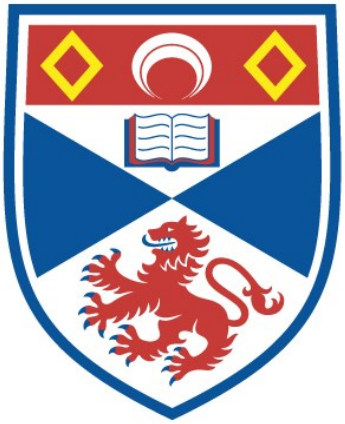
Unravelling the complex reproductive tactics of male humpback whales: an integrative analysis of paternity, age, testosterone, and genetic diversity

Franca Eichenberger

A thesis submitted for the degree of PhD
at the
University of St Andrews



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General Abstract

How the underlying forces of sexual selection impact reproductive tactics including elaborate acoustic displays in cetaceans remains poorly understood. Here, I combined 26 years (1995-2020) of photo-identification, behavioural, (epi)genetic, and endocrine data from an endangered population of humpback whales (New Caledonia), to explore male reproductive success, age, physiology, and population dynamics over almost a third of the lifespan of a humpback whale. First, I conducted a paternity analysis on 177 known mother-offspring pairs and confirmed previous findings of low variation in reproductive success in male humpback whales. Second, epigenetic age estimates of 485 males revealed a left-skewed population age structure in the first half of the study period that became more balanced in the second half. Further, older males (> 23 years) more often engaged in certain reproductive tactics (singing and escorting) and were more successful in siring offspring once the population age structure stabilised, suggesting reproductive tactics and reproductive success in male humpback whales may be age-dependent. Third, using enzyme immunoassays on 457 blubber samples, I observed a seasonal decline in male testosterone in the population over the breeding season. Testosterone levels appeared highest during puberty, then decreased and levelled off at the onset of maturity, yet were highly variable at any point during the breeding season and across males of all ages. Lastly, I investigated the influence of genetic diversity at the major histocompatibility complex (MHC) class I and class IIa (DQB and DRB-a) on patterns of male reproductive success in humpback whales. Mating pairs shared fewer alleles than expected under random mating at MHC class I and IIa, thus, providing evidence of an MHC-mediated female mate choice in humpback whales. This thesis provides novel, critical insights into the evolutionary consequences of commercial whaling on the demography, patterns of reproduction and sexual selection of exploited populations of baleen whales

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Ethical statement

This study was approved by the University of St Andrews School of Biology Ethics Committee (ref: SEC2018004). Humpback whales were sampled by the NGO Opération Cétacés (1995-2014) and IRD (2015-2019) under licenses by the Province Sud, Province Nord and the government of New Caledonia.

Digital Output access statement

Supplementary material S2 – S5 for each data chapter underpinning this thesis are available at [https://github.com/francae/PhD-Thesis_FrancaEichenberger].

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1: John Steinbeck in 'The Log from the Sea of Cortez'

“The compass simply represents the ideal, present but unachievable, and sight-steering a compromise with perfection which allows your boat to exist at all.”

- John Steinbeck, *The Log from the Sea of Cortez*

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Chapter 1

General Introduction

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1.1 Abstract

While a variety of reproductive tactics are readily witnessed in the odontocetes, such behaviours can be far more elusive, and in some cases, are yet to be observed, in baleen whales. This leads researchers to employ a variety of research methods, some of which have improved greatly in recent decades, to study reproductive behaviours in mysticetes. Genetics and genomics tools can provide invaluable information on maternity, paternity, age, diversity, and kinship, while acoustic tools can provide new insights into the function of sexual displays such as song. In this chapter, I explore what is known about the reproductive strategies and tactics of baleen whales, with a particular focus on the comparatively well-studied right whales (*Eubalaena* spp.) and humpback whales (*Megaptera novaeangliae*). Finally, I showcase that by integrating multiple data types, we can explore the interactions between anatomy, physiology, reproductive success, age, population dynamics, and acoustic displays to better understand the mating systems of baleen whales.

1.2 Mating systems and reproductive strategies of baleen whales

There are many gaps in our understanding of mating systems and strategies of marine mammals, in particular for many of the mysticete suborder (baleen whales). The species are often rare, endangered, or otherwise difficult to observe; few observations may have therefore been made of their mating behaviour, especially at the temporal scale necessary to evaluate lifetime reproductive success. When mating behaviours are observed, understanding the full behavioural repertoire and its context is challenging due to the elusive nature of mammals that spend most of their time submerged.

Baleen whales undertake seasonal migrations to feed, mate, and give birth. The distances of these migrations and the extent to which breeding and feeding areas are separated from each other vary greatly across species, sometimes even across populations (e.g., non-migratory Arabian sea humpback whales, Megaptera novaeangliae: Mikhalev 1997). While some of the largest lunge feeders (rorquals), blue and fin whales (*Balaenoptera musculus* and *B. physalus*), appear to breed dispersed across unobserved offshore areas (Simon et al. 2010; Sears et al. 2013), other baleen whales aggregate on distinct breeding grounds (e.g., humpback whale; gray whale, *Eschrichtius robustus*; southern right whales, *Eubalaena australis*). The reproductive behaviours of most baleen whales indicate a polygynous mating system (successful males mate with multiple females), yet variance in male reproductive success is relatively low in comparison to polygynous mammals on land (Cerchio et al. 2005; Frasier et al. 2007; Carroll et al. 2012). Parentage analyses have further revealed that females mate with different males across years (e.g., Clapham and Palsboll 1997; Frasier et al. 2007), thus hinting towards a polygynandrous mating system (both males and females mate with multiple partners). However, direct observations of females mating with multiple males within the breeding season have, so far, only been reported in bowhead whales (Balaena mysticetus, Tarpley et al. 2021), gray whales (Swartz 1986), and North Atlantic right whales (Eubalaena glacialis, Mate et al. 2005). Despite the similarities in the reproductive strategies (i.e., polygynandry, polygyny) across species, the behaviours individuals engage in within their species' mating system can vary considerably (Table 1.1).

Table 1.1. Overview of the male reproductive tactics and the potential for female choice in baleen whales. 'Distribution' refers to the distribution of individuals in space during the breeding season. 'Pre-copulatory trait investment' was based on the presence of elaborate vocal displays (i.e., song) with higher investment for more complex songs (see Table 2). 'Post-copulatory trait investment' was based on whether the phylogenetically controlled residuals of maximum testes mass regressed onto maximum body length were lower than expected (low), as expected (medium), or higher than expected (high) based on Dines et al. (2015). 'Potential for female choice' indicates the hypothetical possibility for female choice to occur based on the species' mating system and the observed or inferred male reproductive tactics, and whether female choice likely takes place before and/or after copulation. Abbreviations: aggr: breeding aggregations; dsp: dispersed; C: contest competition; Sc: scramble competition; E: endurance competition; Sp: sperm competition; S: singing; preC: pre-copulatory; postC: post-copulatory; u: unknown or unclear; () for inferred or hypothesized.

Family	Species	Distribution	Pre-copulatory trait investment	Post-copulatory trait investment	Male reproductive tactics	Potential for female choice	Source
Balaenopteridae	Bryde's whale, *Balaenoptera edeni*	(dsp)	(low)	medium	(Sc, Sp)	u	1-3
	Omura's whale, *B. omurai*	(dsp)	low-medium	u	(Sc, S)	u	1-4
	Sei whale, *B. borealis*	(dsp)	(low)	medium	(Sc, Sp)	u	1-3
	Blue whale, *B. musculus*	disp	high	medium	Sc, S	preC(+postC)	1-3,5
	Fin whale, *B. physalus*	disp	high	medium	Sc, S	preC(+postC)	1-3,10
	Humpback whale, *Megaptera novaeangliae*	aggr	high	low	C, S, Sc, E	preC	1-3,6-11
	Antarctic minke whale, *B. bonaerensis*	(dsp)	(low)	u	(Sc)	u	
	Common minke whale, *B. acutorostrata*	dsp	medium	medium	Sc, S	(preC+postC)	1-3
Eschrichtiidae	Gray whale, *E. robustus*	aggr	low	high	Sp, Sc	postC	1-3,12
Balaenidae	Pygmy right whale, *Caperea marginata*	(dsp)	(low)	(medium)	(Sc)	u	3
	NA right whale, *Eubalaena glacialis*	aggr	low	(high)	Sp, Sc, E	postC	13-16
	NP right whale, *E. japonica*	(aggr)	low-medium	high	Sp, Sc, S	postC(+preC)	1-3
	S right whale, *E. australis*	aggr	low	(high)	Sp, Sc	postC	17-19
	Bowhead whale, *Balaena mysticetus*	aggr	high	high	Sp, S, Sc	preC+postC	1-3,20-21

References: 1: Ralls and Mesnick (2019) 2: Dines et al. (2015); 3: Brownell and Ralls (1986); 4: Cerchio et al. (2022); 5: Sears et al. (2013); 6: Tyack and Whitehead (1982); 7: Clapham and Palsboll (1997); 8: Cerchio et al. (2005); 9: Pack et al. (2012); 10: Simon et al. (2010); 11: Herman (2017); 12: Swartz (1986); 13: Kraus and Hatch (2001); 14: Mate (2005) 15-16: Frasier et al. (2007, 2013); 17: Carroll et al. (2012); 18: Burnell (2001); 19: Rowntree et al. (2001); 20: Würsig and Clark (1993); 21: Tarpley et al. (2021)

Much of what we know about mysticete reproductive behaviour comes from humpback, right, and gray whales. There are similarities in the reproductive behaviours between these species: males typically aggregate in groups of a few to a few dozen individuals, where they physically compete to be closest to a single female at the centre of the group (Tyack and Whitehead 1982; Norris et al. 1983; Kraus and Hatch 2001; Parks et al. 2007). However, while male humpback whales produce one of the most complex acoustic and culturally transmitted displays in the animal kingdom (Payne and McVay 1971; Payne and Payne 1985; Noad et al. 2000; Garland et al. 2011), the acoustic display of right and gray whales is much simpler (Crance et al. 2019; Matthews and Parks 2021; Parks 2022) or absent in the latter. Right whales, on the other hand, have the largest testes to body mass ratio of any baleen whale, indicating the important role of sperm competition as their reproductive tactic (Brownell and Ralls 1986). Despite being in the same taxon and exposed to similar environmental pressures, baleen whales seem to have evolved different reproductive strategies and tactics. This raises the question of what behavioural strategies the lesser-studied baleen whales have evolved and what are the underlying ecological and social drivers that led to the variation in reproductive behaviours across baleen whales. The unique evolutionary history of the transition from land to sea, well-resolved phylogeny, and trait variation of cetaceans offer a great opportunity to test hypotheses on the evolution of mating systems and reproductive behaviours.

1.3 The theoretical basis of reproductive tactics

Mating systems are typically defined as species-specific, broad reproductive behaviours (e.g., monogamy, polygyny, polyandry, polygynandry) and describe an evolved set of decision rules (e.g., Dominey 1984; Gross 1996; Clutton-Brock 2016; Ralls and Mesnick 2019). Individuals might express different reproductive tactics (i.e., behavioural phenotypes) resulting from a strategy (Dominey 1984; Gross 1996) that can depend on their own condition (e.g., body size) or experience (e.g., level of maturity, age) and/or that of their potential mates (e.g., assortative mating).

