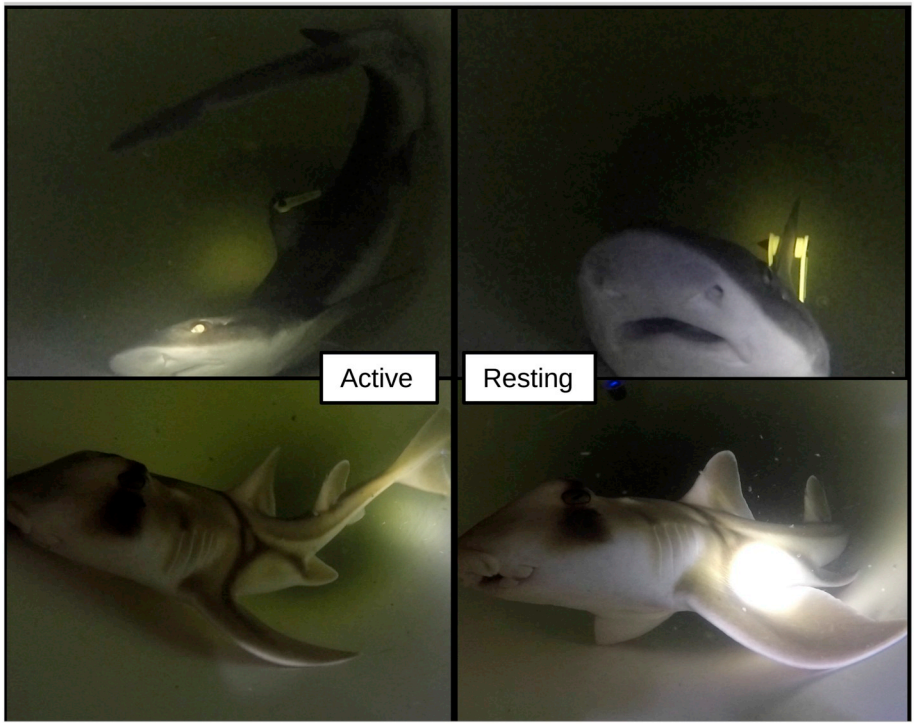

Fig. 3. Activity inside the respirometer chamber. Active (left panels) and resting (right panel); *Mustelus antarcticus* (top) and *Heterodontus portusjacksoni* (bottom).

not to detect any background respiration, with DO not varying from 8.5 ± 0.1 mg O_2 L^{-1}.

2.4. Gillnet capture simulation

The condition of sharks caught by gillnets during normal commercial fishing operations depends on factors such as sensitivity intrinsic to the animal or its species, respiratory mode (Dapp et al., 2016b), the time the animal is captive in the gillnet before it is removed (Frick et al., 2010a), and type and location of constraint by the gillnet mesh while held captive. To limit the variability in the metabolic response to capture resulting from differing locations and severity of enmeshment, in the present study we carefully applied the gillnet capture treatment by enmeshing the animals around the head without contact with the gills. We acknowledge that this does not represent all types of entanglement resulting from demersal gillnet fishing. We chose to investigate the least invasive form of enmeshment possible to provide a conservative estimate of the effect of gillnetting on sharks' metabolic rates. Additionally, our main aim was to evaluate the energetic cost associated with the stress response and gauge the possible subsequent long-term effects on other biological activities, not the prevalence of delayed mortality in commercial fishing operations.

For gillnet capture simulation trials, animals were not fed for 24 h prior to the trials to ensure a postabsorptive state. To simulate gillnet capture, a 1.5 m long x 1.5 m deep section of monofilament mesh was attached to a metal frame suspended by ropes from the top of a 5000-L experimental capture tank. To allow the enmeshment of animals of different size, three different sections with mesh sizes 100, 130 and 180 mm (between opposite knots of stretched diamond-shaped mesh) were used. Each animal was transferred by dip net from its holding tank to the adjacent experimental capture tank (although this procedure takes only several seconds to complete, we acknowledge it may introduce a small amount of stress). The animal was left to swim freely in the experimental tank until it was calm (~5 min), and was then enmeshed firmly around the head without contact with the gills. Flow-through seawater passed from the holding tank directly to the experimental capture tank to avoid stressing the animals with the transfer to different water (Conte, 2004). In preparation for the fishing capture trial of each animal, the experimental capture tank was flushed and refilled to remove chemical stress cues lingering in the water from the previous individual.

For each trial of the gillnet capture treatment, the animal was enmeshed for a 20-min period. During this time the animal which was placed in the experimental tank was constantly observed to ensure its welfare and to avoid hypoxia induced by filaments of the gillnet webbing obstructing the water flow through its gills, which would prevent completion of the trial. At the end of this 20-min period the animal was transferred to the respirometer chamber in the holding tank, to measure post-stress oxygen consumption. The average time between the end of the gillnet capture trial and the start of the respirometer measurement was ~1 min. During these measurements, care was taken to minimize the time between exposure to the gillnet capture treatment and the measurement in the respirometer chamber, given the diversity of metabolic responses to stress (Clark et al., 2013).

After finalising the experiments, the animals were allowed a recovery period of 7 days before being released to the wild after veterinary examination. All experiments were performed under Monash University Animal Ethics Protocol BSCI/2017/19 and complied with current Australian law.

2.5. Data analysis

Dissolved oxygen level in the respirometer chamber was plotted against time for each respirometry cycle of an animal and a regression slope was obtained. An ANCOVA test was performed to detect differences in the slopes between the cycles for each individual animal (Fox, 2015) (Fig. 4). These regression slopes were used to calculate the MR (estimated as oxygen consumption), modified from Clark et al. (2013) as:

$$MR = O \times VR \times M^{-0.67} \times T^{-1}$$

where MR is the metabolic rate of the fish measured as mg O_2 $kg^{-0.67}$ h^{-1}, O is the slope of the linear model fitted to the oxygen concentration (mg L^{-1}) data from the start through to the end of each

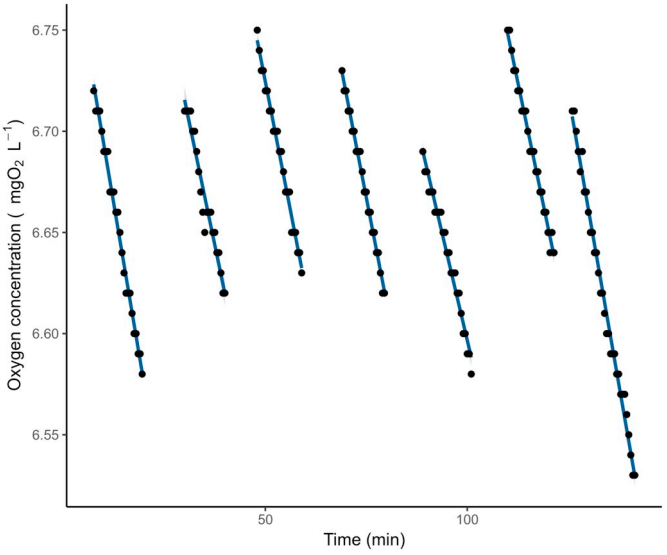

Fig. 4. Sample data of concentration of oxygen (mg L^{-1}) inside the respirometer chamber vs time for *Mustelus antarcticus* n°2 (female, 720 mm TL, 0.9 kg of body mass). Dots are the measured oxygen concentrations, and each line is a linear regression fitted to one of a series of measuring cycles.

cycle, VR is the volume of the respirometer chamber (L) calculated as the total volume of the chamber minus the volume of each animal, M is the total wet body mass of the animal (kg) and T is the duration (hours) of the cycle. The volume of each animal was measured using the water displacement technique, which consists of measuring the volume of water displaced by submerging the animal in a container of known volume. The mass exponent of 0.67 was suggested for elasmobranchs by Hopkins and Cech (1994) and Meloni et al. (2002) to correct for the allometric relationships between MR and M for quiescent sharks. We use the allometrically corrected body masses in reported analyses unless noted otherwise.

As a reference for future work, we describe the relationships between SMR and body mass for each species. Linear and power regressions were fitted to the data of SMR for all the cycles of the control treatment to obtain predictive equations (Crawley, 2007; Fox, 2015). See supplementary material for model validation.

The normality of the error structure of the data set was assessed with a Quantile-Comparison Plot, and kurtosis and skewness test. Given that the data fit well to a normal distribution, we employed Linear Mixed Effects models (LME) (Crawley, 2007; Bates, 2010; Bates et al., 2015) to explore which sources of variance in metabolic rate were significant (i.e., control/gillnet capture treatment, routine/standard metabolic rate, species, individual animal, occurrence/absence of delayed mortality). A backward stepwise model selection process was employed to find a model that best described the effects of simulated gillnet stress on the MR of *M. antarcticus* and *H. portusjacksoni.* Model fit and validation is described in the supplementary material. Temperature was not included in the modelling protocol as we found no differences in the water temperature between treatments, individuals nor species (Supplementary material section contains details).

All tests used a significance level of $\alpha \leq 0.05$.

Data were processed using R statistical software (R Core team, 2018), using scripts specifically developed by us for this purpose and functions in FSA (Ogle, 2018) and lme4 (Bates et al., 2015) packages.

3. Results

The SMR (using the originally measured body mass, expressed as $mgO_2kg^{-1}\ h^{-1}$) for the control treatment showed an inverse relationship with body mass (M) for both *M. antarcticus* and *H. portusjacksoni*. This relationship is described by the linear equation.

SMR = 247.49 (± 6.59) –18.19 (± 1.24) x M for *M. antarcticus* and.

SMR = 161.21 (± 7.08) –8.97 (± 3.12) x M *for H. portusjacksoni*.

Power regressions are SMR = 256.79 (± 6.78) x $M^{-0.403(\pm 0.026)}$ and.

SMR = 151.60 (± 5.72) x $M^{-0.109(\pm 0.057)}$, respectively.

The power equations provided the least biased estimate of SMR predicted from total body mass for *M. antarcticus* (Fig. 5 top), whereas the SMR estimates are similar in both equations for *H. portusjacksoni* (Fig. 5 bottom). The comparatively large range of variation in SMR for *H. portusjacksoni* could be due to the relatively low sample size ($n = 5$) (Table 1). Time spent inactive/active in the SMR/RMR measuring is displayed as percentage of total measuring time in Table 1.

Both mean SMR and mean RMR were lower for the gillnet capture treatment than the control treatment in *M. antarcticus*, whereas conversely, both were higher for the gillnet capture treatment than the control treatment in *H. portusjacksoni* (Table 1).

Through stepwise elimination of non-significant interaction and main effect terms, linear mixed effects model simplification produced Model 7 (Table 2), with all its parameters being significant. The final model incorporates variance from treatment, activity (routine or standard metabolic rate), interaction between treatment and species, activity and delayed mortality. Variation in the individual animal responses to stress represented an important portion of the variance (Table 3).

The stress treatment had a significant effect on the MR of sharks, and this effect was different for each species. The stress treatment decreased the MR of *M. antarcticus* by 59.3% and increased the MR of *H. portusjacksoni* by 21.6% (Table 3). Additionally, we found differences between SMR and RMR in *M. antarcticus* (SMR was 13.8% lower than RMR; Table 3). In the case of *M. antarcticus,* individuals that survived the experimental procedure had lower MR in the control treatment and higher during the stress treatment than those that died after the stress treatment (Table 3). Fig. 6 shows the fitted models to the total body mass of the individuals of *M. antarcticus*. The effect of the stress from the gillnet capture treatment on MR was more pronounced in smaller individuals. For *H. portusjacksoni* (Fig. 6) it is harder to interpret the influence of the animal body mass in the metabolic response to stress. MR increased in response to stress for most individuals, but the largest shark showed the inverse response.

4. Discussion

Fishing-induced stress in chondrichthyans is an emergent area of research. Literature regarding this subject in teleosts is far more extensive (see Skomal and Mandelman, 2012), and contributions to further our understanding of these processes in chondrichthyans are needed to enable higher survival of bycatch species through improved fisheries management. In this study, we measured the response of MR of *M. antarcticus* and *H. portusjacksoni* to gillnet capture for the first time to address partially this knowledge gap, as well as to provide the first estimate for the SMR for these species. Studies on the changes of RMR in response to shifts in salinity have been conducted on *M. antarcticus* by Morash et al. (2016) and Tunnah et al. (2016) and on *H. portusjacksoni* by Cooper and Morris (2004).

Mustelus antarcticus decreased oxygen consumption after being subjected to simulated gillnet capture, compared with before the capture stress, using 42.6% less energy at rest and 55.5% less energy while active in the respirometer chamber. Resting could potentially help sharks cope with the aerobic demand of capture, as was suggested for this species under simulated longline capture (Guida et al., 2016a). Morash et al. (2016) and Tunnah et al. (2016) found that the RMR of this species also decreased with osmolarity-induced stress. The authors hypothesised that the decrease was due to a lower osmolality/urea concentration in the plasma, which would in turn decrease the affinity

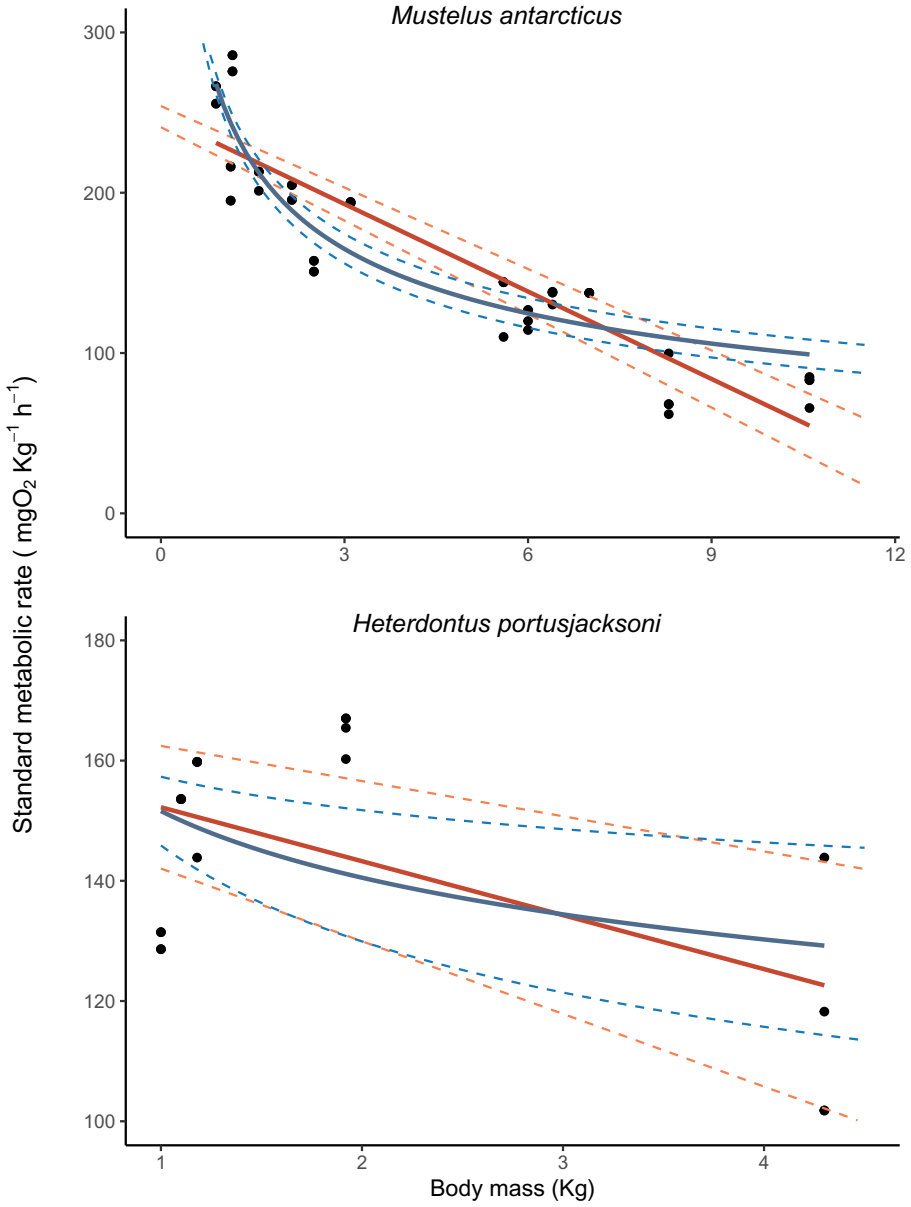

Fig. 5. Standard metabolic rate (mg O_2 kg^{-1} h^{-1}) vs originally measured body mass (kg) of *Mustelus antarcticus* (top) and *Heterodontus portusjacksoni* (bottom) of the control treatment. Dots are the individual data points, the blue line is the power and the red is the linear model fitted to the data. Dashed lines represent standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Mean and standard error (SE) metabolic rates (MR,) by species, treatment and MR type (SMR: Standard metabolic rate, RMR: Routine metabolic rate. Numbers in parenthesis indicate the mean percentage of the time per cycle spent active (RMR) or resting (SMR) during the measuring phase).

Species	Treatment	MR type	MR ± SE $mgO_2kg^{-0.67}$ h^{-1}
Mustelus antarcticus (n: 13)	Control	RMR (72.1%)	202.6 ± 63.5
		SMR (83.3%)	168.5 ± 61.9
	Gillnet capture	RMR (67.3%)	112.4 ± 54.4
		SMR (79.6%)	71.7 ± 45.3
Heterodontus portusjacksoni (n: 5)	Control	RMR (65.8%)	166.4 ± 11.2
		SMR (89.2%)	144.1 ± 20.4
	Gillnet capture	RMR (67.4%)	208.9 ± 44.1
		SMR (85.2%)	163.4 ± 33.7

of haemoglobin for O_2 and therefore causes a lower O_2 consumption. In our study of *H. portusjacksoni*, capture stress simulation elicited a 13.3% higher oxygen consumption at rest and 25.5% higher while active, a more expected reaction to stress, as found for several other species (Barton and Schreck, 1987). Frick et al., 2010a identified several secondary stress responses in *M. antarcticus* subjected to simulated gillnet capture, indicating that despite the reduction in MR, this species undergoes deleterious physiological stress responses to capture. Metabolic

Table 2
Akaike and Bayesian information criterion values (AIC and BIC, respectively) for the mixed effects linear models applied to the metabolic rates of *Mustelus antarcticus* and *Heterodontus portusjacksoni*. MR: Metabolic rate, Tr: Treatment, SP: Species, R: Routine/Standard metabolic rate, D: Delayed mortality, I: Individuals. * denotes effect and interaction term,: denotes only interaction term.

Model	Degrees of freedom	AIC	BIC
1) MR ~ Tr*SP*R*D+(1 + Tr + SP + R + D | I)	28	2678,3	2765,9
2)MR ~ Tr + SP + R + D + Tr:SP + Tr:R + SP:R + Tr:D + R:D + Tr:SP:R + Tr:R:D +(1 + Tr + SP + R + D | I)	28	2636.1	2738.6
3) MR ~ Tr + SP + R + D + Tr:SP + Tr:R + SP:R + Tr:D + R:D + Tr:SP:R +(1 + Tr + SP + R + D | I)	27	2634.1	2733.0
4) MR ~ Tr + SP + R + D + Tr:SP + Tr:R + Tr:D + R:D + Tr:SP:R +(1 + Tr + SP + R + D | I)	27	2634.1	2733.0
5) MR ~ Tr + R + D + Tr:SP + Tr:R + Tr:D + Tr:SP:R+(1 + Tr + SP + R + D | I)	26	2632.9	2728.1
6) MR ~ Tr + R + Tr:SP + Tr:R + Tr:D + Tr:SP:R+(1 + Tr + SP + R + D | I)	26	2632.9	2728.1
7) MR ~ Tr + R + Tr:SP + Tr:R + Tr:D+(1 + Tr + SP + R + D | I)	24	2632.5	2720.4

Table 3
Mixed effects linear model 7 applied to the metabolic rates of *Mustelus antarcticus* (Ma) and *Heterodontus portusjacksoni* (Hp). Tr: Control (C) and Stress (S) Treatment, SP: Species, R: Routine (RMR)/Standard (SMR) metabolic rate, D: Delayed mortality (Dead: D, Alive: A). Intercept factor combines Ma, C, RMR and D.