Within a species' mating system, males and females often follow different reproductive strategies due to the differences in gamete investment between the sexes. In most mammals, females invest considerably more energy in the production of offspring than males. This

includes the production of larger gametes, the constant association and energy supply to the offspring, and the provision of parental care. Female reproductive strategies are thus largely driven by natural selection aiming to maximise their energy storage for reproduction and fitness through the survival of their offspring. As a consequence, females are the sex with the longer reproductive time-out (i.e., inter-birth interval) and are limited in the resources necessary to produce offspring. Females may balance their investment into current reproduction, or their survival and potential future offspring, by adjusting their inter-birth interval based on current energy storage (especially in long-lived species). In female baboons (*Papio cynocephalus*), inter-birth intervals are limited by the energetic constraints resulting from a complex interplay of ecological and social factors (e.g., food availability, costs of increasing intragroup competition; Hill et al. 2000). But female reproductive tactics go beyond the decision of whether to reproduce in a given year. Females may engage in pre-copulatory tactics: (1) selective copulation with preferred males (female mate choice) (reviewed in Rosenthal and Ryan 2022), (2) mating with multiple males to induce sperm competition (Andersson 1994), reduce sexual harassment, or confuse paternity (Furuichi et al. 2014), (3) evasive behaviour to avoid copulation with particular males (Orbach et al. 2015); or in post-copulatory tactics: (4) biased fertilization towards the sperm of specific male(s) (cryptic female mate choice) (Firman et al. 2017). However, male and female reproductive tactics can be strongly interdependent, and thus the behaviour of one sex may alter the behaviour of the other (Bro-Jørgensen 2011; Rosenthal and Ryan 2022).

Compared to females, males usually carry little to none of the reproductive cost after copulation, allowing them to reproduce with little to no reproductive time-out; this may result in at least the possibility of males reproducing multiple times within a year if mates are available, although empirically we do not know how often this occurs. Where females undergo the longer reproductive time-out, the operational sex ratio (OSR, ratio of receptive adults at any time in a population) at the breeding ground is skewed towards males. This leaves males more limited in finding a potential mate than females and increases competition among males (and intra-sexual selection). Ultimately, this can lead to sex differences in lifetime reproductive success and opportunities for selection (Clutton-Brock 1989, 2016).

Variation in reproductive success is a fundamental prerequisite for sexual selection to act upon a trait. Ecological and social circumstances (e.g., habitat, resource distribution, predation

pressure, demographic factors) influence the spatial and temporal distribution of resources and mates and thus influence the degree of control or monopolisation a male can hold over a female. The greater the degree of control, the higher the potential for polygyny, and the larger the possible resulting variation in reproductive success. Ecological and social factors, therefore, influence the intensity and direction of sexual selection. The male-biased OSR in most mammals means that sexual selection is usually strongest in males. This has led to the evolution of a variety of different male mating tactics. Males may engage in pre-copulatory tactics: (1) direct male-male competition over mating access to females or territories (e.g., contest competition, endurance rivalry) or in their efforts to search and locate females more efficiently (scramble competition), (2) indirect competition by attempting to attract females via elaborate displays or ornaments; or post-copulatory tactics: (3) sperm competition by attempting to outcompete with higher quality or quantities of sperm (Andersson 1994).

A key idea is that males with a certain advantage will be more successful in competing against other males for mating access or female fertilization. This may lead to a small proportion of males in a population siring a larger proportion of offspring than other males; in other words, creating a reproductive skew. The resulting reproductive skew impacts population dynamics, and in small populations, can affect population recovery (Sky et al. 2022). Below I explore the drivers of intra- and inter-sexual selection on pre- and post-copulatory reproductive tactics, provide examples across a wide variety of taxa and indicate the potential for female choice to arise within each of them.

1.3.1 Direct male-male competition over mating access

Males may directly compete with other males over the control of a single female, group of females (i.e. harem), or resource (i.e. territory). This competition can come in several forms: (1) physical interactions where males fight over females (contest competition), or in forms carrying less injury risk (Smith and Price 1973); (2) where males perform certain displays without any physical interaction, or in interaction-independent forms; (3) where males compete in their ability to remain reproductively active during a large part of the season (endurance competition), or (4) in their efforts to search and locate females more efficiently (scramble competition) (Andersson 1994). Depending on which strategy males employ and within the basic framework of the species' mating system, different behavioural or

morphological traits can be advantageous in siring offspring and are thus favoured by sexual selection. While large body size is often advantageous in direct contest competition (e.g., red deer, Cervus elaphus: Clutton-Brock et al. 1982), song repertoire size can play a role in the territorial defence of several birdsong species (e.g., Great tits, Parus major; Krebs et al. 1978).

1.3.2 Indirect competition to attract females via male displays

Whereas intra-sexual selection may favour weaponry and large body size in males, inter-sexual selection can lead to the evolution of male ornaments and displays to increase a male's attractiveness as a potential mate (Clutton-Brock and Parker 1992). In many species, males have evolved elaborate displays and ornaments that are driven by female mating preference (pigmentation patterns in male guppies, Poecilia reticulata; Kodric-Brown 1985; e.g., calling rate in field crickets, Gryllus campestris; Holzer et al. 2003) that are typically more complex than displays performed during male-male competition. Inter-sexual selection can lead to the evolution of male traits that appear entirely nonadaptive through the lens of natural selection from abiotic and heterospecific selection pressures (e.g., long-tailed widowbird, Euplectes progne; Andersson 1982), yet they increase a male's chances of siring offspring, and thus, his fitness.

If a preferred trait is an honest indicator of a male's quality, then females might use that information to assess and choose their potential mates, and ultimately, increase the fitness of their offspring. Females might prefer to mate with males that honestly signal their superior genetic quality, resistance to parasites (Hamilton and Zuk 1982), and/or condition (Zahavi 1977; Grafen 1990). In song sparrows (*Melospiza melodia*), song complexity was found to advertise optimal major histocompatibility complex (MHC) diversity, a trait affecting disease and parasite resistance (Slade et al. 2017). By choosing mates with complex song, females may enhance the immunocompetence and disease resistance of their offspring (Slade et al. 2017). Consequently, males that signal their superior MHC diversity by singing more complex songs thus may enjoy a reproductive advantage. Sexual selection may also favour genetic dissimilarity or compatibility rather than simply good genes (Trivers 1972; Puurtinen et al. 2005).

Elaborate displays can also evolve without being honest indicators of male quality. A female preference can evolve through a Fisherian runaway process if females favour mates with an arbitrary trait and whose fitness advantage only exists as a result of its covariance with the preference (Fisher 1930). Despite the absence of any correlation between the preferred trait and a male's quality, by choosing a mate with a particular trait, females gain indirect fitness benefits by increasing the sexual attractiveness of their offspring ("sexy son hypothesis", Weatherhead and Robertson 1979). Alternatively, female preference for a particular trait may simply be a by-product of selection due to male exploitation of a pre-existing bias in the female sensory system (Ryan 1998; van Schaik 2016).

While attempts by males to constrain female choice may mask an existing female mating preference (Clutton-Brock and McAuliffe 2009), the degree of female monopolisation and male-male competition may limit opportunities for female mate choice. In addition, environmental and demographic factors will affect opportunities for individuals to interact and assess several potential mates and ultimately, to exert their mating preference or reproductive choice.

1.3.3 Sperm competition over successful female fertilization

Male-male competition and female mate choice can also take place after copulation. In species where females follow a polyandrous reproductive strategy, post-copulatory selection for sperm can arise within the timeframe of sperm survival. By mating with multiple males, a female might gain direct benefits such as insurance against male infertility or male protection against predators or harassment from other males (van Schaik 2016), and/or indirect (genetic) benefits increasing the fitness of her offspring (Zeh and Zeh 2001).

Across the animal kingdom, relative testis size is strongly correlated with the level of polygynandry (MacLeod and MacLeod 2009) and is a good predictor of ejaculation size which influences a male's chances of successfully fertilising a female egg in competition against other males' sperm. Both large ejaculation size and high sperm mobility can increase a male's chance of successful fertilisation (Birkhead et al. 1999; García-González and Simmons 2005).

If a female can discriminate among the ejaculates of different males and thus bias fertilization, indirect female mate choice can take place. Under such a scenario, sperm from

different males compete within the female's reproductive tract for fertilization (sperm competition) and females may choose the sperm of preferred males (cryptic female choice; Firman et al. 2017). Post-copulatory mate choice gives females the opportunity to exert their mating preference allowing them to evade attempts of male monopolization. For example, female red junglefowl (*Gallus gallus*) were found to favour the sperm of males with a dissimilar MHC haplotype (Løvlie et al. 2013), thus increasing the MHC diversity and fitness of their offspring. This selection for dissimilar MHC haplotypes was however lost after artificial insemination, indicating that females might require male phenotypic cues to choose sperm (Løvlie et al. 2013).

1.4 The ecological and social factors shaping reproductive tactics in baleen whales

Baleen whales share many of the life history characteristics of their phylogenetic terrestrial relatives, yet their locomotion and sensory system are strikingly different. Over more than 50 million years of evolution (Uhen 2010), the anatomy and physiology of marine mammals became specialized for the marine environment. Thus, it seems not unreasonable to assume that the reproductive tactics of baleen whales too are adapted for a life in the ocean.

Baleen whales are highly mobile and undertake some of the longest migrations observed in any mammal. For example, Oceania (South Pacific) humpback whales travel more than 7,000 kilometres between their breeding grounds and their Antarctic feeding grounds (Riekkola et al. 2019). While some species show clearly defined migration strategies and large-scale seasonal movements from their polar feeding grounds to their clearly distinct breeding grounds closer to the equator (e.g., humpback, blue, and grey whales), others undertake shorter migrations, do not breed near the equator (e.g., fin and right whales) and/or remain at similar latitude year-round (e.g., Bryde's whale, Balaenoptera edeni; bowhead whale; pygmy right whale, Caperea marginata; Bannister 2018). These migration strategies may even vary across populations (e.g., Bering-Chukchi-Beaufort Sea bowhead whales: Insley et al. 2021). While seasonal migration to warmer waters with fewer predators could represent a female tactic to increase offspring survival (Whitehead and Moore 1982; Corkeron and Connor 1999), the exact reasons why baleen whales travel these sometimes vast distances remain

unclear. Considering the diversity of migratory tactics across baleen whale species and populations, the driving forces underlying their movement patterns might vary similarly (Horton et al. 2022).

Many baleen whales are capital breeders; after migrating from productive feeding grounds, individuals at the breeding ground generally go through an elongated fasting period (Costa and Maresh 2018). Females provision themselves and their offspring by feeding on seasonally abundant food sources, often thousands of miles from where they give birth. The long migration and elongated fasting period further increase the costs of reproduction for female baleen whales as female body condition affects fetal and calf growth (Christiansen et al. 2014, 2018). The capital breeding strategy also means nursing a calf leads to rapid depletion of a female's fat stores and body condition; in southern right whales, females lose an estimated 25% of their body volume in their calves' first few months of life due to lactation (Christiansen et al. 2018; Figure 1.1). At this stage of development, the calf grows up to one meter a month, highlighting the effectiveness and cost of this provisioning (Best 1994; Christiansen et al. 2018). Females may build up the energy reserves required for reproduction over multiple feeding seasons, resulting in the need for longer inter-birth intervals. This likely reflects a female reproductive tactic in which the female delays reproduction to build up sufficient energy storage that may ultimately increase the survival of herself and that of her future offspring. Flexibility in reproductive timing may provide females with a buffer for poor prey conditions in a single year (Christiansen et al. 2022b). It may be that many females can become pregnant annually but carry the fetus to term only if conditions allow. The early stages of pregnancy (first and second trimesters) only incur about 5% of the total energetic cost of gestation (Fredrik Christiansen et al., 2022). It could therefore be that females can 'decide' if the amount of energy resources obtained during the summer feeding period are sufficient to continue with the pregnancy. There is evidence that calving rates of southern right whales relate directly to environmental conditions that impact prey availability at offshore feeding grounds (Leaper et al. 2006; Seyboth et al. 2016). Similarly, the annual pregnancy rates of humpback whales along the Western Antarctic Peninsula may represent a response to favorable ecological conditions at these feeding grounds (Pallin et al. 2018a).

Figure 1.1. Southern right whale cow-calf pair, taken in the Auckland Islands Maungahuka in the Aotearoa New Zealand subantarctic by the University of Auckland Waipapa Taumata Rau southern right whale research team, under New Zealand Department of Conservation permit 84845-MAR.

Compared to females, male baleen whales carry little of the reproductive costs, and their mating and reproductive success are mainly limited in the distribution of mating partners in space and time. Due to the lack of stable groups in mysticetes and the absence of prey resources at their calving grounds, individuals are typically widely distributed across space. To combat this, many baleen whale species aggregate on breeding grounds every year (Table 1.1), many show site fidelity to these locations (Baker et al. 2013; Carroll et al. 2013), and produce acoustic displays audible across vast distances to find mates (section 1.4.2). The variable inter-birth intervals of females can produce a male-biased operational sex ratio (ratio of receptive adults at any time in a population) at these breeding grounds in some species, and further increase male competition for breeding opportunities (Boness et al. 2002). The 3D underwater habitat and the great dispersion of individuals across the breeding ground, or the absence of distinct breeding grounds in some species, make it challenging for males to monopolize and defend groups of females or territories against other male competitors. Considering that fasting at the breeding ground is typical of baleen whales, food sharing and resource defense are also unlikely tactics. By process of elimination, (1) direct male-male competition over mating access in the form of male contest, endurance, and/or scramble competition; (2) indirect competition by attempting to attract females via elaborate displays

(e.g., song); or (3) sperm competition over successful female fertilization are all possible and non-mutually exclusive reproductive tactics. All of these reproductive tactics are inferred or have been observed in baleen whales, and below we discuss each of them in turn.

1.4.1 Direct male-male competition over mating access

In some species, direct male-male interactions are readily observed and allow insights into mating tactics. The temporary group formations of three or more adults as observed in right whales, gray whales, and humpback whales often peak around the breeding season and offer opportunities for direct competition between males over female mating access despite a commonly scattered distribution and solitary behaviour (Norris et al. 1977, 1983; Everitt and Krogman 1979; Tyack and Whitehead 1982; Clark 1983; Clapham et al. 1992; Würsig and Clark 1993; Kraus and Hatch 2001) (Figure 1.2 and Figure 1.3). The level of aggression and intensity of male-male interactions within such groups varies across species. In humpback whales, males often engage in agonistic fights to gain or maintain the privileged position closest to the female of the group (Tyack and Whitehead 1982; Clapham et al. 1992) and show high levels of surface activity and behavioural displays (e.g, charges and peduncle strikes) indicating the aggressive nature and intensity of these interactions (Baker and Herman 1984; Figure 1.3). Males can also be observed escorting a single female (with or without her newborn calf) to form a pair. It is unclear whether the male's defence and the escorting of the female results in copulation or whether it reflects mate guarding following earlier copulation (Clapham 1996). In right, gray, and bowhead whales, male-male interactions within these temporary group formations appear to be much less aggressive.

Figure 1.2. A group of ten socializing southern right whales captured by drone in the Auckland Islands Maungahuka in the Aotearoa New Zealand subantarctic by the University of Auckland Waipapa Taumata Rau southern right whale research team, under New Zealand Department of Conservation permit 84845-MAR.

Figure 1.3. Humpback whale competitive group on their breeding grounds off the coast of New Caledonia in the South Pacific. The photo was captured by Opération Cétacéas, under a permit issued by the Province Sud.

Although the aggregations of several males within these temporary group formations are most likely driven by intra-sexual selection among males; they may also offer females the possibility to assess multiple potential mates. In North Atlantic right whales and humpback whales, females may facilitate the formation or increase the size of male aggregations to incite competition among males using surface active displays (Clapham 2000) or vocalizations (Kraus and Hatch 2001; Parks 2003; Parks and Tyack 2005; Parks et al. 2007). Females may thus use aggregations of competing males to secure the highest quality male by mating with (or being fertilized by, see section on sperm competition) the winner of the competition or through active mate choice.

The traits that allow a male to outcompete rivals underwater are likely different from the traits determining the outcome of male-male competition on land. While large body size is often correlated with increased strength on land and a clear advantage in fighting, a large size might come at the cost of reduced manoeuvrability underwater (Le Boeuf 1991; Segre et al. 2022). Better agility due to small size may be more advantageous in male-male competition in baleen whales considering the 3D underwater habitat in contrast to terrestrially-mating mammals (Mesnick and Ralls 2018a). However, large body size could increase the duration a male remains on breeding grounds, which are devoid of food (Craig et al. 2003), therefore increasing mating opportunities. Apart from manoeuvrability, male endurance and stamina likely also play an important role in determining a successful competitor, considering the hours-long duration of competitive group formations. Interestingly, mature-sized females at times have a preference for large males (Pack et al. 2012), suggesting that sexual selection could still favour large body size in males through female mate choice. Large male body size may convey other advantages, such as large offspring size, which has been correlated with low mortality in the first year of life in southern right whales (Best and Rüther 1992). Considering the atypical mammalian sexual dimorphism in mysticetes, where females tend to be slightly larger than males, selective pressures for large body size in females resulting from their higher energetic demands for reproduction likely outweigh sexual selection pressures for large body size in males (Ralls 1976).

1.4.2 Indirect competition to attract females via male song

Baleen whales show a high variety of sounds and acoustic displays ranging from the low-frequency sounds of fin and blue whales, some sounds of which are below human hearing, to the star-wars vocalization of dwarf minke whales (*Balaenoptera acutorostrata*), to the more complex and hierarchically structured songs of humpback whales (Clark and Garland 2022). While all baleen whales vocalize, some also produce male-only breeding vocalizations termed 'songs' (Table 1.2); these range in complexity from simple songs (comprised of a few sound types) of North Pacific right whales (*Eubalaena japonica*) to the highly complex songs of the bowhead and humpback whale (Garland and McGregor 2020).

The highly stereotyped and hierarchically structured song of humpback whales is one of the most elaborate and complex vocal displays in the animal kingdom (Figure 1.4). Songs typically last from 5 to 35 minutes; however, males may sing for many hours (Payne and McVay 1971; Winn and Winn 1978; Garland et al. 2013). Although songs change progressively each year through cultural evolution, all males within a population conform to the same song type at any given time (Winn and Winn 1978; Payne and Guinee 1983). In the South Pacific, a population's current song can be rapidly replaced by a novel song during so-called 'cultural revolutions' (Noad et al. 2000; Garland et al. 2011). This indicates that despite the song's high complexity, males are able to learn entirely novel songs very quickly (i.e., within one season).

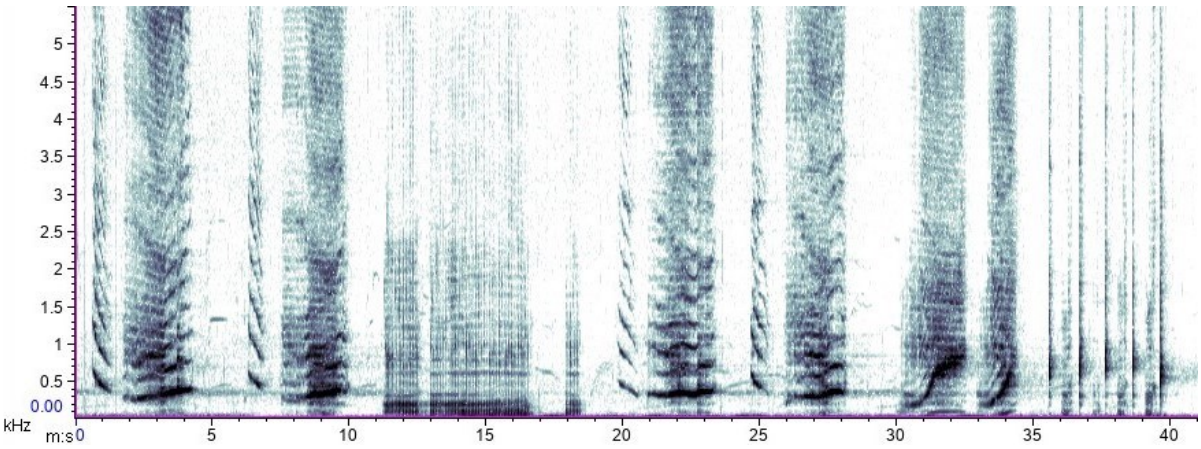


Figure 1.4. Spectrogram of a small, continuous section of humpback whale song showing a variety of units recorded from a lone male on the breeding ground off the coast of New Caledonia in the South Pacific in 2018. Corresponding audio is provided online. The x-axis indicates time in seconds; the y-axis shows frequency in kHz. Spectrogram was generated in RavenPro 1.6 (Hann window, 75% overlap, 2048 point FFT, 16-bit).

Table 1.2. Overview of baleen whale song from species that have been suggested to sing. Seasonality refers to the act of singing within a given year. Estimates of song complexity levels (simple, low complexity, high complexity) are based on the general song structure and sound repertoire (as given in 'Song description') and the temporal and spatial variation of song. Temporal and spatial variation refer to changes in song (e.g., structure, composition, frequency). Abbreviations: u: unknown or unclear; () for inferred or hypothesized.

Family	Species	Song description	Seasonality	Frequency (Hz)	Song complexity	Temporal variation	Spatial variation	Sex of singer	Reproductive function	Source
Balaenopteridae (rorquals)	Omura's whale, *Balaenoptera omurai*	amplitude-modulated vocalizations, rhythmically repeated	u	15-20	Simple but not much known	u	u	u	suggested	1
	Blue whale, *B. musculus*	low-frequency songs that consist of a series of phrases each comprised of 1-5 sounds units	(year-round)	16-100	relatively low	worldwide decline in frequency	geographically distinct	male	suggested, further hypothesized to indicate male body size	2-4
	Fin whale, *B. physalus*	low-frequency pulses arranged into stereotypic sequences at regular intervals (i.e., inter-pulse intervals, IPIs)	20 Hz calls produced mainly during the breeding season	15-40	relatively low	increase in IPIs, decrease in peak frequency	geographically distinct	male	suggested, further hypothesized to attract females to aggregations of prey	5-12
	Humpback whale, *Megaptera novaeangliae*	highly stereotyped and hierarchically structured: a sequence of sounds ('units'), creates a 'phrase', repeated phrases form a 'theme', and several different themes in a particular order make a 'song'	mainly during breeding season, and migration, and to a lesser extent on feeding grounds	50-4000	high	changes in song structure and complexity during cultural evolutions and revolutions	geographically distinct, high population conformity	male	suggested, further hypothesized to serve as a multi-message display and to indicate male quality	13-21

	Common minke whale, *B. acutorostrata*	low-frequency pulse trains with variable IPI structure and peak frequencies	seasonal but unclear if due to changes in vocal behaviour or absence of whales at recording sites	55-150	relatively low	limited variability between years	geographical variability in pulse train duration	(male)	suggested	22-24
Balaenidae	NP right whale, *Eubalaena japonica*	stereotypic sequence of gunshot sounds	(seasonal)	50-1500	simple	u	multiple song types within each region	male	suggested, further hypothesized to encode information on resource availability	25
	NA right whale, *E. glacialis*	long patterned sequences of gunshots (unclear if song due to data deficiency)	(seasonal)	50-1500	(simple)	u	u	(male)	suggested to function as female advertisement or male-male agonistic signal	26-28
	Bowhead whale, *Balaena mysticetus*	high diversity of song types comprised of highly modulated sounds and biphonation	(mainly during breeding season)	50-4000	high	complete renewal of singing repertoire	multiple song types within each region but shared among smaller clusters of animals	u	suggested, further hypothesized to indicate male quality	29-32

References: 1: Cercio et al. (2017); 2: Cummings and Thompson (1971); 3-4: McDonald et al. (2006, 2009); 5: Watkins et al. (1987); 6: Croll et al. (2002); 7: Delarue et al. (2009); 8: Simon et al. (2010); 9: Morano et al. (2012); 10: Oleson et al. (2014); 11: Širović et al. (2017); 12: Weirathmueller et al. (2017); 13: Payne and McVay (1971); 14: Winn and Winn (1978); 15: Payne and Guinee (1983); 16: Noad et al. (2000); 17-18: Garland et al. (2011, 2013); 19: Herman (2017); 20: Allen et al. (2018); 21: Murray et al. (2018); 22-23: Risch et al. (2013, 2014); 24: Risch (2022); 25: Crance et al. (2019); 26: Parks et al. (2005); 27: Matthews and Parks (2021); 28: Parks (2022) ; 29-30: Tervo et al. (2011b, a); 31: Stafford et al. (2018); 32: Erbs et al. (2021)

Humpback whale song has received considerable attention over the past 50 years, yet the underlying function(s) of song and its role within the mating system remains debated. There is clear evidence that singing is displayed solely by males which sing during the breeding season (including on migration and occasionally on the feeding grounds), and consequently singing is recognized as a male mating behaviour (Glockner 1983; Baker and Herman 1984; Darling et al. 2006; Smith et al. 2008). Most studies have investigated the function of humpback whale song either in the context of intra-sexual selection: (1) mediator of male-male interactions or male dominance relationships (Darling and Berube 2001; Cholewiak et al. 2018), (2) a spacing mechanism (Tyack 1981, 1983; Frankel et al. 1995), and (3) an index of association (Darling et al. 2006), while others suggest it is directed at females (inter-sexual selection): (4) female attraction to individual males (Winn and Winn 1978; Tyack 1981; Frankel et al. 1995), and (5) female attraction to an aggregation of communally singing males within the postulated lek mating system (Herman and Tavolga 1980; Herman 2017). Although most studies on song function have focused on either intra-sexual or inter-sexual drivers, many conclude that both selective pressures are likely at play (Frankel et al. 1995; Clapham 2000; Darling and Berube 2001; Craig et al. 2002; Herman 2017; Cholewiak et al. 2018; Murray et al. 2018); song may thus serve more than a single function.