Factor		
Random	***Variance %***	**SE**
Individuals	24.4	0.1
Tr	17.4	0.0
SP	21.5	0.0
R	3.3	0.2
D	30.7	0.3
residuals	2.8	0.2
Fixed	***Estimate***	**SE**
Intercept	227.1	15.7
Tr(S)	−134.7	13.0
R (SMR)	−31.5	5.9
Tr (C):SP (Hp)	−17.5	18.6
Tr (S):SP (Hp)	45.4	18.7
Tr (S):R (SMR)	−11.2	4.0
Tr (C):D (A)	−41.4	22.6
Tr (S):D (A)	60.2	21.9

stress responses such as the ones described in our study are highly species-specific (Skomal and Mandelman, 2012), so the two different responses exhibited by our experimental species are possible. Regardless of the primary and secondary stress mechanism underlying the effect observed in *M. antarcticus,* we think that the reduction of O_2 consumption was either indicative of exhaustion of readily available energetic resources, or some compensatory response to the allostatic state. It has been observed in other organisms that acute stress produces a reduction in extracellular pH, up to a point in which normal aerobic compensatory mechanisms fail and the metabolic rate is depressed (Reipschläger and Pörtner, 1996; Guppy and Withers, 1999; Guppy et al., 1994). For *M. antarcticus,* the simulated gillnet capture could have produced a decrease in extracellular pH below the threshold for metabolic depression. However, this response was unable to return all animals to homeostasis because not all animals ultimately survived. In contrast, all *H. portusjacksoni* survived following the gillnet capture treatment, despite showing increased MR. *Heterodontus portusjacksoni* could potentially have a lower pH threshold for metabolic depression or an intrinsic resilience to extracellular pH oscillations. A similar outcome was found by Frick et al., 2010a when studying the post-release survival of these same species under laboratory conditions, with high *M. antarcticus* mortality, and no *H. portusjacksoni* mortality following capture stress. The increase in aerobic energy expenditure of *H. portusjacksoni* after the capture simulation (~20%) is relatively low compared to other species, such as the lemon shark *Negaprion brevirostris* (~60%) (Bouyoucos et al., 2017), which might explain its high resilience to fishing capture. There is a caveat in our procedure, however, that might have introduced some variability. We performed the estimation of baseline MRs almost without handling and without air exposure, but animals in the capture treatment were handled and exposed to air briefly in order to transfer them to the experimental tank. While we consider this extra stress would not be significant compared with 20 min of simulated gillnet capture, we acknowledge that the lack of sham trials prevent us from eliminating this source of stress in our analysis.

Natural variation among individuals of a species affects their energy use during and after stress and ultimately the outcome of that stress (Biro and Stamps, 2010). Our methods allowed us to identify and quantify the relative importance of individual components (up to 24.4% of the total variance) in the variation of MR in each of the two species (Table 3, Random factors section). Furthermore, the *M. antarcticus* that died after the capture procedure showed significantly higher pre-stress SMR and RMR, and lower post-stress SMR and RMR. Some of this difference might be explained by bold vs shy behaviour, presented in Byrnes and Brown (2016), in which the authors describe how bolder individuals exhibit higher stress responses to handling. These individual differences influence behaviour and energy output, and can mask overall trends (Biro and Stamps, 2010) if the experimental design and statistical methods used are inadequate to identify them. This can occur when the intrinsic variation among individuals in their responses to stress is different between the experimental control and stress-induced treatment groups, but animals are assumed to respond equally. This traditional design has been employed extensively in experimental biology without testing for individual differences (for an extended discussion on the subject, see Biro and Stamps, 2010). Our work highlights the need to take a different approach to designing experiments with wild-caught species, in which the inter-individual differences should not be assumed to be non-existent.

Temperature is one of the most influential variables affecting the MR of fish (Schmidt-Nielsen, 1997). We did not detect any appreciable variation in the MR in relation to temperature, given that the temperature variation was < 4 °C.

The degree of entanglement can also introduce variability in the estimation of MR, and potentially lead to suffocation and death. In a previous experiment, four of five gummy sharks that died during a 120-min gillnet capture simulation were heavily enmeshed around the gill region (Frick et al., 2010a). Similarly, Manire et al. (2001) connected lower mortality during gillnet capture in bull sharks *Carcharhinus leucas* than in blacktip sharks *C. limbatus* and bonnethead sharks *Sphyrna tiburo* to a lower degree of obstruction of the gills when the bull sharks were enmeshed. A high degree of mesh contact around the gill region of enmeshed sharks accelerates oxygen deprivation and thus increases risk of mortality. We took care to avoid enmeshing the experimental animals in the gill region, ensuring all our animals were capable of buccal pumping while captured, but still 8 of the 13 *M. antarcticus* died 3–8 h after the gillnet capture. This suggests that even when able to perform buccal pumping effectively, this species suffers deleterious effects when immobilised that lead to delayed mortality, as this ventilation mechanism might not be very effective at relieving the effects of capture stress (Guida et al., 2016b). We conclude *M. antarcticus* might rely on ram ventilation more than was previously thought. Bouyoucos et al.

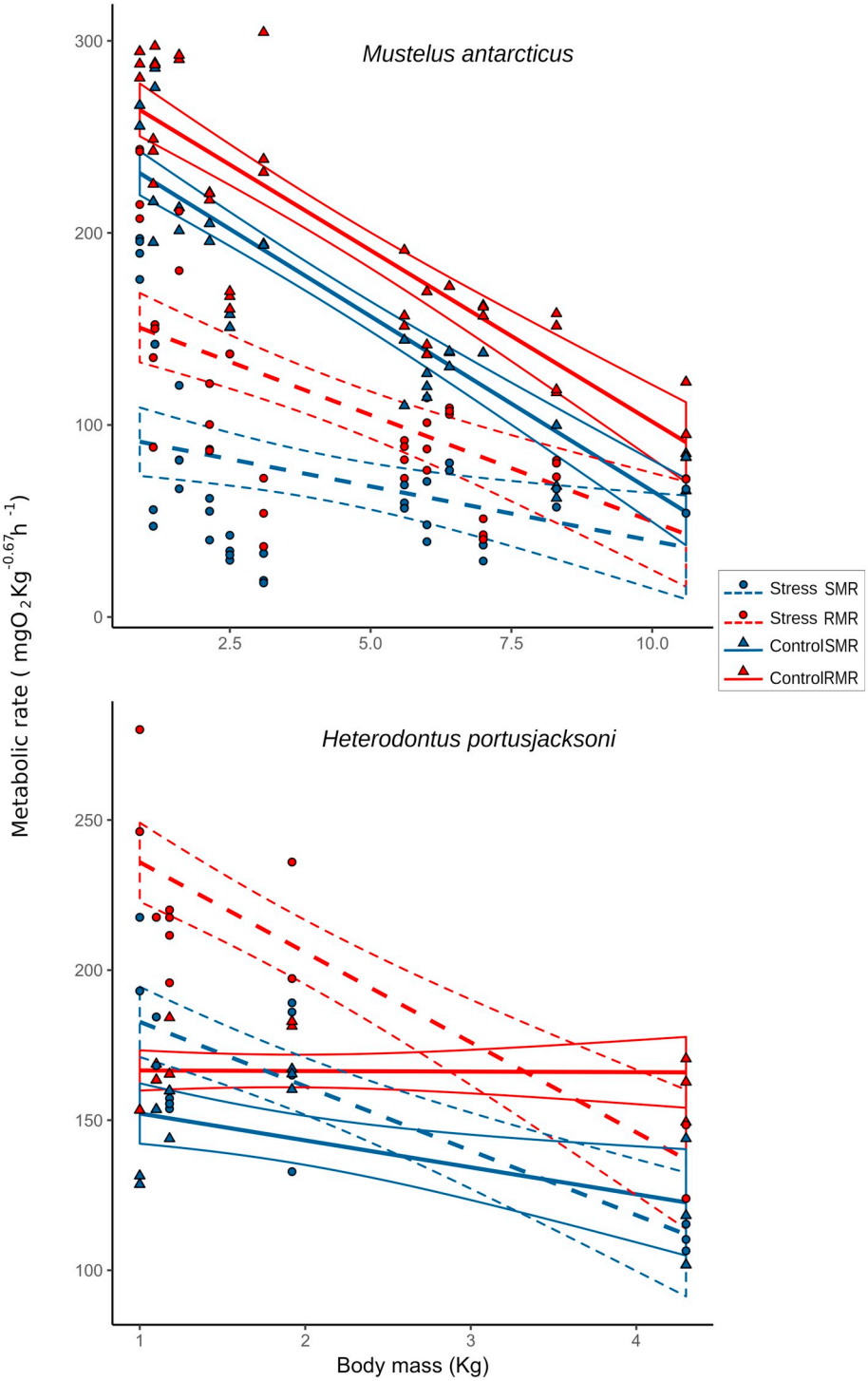

Fig. 6. Linear mixed-effects models fitted to the metabolic rates of *Mustelus antarcticus* (top) and *Heterodontus portusjacksoni* (bottom) according to treatment and metabolic rate type.

(2017) found a reduction in resting time for simulated longline-captured *N. brevirostris,* and argued that this likely indicates that ram ventilation can enhance gas exchange and post-stress recuperation, compared to buccal pumping (Clark and Seymour, 2006). Conversely, Guida et al. (2016b) found the resting time increased in hooked *M. antarcticus,* which suggests that resting behaviour supersedes the need for effective ram ventilation when reducing oxygen demand is advantageous. Interestingly, the mortality rate we observed (61.53%) was quite close to the immediate mortality that Dapp et al. (2016a) estimated for obligate ram-ventilating shark species, and higher than that of stationary-respiring species. In our study, the restraint imposed by gillnetting did not allow *M. antarcticus* to ram ventilate, compared to longline capture in Guida et al. (2016b), and therefore probably produced physiological disruptions as a consequence of accumulated metabolites of anaerobic metabolism, that likely lead to delayed mortality. Such a consequence of capture would suggest that *M. antarcticus* is a very active demersal shark, and would explain the species' sensitivity to highly restraining fishing gear, such as gillnets and trawl nets. Field underwater observations revealed that this species commonly rests on the bottom orienting its head into the current, which might aid in

Table 4
Comparison of standard and routine metabolic rates (SMR and RMR respectively) estimations among shark species. SMR and RMR were temperature-corrected to 17 °C using a Q_{10} of 2.3 for consistency with the literature (see Whitney et al., 2016). SMR and RMR are reported in mg O_2 $kg^{-0.67}$ h^{-1}. T °C: Temperature, N: number of individuals used for the estimations.

Species	T °C	N	SMR	RMR	SMR (17 °C)	RMR (17 °C)	Reference
Carcharhinus acronotus	28.00	10	240.00	395.00	96.01	158.01	Carlson et al. (1999)
Carcharhinus limbatus	29.40	7	246.00	362.00	87.58	128.87	Lear et al., 2017
Carcharhinus plumbeus	24.00	16	120.00	213.00	66.98	118.90	Dowd et al. (2006)
Cephaloscyllium ventriosum	16.00	4	44.30	93.70	48.15	101.84	Ferry-Graham and Gibs (2001)
Cetorhinus maximus	15.00	20	62.50	91.10	73.83	107.61	Sims (2000)
Ginglymostoma cirratum	23.00	8	36.00	95.00	21.84	57.64	Whitney et al. (2016)
Heterodontus francisci	22.00	10	159.71	–	105.31	–	Luongo and Lowe (2018)
Isurus oxyrinchus	18.30	9	124.00	344.00	111.27	308.70	Sepulveda et al. (2007)
Isurus oxyrinchus	18.25	6	124.10	325.70	111.83	293.50	Wegner et al. (2012)
Negaprion brevirostris	5.00	7	153.00	240.00	78.58	123.26	Scharold and Gruber (1991)
Negaprion brevirostris	23.00	3	175.60	210.20	106.53	127.53	Nixon and Gruber (1988)
Negaprion brevirostris	29.00	10	155.19	398.02	57.12	146.50	Bouyoucos et al. (2017)
Scyliorhinus stellaris	18.30	8	94.00	165.00	84.35	148.07	Piiper et al. (1977)
Sphyrna lewini	26.00	17	189.00	275.00	89.31	129.95	Lowe (2001)
Sphyrna tiburo	26.00	17	173.00	235.00	81.75	111.05	Carlson and Parsons (2003)
Squalus acanthias	10.00	8	32.00	88.00	57.33	157.65	Brett and Blackburn (1978)
Stegostoma fasciatum	27.00	1	194.30	–	84.48	–	Payne et al. (2015)
Triaenodon obesus	27.00		187.08	343.78	81.34	149.47	Barnett et al. (2016)
Triakis semifasciata	22.00	18	105.00	167.00	69.23	110.12	Graham et al. (1990)
Triakis semifasciata	17.00	10	193.05	–	193.05	–	Miklos et al. (2003)
Mustelus antarcticus	16.80	5	–	280.00	–	281.35	Tunnah et al. (2016)
Heterodontus portusjacksoni	17.00	5	144.16	166.40	144.16	166.40	This study
Mustelus antarcticus	17.00	13	168.52	202.65	168.52	202.65	This study

ventilation in their natural environment (Walker, pers. obs).

Body size influenced the rate of change of the MR of *M. antarcticus* and *H. portusjacksoni*, and larger individuals of both species showed less change in their MR when stressed by the gillnet capture treatment. A greater range of change in MR led to a higher chance of mortality in *M. antarcticus* due to stress; therefore, we infer that smaller animals are more likely to suffer lethal outcomes from stressful events. One implication of our finding is that larger animals could better withstand capture stress, while smaller ones, typically those that are more likely to be discarded because of legal minimum length restrictions, are more vulnerable to mortality. Dapp et al. (2017b) also suggest that juvenile *Carcharhinus melanopterus* experience higher mortality than larger individuals, after being captured by gillnets. Increasing the legal mesh-size range of gillnets should reduce post-release mortality of small animals, but this benefit would need to be evaluated against the additional loss of large breeding animals from the population. The results presented here highlight the importance of experimental studies for the conservation of sharks.

Compared with other species of sharks (Table 4), the temperature-corrected SMRs of *M. antarcticus* and *H. portusjacksoni* are comparatively high, surpassing even that of shortfin mako shark (*Isurus oxyrhinchus)*. This might imply that holding tanks might have imposed a continuous stress to our animals, or that they did not fully recover from the initial fishing capture from the wild or acclimate to captive conditions quickly enough, and our estimation of SMR is actually a low-end RMR. However, this does not change the interpretation of our findings, as responses of both SMR and RMR under stress were similar. Examining the temperature-corrected RMR in Table 4, the values obtained for *M. antarcticus* are higher than those of the bottom-dwelling sharks but considerably lower than that of the active swimming shark *I. oxyrinchus*. Tunnah et al. (2016) and Morash et al. (2016), found similar values for the *M. antarcticus* they employed in their studies, but the latter do not present the temperature at which their estimations were performed, so their results are not included in Table 4. Regardless, given that both studies used the same animals, it would be reasonable to assume temperatures were close to those described in Tunnah et al. (2016). Our findings imply that, metabolically speaking, *M. antarcticus* is not a sluggish bottom-dweller, but an active shark. RMR of *H. portusjacksoni* was lower and consistent with its described behaviour of remaining inactive in holes and caves in groups, to hunt for benthic prey at night (Byrnes and Brown, 2016; Guttridge and Brown, 2014; Powter and Gladstone, 2008). A species with a close RMR is the nursehound *Scyliorhinus stellaris,* which spends half of its time secluded in rocky holes and refuges along with other congeners, a remarkably similar behaviour to that of *H. portusjacksoni* (Sims et al., 2005). It is worth noting that extrapolating results from other studies conducted at other temperatures and size range of animals raises uncertainty about the extrapolated estimates obtained. Temperature-corrected MR can be affected by the Q_{10} value used in a non-linear fashion, and the mass exponent for a mass-independent MR value varies according to the species, and is highly sensitive to change (Payne et al., 2015). Furthermore, some studies use a standard mass exponent that does not fit the MR-Mass relationship of the species under study. In our study, we employed the mass exponent employed in most MR studies in sharks, and we used a Q_{10} of 2.3 for all our comparisons as used by Whitney et al. (2016) for the sake of consistency with the literature.

It is important to note that our gillnet capture treatment of only 20 min of enmeshment around the head without mesh contact with the gills was relatively brief and less stressful compared with the typical commercial operations off southern Australia. The magnitude of the stress imposed by commercial gillnet fishing, with mean soak times of 8.2 h (Walker et al., 2005) and high chance of gill obstruction when enmeshed, is far greater, therefore the negative physiological effects arguably would also be more profound. Indeed, in their study of the shark fishery of south-eastern Australia, Walker et al. (2005) found immediate mortality of 60% of 3697 *M. antarcticus* landed in Bass Strait and 53% of 928 *M. antarcticus* landed off South Australia on commercial vessels. The mortality rate would be much higher if delayed mortality was also taken into account, which in itself is above 60%, even in controlled, simulated environments (Frick et al., 2010a, 2010b and the results presented in the present study). An estimate of initial mortality (26%) for 5578 *M. antarcticus* tagged and released provides a reasonable estimate of delayed mortality for animals caught in gillnets aboard commercial fishing vessels and judged as lively and likely to survive after prompt release at sea (Walker, 2010). This compares with an estimate of 44% for the ram-ventilating species *Galeorhinus galeus* for 2054 animals similarly tagged and released (Walker et al., 2008).

Furthermore, several biotic and abiotic factors can impact the

resilience of the individuals in field conditions (Davis, 2002), which might complicate direct field vs laboratory comparisons of MR (Frick et al., 2010b; Bouyoucos et al., 2017; Dapp et al.,2016b, Dapp et al., 2017a). For example, the presence of strong tidal flow on the fishing grounds may reduce and delay hypoxia in enmeshed animals. We tried to emulate this at the best of our logistical capabilities, but constant flowing seawater might have not been as strong as those on the sea-floor. Nevertheless, our study provides valuable information for the management of fishery by-catch and evaluation of the conservation status of these species, highlighting the magnitude and importance of broadening our understanding of species-specific and individual-specific variations in MR in response to stress. The energetic costs resulting from capture stress must be paid from a finite energy budget, and therefore impact the resources for reproduction, growth and other vital biological demands. Some quantification of these other costs, which currently remain unknown, will provide important context for the significance of capture stress to surviving animals in an energetic sense and should be a focus of investigation.

5. Conclusions

Our findings provide new information on the responses of sharks to capture stress. We conclude that *M. antarcticus* are active demersal sharks exhibiting relatively high MRs, and that, although they perform buccal pumping when at rest, they also rely on ram ventilation to maintain homeostasis in stressful situations such as fishing capture. Confronted with the gillnetting simulation employed in our study, this species presented an unusual and unexpected response by decreasing its metabolic output by half. We interpret this phenomenon as a compensatory mechanism to reduce oxygen consumption to cope with the stress imposed. This species also presented notable individual variations in their responses to stress, with mortality being associated with higher pre-stress MR, and lower post-stress MR. The effects of capture stress were also more pronounced in smaller individuals of the species.

Heterdontus portusjacksoni are bottom-dwelling sharks that showed a mild increase in their MR after the simulated gillnetting, in comparison with other more fisheries-vulnerable counterparts. Their resilience to capture stress most likely relies on effective buccal pumping and lower MRs.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

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5.8. COMMENT ON CHAPTER 5

The limitations of two-species comparisons, both in evolutionary and physiological/ecological studies, have been highlighted by several authors (Garland and Adolph, 1994; Feder et al., 2000). Specifically, inferring that an observed physiological response is the direct consequence of a difference existing between the two species in the ecological traits investigated, can be a flawed assumption. Drawing this conclusion presumes that the two species are physiologically and/or ecologically identical apart from the investigated traits, an assumption that, especially for wild populations and animals, might be incorrect (Garland and Adolph, 1994). In this context, the conclusion that the differences in basal MR observed between *Mustelus antarcticus* and *Heterdontus portusjacksoni* are derived from differences in their lifestyles and swimming regime might not have strong support as it is derived from an experiment investigating only two species. The inference that the opposite metabolic response observed in these same species after the exposure to the same fishing-capture stress is a result of a difference in species sensitivity towards the specific stress (Dapp et al., 2015) might be similarly affected. However, the existence of a positive correlation between baseline MO_2 and the activity level/swimming behavior of a species is now an established concept, even for chondrichthyans (Bernal et al., 2012). Moreover, the results presented in Chapter 3, 4 and 5 and data previously published (Bouyoucos et al., 2017, 2018) all support the hypothesis that the opposite metabolic responses observed in different species following different fishing-capture stress might be related to the interaction between species sensitivity and stress severity. Therefore, while the inference of causations obtained from two-species studies need to be cautious, in this case, the support given by other studies allows us to propose these correlations with confidence.

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Chapter Six

Prolonged alteration of neonate traits following maternal exposure to fishing-capture stress during late pregnancy in a chondrichthyan species

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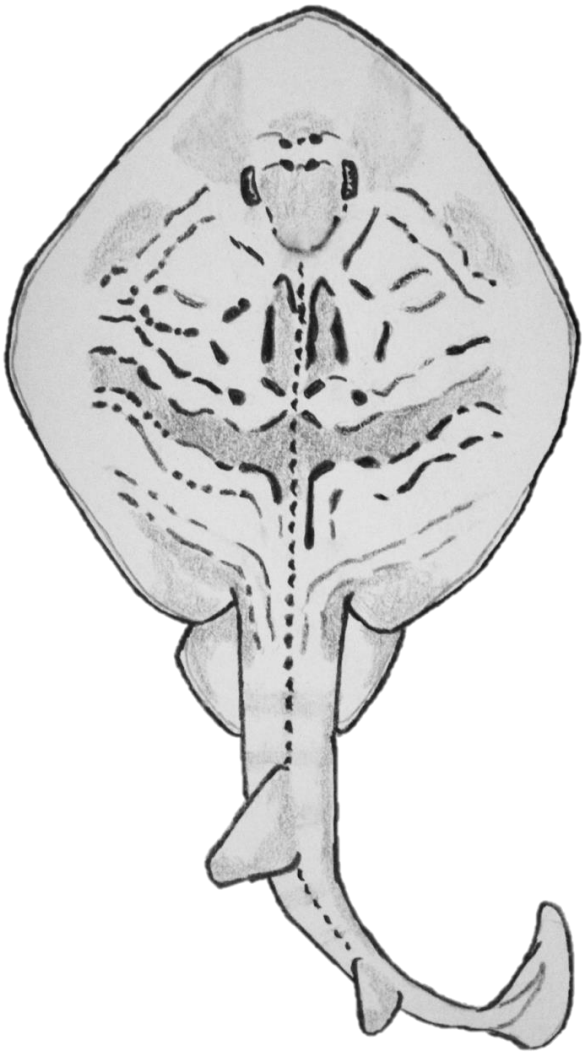

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Drawing by Licia Finotto

ABSTRACT

For animals discarded after capture (unwanted bycatch), the effects of fishing-capture stress can extend beyond immediate or delayed death, causing long-term, sub-lethal effects such as injuries and physiological, behavioural, immune, energetic and reproductive consequences. Given the importance of successful reproduction for population recruitment, investigating reproductive impairment is essential, even more so for animals with low reproductive rates such as chondrichthyans. However, data for these species are lacking, even though the poor conservation status of many chondrichthyan populations and the threats from overexploitation and discard require a better understanding of fishing-capture stress consequences. In this study, we investigated the reproductive consequences of trawling and air exposure stress when experienced by southern fiddler rays (*Trygonorrhina dumerilii*) during late pregnancy, with a focus on neonatal traits. Compared to neonates from unstressed mothers, neonates born from mothers subjected to capture simulation had significantly lower body mass and yolk sac volume at birth, showed a granulocyte to lymphocyte ratio indicative of a stressed condition, reduced growth, altered burying behaviour, reduced boldness and swam for shorter distances after a simulated predator attack. Smaller size and lower growth rate might expose neonates to a higher rate of predation, and similarly, the altered burying and swimming behaviour may reduce their ability to escape from predators. Decreased boldness could impact neonate survival by reducing their ability to compete and obtain food and resources. Further investigations are needed, but these initial results suggest that capture stress suffered by pregnant females may alter traits and survival of their offspring with consequences for recruitment and population abundance.

6.1. INTRODUCTION

Chondrichthyans (sharks, rays and chimeras) are among the most threatened groups of vertebrates (Dulvy et al., 2008, 2014; IUCN, 2020). The high vulnerability of these species originates from their peculiar life-history traits of late sexual maturity, long reproductive cycles, long gestation or embryonic development and low fecundity (Camhi et al., 1998), resulting in low reproductive rates and low ability to recover from population decreases. Among various threats, the most severe and frequent are overfishing and bycatch, i.e., the discard of unwanted captured animals due to management measures or low economic value (Dulvy et al., 2008; Ferretti et al., 2010; Dulvy et al., 2014). For chondrichthyans, bycatch is a serious problem (Molina and Cooke, 2012) because fishing procedures can cause injuries and extreme homeostatic alterations resulting in immediate or delayed death (Frick et al., 2010; Braccini et al., 2012). Even when animals survive, the elicited stress response, which is necessary to maintain or restore homeostasis and increase survival (Skomal and Mandelman, 2012), can also be detrimental if fishing stressors are severe or prolonged. Indeed, chronic alterations of homeostasis, increased stress hormone levels and altered energetic allocation may result in long-term consequences such as altered growth, immune competence, behaviour and reproduction, which ultimately affect survival and fitness (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012; Watson et al., 2020). Given the importance of successful reproduction to the maintenance of sustainable populations, reproductive impairments that affect the individuals' ability to contribute to increasing population abundance through recruitment or affect the survival of their offspring need to be understood (Schreck et al., 2001; Sopinka et al., 2016a). This is especially important for chondrichthyans, not only because of the poor conservation status of many populations, but also because of the generally large energy investment in a single reproductive event (Hamlett et al., 2005; Trinnie et al., 2012) and long reproductive cycles (up to 3 years in school shark *Galeorhinus galeus*; Hamlett

et al., 2005; Walker, 2005b). Therefore, reproductive impairments could jeopardize the large investment already allocated to reproduction over the several prior years, further hindering population recovery.

In teleost fishes, stressors can affect a large variety of reproductive traits, both in parents, including reproductive hormone concentrations, reproductive behaviour, vitellogenesis, gamete quality, fecundity and ovulation timing, and in offspring of stressed parents, such as behaviour, hatching success, size, growth, swimming performance, feeding ability and survival (Chapter 2). Few data exist for chondrichthyans and almost entirely concern observations of capture-induced premature parturition and abortion (Adams et al., 2018; Wosnick et al., 2019; de Sousa Rangel et al., 2020). Given the low probability of neonates surviving after premature parturition (Charvet-Almeida et al., 2005; Campbell et al., 2018), this is the most severe outcome representing the likely complete failure of the reproductive event. However, even when not causing premature parturition, in southern fiddler rays (*Trygonorrhina dumerilii*), trawling capture stress experienced by mothers during pregnancy affects neonate size at birth and causes a state of chronic stress in neonates, potentially reducing survival in the long-term (Guida et al., 2017). In teleosts, various other offspring traits associated with survival are affected by parental stress and, given the similarity of many of the stress consequences reported in teleosts and chondrichthyans (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012), further research aimed at verifying whether these alterations occur also in chondrichthyan offspring is needed. These data will help better assess the extent of the fishing-capture consequences and the potential need for management measures. Moreover, studying stress effects in wild animals is intrinsically difficult due to various confounding factors that cannot be controlled and, especially for poorly researched areas, reliable conclusions can be drawn only from investigating different stress indices and their

consequences (Johnstone et al., 2012, 2017), calling for further research on this topic in chondrichthyans.

We investigated the consequences of maternal exposure to trawling capture simulation and air (Frick et al., 2010) during late pregnancy on neonatal traits in *T. dumerilii*. This species is commonly captured by commercial and recreational fisheries in southern Australian waters and, due to low market values, most animals, including pregnant females (Marshall et al., 2007), are released after capture (Walker and Gason, 2007; Last and Stevens, 2009). The reproductive impairments previously observed by Guida et al. (2017) in pregnant mothers and neonates of this species indicate the need for further research. We focused on investigating alteration in traits that persist in neonate life, including growth rate, residual yolk sac volume and its consumption rate, swimming performance, and burying and boldness behaviour.

6.2. MATERIALS AND METHODS

6.2.1. Animal collection and husbandry

In March and April 2018, 14 pregnant *T. dumerilii* females were hand-collected in Swan Bay, Victoria, Australia. Pregnancy was determined in-water by the presence of embryos in the uteri using ultrasound scanning (L6.2 linear transducer probe at 8–5 MHz, Ibex Pro Portable Ultrasound, E. I. Medical Imaging, USA). Upon collection, pregnant animals were placed in a 200-L holding tank, with a continuous flow of ambient seawater and transported to the housing facility (Victorian Fisheries Authority, Queenscliff, Victoria, Australia) in <2h with up to 3 animals (20 kg combined mass) held together in the holding tank. At the facility, females' body mass (BM_F) to the nearest 0.1 kg and total length (TL_F) to the nearest 0.5 cm were measured. Females were tagged applying different colour combinations of cable ties to their tail (loose enough to avoid skin injuries) to allow identification of each individual. Animals were randomly assigned to two experimental groups (see below) and transferred to two separate 9,000-L circular housing tanks (7 animals in

each tank, for a maximum biomass of 45 kg). The tanks were located in the open air, under a covered structure and supplied with continuously flowing ambient seawater and aeration. Animals were exposed to natural photoperiod and water temperature (mean ± SD: 17.2 ± 1.1 °C) and fed sardines and prawns twice a week (~10% of their total body mass per week). Females were maintained in captivity until a maximum of 4 days after parturition and, after veterinary health-check, were released back in Swan Bay. All experiments were performed under Monash University Animal Ethics Protocol BSCI/2016/37, Parks Victoria research permit n° 10008588, VFA general permit n° RP1286 and complied with current Australian law.

6.2.2. Experimental design

The schematic of the experimental design is presented in Figure 6.1. The 14 near-term, pregnant females were randomly assigned to one of two maternal treatment groups. Females in the Trawl Group (n=7) were subjected to trawl simulation with air exposure, while those in the Control Group (n=7) were treated as controls. The consequences for neonates were investigated by assessing stress at birth and monitoring morphometric and behavioural changes during the first 30 days postpartum. We examined the effects of maternal treatment on number of stillborn neonates and neonate size, volume of yolk reserves and stress, indicated by blood parameters, at birth. We measured growth, indicated by length- and body-mass-increments, and consumption of the yolk, indicated by the percentage reduction in yolk sac volume, over 30 days. We also examined the effects of maternal treatment on neonate behaviour expressed as burying success,

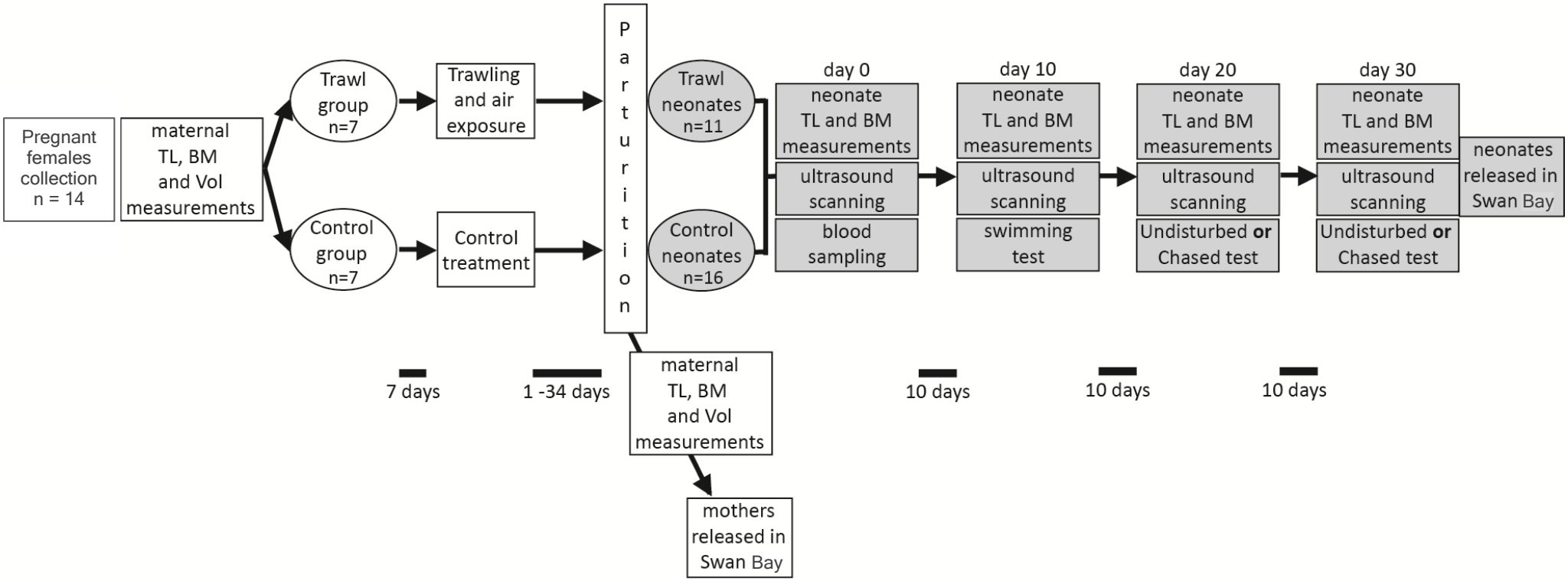

Figure 6.1. Schematic of the experimental design. White and grey squares represent procedures experienced by *Trygonorrhina dumerilii* pregnant females and neonates, respectively. White and grey ellipses represent, respectively, the two different treatment groups in which pregnant females were randomly divided (Trawl and Control) and the neonates that were born from these females. Black arrows indicate the passage from one procedure to the next. The vertical white bar indicates when parturition occurred. Horizontal black segments represent the number of days passing between different procedures/events (not in scale). n indicates the sample size.

time to bury, the time before initial movement, total time spent moving, and speed and distance moved in response to simulated predation attack.

6.2.3. Maternal treatments of trawling simulation with air exposure and control

Trawling capture was simulated at least 12 days after collection to allow recovery from the initial capture stress and habituation to the housing conditions. Females were also used in a metabolic study (Chapter 3) and due to limitations on equipment availability for that study, each female in the Trawl Group was towed one at each time. Females in the Trawl group were dip-netted from the housing tank and individually placed inside a cod-end (a 110-cm long bag constructed of monofilament webbing; 102 mm between opposite knots of diamond-shaped mesh if stretched) mounted on a circular support hanging from a metal bar suspended over a 19,000-L experimental tank. In the centre of the tank, a rotating wood panel (2.0 × 1.0 m; W × H) generated a water current of ~0.6 m s^{-1} in front of the cod-end, simulating the condition of towing a net through the water (Frick et al. ,2010). Each female was towed for 7 h, then retrieved from the cod-end and placed for the 30-min air exposure in an empty tub (2.0 × 1.0 × 0.7 m; L × W × H) outdoors in a shaded area. At the end of the air exposure period, females in the Trawl Group had their metabolic rate measured and then were transferred back to the housing tank where they remained until 4 days after parturition. Each day, concomitantly with each female of the Trawl Group, a female in the Control group experienced the same dip-netting, handling and transfer (to a 5,000-L tank) procedures but was neither towed nor measured for metabolic rate. The fact that Trawl females experienced the confinement in a respirometry chamber during metabolic measurement, while Control females did not, might have influenced their stress load. However, blacktip reef sharks (*Carcharhinus melanopterus*) spending 3 h in a respirometry chamber did not experience the physiological alterations associated with a stress response (Bouyoucos et al., 2018). Therefore, it seems unlikely that the small stress potentially associated with metabolic

measurement significantly impacted Trawl females and subsequent measurements, especially when compared with the highly stressful conditions of the trawling simulation. The experimental tank was supplied with constant seawater flow such that the whole mass of water was replaced between successive trawl simulations of separate days, to eliminate the possibility of stress-related chemical clues influencing the responses of animals treated subsequently.

6.2.4. Neonate morphological measurements

Parturition was indicated by the presence of neonates in the two separate tanks holding the Trawl mothers and Control mothers observed during daily inspections and the numbers of live and stillborn neonates were recorded. *Trygonorrhina dumerilii* litter size ranges from 2 to 5 pups (Marshall et al., 2007), so the presence of 4 or more pups indicated that potentially two females gave birth the same night. The identification of the female(s) that gave birth was initially based on visual inspection of females' body appearance because near-term pregnancy is evident from dorsal bulges on the two sides, which disappear immediately after parturition (de Sousa Rangel et al., 2020). To confirm parturition, females suspected to have given birth were dip-netted, drawn to the side of the housing tank and, while lightly restrained, checked with the ultrasound. This protocol allowed assigning a female to each parturition event. On one occasion, two females in the same housing tank gave birth the same night. From ultrasound scanning performed at initial capture, an estimate of the number of embryos carried by each female was available. Moreover, data collected during a preliminary experiment highlighted that neonate TL at birth was positively correlated with maternal size (LME: $TL_N \sim TL_M + (1|\text{MotherID})$; $TL_N \sim 21.95 + 0.04 * TL_M$; $F_{1,20} = 4.35$, $p = 0.037$) and that siblings showed similar coloration patterns (Figure 6.2). Based on the similarity of the coloration patterns among siblings, on maternal and neonate sizes and on data on the number of the embryos determined by ultrasound scanning the mothers when initially captured, it was possible to assign all neonates to the respective mother.

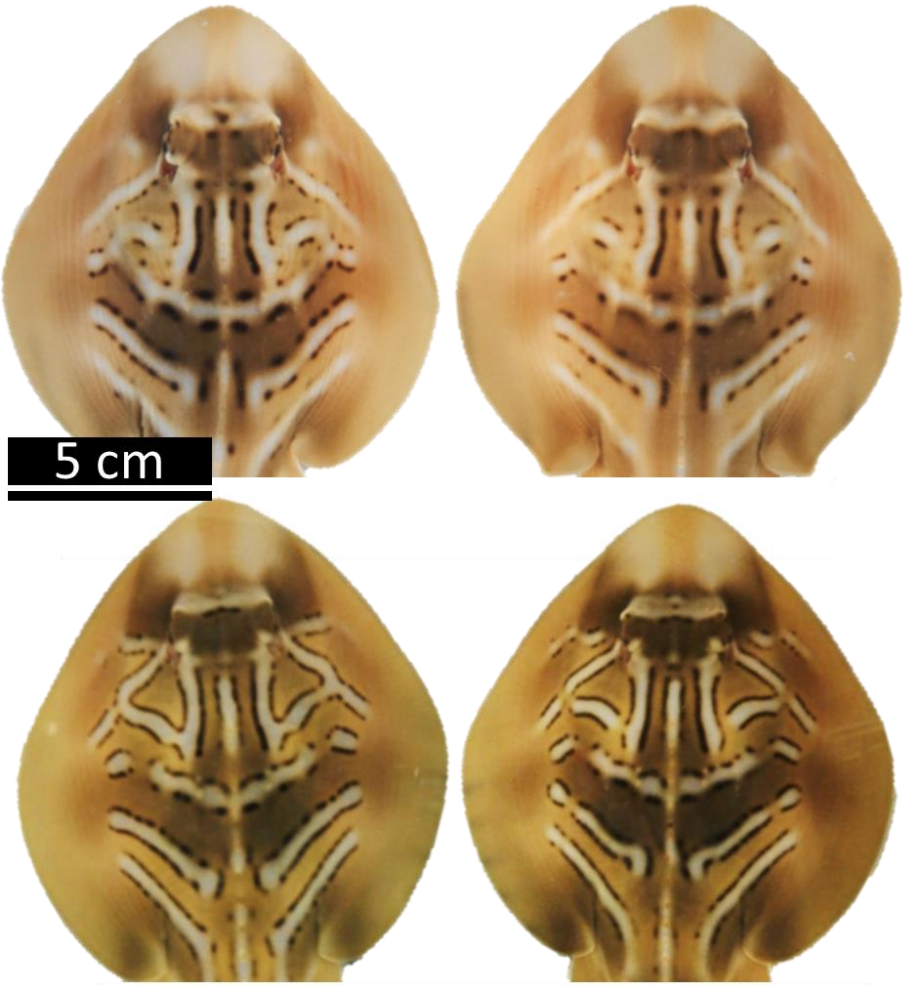

Figure 6.2. Dorsal photos of southern fiddler ray (*Trygonorrhina dumerilii*) neonates showing the similarity in coloration and patterning between pairs of siblings (pair A: top two and pair B: bottom two).

At birth (day 0), living neonates were individually removed from the housing tanks, transferred to a bucket filled with a known mass of water and body mass (BM_N) to the nearest 0.1 g and total length (TL_N) to the nearest 0.1 cm were measured. A blood sample (500 µl) was collected in <1 min through caudal venipuncture using a 27 gauge needle and a 3ml heparinized syringe. The dorsal side of each neonate was photographed, allowing later identification by checking its coloration pattern (Figure 6.2). Neonates underwent longitudinal and transversal ultrasound scanning (Figure 6.3) and from still images, using the measuring tool provided by the ultrasound machine software, the length, height and width of neonate internal yolk sac (L_Y, H_Y and

W_Y; mm) were estimated and used to calculate yolk sac volume (see Data analysis). Afterwards, neonates from both maternal treatment groups were transferred to a single 5,000-L housing tank (maximum of 20 neonates for a biomass of 2273.4 g), fed chopped prawns every other day (~10% of their body mass weekly), maintained in captivity for 30 days and then, after a veterinary health check, released in Swan Bay. All the neonates born live survived the period in captivity.