Humpback whale song contains both simple and complex phrase types, suggesting it might act as a multi-message display (Murray et al. 2018). Simple phrase types typically contain low-frequency sounds suitable for transmitting a signal across long distances and may thus facilitate a female's and/or male's ability to locate a singer over large distances (Bradbury and Vehrencamp 1998; Murray et al. 2018). The high-frequency sounds typical of complex phrase types convey information over a shorter range; thus, these shorter-range signals may be directed at females akin to how courtship usually occurs once potential mates are within close proximity (Bradbury and Vehrencamp 1998). Further, the high structural variability found in complex phrase variants appears ideal for conveying information on male quality, thus allowing the possibility of female mate choice to be the driver of song complexity (Hebets and Papaj 2005; Murray et al. 2018). However, female preference for any humpback whale song characteristic has yet to be investigated.

Compared to humpback whales, much less is known about the songs of other baleen whales, but several commonalities and differences across mysticete song are becoming

apparent. The extraordinary diversity and variability of bowhead whale songs (Stafford et al. 2018; Erbs et al. 2021) suggest a complexity not dissimilar to the better-known humpback whale song. The songs of blue whales, fin whales, minke whales, North Pacific right whales, and Omura's whales are structurally simple, especially in the case of the latter two (Table 1.2). Although the songs of mysticetes show diverse levels of complexity and variability, they share several commonalities: (1) songs contain elements that aid long-distance communication across the ocean (e.g., contain low-frequency sounds and/or high redundancy; Payne and Webb 1971; Bradbury and Vehrencamp 1998; Clark and Ellison 2004; Risch 2022); (2) show some level of change across time (Noad et al. 2000; McDonald et al. 2009; Garland et al. 2011; Širović et al. 2017; Weirathmueller et al. 2017; Helble et al. 2020); (3) in at least the rorquals, songs show some level of conformity within geographically distinct groups (Payne and Guinee 1983; McDonald et al. 2006; Garland et al. 2011, 2013; Darling et al. 2014; Risch et al. 2014; Širović et al. 2017; Weirathmueller et al. 2017); (4) song was proposed to serve a reproductive function (Croll et al. 2002; Tervo et al. 2011a; Risch et al. 2013; Cerchio et al. 2017; Tyack 2022); and (5) for several species, song may convey individual-specific information and/or serve as a potential indicator of male quality (McDonald et al. 2006; Tervo et al. 2011a; Herman 2017; Clark et al. 2019; Crance et al. 2019; Erbs et al. 2021). For more detailed information by species, we direct readers to a recent review of baleen whale songs (see Clark and Garland 2022).

For species where the sex of the individual was determined, all singers were male (humpback whales: Payne and McVay 1971; fin whales: Croll et al. 2002; blue whales: McDonald et al. 2006; North Pacific right whales: Crance et al. 2019), and song mainly occurred during the breeding season (e.g., Smith et al. 2008), thus indicating that mysticete song likely serves a reproductive function and may therefore be under sexual selection. However, several species sing on the feeding grounds and during migratory stopovers (e.g., Owen et al. 2019). Singing outside the main breeding season might be driven by elevated testosterone levels during the spring or fall season while individuals are still on their high-latitude feeding grounds, as reproductive conditioning likely starts months before the peak breeding time (Vu et al. 2015). Such singing may represent a low-cost opportunistic advertisement by males to court females that failed to conceive, and/or possibly an intra-sexual display (Clark and Clapham 2004).

The 3D underwater habitat, the slightly larger body size of females relative to males (Ralls 1976), and the absence of organs to grab and force females into mating, all promote female behavioural freedom, and thus allow for a relatively strong influence of female mate choice compared to other mammals. Male-male competition and female mate choice are possible and non-exclusive drivers for the function of whale song. More research is needed to better understand whether song signals the singer's quality and whether males and/or females adapt their reproductive choices or behaviour upon receiving that signal.

1.4.3 Sperm competition over successful female fertilization

Except for bowhead, right, and grey whales, few matings have been observed by humans, and it is not known whether females mate with multiple males. Relative testes size and penis length serve as a proxy for the role of sperm competition (Würsig et al. 2023) and can shed light on the reproductive tactics of baleen whales. The relative testes size and penis length of right, bowhead, and grey whales are larger than those of all other baleen whales, and larger than expected based on their body mass, indicating the importance of sperm competition as their main reproductive tactic (Brownell and Ralls 1986; Dines et al. 2015). As mentioned in section 1.4.1, interactions among males in these species are relatively unaggressive and females mate with multiple males during the breeding season (Swartz 1986), sometimes even simultaneously (Mate et al. 2005), suggesting that males are unlikely to monopolize access to females. Further, the higher-than-expected microsatellite heterozygosity in offspring of North Atlantic right whales indicates post-copulatory competition among males (Frasier et al. 2013). As relatedness of mating pairs was not lower than expected under random mating, this excess of heterozygous offspring does not appear to result from pre-copulatory mate choice for dissimilar mates (Frasier et al. 2013). Instead, the observed patterns indicate the presence of post-copulatory selection for dissimilar gametes. However, it remains unclear whether these patterns are due to biased fertilization (e.g., cryptic female choice for dissimilar sperm) or biased mortality of zygotes (Frasier et al. 2013).

In comparison, the relative testes size of male humpback whales is lower than expected based on their body mass (Dines et al. 2015). Male humpback whales appear to engage in direct contest competition (section 1.4.1) which suggests that males attempt to monopolize and defend access to females, thus reducing opportunities for sperm competition

(Lüpold et al. 2014). Together with their elaborate acoustic displays (section 1.4.2), this indicates their investment in and reliance on pre-copulatory reproductive tactics. In most other rorquals (blue whale, fin whale, minke whale, Bryde's whale, and sei whale), relative testes size is within the range expected based on their body mass (Dines et al. 2015), and males are unlikely to be able to monopolize access to females due to their dispersed distribution (Table 1.1). Thus, sperm competition remains a possible male reproductive tactic in most rorquals.

1.5 Toolkit for studying reproductive tactics

Considering the long lifespan of baleen whales, long-term data collection is crucial to not only cover a wide range of the species' life history but to make inferences on sexual maturity and how reproductive tactics may change with age, experience, and/or condition. Many long-term studies on baleen whales are generally focused on the assessment of the population, rather than focal follows of individual whales. However, the identification of individual whales enables researchers to follow them through their lives to learn more about their life history patterns and ground truth and calibrate tools to study mating systems, reproductive tactics, and other factors such as population dynamics (e.g., epigenetic ageing, photogrammetry).

An example of a long-term study that has shed light on changes in reproductive patterns is the extensive long-term monitoring program on Oceania humpback whales that has allowed for the reconstruction of recapture histories and the modelling of the reproductive parameters in females (Chero et al. 2020). The relatively high calving rates of females at their breeding ground in New Caledonia are consistent with the high pregnancy rates inferred by blubber progesterone levels on the migratory corridors (Riekkola et al. 2018) and the feeding grounds (Pallin et al. 2018a), and may partially be driven by an increased reproductive capacity of this population (Chero et al. 2020). Epigenetic ageing of individuals at this breeding ground could reveal whether this increased reproductive capacity is related to the age structure of the population, and/or if the anthropogenic pressures caused by commercial whaling led to the modification of breeding parameters (i.e., age at maturity or birth interval) (Chero et al. 2020).

Combining long-term behavioural observations with molecular data is also a powerful approach. For example, paternity analysis using a long-term dataset of photo-identification and molecular data on the endangered North Atlantic right whale revealed low variation in male reproductive success (Frasier et al. 2007). Combining the paternity data with measures of neutral and functional genetic diversity further indicated the presence of post-copulatory selection for dissimilar gametes that may represent cryptic female choice (Frasier et al. 2013). This integration of genetic parentage and genetic diversity also unveiled a possible mechanism to mitigate the loss of genetic diversity after population exploitation (Frasier et al. 2013).

Research methods and technologies have greatly improved in recent decades resulting in a variety of tools for data collection and analysis offering new and deeper insights into the life of mysticetes. The examples above integrated long-term observational datasets, genetics, and hormonal (physiology) datasets. Building on these multi-disciplinary approaches will allow us to explore the interactions between anatomy, physiology, reproductive success, age, and vocal displays, to better understand the reproductive tactics of baleen whales. In Table 1.3 we highlight tools that can increase our understanding of the reproductive strategies and tactics of baleen whales and thereby direct readers to studies on their methods and examples in baleen whales.

Table 1.3. Tools that can be used to delve deeper into the reproductive tactics of baleen whales.

Tool	**Description**	**Examples in baleen whales**
Individual identification	Baleen whales can be individually identified by photo-identification of unique markings (e.g., ventral fluke patterns of humpback whales: Katona and Whitehead 1981; callosity patterns in right whales: Payne et al. 1983; Kraus et al. 1986; flank pigmentation patterns in blue whales: Sears et al. 1990) and/or genetic identification (e.g., microsatellite genotyping: Garrigue et al. 2004; Olavarría et al. 2007; Wade et al. 2011; Wiig et al. 2011; Baker et al. 2013; Carroll et al. 2013).	Reproductive histories of female humpback whales (Baker et al. 1987) Reproductive capacity of an endangered and recovering population of humpback whales (Chero et al. 2020)
Sex identification	Identifying the sex of individuals can be difficult in wild marine mammals; this can be done using behavioural observations and genetic markers.	Identifying the sex of focal animals in southern right whale social groups (Best et al. 2003)
Genetic parentage analyses	Using Mendelian inheritance patterns of genetic markers to infer maternity and paternity patterns (see Chapter 4).	Patterns of maternity and paternity can provide information on the reproductive skew, and variation in reproductive success (Cerchio et al. 2005; Frasier et al. 2007), the strength of sexual selection, as well as reproductive interchange among populations (Garrigue et al. 2004; Carroll et al. 2012).
Genetic diversity	Genetic diversity not only offers valuable insights into the demography and gene flow across populations, it further can be used to identify genes under selection and to assess the role of female choice within a species' mating system (e.g., genetic compatibility: Mays and Hill 2004; Puurtinen et al. 2005). Untangling molecular patterns of non-random	Diversity and duplication of MHC genes in several mysticetes suggest that these genes are under positive selection (Baker et al. 2006; Moreno-Santillán et al. 2016).