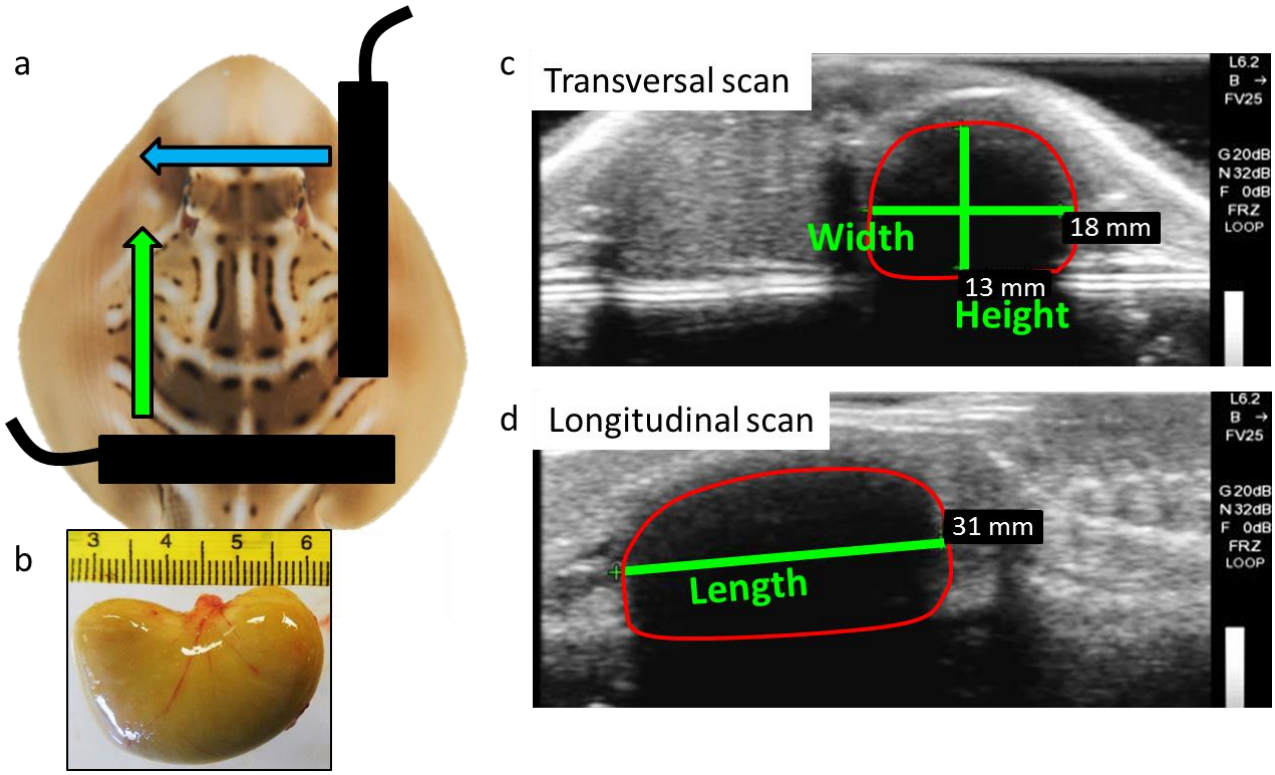

Figure 6.3. Schematic of neonate yolk sac measurements. a) schematic representation of ultrasound scans performed on neonates to obtain a longitudinal view and a transversal view of the yolk sac. Black bars represent the ultrasound probe, blue and green arrows represent the movement of the probe during the longitudinal and the transversal scan, respectively. b) photo of the bean-shaped yolk sac excised from a stillborn neonate. c) still image obtained from the transversal scan and used to measure yolk sac height and width. d) still image obtained from the longitudinal scan and used to measure yolk sac length. The red shape in c) and d) delineates the yolk sac.

6.2.5. Neonate growth and yolk sac consumption rates

For a month after the birth of each neonate, every 10 days (day 10, day 20 and day 30), measurements of body mass, total length and ultrasound, to monitor the volume of the remaining internal yolk sac, were performed as described above. To reduce the number of times neonates had to be dip-netted from the tank and the minor stress associated with the procedure, neonate swimming performance and behaviours (see below) were tested concomitantly with these measurements.

6.2.6. Swimming test

Swimming was tested on day 10. Neonates were individually placed at the entrance of a 2-m long corridor set inside a 5,000-L tank. The corridor was built shaping two portions of plastic-coated metallic wire mesh weighted down on the bottom of the tank. An enclosed funnel-shaped space, separated from the main section of the corridor by a removable panel, served as a habituation area. The neonate was allowed to settle on the bottom of the tank in the habituation area (<2 min) and, after removal of the panel, it was gently touched on the tail to simulate a predator attack and stimulate its movement (Gyssels and Stoks, 2005). Three consecutive trials were performed and video-recorded. Most neonates did not swim from the entrance to the end of the corridor in a single movement but stopped at various distances from the entrance. On these occasions, neonates were again touched on the tail to stimulate swimming. If movement did not resume within 5 seconds, the test was concluded. At the end of the test, neonates were transferred back to their housing tank.

For each neonate and trial, the length of the segment covered during the first burst of swimming (Swimming distance, m) was measured from a still image of the video analysed in ImageJ (National Institute of Health, USA, 1997). The time taken for the neonate to swim to the

end of the corridor, measured starting after the first stimulus and including any time the neonate stopped, was recorded to calculate completion speed (see Data analysis).

6.2.7. Burying and boldness tests

Each neonate underwent two different tests, one on day 20 and one on day 30. Neonates were individually transferred to a separate tank (70cm x 160cm x 35cm, 340 L) with a 5-cm deep layer of sand. After a 2-min habituation, neonates were either left undisturbed for 20 min in the 'Undisturbed test', or chased with a dip net for 30 sec to simulate a predatory attack eliciting an escape response and then left undisturbed for 20 min, in the 'Chased test'. Tests were video-recorded and, at the conclusion, neonates were transferred back to their housing tank.

From the video for each neonate, two dichotomous categorical variables (Burying success and Moving success) and three continuous variables (Burying time, Latency time and Total moving time) were quantified for each of the Undisturbed test and Chased test. Following the habituation period in the Undisturbed test and after the chasing procedure in the Chased test, 'Burying success' and 'Moving success' related to neonate success in submerging in the sand and moving from its initial position, respectively. The neonate's 'Burying time' was the elapsed time before the neonates started the burying process, the 'Latency time' was the elapsed time before the first movement and 'Total moving time' was the time spent moving during the first 5 min following habituation or the chasing procedure. When neonates did not bury or move before the end of the test, the maximum time (20 min) was recorded as Burying time or Latency time. In preliminary experiments, these variables did not differ between the first and the second time the neonates were tested. Nevertheless, to minimize potential systematic error and habituation, whether a neonate was tested first in the Chased or the Undisturbed test was randomly allocated.

6.2.8. Granulocyte to lymphocyte ratio

Smears were prepared of neonate blood samples collected on day 0, air-dried in a closed container and then fixed by immersion in 100% methanol for 10 min. Blood smears were stained with May-Grünwald (15 min; solution diluted 1:1 with water; Australian Biostain, Traralgon, Victoria, Australia) and Giemsa (15 min; solution diluted 1:9 with water; Australian Biostain) stains, rinsed three times in distilled water and then immersed in fresh distilled water for 5 min. Slides were examined, with hidden identity, using a compound microscope at × 400 magnification. Granulocytes (neutrophils, heterophils, eosinophils and basophils) and lymphocytes were identified according to available descriptions (Van Rijn and Reina, 2010; Haines and Arnold, 2015; Guida et al., 2017). Leukocytes were counted in at least 4 fields of view moving diagonally along the slide and avoiding the edges of the blood smears. Counting ceased when a minimum of 350 leukocytes were counted and all the cells in the last field of view were identified (Van Rijn and Reina, 2010; Johnstone et al., 2012). A random subsample of eight slides was counted twice to test for the repeatability of the leukocyte counting profile (see Data analysis).

6.2.9. Data processing and analysis

Yolk sac measurements obtained from the ultrasound scanning were used to calculate the volume of the bean-shaped yolk sac (Figure 6.3) through the equation:

$VOL = (2*W_Y*L_Y*0.45*H_Y)*10^{-3}$

where VOL is the estimated yolk sac volume in ml, W_Y is the width of the yolk sac measured at its widest part, L_Y is the length and H_Y is the height, all expressed in mm, and 10^{-3} is the factor for converting volume expressed in mm^3 to ml. In four stillborn neonates, yolk sac measurements obtained indirectly from the ultrasound video (Figure 6.3c, d) did not significantly differ from direct measurements of excised yolk sacs (data not shown; Figure 6.3b), supporting the validity of indirectly measuring yolk sac volume from ultrasound scanning.

The normality of the datasets was assessed with the Shapiro-Wilk test and the homoscedasticity with Levene's test. T-tests were used to investigate differences in adult female total length and body mass (TL_F and BM_F) at capture between pregnant females of the Trawl and the Control treatment groups (Treatment).

Differences in the occurrence of stillborn neonates between treatments were analysed both in terms of number of stillborn neonates and number of parturitions with stillborn neonates, using Fisher's test. Linear mixed effects models (LME) were used to examine differences in neonate total length (TL_N), body mass (BM_N) and G:L ratio at birth, including Treatment as a fixed effect and Mother ID as a random effect. To test for G:L counting repeatability, the two readings obtained from the eight re-counted slides were compared, after log transformation, using a paired t-test. To test for the presence of a learning process in the ability to identify different cells, a GLME was used to compare the G:L readings of the first eight versus the last eight slides examined including Mother ID as a random effect. Using only data from Control neonates, the relationship between yolk sac volume (VOL) at birth and BM_N was investigated using a LME including Mother ID as a random effect. To analyse difference in VOL at birth between Control and Trawl neonates, a LME was used, including Treatment and the interaction between Treatment and BM_N as fixed effects and Mother ID as a random effect (VOL ~ Treatment + Treatment:BM_N + (1|MotherID)). Data and generated residuals were visually assessed for normality and homoscedasticity. Significance of the fixed terms was evaluated using a Wald test.

To investigate TL_N and BM_N increments over time (as continuous variables; days 0, 10, 20 and 30), generalized linear mixed effects models (GLME) were used, including the interaction between Treatment and Time as a fixed effect and Neonate ID as a random effect. Yolk consumption rate was investigated with a similar GLME, but to account for potentially faster consumption rates in

larger neonates, the proportion of yolk volume remaining relative to the initial value (VOL_L) was used in the analysis. VOL_L was calculated using the equation:

$VOL_L = VOL_t * VOL_i^{-1}$

where VOL_L is the proportional volume left at time t (days 0, 10, 20 or 30), VOL_t is the volume at time t in ml and VOL_i is the initial volume at d0 in ml. Before running the model, VOL_L data were transformed as the arcsin of the square root, but for clarity results are reported as percentages.

Completion speed (m s^{-1}) was calculated dividing the length of the corridor (2 m) by the total elapsed time taken to swim this distance, including the duration of any potential stop (s). For each neonate, Swimming distance (m) and Completion speed were averaged between the three different trials. The relationship between TL_N and Swimming distance and Completion speed was investigated using only data from Control neonates and two LMEs including Mother ID as a random effect. To investigate the effect of Treatment on these two swimming variables, GLMEs were used, including Treatment as a fixed effect and Mother ID as a random effect.

Differences in Burying success and Movement success were determined using Fisher's tests. To investigate differences in Burying time, Latency time and Total moving time, GLMEs were used. Differences in these variables were investigated both between Treatment, within each Test type (Undisturbed or Chased test), including Treatment as a fixed effect and Mother ID as a random effect, and between Test type, separately for Control and Trawl Treatments, including Test type as a fixed effect and Neonate ID as a random effect.

Data presented are derived from the most parsimonious models, obtained from the full models through step-wise backward elimination of non-significant terms. Results are reported as mean ± standard error (SE). All tests used a significance level of $\alpha \leq 0.05$. Data were processed using R statistical software (R Core team 2019).

6.3. RESULTS

6.3.1. Female morphological traits and parturition time

There was no significant difference between treatments in maternal TL_F (Control: 96.9 ± 1.9 cm; Trawl: 93.4 ± 3.1 cm; $t_{1,11} = 0.83$, $p = 0.43$) and BM_F (Control: 6.3 ± 0.3 kg; Trawl: 5.8 ± 0.4 kg; $t_{1,11} = 0.85$, $p = 0.41$). Females in the Control and Trawl groups gave birth to an average of 2.8 and 2.0 pups, respectively. Neonates were born an average of 21 days after trawling-capture simulation for Trawl females (1–34 days). One Control female never gave birth and ultrasound indicated that the embryos were likely non-viable because no mouth movements associated with gill oxygenation was observed. One Trawl female gave birth to two neonates the night after trawling simulation but was not considered a premature, stress-induced parturition because pregnancy was in an advanced state and occurred within the normal parturition period (Marshall et al., 2007). Excluding this female, parturition occurred an average of 24 days after trawling simulation (6–34 days).

The number of stillborn neonates (Control: 1 of 17 neonates; Trawl: 3 of 14 neonates; Fisher's test $p = 0.30$) or of parturitions with stillborn neonates (Control: 1 of 6 parturition events; Trawl: 2 of 7 parturition events; Fisher's test $p = 1$) did not differ between treatments.

6.3.2. Neonate morphological traits

Neonate TL_N at birth did not significantly differ between treatments (Control: 27.3 ± 0.2 cm; Trawl: 26.4 ± 0.5 cm; $F_{1,25} = 0.004$, $p = 0.95$) but Control neonates were significantly heavier (BM_N Control: 118.9 ± 3.3 g; BM_N Trawl: 102.8 ± 5.0 g; $F_{1,25} = 3.99$, $p = 0.04$). In Control neonates, yolk sac volume at birth was significantly and positively correlated with neonate BM_N ($VOL = –8.72 + 0.12*BM_N$; $F_{1,14} = 18.89$, $p < 0.0001$). Both Treatment ($F_{1,25} = 5.48$, $p = 0.02$) and the interaction between Treatment and BM_N ($F_{2,24} = 32.14$, $p < 0.0001$) significantly influenced yolk sac volume at

birth, with Trawl neonates having a smaller yolk sac (Control: 5.41 ± 0.46 ml; Trawl: 4.39 ± 0.43 ml) and manifesting a lower increase in yolk sac volume with BM_N (Figure 6.4; Table 6.1).

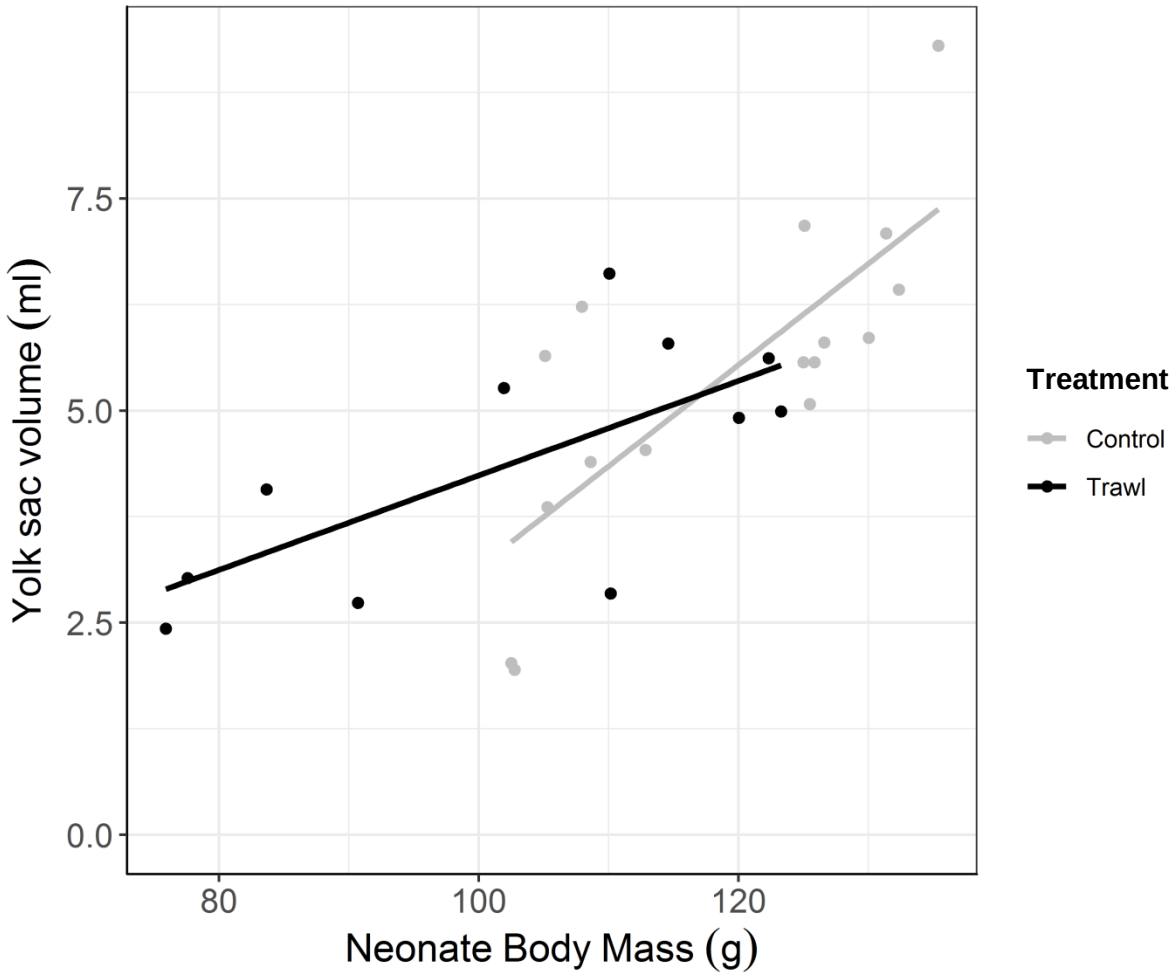

Figure 6.4. Relationship between neonate yolk sac volume (ml) and neonate body mass (g) at birth (day 0) for Control (grey; n=16) and Trawl neonates (black; n=11). Points represent original data and lines represent the linear regressions fitted to the data.

Table 6.1. Effects obtained from the most parsimonious linear mixed effects model illustrating the influence of neonate body mass (BM_N) and treatment group (Control (n=16) or Trawl (n=11)) on yolk sac volume at birth.

Yolk sac volume	Effect Mean ± SE (ml)
Intercept (Control)	–8.75 ± 2.90
Treatment (Trawl)	7.46 ± 3.54
Control:BM_N	0.12 ± 0.02
Trawl:BM_N	0.06 ± 0.02

6.3.3. Granulocyte to lymphocyte ratio

Trawl neonates showed significantly higher G:L ratio values (Control: 0.05 ± 0.01; Trawl: 0.22 ± 0.09; $F_{1,25}$ = 2.57, p = 0.02). No difference between repeated counts (First: 0.10 ± 0.03; Second: 0.14 ± 0.07; $t_{1,14}$ = 0.77, p = 0.74) and no learning process (Early: 0.16 ± 0.05; Late: 0.14 ± 0.07; $F_{1,14}$ = 1.22, p = 0.27) were observed.

6.3.4. Neonate growth and yolk sac consumption rates

Time ($F_{1,102}$ = 8.24, p < 0.0001) and the interaction between Treatment and Time ($F_{1,102}$ = 2.33, p = 0.02) significantly influenced neonate TL_N. TL_N increased with time, and length-increments with time were smaller in neonates from Trawl mothers, indicative of slower growth (TL at day 30: Control: 28.0 ± 0.2 cm; Trawl: 26.9 ± 0.5 cm; Table 6.2, Figure 6.5a). Neonate BM_N was not influenced by Time ($F_{1,102}$ = 1.32, p = 0.19), Treatment ($F_{1,102}$ = 2.02, p = 0.06) or their interaction ($F_{1,102}$ = 0.83, p = 0.41; BM at day 30: Control: 119.1 ± 3.2 g; Trawl: 107.8 ± 6.1 g; Table 6.2, Figure 6.5b). Only Time significantly influenced the proportion of yolk sac volume remaining ($F_{1,102}$ = 16.36, p < 0.0001), which decreased over time at a similar rate for both Trawl and Control

neonates ($F_{1,102}$ = 1.03, p = 0.31; Yolk sac volume remaining at day 30: Control: 2.47 ± 0.22 ml; Trawl: 2.36 ± 0.35 ml, corresponding to the 47 ± 3% and 55 ± 5% of the initial volume, respectively; Table 6.2, Figure 6.6).

Table 6.2. Effects obtained from the most parsimonious linear mixed effects model illustrating the influence of time and maternal treatment group (Control (n=16) or Trawl (n=11)) on neonatal total length (cm), body mass (g) and proportion of yolk sac volume remaining, as percentage (related to initial volume at birth (d0 = 100%)). * indicates significant effect (p < 0.05).

Total length	Effect Mean ± SE (cm)
Intercept (Control) *	27.04 ± 0.27
Time *	0.02 ± 0.01
Treatment(Trawl):Time *	–0.01 ± 0.01
Body mass	Mean ± SE (g)
Intercept (Control)	118.92 ± 3.99
Treatment(Trawl)	–11.72 ± 6.911
Time	0.02 ± 0.02
Treatment(Trawl):Time	0.02 ± 0.03
Proportion of Yolk Sac remaining	Mean ± SE (%)
Intercept (Control) *	97.04 ± 2.68
Time *	–1.80 ± 0.21
Treatment(Trawl):Time	0.28 ± 0.17

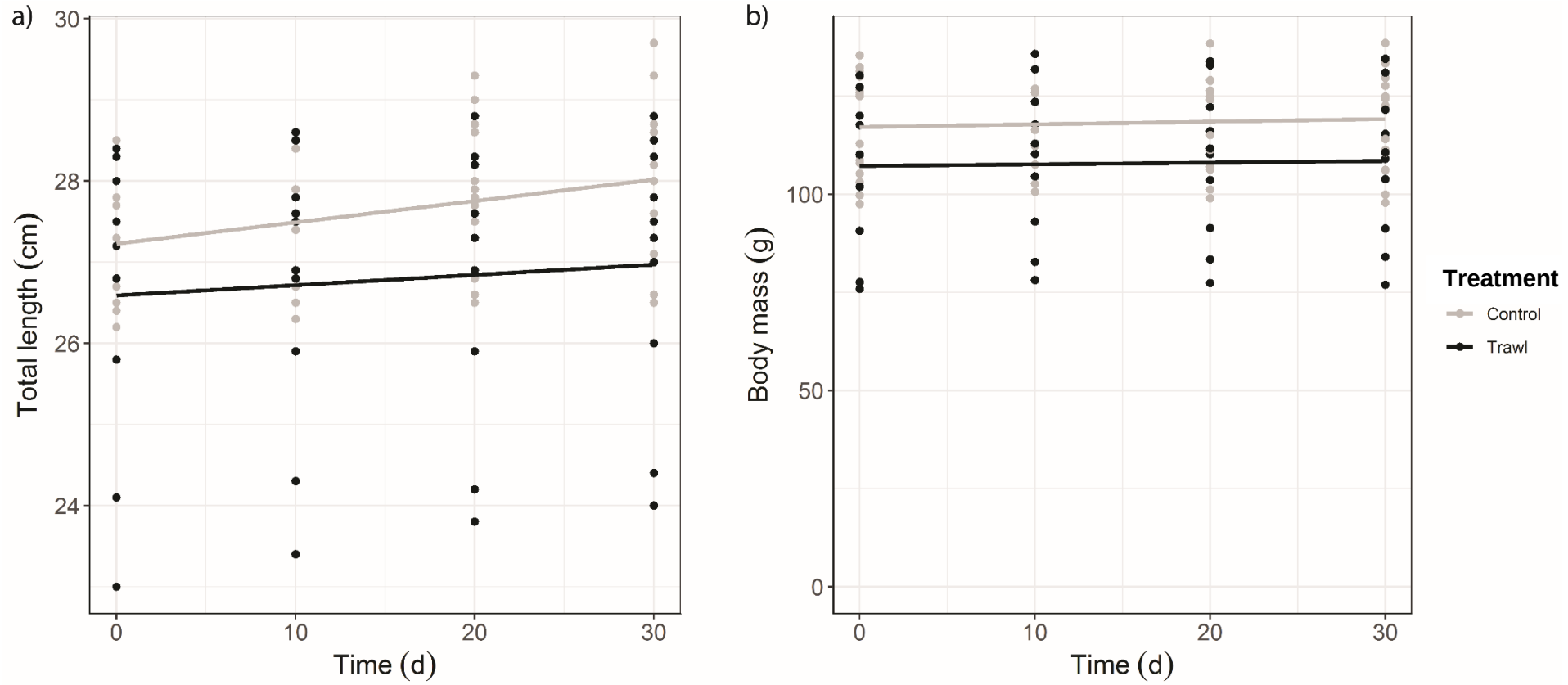

Figure 6.5. Neonate total length (Note that y axis starts from 23cm) (a) and body mass (b) growth-increment with time. Total length and body mass data are plotted against time separately for each maternal Treatment: Control (grey; n=16) and Trawl (black; n=11). Points represent original data and the lines were fitted by linear regression.

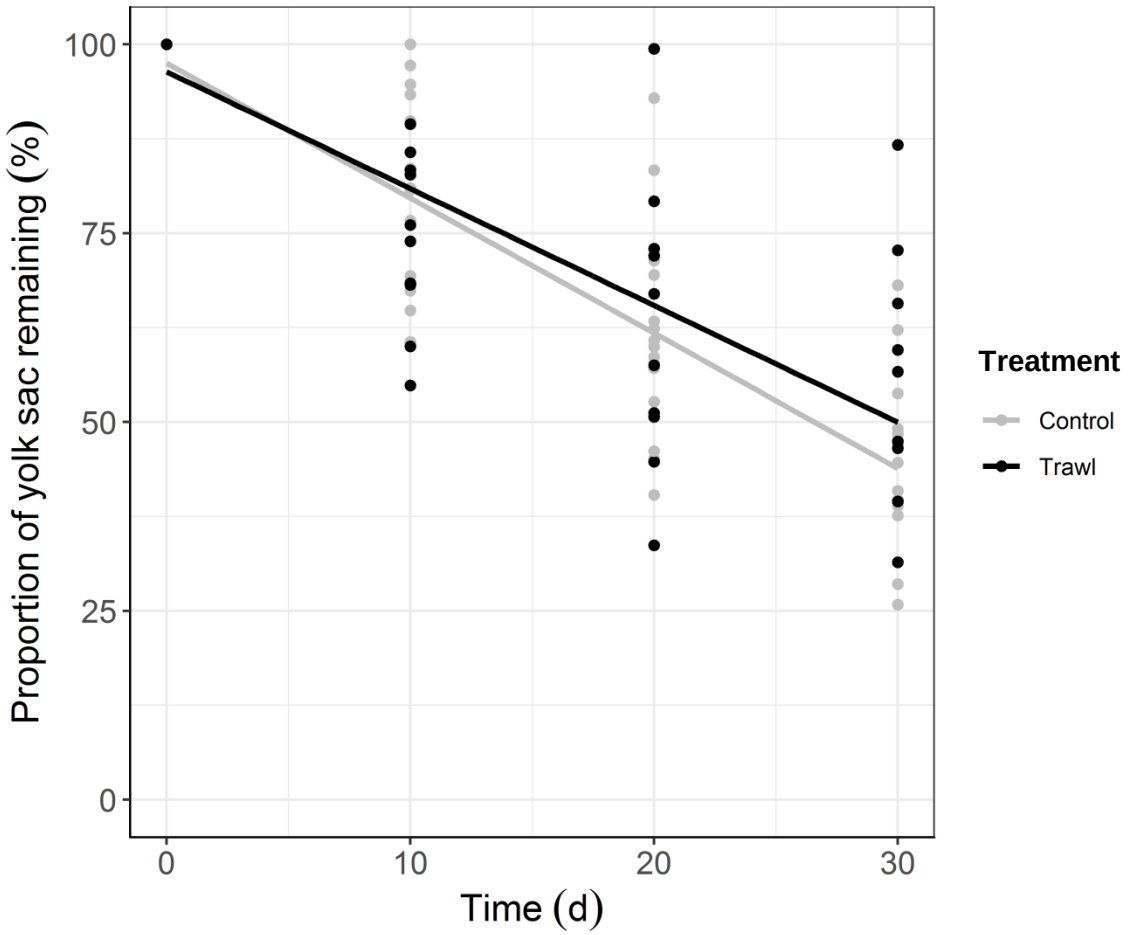

Figure 6.6. Yolk sac consumption rate. The proportion of neonate yolk sac volume remaining as a percentage (related to initial volume at day 0 = 100%) is plotted against time separately for each maternal Treatment: Control (grey; n=16) and Trawl (black; n=11). Points represent original data and the lines were fitted by linear regression.

6.3.5. Swimming performance

In Control neonates, Completion speed (Speed = -0.58 + 0.03*TL_N; $F_{1,14}$ = 5.65, p = 0.02) but not Swimming distance ($F_{1,14}$ = 0.04, p = 0.84), was significantly and positively correlated with neonate TL_N. Treatment significantly influenced Swimming distance ($F_{1,25}$ = 3.96, p = 0.04), with Control neonates swimming for longer distances (Control: 0.97 ± 0.06 m; Trawl: 0.71 ± 0.09 m), while it did not affect Completion speed (Control: 0.14 ± 0.01 ms^{-1}; Trawl: 0.13 ± 0.01 ms^{-1}; $F_{1,25}$ = 0.32; p = 0.57).

6.3.6. Burying and boldness behaviour

Results are reported in Table 6.3 and Figure 6.6. No difference emerged in Burying success or Burying time between treatments in the Undisturbed test (Burying success: Fisher's test p = 0.26; Burying time: $F_{1,24}$ = 0.02, p = 0.88) or the Chased test (Burying success: Fisher's test p = 0.69; Burying time: $F_{1,24}$ = 0.88, p = 0.35).

Comparing Burying success between the two types of test (Chased test or Undisturbed test), no significant difference emerged within Trawl neonates (Fisher's test p = 0.37), but Control neonates had a significantly higher Burying success in the Undisturbed test (Fisher's test p = 0.04). Test type was significant for Burying time, which was higher for the Chased test than the Undisturbed test, both in Control ($F_{1,24}$ =22.49, p < 0.0001) and Trawl neonates ($F_{1,24}$ = 17.68, p < 0.0001).

Table 6.3. Influence of Test type (Undisturbed or Chased) and maternal Treatment group (Control or Trawl) on neonate burying and boldness variables. Number of neonates that did or did not bury (Burying success) or moved (Moving success) before the end of the test. Mean ± SE (min) Burying time, Latency time and Total moving time. For each investigated variable, different lowercase superscript letters indicate significant differences between Treatment groups within the Undisturbed test, uppercase superscript letters indicate significant differences between Treatment groups within the Chased test, * indicates significant differences between Test types, within Control group and # indicates significant differences between Test types within Trawl group ($p < 0.05$). n indicates sample size. Sample size are sometimes lower than the total number of neonates tested (Control = 16 and Trawl = 11) because some neonates buried before the end of the acclimation period or before the end of the observation period for the boldness evaluation (5 min) and were therefore excluded from the analysis.

Variable		Control neonates		Trawl neonates	
		Undisturbed	Chased	Undisturbed	Chased
Burying success (n°)	Buried	15 ^{a} *	9 ^{A}	7 ^{a}	4 ^{A}
	Not buried	1	7	3	6
Burying time (min)		8.21 ± 1.74 ^{a} *	13.84 ± 1.66 ^{A}	9.73 ± 2.10 $^{a\#}$	16.64 ± 1.51 ^{A}
Moving success (n°)	Moved	14 ^{a}	11 ^{A}	9 $^{a\#}$	2 ^{B}
	Not moved	2	5	1	8
Latency time (min)		2.57 ± 0.92 ^{a} *	6.84 ± 1.21 ^{A}	6.14 ± 1.42 $^{a\#}$	14.84 ± 1.67 ^{A}
Total moving time (min)		1.21 ± 0.16 ^{a} *	0.30 ± 0.10 ^{A}	0.66 ± 0.33 $^{b\#}$	0.67 ± 0.38 ^{A}

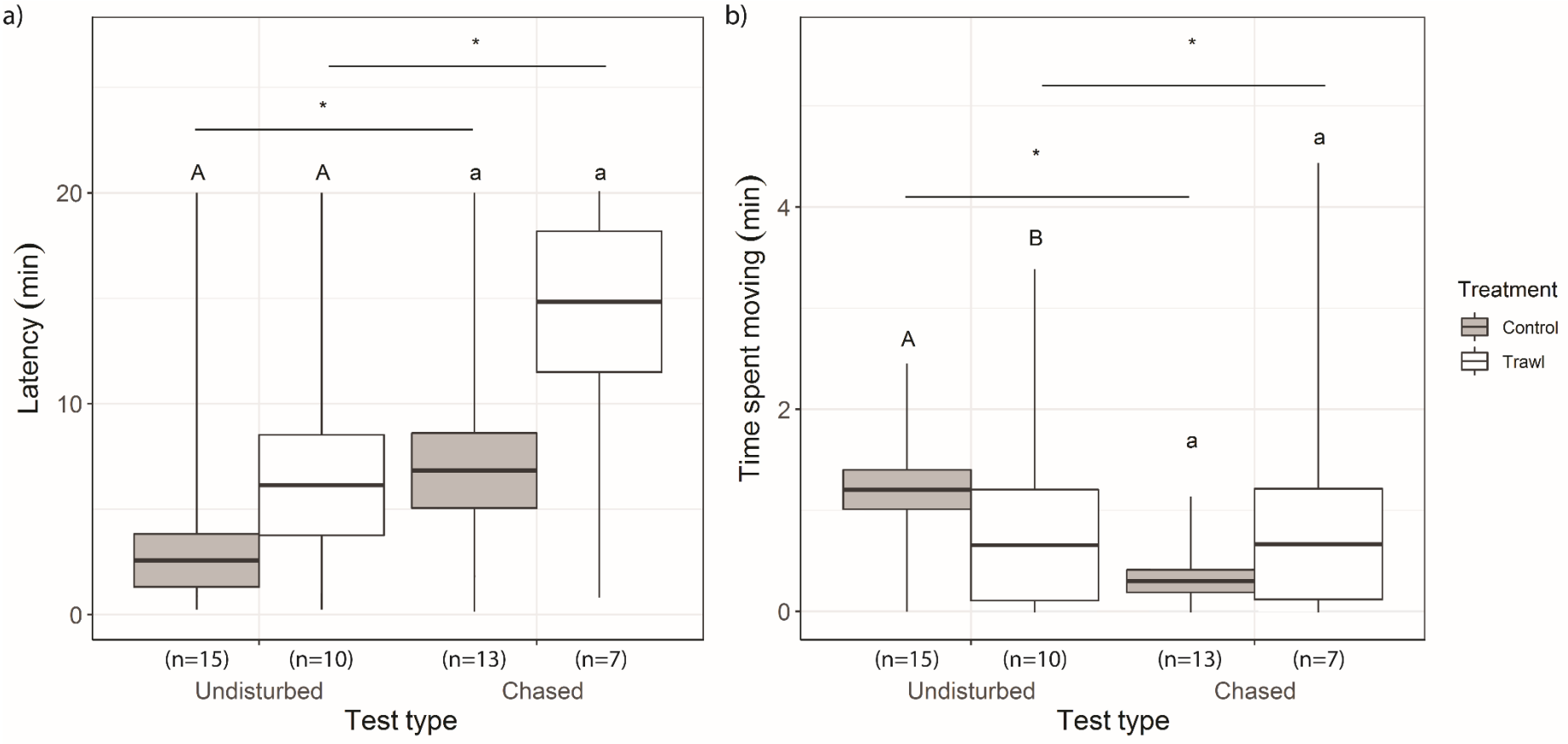

Figure 6.7. Mean ± SE time elapsed before the first movement (Latency time; min) (a) and total time spent moving around the experimental tank (Total moving time; min) during the first 5 min of the test (b) recorded in Control (grey boxes) and Trawl (white boxes) neonates (Treatment) during the Undisturbed and the Chased Test (Test type). Different uppercase and lowercase letters indicate significant differences between Treatments, within the Undisturbed and within the Chased test respectively. * indicates significant differences between different Test types within Trawl or Control neonates. Sample sizes are reported in parenthesis. Sample size are sometimes smaller than the total number of neonates tested (Control = 16 and Trawl = 11) because some neonates buried before the end of the observation period for the boldness evaluation (5 min) and were therefore excluded from the analysis.

No difference was detected in Movement success between treatments in the Undisturbed test (Fisher's test p = 1), while Control neonates had a significantly higher Movement success in the Chased test (Fisher's test p = 0.04). No difference in Latency time was found between Treatments in the Undisturbed test ($F_{1,23}$ = 0.98, p = 0.32) or the Chased Test ($F_{1,18}$ = 1.19, p = 0.27). In the Undisturbed test, Control neonates had a significantly higher Total moving time compared to Trawl neonates ($F_{1,16}$ = 4.09, p = 0.04) while no difference emerged in the Chased test ($F_{1,19}$ = 0.69, p = 0.41).

Comparing Movement success between the two types of test, no difference was found within Control neonates (Fisher's test p = 0.39), but within Trawl neonates (Fisher's test p = 0.01) success was significantly lower in the Chased test. Within both Control ($F_{1,26}$ =22.77, p < 0.0001) and Trawl neonates ($F_{1,15}$ = 16.44, p < 0.0001), neonates showed a lower Latency time in the Undisturbed test. Test type significantly affected also Total moving time in both Control ($F_{1,23}$ = 293.65, p < 0.0001) and Trawl neonates ($F_{1,12}$ = 11.95, p < 0.0001). Control neonates moved for a longer time when tested in the Undisturbed test, while the opposite was observed for Trawl neonates. This contrasting result seems to be driven by a single Trawl neonate that in the Chased test largely increased its Total moving time, while four neonates reduced it.

6.4. DISCUSSION

Given that successful reproduction is essential in maintaining sustainable populations, studying the reproductive consequences of stress is important, especially for chondrichthyans given their poor conservation status (Dulvy et al., 2014) and low reproductive rates (Camhi et al., 1998). To contribute to addressing the need for improved understanding of the intergenerational effects of capture stress in chondrichthyans, we evaluated the consequences of maternal exposure to fishing-capture stress persisting in neonates up to one month after birth.

At birth, neonates born from trawled mothers (stressed neonates) had lower body mass than control neonates. This was previously reported by Guida et al. (2017) who, in contrast to our study, also observed a smaller total length in stressed neonates, the difference likely resulting from differences in stress timing. While in Guida et al. (2017) trawling was simulated 40–86 days before parturition, in our study, it occurred as little as 1 day before. Stressors applied so close to parturition, once embryonic development had almost concluded, would be unlikely to affect total length, but could have impacted body mass, a more plastic trait.

In our study and that of Guida et al. (2017), neonates born from stressed mothers had higher G:L ratios than control neonates, suggesting a state of stress (Van Rijn and Reina, 2010) persisting in the offspring after trawling simulation and lasting at least till birth, potentially due to neonate exposure to maternal stress hormones (Skomal and Mandelman, 2012). The increase in G:L ratio is an important response to stress that minimises the chances of infection by mobilizing different leukocyte populations towards areas of the body with a higher probability for pathogen entrance (Van Rijn and Reina, 2010; Heard et al., 2014). However, a continuous alteration of the immune strategy might induce an immunosuppressed state impairing disease resistance and causing a generally lowered health status (Wendelaar-Bonga, 1997). Additionally, immune responses are energetically expensive (French et al., 2007) and a prolonged reaction might impact the energy reserves, reducing the amount the neonates can allocate to other biological activities.

At birth, compared to control neonates, stressed neonates had smaller yolk sacs and the difference became more pronounced as body mass increased. Alterations in energy allocation to oocytes during vitellogenesis are suggested as the cause of the reduction in offspring energetic reserves (yolk sac and oil globules) observed following parental exposure to stress in Atlantic salmon (*Salmo salar*; Eriksen et al., 2006) and damselfish (*Pomacentrus amboinensis*; Gagliano and McCormick, 2009). In our study, stress could not have impacted vitellogenesis because capture

was simulated after its conclusion. The increased energy allocation towards the immunological response (French et al., 2007) hypothesized for stressed neonates, is a more likely explanation for the reduction in yolk sac volume. The smaller residual yolk sac reserves might impact stressed neonate fitness given that these reserves sustain offspring in the first period after birth and a decreased quantity of yolk likely slows growth rate (Rothschild, 1986) and reduces the length of the starvation period that offspring can endure (Miller et al., 1988; Leggett and Deblois, 1994).

Compared to control neonates, stressed neonates manifested a smaller increase in total length over time. In contrast, in teleost fishes, stressed neonates manifest faster growth (Li et al., 2010; Eriksen et al., 2013; Nesan and Vijayan, 2016), but the opposite response we observed, given that our study is the only report for chondrichthyans, cannot yet be ascribed to taxonomic differences. Stressed and control neonates of *T. dumerilii* showed a similar rate of yolk sac consumption. Despite stressed neonates using the same amount of resources, this is fuelling a smaller increase in length, suggesting that energy is allocated to different activities, such as prolonged stress or immunological responses (French et al., 2007). Such alteration in energy allocation, occurring to the detriment of growth, might present severe drawbacks because faster growth promotes a more rapid passage through critical life stages when small body size is associated with slower swimming performance, thus impaired prey capture and predator escape abilities (Bergenius et al., 2002; Wilson and Meekan, 2002). Body mass did not change with time in our control or stressed neonates, possibly because neonates did not consume much food (LF personal observation) and growth was mainly supported by the energy acquired from the yolk sac, further confirming the importance of these reserves for neonate sustenance.

Given that two stressed neonates were born only one day after maternal exposure to fishing-capture stress, we recognize that there might not have been enough time for the stress consequences to impact some neonate morphological traits, masking potential differences. In

contrast, more plastic traits, such as behavioural, physiological and immunological responses (Mandelman and Skomal, 2009; Frick et al., 2010; Harris et al., 2010; Van Rijn and Reina, 2010), would have likely been impacted despite the short time elapsed from the stressful event. If we exclude these neonates from the analysis, yolk reserves consumption rate was slower in stressed neonates as also observed in offspring of stressed *S. salar*, likely a response originating from the need to conserve the maternally-derived reserves (Eriksen et al., 2006). Nevertheless, we decided to include these neonates in all analysis and accept the results as such to avoid drawing untested assumptions and to maintain a more balanced sample size.

In teleosts, maternal stress alters offspring swimming variables (Sopinka et al., 2014, 2016b). Similarly, our stressed *T. dumerilii* neonates swam for shorter distances after a simulated predator attack. This species relies on camouflage and burying to escape predators (LF personal observation) and animals' inability to distance themselves enough from predators, thus remaining in their field of view, might increase predation risk. Completion speed is a measure of the combination of swimming speed and neonate responsiveness to predator simulation (i.e., willingness/reluctance to move), which relates to predator escape abilities (Gyssels and Stoks, 2005). Neonate completion speed was not affected by the trawling stress experienced by the mothers, but it was positively correlated with neonate total length. Stressed neonates have a slower growth rate and a difference in completion speed might appear later in life when differences in total length emerge, and should be further investigated. Indeed, impaired swimming performance, and specifically reduced completion speed with the associated delayed response to predator and reduced swimming speed, might impact prey capture and predator escape abilities (Sopinka et al., 2016b).