	fertilization in the context of post-copulatory sexual selection can shed light on the role of female choice and the resulting impacts on population biology and evolutionary genetics (gamete compatibility: Springate and Frasier 2017).	Linking parentage with genetic diversity revealed the post-copulatory reproductive strategy in North Atlantic right whales which might indicate cryptic female choice (Frasier et al. 2013).
Molecular age biomarker	Measurable changes in DNA or RNA abundance or sequence that change over the lifespan of an animal can be used to estimate age (Jarman et al. 2015). Epigenetic clocks make use of age-related changes in DNA methylation levels to estimate the age of living whales using skin biopsy samples. Such epigenetic clocks need to be calibrated using individuals of known age, thus highlighting the crucial role of long-term data collection for the assessment and ground-truthing of such methods.	Epigenetic age estimation has been applied to several baleen whale species (Polanowski et al. 2014; Tanabe et al. 2020; Goto et al. 2020; García-Vernet et al. 2021), and other cetaceans (Bors et al. 2021; Robeck et al. 2021). If related to other factors such as behaviour, body size, hormone levels and reproductive success, an individual's estimated chronological age can offer new insights into the reproductive strategies and life history parameters that, considering the life span of these animals, are out of reach of most datasets.
Endocrinology	Estimating hormone levels in individuals can tell us more about their sexual maturity or reproductive state (see Hunt et al. 2017). Hormone concentrations can be measured using multiple matrices: blubber, respiratory vapour ('blow'), and faecal samples (Rolland et al. 2005; Hunt et al. 2013), and for the retrospective and longitudinal assessment of reproductive hormones: baleen plates (Hunt et al. 2014, 2016).	Progesterone: inference of pregnancy status and rates (e.g., Kellar et al. 2013; Hunt et al. 2016; Pallin et al. 2018b, a; Kershaw et al. 2021). Testosterone: can be used to infer reproductive maturity and status of individuals or seasonal changes in reproductive state (e.g., Kellar et al. 2009; Vu et al. 2015; Cates et al. 2019; Mingramm et al. 2020; Melica et al. 2021) Estradiol: can provide information on female reproductive maturity and receptivity (e.g., Mingramm et al. 2020; Lowe et al. 2022)

Bioacoustics	Baleen whale vocalizations can be recorded using handheld equipment taken at the individual whale through to passive acoustic monitoring using autonomously deployed recorders that are anchored to the seafloor. Sound units are typically quantified for multiple acoustic parameters to ensure consistent classification of sound types (Dunlop et al. 2007; Garland et al. 2017; see also Clark and Garland 2022)	A quantitative comparison of the similarity in arrangement, structure, and complexity in humpback whale song (Garland et al. 2012, 2013, 2017; Allen et al. 2018) can uncover song dynamics at large spatial scales such as the unidirectional cultural revolutions (discussed in section 3; Garland et al. 2011), through to intricate intra- and inter-individual differences (Murray et al. 2018; Allen et al. 2018). By uncovering song differences, whether large-scale or extremely subtle, we may be able to tease apart aspects of the song that signal male quality and thus may serve in female mate choice.
Animal-borne tags	There are a wide variety of tag types ranging from high-resolution behaviour loggers to satellite tags that provide tracking data over large spatial and temporal scales (Goldbogen et al. 2013). Biologgers are tags equipped with additional sensors (e.g., accelerometer, hydrophones, video cameras, magnetometers) making them a powerful tool to simultaneously track the behaviour and environment of individuals (Watanabe and Goldbogen 2021). Such tools are extremely valuable for species that are more located offshore, deep diving, or live in environments that are otherwise hard to reach (e.g., ice shelf).	Satellite tracking can tell us about the migratory routes and spatial usage of species and individuals and can reveal migratory and reproductive strategies (e.g., Garrigue et al. 2015; Derville et al. 2018; Mackay et al. 2020). Biologgers, regularly used to study the foraging ecology and diving behaviour of marine mammals, could reveal further insights into the cost of reproduction and vocal communication of marine mammals.
Drone technology	Drones, or Unmanned Aerial Vehicles (UAVs), provide a cost-effective option for monitoring, photogrammetry, and behavioural observations of free-ranging marine species. Aerial photogrammetry can be used to estimate	As capital breeders, baleen whale body condition and reproductive costs likely play an important role in their reproductive strategies. UAV photogrammetry and long-term sighting histories can be used to establish

	the body size and condition of individuals (e.g., Dawson et al. 2017; Burnett et al. 2019; Christiansen et al. 2019; Aoki et al. 2021). UAVs can be used to obtain acoustic measurements close to the whales (Lloyd et al. 2016; Frouin-Mouy et al. 2020) and respiratory blow samples for genetic, endocrine, and microbiological analyses (Atkinson et al. 2021).	growth patterns to estimate age based on body mass, explore the energetic costs of female reproduction (e.g., Christiansen et al. 2014, 2022a, b), or the relationship between migratory timing and body condition (Russell et al. 2022). Paring acoustic recordings and overhead visual observations could shed light on the behavioural context and function of acoustic displays (e.g., song).

1.6 Conclusion and future directions

While the *Balaenidae* and *Eschrichtiidae* species appear to rely heavily on post-copulatory reproductive tactics by competing over successful fertilization rather than mating access; the morphology, behaviour, and distribution of many species within the *Balaenopteridae* (rorquals) suggest their reliance on pre-copulatory tactics. The often aggressive interactions among males within competitive groups suggest that male humpback whales compete primarily via direct contest competition by attempting to prevent matings by other males. The more widely dispersed distribution and lack of breeding aggregations of blue and fin whales could indicate scramble competition, where males directly compete in their efforts and efficiency of searching for and locating receptive females as their reproductive tactic, and highlights the importance of acoustic cues to find mates. Based on the trade-off between pre- and post-copulatory trait investment, the lack of prominent pre-copulatory traits of many baleen whales (Dines et al. 2015), except for a few species with elaborate male songs (see section 1.4.2), and the apparent lack of direct contest competition in most baleen whales (apart from humpback whales; section 1.4.1) suggests that polygynandry (and thus sperm competition) may be more common within mysticetes than the lack of direct observational evidence to date outside of the *Balaenidae* and *Eschrichtiidae* families may suggest. Thus, irrespective of the reproductive tactics males employ, the elaborate acoustic displays and large testes size observed in several mysticete species suggest that female baleen whales may be able to exert a certain level of choice, before and/or after copulation.

Much can be learned when taking a comparative perspective across marine mammals to understand reproductive tactics. While some species of pinnipeds and odontocetes show extreme levels of male-biased dimorphism in both body size (e.g., elephant seals, *Mirounga* spp.; killer whales, *Orcinus orca*) and weaponry (e.g., walrus, *Odobenus rosmarus*; narwhals, *Monodon monoceros*) (Mesnick and Ralls 2018b), sexual size dimorphism in baleen whales is relatively moderate and female-biased and further characterized by an absence of any dangerous male-specific weaponry. The temporal and spatial distribution and social structure of females increase the potential for single males to monopolize groups of females in land-breeding pinnipeds, and led to the evolution of male alliances and temporary courtships in several odontocetes (see Ralls and Mesnick 2019). The reproductive strategies and tactics across pinnipeds, odontocetes, and mysticetes are highly variable, yet the reproductive tactics

within each suborder vary similarly. Understanding how the diversity of ecological and social factors across and within each suborder shape the reproductive behaviours of individuals will shed light on the evolution of reproductive strategies and tactics of baleen whales, and marine mammals in general.

The emergence of new technologies has greatly enhanced the detail and scope of investigations that are now possible. Future directions include the employment of animal-borne tags and drone technology to study the behaviours of species and individuals that are difficult to observe or approach. The use of genomics, epigenomics, and endocrinology offers insights into the genetic quality, reproductive maturity and status, and physiological condition providing a comprehensive picture at the level of the individual needed to untangle the multifaceted factors shaping their reproductive tactics and the role and mechanisms of female choice. Understanding the function(s) of baleen whale song is a largely unanswered question that is ripe for exploration through a multi-disciplinary approach that offers insights into the proximate and ultimate causes of singing. This could then be expanded into a comparative perspective to investigate the evolution of song and complex communication in multiple taxa both marine and terrestrial. By understanding the reproductive tactics employed by the large whales we provide invaluable contributions to the wider understanding of mating behavior across taxa.

1.7 Thesis outline

This thesis focuses on patterns of male reproduction and sexual selection in humpback whales. Due to their complex acoustic male-only display, humpback whales have received considerable attention over the past 50 years. Yet, much about the species reproduction is left unknown and the song's underlying function(s) remain debated. The aim of this thesis is to improve our understanding of the complex mating behaviours of humpback whales and their role within the species' proposed polygynandrous mating system. To achieve this, I employed a variety of research methods using a 26-year-long dataset on humpback whales on their breeding ground in New Caledonia, South Pacific. The long-term monitoring of this breeding population offered me the unique opportunity to conduct an integrative analysis of

behavioural, (epi)genetic and endocrine data to explore the interactions between reproductive success, age, physiology, and population dynamics across time.

This thesis consists of four data chapters which all focus on different aspects of male reproduction by applying different techniques and data types. All data chapters contain analyses at the level of both the population and the individual. This further allowed me to assess the recovery, status, and connectivity of the New Caledonian population in the South Pacific. Detailed information on the analyses performed is presented in the Appendix at the end of each chapter.

Chapter 2 investigates the strength of sexual selection and reproductive autonomy of male humpback whales on their breeding ground in New Caledonia. A paternity analysis was conducted to assess the variation in male reproductive success and discuss findings in the context of the species' polygynous mating system. Paternity assignments were used to estimate the abundance of the male population using gametic mark-recapture and to investigate levels of gene flow between the New Caledonian humpback whales with other Oceanian breeding grounds. This chapter provides insights into the reproductive skew of humpback whales and the population dynamics across Oceanian breeding populations, two important factors affecting the recovery of humpback whales in the South Pacific.

Chapter 3 explores age-specific changes in sexual selection in light of the population's recovery from commercial whaling. Using epigenetic ageing, the age structure of the male population was assessed over time implementing published information on population growth throughout the study period. Building on the results from Chapter 2, the presence and extent of age-related changes in male reproductive tactics and reproductive success were investigated. This chapter ends with a discussion of its underlying limitations and highlights the potential of epigenetic ageing. It illustrates how sexual selection is currently acting on the complex male mating behaviours in a recovering population of humpback whales.

Chapter 4 assesses the seasonal and age-related changes in the reproductive physiology of male humpback whales. Using enzyme immunoassays, male blubber testosterone was measured and combined with age estimates derived in Chapter 3. Seasonal changes in male testosterone at the breeding ground were investigated at the level of the population and the individual within each year. Male blubber testosterone was highly variable at any point during

the breeding season and across males of all ages. The possible biological, environmental and/or social factors contributing to the observed large variation in male testosterone are discussed. This chapter demonstrates the integration of endocrine and molecular age markers in long-term datasets to be a powerful tool in the assessment of species' life-history trends and mating systems.

Chapter 5 investigates the influence of the major histocompatibility complex (MHC) diversity on patterns of male reproductive success in humpback whales. Applying a recently developed and validated cetacean MHC amplicon sequencing panel, the MHC diversity of the population was assessed at three MHC regions (class I, class IIa DQB and class IIa DRB). Results were put in the context of the possible natural selection pressures of the marine environment acting on MHC diversity in cetaceans. By building upon the results of Chapter 2, this chapter further tests the hypothesis of an MHC-mediated mate choice in humpback whales. It contributes to the reflection that female humpback whales may shape observed patterns of male reproductive success through pre-copulatory reproductive strategies.

Chapter 6 summarizes the major results from this thesis, which are put in the context of predictions of sexual selection theory and compared with other species. It includes an integrative assessment of the viability and recovery of the humpback whale breeding population in New Caledonia. I conclude by suggesting future areas of research that may be undertaken.