No difference emerged between control and stressed neonates in the success with which they buried themselves or in the time taken before burying, indicating that burying ability is not

impacted by maternal stress or is not a reliable indicator of a stressed state. After a simulated predatory attack (Chased test), both groups of neonates delayed burying and control neonates manifested also a lower burying success. Burying is an essential anti-predatory tactic (Hobson, 1965; Stein and Magnuson, 1976; Couffer and Benseman, 2015), but if a predator observes the burying event the benefit of hiding is lost. It seems that neonates perceived the chasing procedure as a predatory attack and postponed or avoided burying, perhaps waiting for the predator to leave the area. Stressed neonates, not reducing their burying success, might experience a higher predation risk. Indeed, it is known that fishes can modify their behaviour in response to the presence of predators so as to reduce the chances of being preyed upon (Harris et al., 2010) and stressed *T. dumerilii* neonates, not reducing their burying success, might experience a higher predation risk.

Spending a higher amount of time moving around the tank (Total time moving in the Undisturbed test) and with a higher proportion of animals that started movements (Movement success in the Chased test), control neonates manifested behaviour consistent with higher boldness. In contrast, teleost offspring produced by stressed parents manifested an increased boldness (Sopinka et al., 2015; Best et al., 2017). Higher boldness allows exploring larger areas, increasing chances of finding food and resources, suggesting a competitive advantage for control *T. dumerilii* neonates. Nevertheless, the higher activity associated with higher boldness might also increase the chances of being located by predators (Martel and Dill, 1993; Fuiman and Magurran, 1994; Archard and Braithwaite, 2003), and, for control neonates, the advantages of easier access to resources might be offset by higher predation risk. However, both stressed and control neonates, when chased, potentially recognising the presence of a predator, adopted a more cautious behaviour reducing boldness by increasing Latency time and reducing Movement success and Total moving time, and the behavioural difference observed between undisturbed neonate

groups disappeared, eliminating any potential higher vulnerability of control neonates to predation risk. Despite control neonates not decreasing movement success when chased, they delayed the first movement, likely waiting to ascertain whether the predator left the area. Similarly, in perch (*Perca fluviatilis*), animals experiencing higher predation occurrence show decreased boldness indicated by an increased time spent in a refuge, an increased latency to start feeding and a reduced duration of the feeding event (Magnhagen and Borcherding, 2008).

Methodologies similar to the one used in this experiment have been previously adopted in other studies to simulate trawling (Frick et al., 2010; Guida et al., 2017; Martins, 2018). The fishing-capture treatment towing the animals individually for 7 h is slightly longer than typical commercial durations of fisheries in the general area of our study, which range from 4–6 h (Frick et al., 2010). However, the treatment excludes a variety of other highly variable stressors such as compression, injury, and associated hypoxia from the presence of other animals and debris in the cod-end, and environmental changes in temperature and pressure, which relate to towing speed and environmental and weather conditions dependent on locality, depth, and time of year. Similarly, the conditions encountered after the end of the capture simulation are mild, with an absence of predators and occurring in an optimal environment with abundant food. All this considered, and despite longer tow durations, our capture simulation was unlikely to have overstressed the animals, rather we think it represented a best-case scenario compared to commercial procedures. Moreover, despite being a mild to moderate trawl treatment, this capture simulation measurably affected neonate traits, indicating the importance of these first data and the need to investigate the effects on offspring fitness from some of the more extreme conditions that arise during normal trawl operations.

A trawling capture stress experienced by near-term pregnant *T. dumerilii* neither induced premature parturition or abortion, nor increased the occurrence of stillborn neonates.

Nevertheless, despite the capture simulation occurring shortly before parturition, when embryonic development was almost concluded, capture stress still impacted neonate traits, potentially affecting their competitiveness and survival. *Trygonorrhina dumerilii* is a lecithotrophic viviparous species (Marshall et al., 2007) and, during development, embryos rely solely on yolk sac reserves. In matrotrophic species, mothers supply additional nutrients as histotroph to the embryos (Hamlett et al., 2005) and reproductive consequences will likely be more severe. Indeed, due to the reduced energy allocation to pregnancy observed in *T. dumerilii*, together with potentially affected oxygen delivery to the embryos and waste removal (Chapter 3), the embryos of stressed matrotrophic species are to experience the additional burden of reduced supplies of nutrient. Moreover, being a resilient species (Martins, 2017), consequences in *T. dumerilii* are probably milder than those that might be observed in species with higher sensitivity to fishing-capture stress (Dapp et al., 2015).

Trygonorrhina dumerilii is not classed as at high extinction risk (IUCN, 2020) and the observed neonatal impairments might seem of minor concern to this species from a management perspective. However, taxonomically close species, such as the shortnose guitarfish (*Zapteryx brevirostris*) and the spotted shovelnose ray (*Aptychotrema timorensis*), have poor conservation status (IUCN, 2020) and the observations recorded in our study might be helpful for their management. Although more investigation is needed to confirm whether the alterations we observed affect neonate survival in the natural environment, the potentially severe outcomes hypothesized call for a precautionary approach to fishing effort management. In teleosts, and potentially also in chondrichthyans, a reduction in the severity of the fishing-capture stress can lessen some of the observed reproductive consequences (Pettit, 1977; Booth et al., 1995; Lowerre-Barbieri et al., 2003; Hall et al., 2009, 2017). Therefore, reductions in fishing time, air exposure periods and handling, and the adoption of best handling and releasing practices (AFMA,

2014), are measures that could be easily implemented to reduce stress load and likely the severity of its reproductive consequences. Actions to reduce the likelihood of encountering and/or capturing pregnant females at certain times and using gear or handling practices to minimise stress in areas where they are in high abundance would also be beneficial. Ultimately, additional to quantitative measures of the direct effects of fishing-capture stress on the survival of released animals, the effects of stress on the reproductive capacity of populations also need to be accounted in population dynamics and risk assessment models used for assessing fishery sustainability (Punt and Walker, 1998; Pribac et al., 2005; Walker, 2005b, a) to develop effective management measures.

6.5. CONCLUSIONS

Unlike fishing-induced parturition, the neonatal consequences observed in this study will probably go unnoticed in the absence of specific research, highlighting the need to investigate reproductive consequences of fishing-capture stress in more species, characterized by varying sensitivity to stress and different reproductive modes. Alterations in offspring foraging skills, stress response, metabolic rate, cardiac performance and reproductive output after fishing-capture stress experienced by the parents should also be researched. Despite the merit of having highlighted impairments to neonate growth, swimming and behaviour, to understand the full extent of the consequences of fishing-capture stress, the impact that alterations observed in captive conditions have on survival, recruitment and fitness of neonates in the natural environment must be evaluated.

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Chapter 7. GENERAL DISCUSSION

Capture-induced energetic and reproductive impairments are an overlooked area of research in chondrichthyans and the results of my study contribute to improve our understanding of the long-term consequences of fishing-capture stress. The observed energetic and reproductive impairments point to potentially severe consequences that could reduce survival and reproductive output of the captured animals and recruitment of the neonates born from stressed mothers. These data are important and should be included in population models to obtain a sound assessment of fishery sustainability and to develop effective management strategies. A summary of my study's main aim, objectives, main results and implications is presented in Figure 7.1.

7.1. OBJECTIVE 1: IDENTIFY CAPTURE-INDUCED REPRODUCTIVE CONSEQUENCES

Despite the importance of successful reproductive events in maintaining population abundance (Schreck et al., 2001; Sopinka et al., 2016), up until now, data on reproductive impairments caused by fishing-capture stress in chondrichthyans have been limited to observations of premature parturition or of neonate traits recorded at birth (Guida et al., 2017; Adams et al., 2018). The results of my experiments highlighted that several other neonate traits are impacted, once again confirming the similarity in stress consequences between teleosts and chondrichthyans.

Neonate *T. dumerilii* born from mothers experiencing capture stress during pregnancy showed a smaller size at birth, as previously reported by Guida *et al.* (2017), and also had a slower growth rate despite consuming the yolk sac reserves at the same rate. Smaller size is disadvantageous given that predation in the aquatic environment is usually size-dependent (Kamler, 2005) and slower swimming speeds are associated with smaller sizes, reducing both predator-escape and prey-capture skills (Miller et al., 1988; Leggett and Deblois, 1994; Sogard, 1997). Moreover, the

swimming performance of stressed neonates was impaired and their boldness was reduced. A lower boldness reduces the chances of finding food and resources, impacting animals' competitiveness (Martel and Dill, 1993; Fuiman and Magurran, 1994; Archard and Braithwaite, 2003). Along with the hypothesized reduced foraging ability related to small size, stressed neonates were also born with a smaller residual yolk sac. Yolk sac reserves are an essential resource to sustain neonate growth following birth and smaller amounts are associated with lower growth rates (Rothschild, 1986) and shorter starvation periods that neonates can survive (Miller et al., 1988; Leggett and Deblois, 1994). Together these findings point to a potential reduction in neonate survival that might ultimately impact recruitment and population abundance (Schreck et al., 2001). These results pose a challenge on how to account for reduced survival and sub-lethal effects of fishing diminishing the fitness of newborn chondrichthyans in population dynamics and fishery assessment models but are essential to improve predictions (Punt and Walker, 1998; Pribac et al., 2005; Walker, 2005a, b).

In *T. dumerilii*, due to the triggering of a metabolic decline by the trawling-capture simulation (Chapter3; see below for further discussion), metabolic rates estimated post-parturition are similar to values recorded in pregnant females immediately after fishing-capture stress, suggesting a possible capture-induced suppression of the energy allocation to activities involved in sustaining pregnancy. Among other causes, it is likely that the fishing-induced consequences recorded in neonates are originating from the metabolic decline and the hypothesized suppression of energy allocation to pregnancy sustenance observed in their mothers. A large part of the cost of pregnancy is allocated to the maintenance of suitable *in utero* conditions. The maintenance includes eliminating the metabolic waste produced by the embryos and supplying them with oxygen (Carrier et al., 2004; Hamlett et al., 2005), the latter likely being the main energetic expense (Boehlert et al., 1991). Moreover, pregnant females have to sustain the increased cost of

swimming (Makiguchi et al., 2017). The hypothesized reduced allocation of energy to pregnancy following the trawling-capture simulation might reduce the delivery of oxygen to the embryos, potentially reducing their growth and the development of different organs, and might slow down the removal of toxic byproducts of metabolism that could affect the health of embryos (Hamlett et al., 2005). Metabolic recovery has not been investigated in *T. dumerilii*, but if the recovery time were similar to the one observed in *C. milii* (Chapter 4), Inactive MR and the energy supply to pregnancy will remain suppressed for up at least seven days. For species characterized by rapid embryonic development, as for *T. dumerilii* (Marshall et al., 2007), the hypothesized 7-day suppression of energy to pregnancy will likely severely impact this process and is, along with the exposure of embryos to maternal stress hormones, one of the probable causes of the observed neonatal consequences.

7.2. OBJECTIVE 2: QUANTIFY CAPTURE-INDUCED CHANGES IN ENERGY EXPENDITURE

In Port Jackson sharks (*Heterodontus portusjacksoni*), gillnet capture caused an increase in energy consumption of 13.3% at rest and 25.5% while swimming. These results match observations in other species of sharks (Bouyoucos et al., 2017, 2018) and confirm the impact that fishing-capture stress can have on the amount of energy available for other biological activities, that ultimately can cause measurable reductions in growth and fecundity (Watson et al., 2020). These observations further confirm the similarity in the responses to stress between teleosts and chondrichthyans given that an increase in energy expenditure is the common consequence recorded in teleost fishes exposed to fishing capture (Brick and Cech, 2002; Clark et al., 2012; Cooke et al., 2014).

Unexpectedly, after fishing-capture simulation, a decrease in the oxygen consumption was observed in the gummy shark (*Mustelus antarcticus*), 42.6% at rest and 55.5% while swimming, in the southern fiddler ray (*Trygonorrhina dumerilii*), 32.1% while active, and in the elephant fish

(*Callorhinchus milii*), 43.1% at rest and 27.5% while swimming. This is the first report of metabolic decline caused by fishing-capture stress both for teleosts and chondrichthyans. However, metabolic depression, a similar response, has been frequently reported in cases of naturally severe environmental stress, including drought, salinity changes, temperature changes and low oxygen concentration, both in teleosts (Hand, 1996) and in chondrichthyans (Mulvey and Renshaw, 2000; Routley et al., 2002; Morash et al., 2016; Tunnah et al., 2016). This depression serves to reduce the energetic allocation to non-essential activities such as movement, preserving it for life sustenance and thereby increasing survival probability (Renshaw et al., 2002). On the other hand, this decrease in metabolic rate, sacrificing non-essential activities, potentially affected reproduction given that, in pregnant *T. dumerilii*, fishing-capture stress decreased metabolic rates to values similar to those estimated in females post-parturition, suggesting a possible suppression of the energy allocation to the sustainment of pregnancy. This energetic alteration, potentially associated with impairments in oxygen provisioning to the embryos and metabolic waste removal (Hamlett et al., 2005), is likely to cause embryonic impairments. Indeed, along with other mechanisms, the change in energy allocation is likely one of the causes of the neonatal impairments recorded in this species after pregnant females were exposed to trawling simulation (Guida et al., 2017; Chapter 6). Similarly, the reduced allocation of energy to sustain movements caused a significant decrease in swimming activity in *C. milii* that might increase predation occurrence when animals are released (Ryer, 2004; Raby et al., 2014).

Moreover, in all species manifesting metabolic decline, a reduction in the Inactive MR (a proxy for standard MR, SMR) was observed, and, given that this component of the energy expenditure is the one associated with life sustenance, any decrease might have negative consequences (Chabot et al., 2016). The suppression of Inactive MR persisted for up to seven days after fishing-capture simulation in *C. milii*. Previous studies reported a maximum estimated full recovery time of 19.8 h

from a capture-induced increase in metabolic rate for lemon sharks *Negaprion brevirostris*, and blacktip reef sharks *Carcharhinus melanopterus* (Bouyoucos et al., 2017, 2018). On the other hand, metabolic rates were still depressed 24 h after the osmotic stress that caused the initial metabolic depression in school shark (*Galeorhinus galeus*) and *M. antarcticus* (Morash et al., 2016; Tunnah et al., 2016). These observations seem to confirm that in contrast to recovery from an increase in metabolic rate, metabolic decline is longer lasting. Given that the components of the metabolic rate that remains suppressed in *C. milii* are associated with activities fundamental to sustain life, the prolonged depression might have severe, long-term consequences on growth, health, life-span and survival (Chabot et al., 2016).

7.3. OBJECTIVE 3: VARIABILITY OF THE ENERGETIC RESPONSES TO FISHING-CAPTURE STRESS

Investigating the energetic responses in several species exposed to different fishing capture scenarios revealed contrasting metabolic responses of both an increase and a decrease in the energy expenditure, which are likely related to different species sensitivity and differences in severity of the stress elicited by the various fishing gears. Animals initially respond to the fishing-capture stress investing more energy in sustaining the stress response and restore homeostasis (Mulvey and Renshaw, 2000; Routley et al., 2002; Bouyoucos et al., 2017, 2018), but, when the stress is too prolonged or when it exceeds an animal's ability to tolerate it, the initial excessive energy use triggers the metabolic decline to preserve the remaining energy resources in order to sustain vital activities (Renshaw et al., 2002). The initial increase in energy use likely went unobserved in *M. antarcticus*, *T. dumerilii* and *C. milii* because it occurred when animals were still trapped in the net. Among the studied species, *H. portusjacksoni*, a resilient species (Frick et al., 2010a, 2010b), showed an increase in metabolic rate when exposed to gillnet simulation. On the other hand, the same fishing capture gear caused a decrease in metabolic rate in *M. antarcticus* and *C. milii*, two fairly sensitive species (Frick et al., 2009, 2010a; Martins et al., 2018). Despite

being a resilient species (Guida et al., 2017; Martins, 2017), *T. dumerilii* showed a metabolic decline, but in this case animals were exposed to trawling simulation, a much more severe fishing-capture stress compared to gillnet capture (Dapp et al., 2015).

As previously observed, both increased and decreased metabolic rates can have severe repercussions for other biological activities, diverting energy from these activities, in the first case, or temporarily suppressing them to save energy, in the second case. However, animals that manifested metabolic decline also experienced an initial increase in energy consumption that was unsuccessful in restoring homeostasis due to the severity of the stress suffered. Therefore, the metabolic decline seems to be the last option available for animals to avoid incurring even more severe, life-threatening consequences and is likely associated with more serious repercussions than those connected to the temporary increase in metabolic rate alone.

Investigating energetic responses in species belonging to the shark, ray and holocephalan taxonomic groups allowed recognizing that the metabolic decline is a response common to all the main chondrichthyan groups, originating after severe fishing-capture events. Given the similarity of teleost and chondrichthyan responses to stress (Wendelaar-Bonga, 1997; Skomal and Mandelman, 2012), metabolic decline might also characterize the teleost response to severe fishing-capture stress yet to be observed. Indeed, the simulated fishing-capture conditions that caused the increase in metabolic rate observed in the various energetic studies were mild (Brick and Cech, 2002; Clark et al., 2012; Cooke et al., 2014) and might not have been severe enough to trigger the metabolic decline.

7.4. IMPLICATIONS FOR FISHERY MANAGEMENT

Most of the species I investigated are not at risk of extinction (IUCN, 2020) and the detected severe energetic and reproductive impacts might seem of minor concern; nevertheless, understanding these impacts is fundamental for the management of other endangered species.

Indeed, having demonstrated that species of all three main chondrichthyan groups respond similarly to severe fishing-capture stress suggests that the energetic responses and associated impairments to reproduction observed in the studied viviparous species are a common feature of all chondrichthyans. Therefore, the severe consequences experienced by threatened species will likely substantially affect survival, recruitment and population abundance, calling for management measures and reliable fishery sustainability assessment. In teleosts, the incorporation of the energetic consequences of a capture event in statistical models has proven useful to predict the consequent loss in growth and fecundity (Watson et al., 2020) and is a promising approach for chondrichthyan population studies. The predictions obtained will be necessary information for the development of accurate population dynamics and risk assessment models to evaluate fishery sustainability. Unfortunately, data on the energetic and reproductive consequences of fishing-capture stress in chondrichthyans are still scarce and further research is needed.

While waiting for more specific information, a precautionary approach should be applied by developing management measures aimed at avoiding or reducing the severity of the consequences. Both viviparous (Walker, 2005b; Marshall et al., 2007; Walker, 2007) and oviparous (Tovar-Ávila, et al., 2007; Bell, 2012) species, particularly those inhabiting the continental shelf, have reproductive cycles highly synchronized among the animals in the population. This synchronization provides the opportunity for strategically designed closed areas and closed seasons aligned with the critical periods of the reproductive cycles to minimise reproductive impairments (de Sousa Rangel et al., 2020). For example, closing areas where pregnant female occurrence is high or areas where animals aggregate to give birth or lay eggs will reduce the likelihood of reproductively-active animals being captured. However, this might not be applicable where these areas overlap with areas important for fishing activity. Nevertheless, the suggestions that the consequences of a metabolic decline are more severe than those of the transitory

increase in metabolic rate suggest that, at least, it should be prevented from reaching the point when the metabolic decline is triggered. Given that metabolic decline seems to be caused by the most severe stressors, reducing the stress load animals suffer during capture might lessen its long-term consequences. Similarly, data from teleosts (Booth et al., 1995; Hall et al., 2009; Chapter 2) and chondrichthyans (Manire and Rasmussen, 1997) confirm that mild fishing-capture operations do not cause or cause only minor reproductive consequences. A growing body of research, confirmed also by my results, highlights that different types of fishing gears (Dapp et al., 2015) and different fishing conditions (Frick et al., 2010b; Heard et al., 2014; Martins, 2017) elicit stress with different severity. Generally, reducing the duration of fishing capture, of air exposure and handling and adopting best handling and releasing practices all reduce the severity of the stress suffered by the captured animals (Frick et al., 2010a, 2010b; AFMA, 2014; Heard et al., 2014; Martins, 2017; Campbell et al., 2018; Martins et al., 2018) and could be easily applied as a precautionary approach to lessen the energetic and reproductive consequences of fishing-capture stress. A precautionary approach is recommended particularly for those fisheries that record catches with a high occurrence of sensitive species, manifesting the most severe responses, and catches of threatened species, suffering most from the negative consequences on population abundance.