Chapter 2

Ecological and demographic factors shaping the patterns of male reproductive success in a singing cetacean

2.1 Abstract

Assessing the variation in reproductive success – the fundamental prerequisite for sexual selection to act upon a trait – is crucial in understanding a species' mating system and can provide insight into population growth. Parentage analyses in cetaceans are rare, and the underlying forces of sexual selection acting on their mating behaviours, including elaborate acoustic displays, remain poorly understood. Here, I combined 25 years of photo-identification and genetic data to assess variation in male reproductive success and population recovery of an endangered humpback whale breeding population located in New Caledonia, in the South Pacific. Paternity analysis of 177 known mother-offspring pairs and 936 adult males revealed low variation in male reproductive success with an average of 1.17 offspring per father over the entire study period. The observed male skew was higher than expected under random mating (FET: $p < 0.01$) but low relative to other polygynous species, including other aquatically-mating mammals. Finally, the male breeding population was estimated to consist of 2,058 [95% CI = 1,732 - 2,384] males over the study period. The observed low reproductive skew is in line with findings of other humpback whale populations and further emphasises the discrepancy between genetic estimates of paternity and predictions of the proposed polygynous social mating system in this species. Alternative mating tactics and/or female choice may counterbalance within-sex variation in reproductive success and should be considered when investigating the factors affecting male reproductive success and the underlying function(s) of humpback whale song.

2.2 Introduction

Variation in reproductive success is a fundamental prerequisite for sexual selection to act upon a trait. There are multiple factors that, often intertwiningly, define the extent to which reproductive success among individuals can vary. Ecological, demographic, and social circumstances (e.g., habitat, resource distribution, predation pressure, age structure) influence the spatial and temporal distribution of resources and mates, thus, the degree of control or monopolisation a male can hold over a female (Emlen and Oring, 1977). The greater the control, the higher the potential for polygyny (successful males mate with multiple females), and the larger the potential for variation in reproductive success. However, this influence is bidirectional, as the variation in reproductive success among individuals within a population can reciprocally affect several social factors.

Variation in reproductive success directly relates to the number of reproductively successful individuals, and thus, affects the effective population size (N_e), a common measure of the genetic variation in a population (Hedrick, 2005). High variation in reproductive success can lead to a high reproductive skew, meaning that certain successful individuals are contributing more to the gene pool of a population than others. This in turn means that fewer individuals are successfully reproducing than is expected based on the census population size (N_c). High reproductive skew lowers N_e, which in turn can increase the stochastic effects on the genetic structure of a population (i.e., genetic drift) and the likelihood of inbreeding in the population. This ultimately reduces the genetic diversity of a population. On the other hand, gene flow between populations (i.e., migration) can reduce these effects by introducing alleles, thereby increasing the effective population size and genetic diversity (Crow and Kimura, 1970). Patterns of reproductive success (i.e., variation and skew) within a population and the gene flow between populations, therefore, influence the viability of a population, its rate of growth and recovery (Sky et al., 2022), as well as the strength of (sexual) selection (Hosken and House, 2011).

Compared to terrestrial mammals, variation in reproductive success is often much lower in cetaceans, especially in baleen whales. It is thought that this is due to the difficulty in controlling territory in a 3D underwater habitat and the often dispersed distribution of females lowering the potential for polygyny (see Chapter 1). However, in one species, the humpback whale (*Megaptera novaeangliae*), the male-biased sex ratio on breeding grounds

(Baker and Herman, 1984; Clapham, 1996; Cerchio *et al.*, 2005; Chero *et al.*, 2020), the resulting intense competition among males, and the elaborate acoustic display suggest intense sexual selection. In light of the species' proposed polygynous mating system (Clapham, 1996; Clapham and Palsboll, 1997), these observations suggest high variation in reproductive success. Yet, previous studies on male reproductive success in humpback whales found low levels of variation in reproductive skew (Nielsen *et al.*, 2001; Cerchio *et al.*, 2005) matching the findings in North Atlantic right whales (*Eubalaena glacialis*; Frasier *et al.*, 2007) and southern right whales (Eubalaena australis; Carroll *et al.*, 2012). While the marine habitat undoubtedly explains the lower variation in male reproductive success of baleen whales in comparison to polygynous mammals on land, there are many gaps in our understanding of their reproductive behaviours and mating systems. The elusive nature and low abundance of many baleen whale species make studies on their reproduction extremely challenging, especially at the temporal scale necessary to evaluate lifetime reproductive success.

This study focuses on humpback whales at their breeding ground off the coast of New Caledonia (NC) in the South Pacific. The International Whaling Commission (IWC) recognises this breeding ground as sub-stock E2 as part of the genetically and demographically distinct Oceania metapopulation (that also includes the sub-stocks of Tonga (E3), the Cook Islands (F1) and French Polynesia (F2) (Olavarría *et al.*, 2007; Childerhouse *et al.*, 2008)). Comparisons of photo-identified whales found that the movement of individuals between Oceania and the Eastern Australia population (E1) (Garrigue *et al.*, 2007) and among populations within Oceania (Garrigue *et al.*, 2002, 2011) was limited, which is further supported by low levels of differentiation in mitochondrial (mtDNA) haplotype frequencies among these regions (Olavarría *et al.*, 2007). Due to this genetic differentiation and low levels of individual movement, it has been suggested that Oceanian sub-stocks are demographically independent of each other and thus are referred to herein as 'populations'.

While occasional genetic interchange between breeding populations in the South Pacific takes place (Steel *et al.*, 2018), the level of gene flow remains unclear. Humpback whales in the Southern Hemisphere were decimated to very low numbers due to commercial whaling (Baker and Clapham, 2004). Many populations have since significantly recovered, some to near pre-exploitation abundance (e.g., western South Atlantic: Bortolotto *et al.*, 2016; eastern Australia: Noad, Kniest and Dunlop, 2019). However, the abundance of the Oceania

metapopulation remains low relative to presumed historical numbers (Constantine *et al.*, 2012) and shows lower levels of recovery than its neighbouring population located in eastern Australia (Noad *et al.*, 2011). The movement of individuals, and the genetic material they carry, among breeding populations in the South Pacific affects their N_e; therefore, assessing the level of gene flow between populations is important for understanding the population dynamics and recovery of humpback whales from commercial whaling in this wide region.

Here, I combined 25 years of photo-identification data with genetic data to assess patterns of reproductive success and reproductive autonomy of humpback whales on their breeding grounds in New Caledonia. First, I conducted a paternity analysis on 177 mother-offspring pairs to assess the variation and skew in male reproductive success in comparison to expectations from behavioural observations and sexual selection theory. Second, using gametic mark-recapture (GMR), I estimated male abundance and investigated the level of gene flow between the New Caledonian humpback whale population with other Oceanian populations. My results provide insights into the reproductive skew of humpback whales and the population dynamics across Oceanian populations, two important factors affecting the recovery of humpback whales in the South Pacific.

2.3 Methods

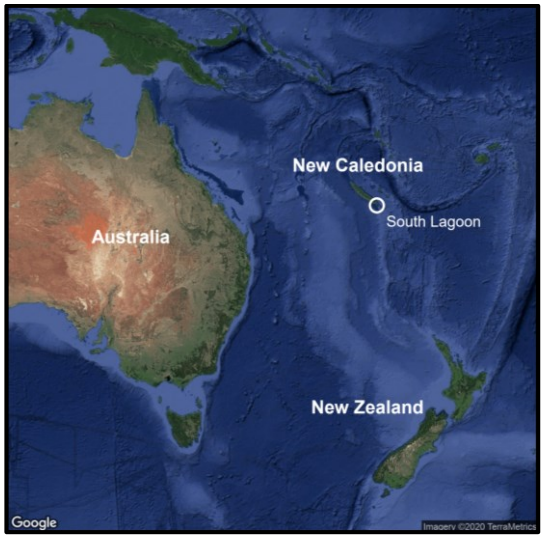
2.3.1 Study site and data collection

Yearly field surveys on humpback whales were conducted on their breeding ground in New Caledonia (E2), South Pacific, between July to September 1995 to 2019, led by Opération Cétacés (Garrigue et al. 2001; Derville et al. 2019). I took part in the field surveys in 2018 and 2019. Survey efforts were primarily focused on the South Lagoon, a shallow area located south of the mainland (Figure 2.1a). Across the study period, several other areas around New Caledonia were additionally studied: the Chesterfield-Bellona coral reef complex; the Southern seamounts of Antigonia and Torch Bank (including the Isle of Pines); the Loyalty Ridge including Orne Bank, Walpole Island, and the Ellet seamount; and several areas closer to the mainland, such as the Loyalty Islands (Lifou, Tiga, Maré, Ouvéa), the East lagoon, North lagoon and West lagoon along the mainland (Figure 2.1b). Field surveys at the main study site (South Lagoon) were conducted daily (weather permitting) where a sea-based team on a 6-m rigid-hulled inflatable boat was in continuous contact with a land (spotting) team that overlooked the study areas from the lighthouse Cape N'Doua (Figure 2.1c). Although boat-based survey efforts varied greatly across study areas and years, survey efforts at the South Lagoon were held largely constant throughout the study period (with exception of the year 2008; Table 2.1).

During field surveys, behavioural, photo-identification, biopsy and acoustic data were collected. For each observed group of whales, the time and location (GPS position) at the start and end of each focal follow, group size, group type (Table 2.3), and any changes in the group composition (group split or individuals joining) were recorded. The behaviour of individuals (e.g., singing, escorting, challenging; Table 2.2) was recorded and tracked throughout observations and individuals were assigned a social type depending on their age class and their behavioural role within the group (Table 2.3). During focal follows, whales were carefully approached to be photographed and biopsied. Skin samples were collected from adults, juveniles and calves using a crossbow with a specially adapted bolt (Lambertsen *et al.*, 1994) or a small dart fired from a modified veterinary rifle (Krützen, 2002). On some occasions, sloughed skin from individuals engaging in surface-active behaviours was also collected (Clapham, Palsboll and Mattila, 1993). Skin samples were stored in 70% ethanol at -20°C. Individual humpback whales were identified based on photo-identification from unique

markings on the ventral surface of their tail flukes (Katona and Whitehead, 1981) and/or their genotypes (see section 2.3.2).

(a)



(b)

(c)

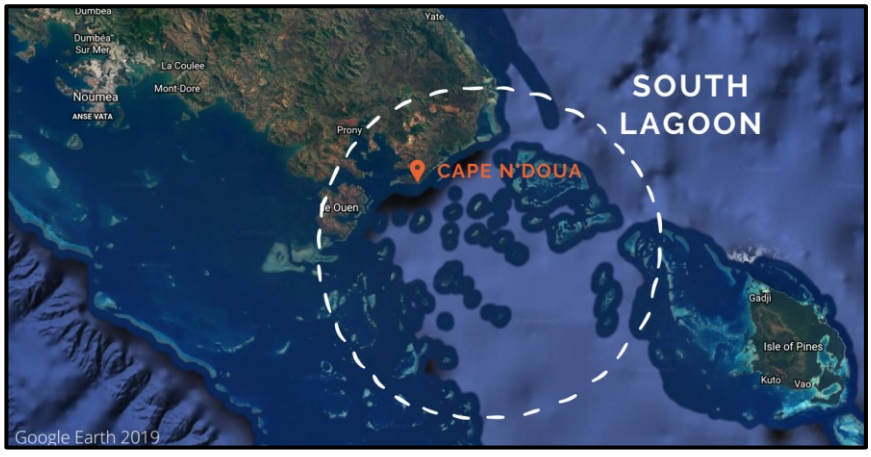


Figure 2.1. New Caledonia is (a) located in the western South Pacific, in the Coral Sea, and (b) is surrounded by several reefs, small islands and seamounts. The South Lagoon at the southern end of New Caledonia is the main study area. The lighthouse at Cape N'Doua (c) serves as a platform for the land-based team to overlook the study area.

Table 2.1. Boat-based survey effort (in days) in each of the study areas between 1995 and 2019. Description of study areas (Figure 2.1): South lagoon: main study site at the southern part of the mainland; Southern seamounts: Antigonia and Torch Bank and including Isle of Pines; Loyalty ridge: Orne Bank, Walpole Island and the Ellet seamount; Loyalty islands: Lifou, Maré and Ouvéa; West lagoon: western part of the mainland; North lagoon: northern part of the mainland; Chesterfield-Bellona coral reef complex: Chesterfield and Bellona atolls and shallow banks located halfway between New Caledonia and Australia; East lagoon: eastern part of the mainland. Shades of blue: 1-15 days = light blue, 16-30 days = medium, 30+ days = dark.

	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
South lagoon	27	55	44	50	46	50	55	35	43	10	44	41	49	3	33	38	39	29	25	29	31	30	39	33	28
Southern seamounts	0	0	0	0	0	0	7	0	0	0	7	4	0	15	5	9	7	0	0	0	0	0	6	2	0
Loyalty ridge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2	4	6
Loyalty islands	0	0	3	7	14	0	0	0	0	0	0	0	0	0	0	0	0	15	16	0	0	0	0	0	0
West lagoon	0	0	0	0	0	0	0	0	0	13	0	0	0	69	16	4	0	0	9	7	0	0	0	0	0
North lagoon	0	0	0	0	0	5	8	0	0	13	0	7	0	0	0	0	0	0	0	1	0	0	0	0	0
Chesterfield Bellona reef complex	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	6	0	0	0	0	0	11	18	0	0
East lagoon	0	0	0	0	0	1	11	0	0	19	5	0	0	13	6	0	0	0	0	16	0	0	0	0	0
Total	27	55	47	57	60	56	81	42	43	55	56	52	49	100	60	57	46	44	50	53	31	45	65	39	34

Table 2.2. Definitions of the multiple reproductive tactics of male humpback whales.

Reproductive tactic	Definition
Physical competition	Direct competition over access to a single mature female (with or without a calf) offered by the temporary formation of competitive groups of three or more individuals. Males often engage in agonistic fights to gain and maintain the position closest to the female of the group (termed 'primary escort') (Tyack and Whitehead, 1982; Clapham et al., 1992).
Escorting	An 'escort' is defined as a male associating with a mature female or a maternal female with calf. Here, I use the term 'solitary escort' to differentiate a single male escorting a female from males escorting a female within a competitive group (e.g., primary escort, challenger secondary escort; see Table 2.3). It is unclear whether escorting a female results in copulation or whether it reflects mate guarding following earlier copulation (Clapham, 1996).
Singing	Humpback whale song is a highly stereotyped, hierarchically structured, culturally transmitted vocal display produced solely by males predominantly during the breeding season (Glockner, 1983; Baker and Herman, 1984; Darling, Jones and Nicklin, 2006; Smith et al., 2008). Although the function(s) of humpback whale song remain debated, it is generally accepted that song functions in sexual selection 1) to attract females, 2) within male-male competition, 3) as an aggregating stimulus within the lekking system, or 4) a combination of these functions (see Herman, 2017).

Table 2.3. Description of humpback whale group and social types.

Group type	Social type	Description
Solitary	Solitary	Single individual (male or female)
Mother-calf	Mother	Female with dependent offspring
	Calf (or yearling)	Calf born this season (yearling, if born in the previous season)
Mother-calf with escort[1]	Solitary escort of a mother	Solitary male and mother with dependent calf
Competitive group (with or without mother-calf)[2]	Nuclear animal	Female (can be accompanied by her calf or be alone) at the centre or front of the group that appears to be leading the direction of movement of the group
	Principle escort	Male closest to the Nuclear animal
	Challenger	Male actively challenging the Principle escort for his position to be closest to the Nuclear animal
	Secondary escort	Any other male within the group actively following the Nuclear animal
	Undefined member of a competitive group	Unclear behavioural role or position within the group
Singer[3]	Singer	Singing male (usually solitary)
Groups of 2, 3 or 4	Member of a group of 2, 3 or 4	A group of 2, 3, or 4 individuals. These groups are much more slow-paced and show low to no levels of aggression in comparison to competitive groups, and no one individual appears to be the lead or at the centre of the group.

References: 1: Brown and Corkeron (1995), Herman et al. (2011); 2: Tyack and Whitehead, (1982); Baker and Herman, (1984); Clapham et al. (1992); 3: Herman (2017)

2.3.2 Genetic profiling

Genomic DNA was extracted from skin samples by digestion with Proteinase K, and standard phenol/chloroform extraction and ethanol precipitation methods (Sambrook, Fritsch and Maniatis, 1989), as modified for small tissue samples by Baker et al. (1994) (protocol in supplementary S2.1). DNA was quantified using a NanoDrop and diluted to a concentration of 20ng/ul with Tris-EDTA (TE) buffer. Individuals were sexed via molecular sexing by multiplex PCR amplification of two sex chromosome specific loci ZFX and SRY using the primers P1-5EZ and P2-3EZ (Aasen and Medrano, 1990) and Y53-3C and Y53-ED (Gilson *et al.*, 1998), respectively. An individual's sex was determined using gel electrophoresis of the PCR product by visualising the stained DNA bands (Midori Green) in UV light. Females show only one band (homogametic: XX) and males show two bands (heterogametic: XY). The mtDNA haplotype of individuals was determined by sequencing an approximately 800 base-pair (bp) fragment of the 5'-end of the mtDNA control region (i.e., D-loop). PCR amplification of the mtDNA control region was carried out using the primer M13Dlp1.5 (tPro-whale, 5'-TGTAAAACGACAGCCAGTTCACCCAAAGCTGRARTTCTA-3'; Baker *et al.*, 1998) and primer Dlp8G (5'-GGAGTACTATGTCCT-GTAACCA-3'; Lento, Patenaude and Baker, 1997) primer following previously published methodologies (Garrigue *et al.*, 2004; Olavarría *et al.*, 2007; Constantine *et al.*, 2012).

Sampled individuals were genotyped using at least 16 previously published microsatellite loci (464/465: Schlötterer, Amos and Tautz, 1991; Ev1, Ev14, Ev21, Ev37, Ev94, Ev96 and Ev104: Valsecchi and Amos, 1996; GATA28 and GATA417: Palsboll *et al.*, 1997; rw31, rw4-10 and rw48: Waldick, Brown and White, 1999; GT211, GT23 and GT575: Berube *et al.*, 2000) (Table S2.1) as described in Garrigue et al. (2004). PCR amplification for each microsatellite loci was conducted separately, and later co-loaded in four sets of multiplex arrangements for further analysis (see thermocycling conditions in Table S2.2 and PCR set-ups in Table S2.3). Multiplexed PCR products (2µl) were mixed with 3.9 µl HiDi formamide and 0.1 µl size standard (GeneScan500LIZ), heat-shocked and then analysed via capillary electrophoresis on an ABI 3730xl DNA sequencer (Applied Biosystems). Allele scoring was done by D. Steel and C. Bonneville using the GeneMapper v5 software (Applied Biosystems). Individuals typed at fewer than 12 loci were discarded from the dataset as a measure of quality control. The mean per-locus genotyping error rate was 0.011, as reported by Constantine et al. (2012).

Microsatellite allele frequencies and analysis of the probability of identity for each locus were conducted using GenAlEx 6.5 (Peakall and Smouse, 2006, 2012). Population genetic tests for Hardy-Weinberg equilibrium, linkage disequilibrium, and null alleles were carried out in CERVUS 3.0.7 (Kalinowski, Taper and Marshall, 2007) and GENEPOP 4.7.0 (Rousset, 2008).

To assess the overall (statistical) power of the microsatellite markers used in this study I analysed the information content of each marker using the Fortran program KinInfor v2 (Wang, 2006). KinInfor provides four informativeness measurements (I_R, I_r, PW_R, RMSD), for each single genetic marker or multiple loci, and incorporates genotyping errors and mutations (Wang, 2006). Informativeness for relationships (I_R) and relatedness (I_r) evaluate the amount of information contributed by each microsatellite locus in inferring pairwise relationships (R) and relatedness (r), respectively (Wang, 2006). The "power for relationship inference" (PW_R) of a given set of loci in differentiating between two relationship categories is obtained via analytical and simulation methods (Wang, 2006). The reciprocal of the mean squared deviations of relatedness estimates (RMSD) measures the amount of information from markers that are actually used by an estimator in estimating relatedness (Wang, 2006). To investigate the gradual increase in power of the whole set of markers I conducted a rarefaction analysis showing the $PW_{R\ (simulation)}$ of each (sub)set of markers and plotted each marker in succession ordered according to decreasing I_R score (see Table S2.4 for detailed data input and parameters used in the analysis of informativeness of markers).

2.3.3 Paternity analysis

I used the microsatellite genotypes to undertake paternity analysis using mother-calf pairs and males sampled over the 25-year study period. Strict exclusion is the simplest method of paternity analysis. If mother-offspring pairs are known, the strict exclusion method applies Mendelian inheritance to identify the paternal alleles inherited by the offspring. Genotypes of all candidate fathers were compared with the paternal alleles in the offspring and males whose genotypes were not consistent with the inferred paternal alleles were excluded as potential fathers. The strict exclusion method is a powerful tool but has two drawbacks. First, the occurrence of genotyping errors such as null alleles, allelic drop-out, and false alleles, as well as germ-line mutations, can lead to the false exclusion of true fathers (Marshall *et al.*, 1998; Jones *et al.*, 2010). This can be accommodated by allowing for one or two mismatches

if genetic markers are powerful enough and if the occurrence of genotyping errors and mutations is rare (Jones *et al.*, 2010). Second, the strict exclusion method does not account for multiple non-excluded males that share alleles by chance alone (Jones *et al.*, 2010). Other more complex paternity methods are necessary to choose amongst multiple non-excluded putative fathers.

Categorical allocation allows the identification of a single most likely father from a group of non-excluded putative fathers. The program CERVUS implements the categorical allocation method by comparing the maximum likelihoods of the two most likely fathers while allowing for genotyping errors and mutations in marker data (Kalinowski, Taper and Marshall, 2007). For each candidate father, CERVUS calculates the natural logarithm of the likelihood-odds ratio (LOD score) as the likelihood of paternity of a particular male relative to the likelihood of paternity of an arbitrary male based on the genotypes of offspring, mother, and alleged father and allele frequencies (Meagher and Thompson, 1986; Marshall *et al.*, 1998). Paternity is assigned to the candidate father with the highest LOD score if the difference between the LOD scores (Δ score) of the two most likely candidate fathers is large enough. Only if the Δ score exceeds a certain critical value, obtained through simulation, is the paternity confidently assigned to the most likely father with the highest LOD score. Simulations are carried out to establish the critical Δ score that determines the confidence of a paternity assignment at two different confidence levels: 95% and 80% (software default). These simulations of paternity inference take into account population parameters (e.g., proportion of males sampled), completeness of the genetic dataset (e.g., proportion of loci typed) and genotyping error rate (e.g., number of loci mistyped). Here, the critical Δ score was established by the simulation of 10,000 offspring genotypes and a genotyping error rate of 0.011 (section 2.3.2). A minimum of 10 loci were compared among mother, offspring, and candidate father (minimum number of overlapping loci). Thus, the 'proportion of loci typed' was conservatively set to this minimum of 10 loci (10/15 loci: 0.67). The 'number of candidate males' was set to 936 males representing the total number of males that were sampled across the entire study period. Considering that the study period ranges over 25 years (1995 – 2019) and that the population has been growing throughout this time but predominantly so from 2008 onwards (Garrigue, Albertson and Jackson, 2012), the number of candidate males will likely be overestimated in the earlier years of the study and potentially underestimated in the later years of the study period. Based on the abundance estimate of the New Caledonian population in 2016 of 1,870

males (male-derived estimate calculated from sex-unspecific estimate by Zicos, Garrigue and Jackson, unpublished data; see also Table S2.13), around 50% of the male population were sampled (936 genotyped males). Assuming a larger population, and therefore a smaller proportion of males sampled, results in a higher critical Δ score, and thus, more conservative paternity assignments. Therefore, considering the unclear levels of genetic interchange of the New Caledonian population with neighbouring populations (see section 2.2) the proportion of candidate males sampled was set to a more conservative estimate of 30%. A sensitivity analysis was carried out to assess the robustness of paternity assignments to altering values of simulation parameters (e.g., number of candidate fathers, proportion of candidate fathers sampled, see Table S2.14). I used CERVUS' default confidence levels: 80% and 95%. A full list of simulation parameter values is provided in Table S2.5 and Table S2.6.