7.5. RESEARCH LIMITATIONS AND FUTURE WORK

Despite the importance of the energetic consequences reported in my research, further work is needed to confirm whether the metabolic decline follows an initial increase in energy expenditure in case the experienced stress is too severe to reestablish homeostasis. Investigating the energetic responses of the same species exposed to different fishing capture procedures, specifically measuring the responses of *H. portusjacksoni* exposed to a more severe capture event such as a trawling simulation, and of *T. dumerilii* exposed to a less stressful fishing-capture simulation such as longlining, could help clarify the issue. A constant monitoring of the MR of

animals, starting while they are still trapped in the net, will be also important. Moreover, combining blood and tissue analysis with MR estimates will help understand the mechanisms triggering the metabolic decline, such as potential thresholds in pH level and the energetic charge of muscle, brain and liver cells (Reipschläger and Pörtner, 1996; Guppy and Withers, 1999; Renshaw et al., 2002; Guida et al., 2016). Metabolic recovery time should be studied in a larger number of species, investigating shorter time frames and prolonging the observation period beyond seven days after the fishing-capture stress to record the complete recovery in Inactive MR. It is also necessary to confirm whether the reduced swimming activity observed in *C. milii* following the reduction of the energy expenditure in non-essential activities is associated with an increased predation occurrence when animals are released in the natural environment. The use of acoustic tags is a feasible, relatively cheap method to monitor animals' movement in confined areas (Martins, 2017) and might be used to monitor *C. milii* long-term survival when they are captured and released during their migration to coastal areas and inshore bays during the egg-laying season (Last and Stevens, 2009; Bell, 2012; Didier et al., 2012).

Despite the importance of having identified several alterations in traits of neonates born from mothers exposed to stress during pregnancy, any impairment to neonate survival needs to be evaluated in the natural environment if the full extent of these consequences and of fishing-capture stress is to be understood. Tagging neonates before releasing them in Swan bay and conducting surveys to identify the neonates in the following months and years might allow quantifying any difference in neonate survival associated with stress experienced by the mother. Another useful approach might be using large mesocosms in which to release the neonates and observe their fate. The reproductive consequences of fishing-capture stress need to be investigated in a larger number of species, prioritizing those characterized by different reproductive modes. Impairments in matrotrophic species (Hamlett et al., 2005) will likely be more

severe given that maternal nutrient supply to embryos might be impacted by the suppression in energy allocation to pregnancy. The effects of capture and handling stress are likely to have the highest impacts on lecithotrophic species during oogenesis and on matrotrophic species during embryogenesis. Thus, studying the effects of stress experienced during these reproductive periods should be prioritized. In oviparous species, maternal stress hormones might be transferred to the gelatin surrounding the eggs during the encapsulation in the egg case (Rubolini et al., 2005) and investigating the consequences of a fishing-capture stress suffered by females during the egg-laying season on hatchlings is also important. The effect of fishing-capture stress also ought to be investigated in association with the exposure to values of seawater temperature and pH foreseen for the end of the century. Indeed, increased temperature and decreased pH can alter several hatchling traits when shark and ray eggs are exposed during embryonic development (Di Santo, 2016; Rosa et al., 2017; Di Santo, 2019) and might act in synergy with the potential effects of fishing capture stress. Different anthropogenic stressors should be investigated together to obtain data that could support the development of reliable ecological vulnerability risk assessments (Chin et al., 2010; Wheeler et al., 2020; Walker et al., in review). Ultimately, all this information on reproductive and energetic consequences will be applicable to develop stock assessment and population dynamics models, in order to assess whether fishery exploitation and bycatch are sustainable or if management measures are needed to improve the conservation status of chondrichthyan populations (Punt and Walker, 1998; Pribac et al., 2005; Walker, 2005a, b).

7.6. REFERENCES

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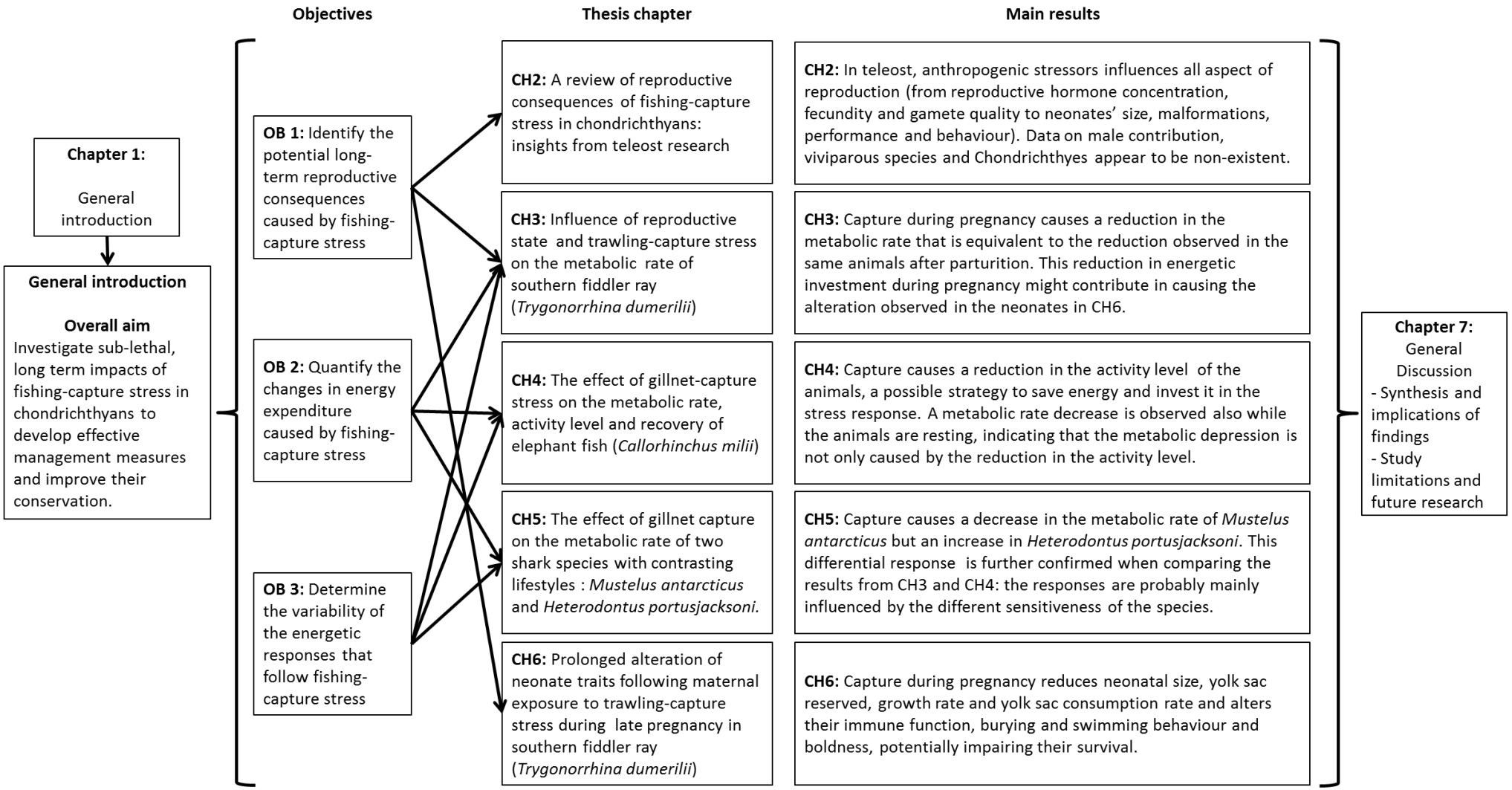

Figure 7.1. Scheme of the study main aim, objectives, main results and implications.

APPENDIX I

SUPPLEMENTARY MATERIAL FOR CHAPTER 3

ADDITIONAL STATISTICAL METHODS

Inactive MO_2 calculation

As suggested when no information on animals' swimming or resting activity is available (Clark et al., 2013; Chabot et al., 2016), we calculated an estimate for Inactive MO_2, a proxy for the energy consumption associated with the activities needed for life sustenance (SMR). Using only dissolved oxygen concentration data (DO) measured in the Post-partum MO_2-class, and separately for each animal, Inactive MO_2 was calculated as the mean of the lowest normal distribution of MO_2s calculated for each 1-min time slot (MR_T) for all the eight measurement cycles pooled together (Chabot et al., 2016). These MO_{2T} values were calculated using the formula:

$$MO_{2T} = [(\Delta DO \times 60) - B] \times Vol \times BM^{-1}$$

where MR_T is the metabolic rate expressed as $mgO_2\ h^{-1}\ kg^{-1}$, ΔDO is the decline in DO occurring in the 1-min time slot (equal to the difference between the DO concentrations measured at the beginning and at the end of the 1-min time slot) expressed as $mgO_2\ L^{-1}min^{-1}$, 60 is the factor by which this value need to be multiplied as to obtain a standardized measure of the decrease in DO occurring in a 1-h period, B is the background respirometry expressed as $mgO_2\ L^{-1}\ h^{-1}$, Vol is the volume of water contained in the respirometry chamber in L and BM is the body mass of the female in kg. Given that MO_2 is strongly influenced by temperature (Schmidt-Nielsen, 1997), calculated MO_{2T} was temperature-corrected to 17°C, the average temperature recorded during the experimental period, using $Q_{10} = 2.3$ (Neer et al. 2006; Whitney et al. 2016).

Relationship between Inactive MO_2 and BM

The normality and homoscedasticity of the Inactive MO2 data were assessed with a Shapiro test and a Levene test, respectively. The relationship between body mass and MO_{2T} was investigated, after log transformation of the data, using a linear model that included body mass as a fixed effect.

Influence of Group on Inactive MO_2

The influence of Group (Trawl or Control) on Inactive MO_2 was investigated using a GLME, including the Group as a fixed effect and the ID of the animal as a random effect (MO_2 ~ Group + (1|ID)).

Results are reported as mean ± standard error (SE). All tests used a significance level of $\alpha \leq 0.05$. Data were processed using R statistical software (R Core team 2019).

Activity cost

The cost of activity (CA_P) was calculated subtracting Inactive MO_2 values, calculated as described above, from Post-partum MO_2, calculated in the main text, dividing the results by Inactive MO_2 and multiplying it by 100 to obtain the percentage increase in energy consumption needed to switch between resting and active state (Whitney et al., 2016).

RESULTS

Treatment group (Control or Trawl) had no significant effect on the values of Inactive MO_2 measured (Control: 35.04 ± 4.52; Trawl: 53.18 ± 9.10; $F_{1,11} = 0.36$, $p = 0.55$).

The cost of activity (CA_P) was an 88.8% increase compared to Inactive MO_2.

DISCUSSION

Baseline metabolic data are essential for ecological (Hove and Moss 1997; Dale et al. 2013; Di Santo and Kenaley 2016; Whitney et al. 2016), behavioural, bioenergetics (Dale et al. 2013) and

trophic studies (Brett and Blackburn 1978; Bushnell et al. 1989; Hove and Moss 1997). Given the paucity of baseline metabolic data existing for elasmobranch, we aimed at discussing Post-partum MO_2s (calculated in the main text), proxies for resting routine MR (RRMR), and the calculated Inactive MO_2 in relation to values available for other chondrichthyan species. Due to some unavoidable limitations in the measurement methodology (discussed below), these must be considered only rough estimates of baseline MO_2, nevertheless they might represent important data that can be used until more reliable estimates of MR are obtained.

Given the allometric, non-linear relationship describing the scaling of MR with body mass, MO_2 are usually mass-corrected (Clarke et al., 1999; Brown et al., 2004; Whitfield, 2004). No agreement exists on a common mass-exponent for all chondrichthyans and the few data available indicate wide variation between different species (Lawson et al., 2019). The size range of *T. dumerilii* females in our study was too narrow (Table 3.1, main text) to detect a significant relationship between MO_2 and body mass and calculate a specific mass exponent. We avoided applying a mass-exponent determined for a species other than *T. dumerilii* because it might bias our MR estimates. At 17°C, the mass-specific RRMR was 84.61 ± 4.74mgO_2 kg^{-1} h^{-1} and Inactive MO_2 was 44.81 ± 5.75 mgO_2 kg^{-1} h^{-1}. These MR estimates are at the lower end of the range of those reported for other batoid species (Table S3.1: MRs were temperature corrected to 17°C using Q_{10} = 2.3; Brett and Blackburn, 1978; Carlson et al., 1999; Neer et al., 2006; Whitney et al., 2016), being similar to values measured in sedentary species (Eastern shovelnose ray *Aptychotrema rostrata*, Speers-Roesch *et al.*, 2012; little skate *Leucoraja erinacea*, Di Santo and Kenaley, 2016 and clearnose skate *Raja eglanteria*, Lauder et al., 2015) and lower than values characteristic of active swimmers (Cownose ray *Rhinoptera bonasus*, Grim et al., 2012 and common eagle ray *Myliobatis aquila*, McEachran, 1990). Being a sedentary species (Last and Stevens, 2009), *T. dumerilii* MRs reflect its ecology. The measurements of RRMR were not obtained from a period of constant movement, but rather from sporadic swimming events.

Nevertheless, *T. dumerilii* CA_P is high probably because sedentary species incur a substantial increase in MR even after very brief periods of movement (Bushnell et al., 1989). Accordingly, the CA_P was well above values of active swimmers (Pelagic stingray *Pteroplatytrygon violacea* and *M. aquila*, McEachran, 1990), while it fell within the range of values expected for sedentary species (Atlantic stingray *Hypanus sabinus*, Grim et al., 2012; *L. erinacea* and *R. eglanteria*; Table S3.1).

The absence of a habituation period in the respirometry chamber potentially influenced MR measurements. Habituation usually occurs within 2–3 h (Piiper et al., 1977; Brett and Blackburn, 1978; Ezcurra, 2001; Tunnah et al., 2016) and the duration of our measurements exceeded this time. Moreover, since only stabilized MO_2s were included in our analysis, values represent reliable unstressed-MR. Furthermore, *T. dumerilii* tolerates severe stress (Martins, 2017) and the short chase, netting and transport were unlikely to have altered MO_2s. Trawl females experienced confinement in the respirometry chamber three times while Control females only twice and this might have influenced their stress load and successive measurements. However, spending up to 3 h in a respirometry chamber did not cause physiological alterations associated with stress in blacktip reef sharks (*Carcharhinus melanopterus;* Bouyoucos et al., 2018) and it is unlikely that the additional respirometry confinement biased the measurements. Therefore, measured MO_2s likely represent reliable unstressed values, closely approaching RMR. However, the short duration of our measurement period (only 4 h) and the fasting period of only 48 h, hinders the possibility of an accurate estimate of SMR. Indeed, SMRs are usually estimated from animals at rest, acclimated to a constant temperature and in a post-absorptive (usually at least 48–72 h from the last feeding event) and non-reproductive state. Moreover, to fully record MO_2 variations associated with circadian rhythm, the measurements are taken during a full 24–48 h period (Clark et al. 2013; Chabot et al., 2016). Therefore, our estimates of Inactive MO_2 are not reliable estimates of SMR and need to be considered only preliminary results.

We calculated MO_2s only from females, however, sex differences potentially exist. Few studies have investigated this relationship, but no difference is reported in most of the studies on teleosts (Wohlschlag et al., 1959; Cech et al., 1985; Lozán, 1992; Lucas, 1994; Adams et al., 2001; Timmerman et al., 2003; Passow et al., 2015) or in the only study on chondrichthyans (Scharold et al., 1991). Reported differences are related to body mass (Kazakov *et al.*, 1981), pronounced sexual dimorphism (Wohlschlag, 1962; Adams et al., 1998; Makiguchi et al., 2017) or to reproductive period, parental care and migration (Beamish, 1964; Cooke, 2004) rather than sex *per se*. In *T. dumerilii*, sexual dimorphism is not extreme and there is no investment in parental care (Last and Stevens, 2009). Oocyte maturation, a highly energetic process (Parker, 1972), is completed by the time of parturition (Marshall et al., 2007), when we measured MRs, and so could not have influenced MR. Although not demonstrated for *T. dumerilii*, in some chondrichthyans, females store sperm in the paired oviducal glands (Pratt, 1993). Therefore, in *T. dumerilii,* the oocytes that are ovulated soon after parturition might be fertilized by stored sperm despite the absence of males and pregnancy status might have influenced MRs. In the initial stages, the cost of pregnancy is negligible (Timmerman and Chapman, 2003). Moreover, in *T. dumerilii*, zygotes enter diapause immediately after fertilization (Marshall et al., 2007) and the energetic cost of these pregnancy phases would have been unlikely high enough to alter MO_2s. However, 4 days after parturition might not be a period long enough to allow for the energy use associated with all the activities and processes involved in pregnancy to return to baseline levels. Therefore, these should be considered preliminary values, and further research investigating metabolic baseline levels in males and non-pregnant females, using MR measurements best practices, are needed to verify whether the MO_2 we measured in post-partum females can be considered reliable estimates of MR values for adult *T. dumerilii* of both sexes .

Table S3.1. Standard and routine metabolic rate (SMR and RMR, respectively) values available for batoid species and calculated proportional cost of activity (CA_P). SMR and RMR were temperature-corrected to 17°C using a Q_{10} of 2.3 (Whitney *et al.*, 2016). CA_P was calculated utilizing the temperature corrected MRs. SMR and RMR are reported in $mgO_2\ kg^{-1}h^{-1}$. T °C: Temperature, n = number of animal used in the study.

Species	**T °C**	**n**	**SMR**	**RMR**	**SMR (17°C)**	**RMR (17°C)**	**CA_P**	**Reference**
Hypanus sabinus	23	11	90.3		55.2			Cameron *et al.*, 1971
Hypanus sabinus	26	7		262.2		125.3	127.0%	Di Santo and Bennett, 2011
Dasyatis lata	20	22	52.8		41.1			Dale, *et al.*, 2013
Pteroplatytrygon violacea	20	8	63.3	117.3	49.3	91.5	85.6%	Ezcurra, 2001
Hypanus americanus	20	6	93.6		72.9			Fournier, 1996
Taeniura lymma	24	10	15.2		8.5			Wilborn, 2007
Myliobatis aquila	10	5	44.0	58.0	78.8	103.9	31.9%	Du Preez *et al.*, 1988
Myliobatis californica	14	6	85.2		109.3			Hopkins *et al.*, 1994
Rhinoptera bonasus	19	4	94.6		80.1			Neer *et al.*, 2006
Leucoraja erinacea	10	7	20.1	65.6	36.0	117.5	226.4%	Hove and Moss, 1997
Raja eglanteria	15	5	45.2	140.2	53.4	165.6	210.1%	Di Santo *et al.*, 2017

Raja clavata	(?)	(?)	57.2					Hughes, 1977
Torpedo marmorata	16		25.5		27.7			Hughes, 1978
Torpedo torpedo	14		70.0		89.9			Winberg, 1956
Acroteriobatus annulatus	15	10	61.0	73.0	72.1	86.2	19.6%	Du Preez *et al.*, 1988
Aptychotrema rostrate	28	8	47.4		19.0			Speers-Roesch *et al.*, 2012
Trygonorrhina dumerilii	17	14	44.8	84.6	44.8	84.6	88.8%	This study

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APPENDIX II

SUPPLEMENTARY MATERIAL FOR CHAPTER 4

ADDITIONAL STATISTICAL METHODS

Inactive MO_2 calculation

For species that swim constantly like *C. millii*, it has been proposed that calculating baseline SMR/Inactive MO_2 for an animal at rest is an uninformative measurement because these animals rarely rest in the wild (Lowe 2001). Instead, the lowest swimming MO_2 (LowestMO_2) is proposed as a more reliable, ecologically relevant estimate of the energetic requirements of organismal maintenance (Lowe 2001; Bouyoucos et al. 2019). To obtain this value it is suggested to calculate MO_2 over shorter time periods (Chabot et al., 2016). Therefore, using dissolved oxygen data recorded during the Unstressed MO_2-class measurements, without separating between resting and swimming periods, for each 1-min time slot, a MO_2-1min value was calculated as:

$MO_2\text{-}1min = [(\Delta DO \times 60) - B] \times Vol \times BM^{-1}$

where MO_2-1min is the metabolic rate expressed as $mgO_2\ h^{-1}\ kg^{-1}$, ΔDO is the decline in DO occurring in the 1-min slot (equal to the difference between the DO concentration measured at the beginning and the end of the 1-min time slot) expressed as $mgO_2\ L^{-1}min^{-1}$, 60 is the factor by which this value needs to be multiplied to obtain a measure of the decrease in DO occurring in a 1-h period, B is the background respiration calculated for each MO_2 trial expressed as $mgO_2\ L^{-1}\ h^{-1}$, Vol is the volume of water contained in the respirometry chamber in L and BM is the body mass of the female in kg. Pooling together values calculated from all the eight measurement cycles constituting a MO_2 trial, the lowest MO_2-1min was recorded, obtaining a single value for each animal (= Lowest-MO_2). Given that MO_2 is strongly influenced by temperature (Schmidt-Nielsen,

1997), calculated Lowest-MO_2s were temperature-corrected to 14°C, the average temperature recorded during the experimental period, using Q_{10} = 2.3 (Neer et al. 2006; Whitney et al. 2016).

Influence of calculation method on Inactive MO_2

To test whether the method used to calculate Inactive MO_2 influenced its values, Lowest-MO_2 values were compared to the values obtained by averaging, within each MO_2 trial, the Inactive MO_2s calculated using equation 1 (MO_2-RESTING; main text). The normality and homoscedasticity of the data were assessed with a Shapiro test and a Levene test, respectively. A linear mixed effect model (LME), including Method (MO_2-RESTING or Lowest-MO_2) as a fixed effect and animal ID as a random effect was used (Inactive MO_2~ Method + (1|ID)).

Results are reported as mean ± standard error (SE). All tests used a significance level of $\alpha \leq 0.05$. Data were processed using R statistical software (R Core team 2019).

RESULTS

The method by which Inactive MO_2 was calculated (MO_2-RESTING or LowestMO_2) had a significant influence on Inactive MO_2 values ($F_{1, 5} = 5.26$, $p = 0.02$) and MO_2-RESTING (58.96 ± 8.23 mg O_2 kg^{-1} h^{-1}) was 36.0% higher than LowestMO_2 (43.35 ± 3.00 mg O_2 kg^{-1} h^{-1}).

DISCUSSION

We aim at discussing the Unstressed Active and Inactive MO_2 values calculated in the main text, which are approximations for SMR and RMR, respectively, in relation to values available for other chondrichthyan species. Due to some unavoidable limitations in the respirometry methodology (see below), these must be considered only rough estimates of baseline MO_2, pending more accurate measurements. Nevertheless, given the paucity of data existing for chondrichthyans, and chimeras in particular as these are the first MO_2 measurements reported for this fish group, these values are important preliminary data. Indeed, baseline metabolic data are essential for ecological (Hove and Moss 1997; Dale et al. 2013; Di Santo and Kenaley 2016;

Whitney et al. 2016), behavioural, bioenergetics (Dale et al. 2013) and trophic studies (Brett and Blackburn 1978; Bushnell et al. 1989; Hove and Moss 1997).

Metabolic rates are often corrected for body mass, given the allometric relationship describing the scaling of MR with body mass. However, no clear agreement exists on a common mass-exponent for all chondrichthyan species, because high interspecific variability exists (Lawson et al. 2019). The size range of our study animals was narrow (mean = 2.1 ± 0.2, range = 1.9 to 2.4 kg), so a significant relationship between MO_2 and body mass was not detectable (see main text) and the calculation of a specific mass-exponent for *C. milii* was not possible. Applying a mass-exponent calculated for a different species is likely to introduce biases and we considered it unsuitable for animals with a similar body mass; thus the un-scaled, mass-specific MO_2 are reported (main text; Figure 4.4). When compared with values available for other chondrichthyans (sharks, Table S4.1, modified from Molina et al., 2020; skates and rays, reviewed in the supplementary materials of Chapter 3, Appendix I), *C. milii* Inactive MO_2, (temperature-corrected to 17°C using Q_{10} = 2.3; Neer et al. 2006; Whitney et al. 2016) was 76.27 $mgO_2\ kg^{-1}h^{-1}$, falling within the range of SMR values characteristic of active swimmers (Blacknose shark *Carcharhinus acronotus*, Carlson et al. 1999; blacktip shark *C. limbatus*, Lear et al. 2017; lemon shark *Negaprion brevirostris*, Scharold and Gruber 1991; scalloped hammerhead *Sphyrna lewini*, Lowe 2001; bonnethead shark *S. tiburo*, Carlson and Parsons 2003; southern stingray *Hypanus americanus*, Fournier 1996; common eagle ray *Myliobatis aquila,* Du Preez et al. 1988; cownose ray *Rhinoptera bonasus*, Neer et al. 2006). The ecological characteristics of *C. milii* are consistent with this observation, given that it is an active species that constantly swims and undertakes long migrations to coastal areas during the egg-laying season (Bell 2012; Barnett et al. 2019). Surprisingly, the temperature-corrected Active MO_2 (150.17 $mgO_2\ kg^{-1}h^{-1}$) was higher than the values of RMR recorded for some of the comparably active species listed above. This difference might originate from the characteristic locomotion mode of holocephalans, which is a combination

of flapping and undulations of pectoral fins that might be associated with a higher drag coefficient (Wilga and Lauder 2004), ultimately increasing the amount of energy needed to sustain movement. Similarly, despite *C. milii* having an active lifestyle, the energetic cost of activity (CA_P; 96.9%, see main text) is high and similar to values measured in sedentary species (Southern fiddler ray *Trygonorrhina dumerilii*, Chapter 3; draughtboard shark *Cephaloscyllium ventriosum*, Ferry-Graham and Gibb 2001; whitetip reef shark *Triaenodon obesus*, Barnett et al. 2016).

Despite incorporating the energetic cost of swimming, the recorded lowest swimming MO_2 (Lowest-MO_2, measured in this Appendix) was 36.0% lower than Inactive MO_2 measured during period in which animals rested on the bottom of the tank (MO_2-RESTING, measured in the main text). This suggest that, for *C. milii*, Lowest-MO_2 might be a better representation of the energetic cost of organismal maintenance than MO_2-RESTING. This result is likely explained by the fact that, when resting, animals expend more energy to actively pump water over their gills (buccal pumping) because they are unable to rely on forward motion to perform ram ventilation (Bushnell et al. 1989; Clark and Seymour 2006) that usually allows up to a 15% metabolic saving relative to buccal pumping (Roberts 1978; Steffensen 1985).

The absence of a habituation period in the respirometry chamber and the potentially incomplete recovery from mild handling stress might have elevated MO_2 measurements (Clark et al. 2013). However, in elasmobranchs, stabilization of MO_2 usually occurs within 2–3 h after placement in the respirometry chamber (Piiper et al. 1977; Brett and Blackburn 1978; Ezcurra 2001; Tunnah et al. 2016). In our study, measurements lasted for 4 h and only stabilized values (generally taking < 1.5 h) were included in the analysis and Active MO_2s likely represent reliable unstressed values, closely approaching RMR. However, the short duration of our measurement period (only 4 h) and the fasting period of only 24 h, hinders the possibility of an accurate estimate of SMR. SMR are usually estimated at a constant temperature from animals at rest and in a post-absorptive (at least 48–72 h from the last feeding event) and non-reproductive state. Moreover, to

fully record MO_2 variations associated with circadian rhythm, the measurements are taken during a full 24–48 h period (Clark et al. 2013; Chabot et al., 2016). Therefore, our measurements of Inactive MO_2 must be considered preliminary.

Only females were included in our study and metabolic differences between sexes might exist. Few studies have investigated this issue in fishes and found differences only in relation to body mass (Kazakov and Khalyapina 1981), sexual dimorphism (Wohlschlag 1962; Adams and Parsons 1998; Makiguchi et al. 2017) or reproductive activities, such as migration and parental care (Beamish 1964; Cooke 2004), rather than sex *per se*. In *C. milii*, no extreme sexual dimorphism or parental care are present (Bell 2012) and our measurements occurred after the end of the egg-laying period and before the beginning of vitellogenesis (Bell 2012). Therefore, these processes, despite having high energetic requirements (Parker 1972), unlikely affected our results and the MO_2s measured from female *C. milii* might be a reliable, preliminary approximation of values of adults of both sexes. However, further research investigating MO_2 in *C. milii* of both sexes in a non-reproductive state adopting best MR practices are needed to confirm this hypothesis.

Table S4.1. Temperature corrected standard metabolic rate (SMR) and routine metabolic rate (RMR) values available for shark species (corrected to 17°C using a Q_{10}=2.3; Whitney et al., 2016) and calculated energetic cost of activity (CA_P). SMR and RMR are reported in $mgO_2\ kg^{-1}h^{-1}$. Table modified from Molina et al. (2020) with permission.

Species	SMR (17°C)	RMR (17°C)	CA_P %	Reference
Carcharhinus acronotus	96.01	158.01	64.6	Carlson et al. (1999)
Carcharhinus limbatus	87.58	128.87	47.1	Lear et al. (2017)
Carcharhinus plumbeus	66.98	118.90	77.5	Dowd et al. (2006)

Cephaloscyllium ventriosum	48.15	101.84	111.5	Ferry-Graham and Gibb (2001)
Cetorhinus maximus	73.83	107.61	45.8	Sims (2000)
Ginglymostoma cirratum	21.84	57.64	163.9	Whitney et al. (2016)
Heterodontus francisci	105.31	-		Luongo and Lowe (2018)
Isurus oxyrinchus	111.83	293.50	162.5	Wegner et al. (2012)
Negaprion brevirostris	78.58	123.26	56.9	Scharold and Gruber (1991)
Scyliorhinus stellaris	84.35	148.07	75.5	Piiper et al. (1977)
Sphyrna lewini	89.31	129.95	45.5	Lowe (2001)
Sphyrna tiburo	81.75	111.05	35.8	Carlson and Parsons (2003)
Squalus acanthias	57.33	157.65	175.0	Brett and Blackburn (1978)
Stegostoma fasciatum	84.48	-		Payne et al. (2015)
Triaenodon obesus	81.34	149.47	83.8	Barnett et al. (2016)
Triakis semifasciata	69.23	110.12	59.1	Graham et al. (1990)
Heterodontus portusjacksoni	144.16	166.40	15.4	Molina et al. (2020)
Mustelus antarcticus	168.52	202.65	20.3	Molina et al. (2020)

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APPENDIX III

SUPPLEMENTARY MATERIAL FOR CHAPTER 5

ADDITIONAL STATISTICAL METHODS

All statistical procedures were performed using R statistical software (R Core team 2018), using scripts specifically developed by us for this purpose and functions in FSA (Ogle, 2018) and lme4 (Bates et al., 2015) packages.

SMR and body mass model validation and error analysis

The predictive models obtained were validated using a jack-knife approach to estimate the prediction error, following Lear et al., (2017), where, each individual was excluded from the analysis in turn and a new predictive equation determined for the remaining individuals pooled. Each new predictive equation was applied using the observed body mass value of the excluded individual to predict its MR value. The difference between the observed and predicted MR values of an individual was then used together with the differences for all the individuals to calculate the standard error on the MR measurements for each species separately. To graphically compare the validation of both models, we plotted the measured SMR vs the predicted SMR by the model, and compared with a perfect fit (a theoretical straight line with slope= 1). The coefficient of variability (COV) and the algebraic percent error (APE) were calculated for each animal and each species, to estimate the modeling uncertainty (e.g., Lear et al., 2017).

Both linear and power relationships were significantly different from 1 for both models of species ($p<0.05$, Figure S5.1), which means our model slightly underestimated SMR at high SMR values and overestimated SMR at lower SMR values. Mean coefficient of variance (COV) in SMR was 4.60% (range 2.71–11.81%) for *M. antarcticus* and 6.06% (5.11–8.39%) for *H. portusjacksoni*.

Algebraic percent error (APE) followed similar trends, being lower in *M. antarcticus* (mean 14.94, range 0.30 – 24.85) and higher in *H. portusjacksoni* (mean 22.11, range 3.71 – 41.18).

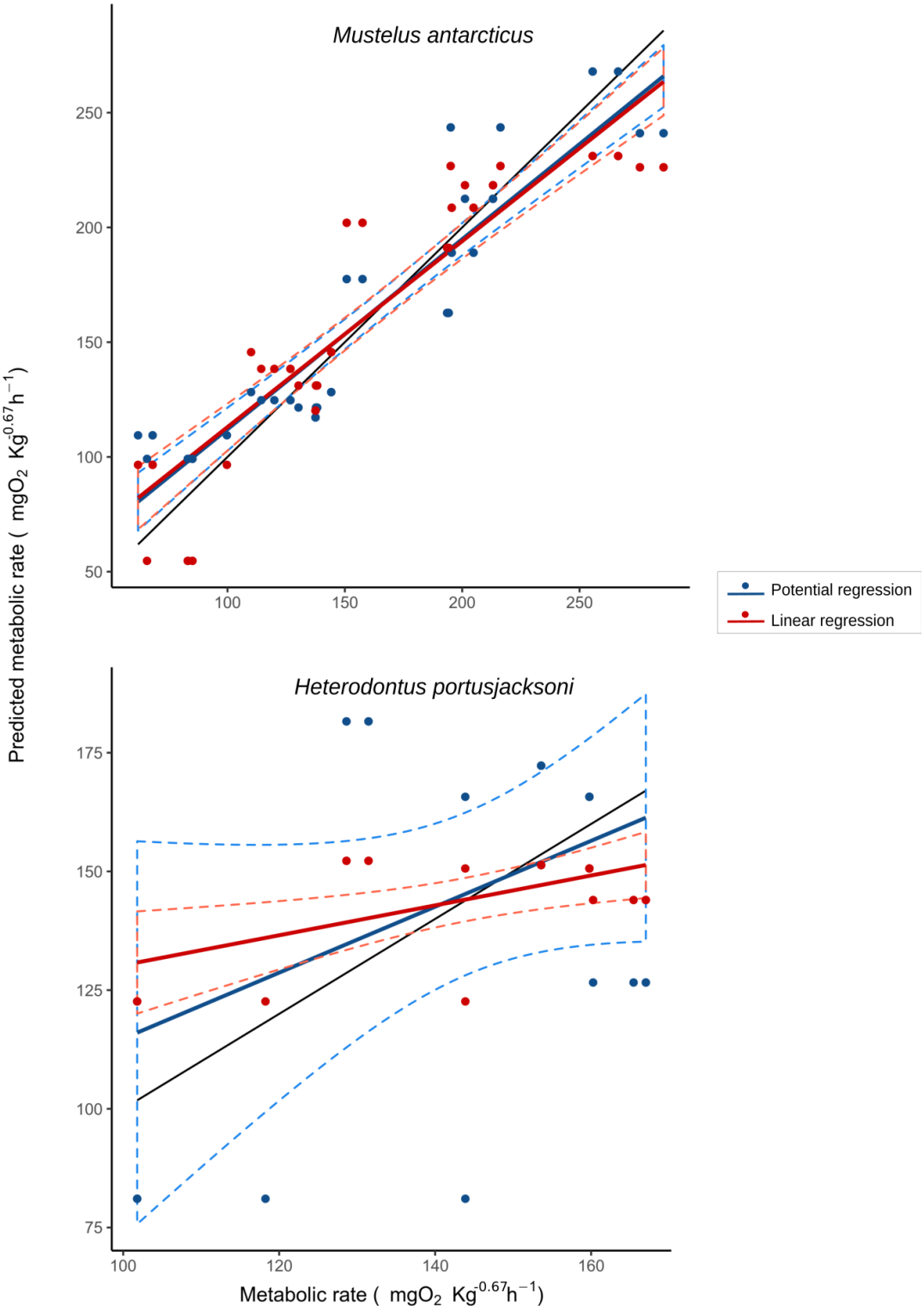

Figure S5.1. Comparison of the error produced by the estimates of both linear and power models for *Mustelus antarcticus* (top) and *Heterodontus portusjacksoni* (bottom). Dashed lines represent standard error.

Temperature

Water temperature was maintained around 16±2°C with a water cooling system, but was measured at the time of the respirometry trials, both for the Baseline MR and the Gillnet capture simulation. Mean water temperature for each individual trial were produced averaging the values logged in the DO sensor meter, and compared between treatments using an ANOVA. When significant differences were found, a Tukey honestly significant difference (HSD) analysis was carried out to identify individual animal significant variations. We found no statistical differences between treatment water temperatures ($F = 1.27$; $P = 0.19$) nor individual trials ($F = 0.52$; $P = 0.48$).

Linear mixed-effect model validation and fit

We fitted a total of 7 models to our data of MR, the most complex of which included a random term for the individuals (I), with factor-dependent slopes, and the four fixed terms, treatment (Tr), species (SP), MR type (R) and delayed mortality (D) (Table 5.1). The simplest model represented the case where none of the fixed parameters were significant. Including the most complex model we built 7 models (Bates, 2010; Ogle, 2015). Significance of the fixed terms in each model was evaluated using a Wald test. Model fit comparison was performed using ANOVA, and model selection was performed according to Akaike and Bayesian information criteria (AIC and BIC, respectively) (Sakamoto et al., 1986; Burnham and Anderson, 2002; Molina et al., 2017).

To reduce inconsistency in the literature, Clark et al. (2013) proposes new research on MR should employ the lowest 10% of all the measured DO points to estimate SMR. We employed the method outlined in Clark et al. (2013), and compared both estimates employing LME, with specific Tr, R, SP and I slopes, as described above.

We fitted a full mixed-effects linear model with all the parameters and their interactions (Model 1, Table 5.1). Through stepwise elimination of non-significant interaction and main effect

terms, model simplification continued until Model 7 was reached, with all its parameters being significant. We therefore chose Model 7 as it explains the observed variance and provides the lowest AIC and BIC values (Table 5.1). Inclusion of the individuals as a random factor in all the models was validated by the high percentage of the variance they represent (Table 5.3). Inclusion of a factor-dependent slope for the random effects of treatment, species and delayed death was also significant, while the variance of R was relatively low, almost as low as the residual variance (Table 5.3).

Our estimates of SMR calculated by employing the "rest" cycles are significantly lower than estimates calculated using the lowest 10% of all the measured DO points, as proposed by Clark et al. (2013) ($P<0.0001$) (Figure S5.2). Despite the statistical differences, the absolute value of the difference was small: 51.97 ± 8.8 $mgO_2kg^{-0.67}h^{-1}$ for *M. antarcticus* and 21.65 ± 19.3 $mgO_2kg^{-0.67}h^{-1}$ for *H. portusjacksoni*.

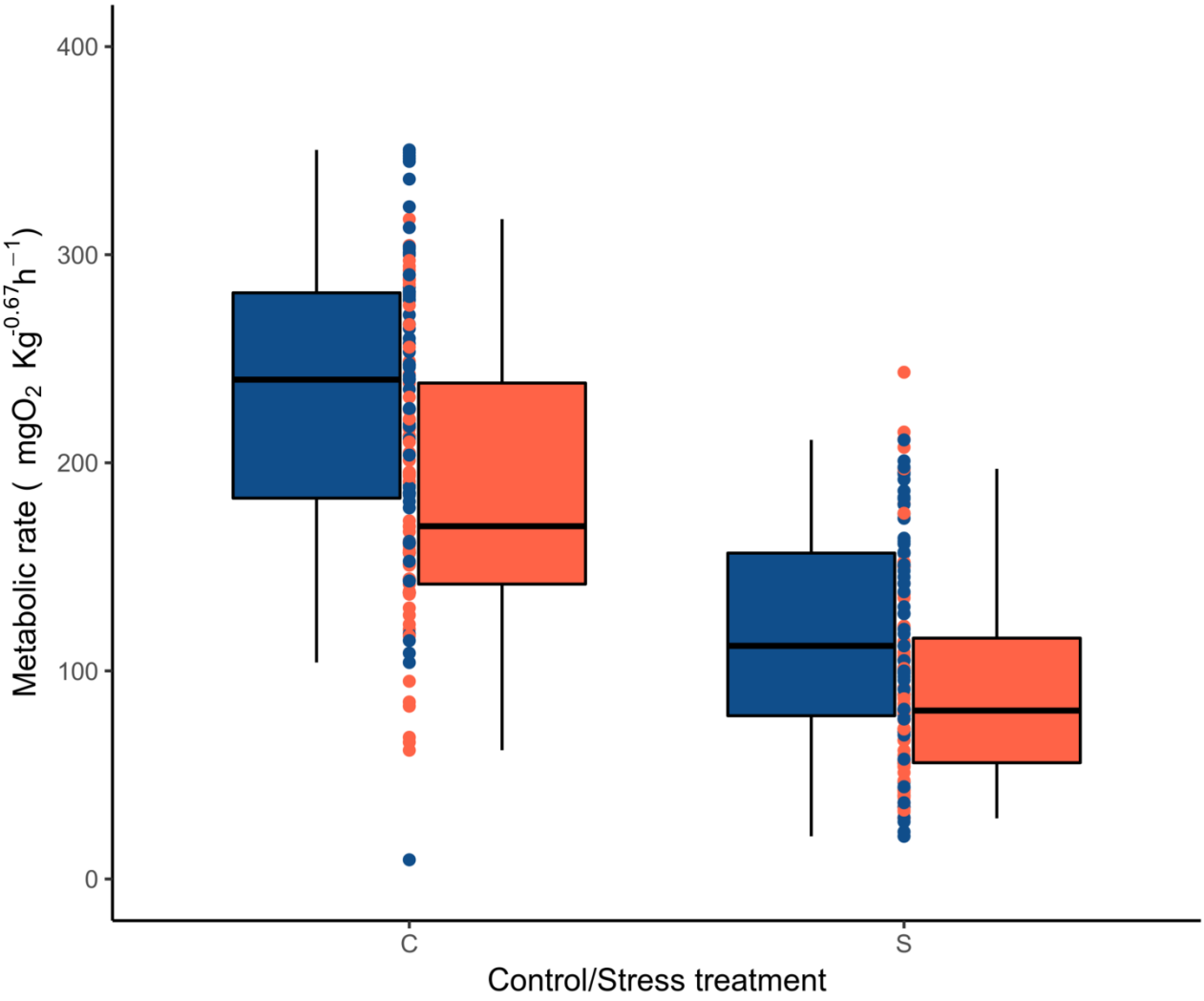

Figure S5.2. Comparison of methods for determining standard metabolic rates in *Mustelus antarcticus.* For control (C) and gillnet capture treatment (S), the blue dots and box plot indicates Clark et al. (2013) method of minimum dissolved oxygen measurements selection, and the red indicates our method. Differences between mean values were statistically significant (ANOVA $P<0.0001$).

It has been fun for sure! Me and a friendly PJ

Photo by Licia Finotto