Considering the relationships among all genotyped individuals jointly, as is the case in full-likelihood methods, is suggested to be a more powerful and accurate approach in paternity analysis than pairwise-likelihood methods (Wang and Santure, 2009). The program Colony follows such a full-likelihood approach to partition sampled individuals according to shared parentage or sibship relationships into family clusters (Wang and Santure, 2009). The likelihood of a partition (relationship configuration) is calculated by the product of the likelihoods of the independent family clusters in the partition (Wang and Santure, 2009). A simulated annealing algorithm is applied to search for the best configuration with the maximum likelihood (Wang and Santure, 2009). This is done by generating an initial configuration that randomly allocates offspring, candidates females, and candidate males into distinct family clusters and then calculates the likelihood of that initial configuration. It then generates a new configuration by randomly changing part of the previous configuration (e.g., reassigning paternity by choosing at random an offspring and a male) and calculates the likelihood of this new configuration. The rate at which a new configuration is accepted is controlled by an annealing temperature that is steadily adjusted as the simulation proceeds so that new configurations with a smaller likelihood than the previous configuration are accepted less frequently (Wang and Santure, 2009). These steps are repeated many times until efforts to improve configurations become sufficiently discouraging, and in the end, the best configuration with the maximum likelihood is reported (Wang and Santure, 2009). Colony accounts for genotyping error and incorporates prior information on the probability of a true father being sampled, similar to CERVUS' proportion of candidate males sampled, and known

sibship inferences (e.g., offspring from the same female). The parameters, their set values and input files for the paternity analysis in Colony are shown in Table S2.7 and Table S2.8.

Here, paternity analysis was conducted using two different methods: (i) categorical allocation using the maximum likelihood (ML) approach implemented in CERVUS (v3.0.7; Kalinowski, Taper and Marshall, 2007), and (ii) the full-likelihood (FL) method of Colony (v2.0.6.5; Wang and Santure, 2009). Paternity analyses were conducted for each year of the study period (1995 – 2018) to account for the growing pool of candidate fathers as calves from earlier years reached sexual maturity. Calves sampled on the breeding ground on a particular year were sired in the previous year by males that were sexually mature in the year they sired. This means that calves born and sampled in the year 2019 were sired in the year 2018. Thus, although individuals sampled in the year 2019 were included in the analysis, the paternity analysis is focused on offspring born and males that sired between 1995 and 2018. The analyses were based on the yearly number of candidate males and the sampled mother-offspring pairs. Genotypes of known mother-offspring pairs were reviewed to confirm their parent-offspring relationship and inspected for mismatches due to possible genotyping errors and null alleles (in collaboration with D. Steel). Males were considered as candidates if they were of unknown age or if they were at least five years old the year prior to sampling the calf. Although a recent update on the age at sexual maturity estimates humpback whales to reach sexual maturity on average at nine to eleven years of age based on a bi-annual accumulation rate of earplug laminations (Best, 2011), some individuals may reach sexual maturity at an earlier age (Gabriele, Straley and Neilson, 2007). Thus, to prevent the false exclusion of younger males as putative fathers, we applied a more conservative estimate of sexual maturity (5 years) for the paternity analysis.

The recommended levels of statistical confidence levels for paternity analyses greatly depend on the research question of interest. While it is crucial for studies on individual reproductive success and behavioural correlations to avoid false paternity assignments through more stringent criteria and high confidence, applying the same confidence criteria for studies on the variation of reproductive success may be problematic due to the increased risk of falsely excluding true fathers (Cerchio *et al.*, 2005). Like in other statistical analyses, the confidence level in paternity analyses acts as a trade-off between type I error (false paternity assignment) and type II error (false exclusion). Increasing the confidence level of paternity

assignments reduces the risk of false paternity assignments (type I error), yet it comes at the cost of an increased risk of missing true parent-offspring pairs (type II error). False exclusion inflates the number of males with no or few offspring, and thus, negatively biases estimates of reproductive success. Here, I followed the recommendations of previous baleen whale paternity studies (Cerchio *et al.*, 2005; Frasier *et al.*, 2007) and created two paternity datasets based on two different confidence criteria for all further analyses on male reproductive success. The relaxed paternity dataset included paternity assignments at a confidence level of at least 80% in CERVUS while allowing for two mismatches of loci. The conservative paternity dataset included only paternity assignments at the 95% confidence level of CERVUS while allowing for zero mismatches of loci. Paternity assignments of offspring for which the two methods (ML and FL) assigned different fathers to the same offspring were excluded from the analysis. Paternity results from Colony were primarily used to confirm the assignment by CERVUS due to limitations resulting from sibling sample size (explored in detail in Discussion section 2.5.3). The true pattern of male reproductive success likely falls somewhere in between the estimates derived from these two paternity datasets.

2.3.4 Patterns of paternity in non-sampled fathers

To assess the male reproductive skew of all sampled offspring we need to account for the fathers that were not sampled. I used DadShare (provided and written by W. Amos; see Hoffman, Boyd and Amos, 2003) to estimate the number of fathers that sired offspring that were left unassigned in the paternity analysis. DadShare estimates how many males may have fathered those calves for which all sampled males were excluded as fathers. DadShare compares the genotypes of known mother-offspring pairs to infer the paternal portion and calculates the pairwise relatedness values (r-values) amongst offspring following the methods of Queller and Goodnight (1989) using only the paternal alleles. A clustering algorithm sequentially links the most closely related individuals to form a dendrogram that is then searched for clusters of offspring that are compatible with a single father. Additionally, the program performs Monte Carlo simulations to generate datasets of different degrees of polygyny (e.g., each male siring one, two, three, four or five offspring). The average r-value of external nodes generated from these simulations provides a reference with which the observed average r-value of the external nodes can be compared to. To evaluate the

robustness of the simulation analysis in DadShare, I conducted the analysis on two different subsets of sampled offspring: offspring that were assigned fathers (assigned paternities) and offspring that were left unassigned likely because their fathers were not sampled (unassigned paternities), for each of the two paternity data sets (conservative and relaxed).

2.3.5 Test of equal reproductive success

To assess whether males share equal chances of siring offspring within the study population, I compared the observed distribution of sampled males assigned as fathers of zero, one, or more sampled offspring as derived from the paternity analysis to the distribution of paternities expected under the assumption of random mating. The expected distribution of paternities was generated using randomised simulations following methods described in Frasier et al. (2007). Simulations were based on the yearly number of candidate males that were used in the paternity analysis and the number of mother-offspring pairs for which paternities were assigned each year to ensure simulation results were directly comparable to the results from the paternity analysis. The simulation analysis (this section) and GMR (section 2.3.6) were conducted using R Statistical Software (v4.0.4; R Core Team, 2021) and the code is available on github (https://github.com/francae/PhD-Thesis_FrancaEichenberger).

The simulation process to establish the expected paternity distribution under random mating had five steps:

(1) For the first year of the analyses, fathers for the number of assigned paternities in that year were randomly selected (with replacement) from the pool of mature males in that year.
(2) This process was then repeated for each year of the study period (1995 – 2018).
(3) The number of offspring sired by each male was then summed across all years to generate the expected number of males assigned zero, one, or more offspring if mating was random and all males had an equal probability of siring offspring.
(4) This process (steps 1 to 3) was repeated 1,000 times to generate the mean and standard deviation (SD) of the number of males assigned zero, one, two, three, or more offspring across all 1,000 iterations.

(5) A two-sided Fisher's exact test (FET) with α = 0.05 was conducted to test whether differences between the observed and expected distributions of paternities were statistically significant. Further to this, I compared the observed and the simulated mean, variance, and standardised variance (SV, see Equation 1, e.g., Coltman, Bowen and Wright, 1998) in male reproductive success for both paternity datasets.

Equation 1 $$\mathrm{SV} = \frac{\mathrm{mean}}{\mathrm{variance}}$$

2.3.6 Gametic mark-recapture (GMR) abundance estimate

I applied gametic mark-recapture (GMR) to estimate the abundance of the male breeding population in New Caledonia following previously published methods (e.g., Pearse *et al.*, 2001; Garrigue *et al.*, 2004; Carroll *et al.*, 2012). The number of assigned paternities in each of the two paternity datasets (conservative and relaxed) formed the gametic recapture of males. Chapman's (1951) modified version of the Lincoln-Peterson two-sample model was adopted for the gametic-recapture estimate:

Equation 2 $$\mathrm{N_m} = \frac{(\mathrm{n_1}+1)(\mathrm{n_2}+1)}{(\mathrm{m}+1)} - 1$$

where N_m is the estimated number of reproductive males, n_1 is the number of mature males sampled over the entire study period (first capture), n_2 is the number of offspring from sampled mother-offspring pairs (second capture), and m is the number of inferred paternities (recapture). The variance of the male abundance estimates (Var_N) was computed as described for the Lincoln-Peterson estimator (Equation 3) and an approximate 95% confidence interval was estimated (Equation 4):

Equation 3 $$\mathrm{Var_N} = \frac{(\mathrm{n_1}+1)(\mathrm{n_2}+1)(\mathrm{n_1}-\mathrm{m})(\mathrm{n_2}-\mathrm{m})}{(\mathrm{m}+1)^2(\mathrm{m}+2)}$$

Equation 4 $$\mathrm{N_m} \pm 1.965 * \mathrm{Var_N}^{0.5}$$

The GMR was conducted across a reduced study period (2000 – 2018). I excluded the first five years of the wider study period (1995 – 1999) from the GMR due to the lower effort to collect data on calves before the year 2000 (Figure 2.2). To assess the sensitivity of our abundance estimates to different levels of confidence in the paternity assignments, I conducted the gametic-recapture analysis on both paternity datasets (conservative and relaxed) separately.

The two fundamental assumptions of the GMR are that (1) the population is closed (geographically and demographically), and that (2) all animals are equally likely to be captured in each sample (Chapman, 1951). During the 19-year-long GMR study period (2000 – 2018), the population may have undergone significant input from births and deaths and is thus not closed. Moreover, both photo-ID and genetic data demonstrate some movement of individuals among Oceanian breeding grounds (Garrigue et al., 2002, 2011; Constantine et al., 2007; Steel et al., 2018). This violation of the GMR's closure assumption can bias estimates upwards as capture probabilities are reduced from the inflated number of marked animals (Boulanger, Mclellan and Boulanger, 2001). In this study (based on the paternity analysis), males become reproductively mature throughout the study period and therefore are eligible for ´recapture´ as they age. In conclusion, my male abundance estimates across the study period (2000 - 2018) might thus be best interpreted akin to a super-population abundance estimate (i.e., the total number of individuals present at the start and entering the population throughout the study period assuming no mortality; Schwarz and Arnason 1996).

2.3.7 Assessing the genetic interchange of the New Caledonian breeding population

Despite the reported low levels of interchange between the New Caledonian breeding grounds and other regional breeding grounds in the South Pacific (Garrigue et al., 2002; Garrigue et al., 2011), occasional gene flow among breeding grounds within Oceania and between Oceania and East Australia takes place (Steel *et al.*, 2018). To assess whether a substantial number of males from other populations were contributing to the paternity of New Caledonian calves, I compared the GMR abundance estimates of the male breeding population to previous photo-ID or genetic abundance estimates of the New Caledonian and other breeding populations within Oceania. Considering the observed migratory interchange of males across neighbouring breeding grounds in Oceania, we expect GMR abundance

estimates to fall somewhere between previous male abundance estimates of the New Caledonian breeding population and the wider Oceanian metapopulation.

2.4 Results

2.4.1 Genetic profiling

In total 1,626 samples from distinct photo-identified individuals were obtained and DNA profiles comprising genetically identified sex, mtDNA haplotype and multi-locus microsatellite genotype were constructed. Of these, 1,606 individuals passed the quality control criterion of at least 12 loci typed. Of the 1,606 individuals that passed the quality control, 962 were identified as male and 640 as female, while molecular sexing for the four remaining individuals failed; this resulted in a sex ratio of 1.5:1 males to females. The number of alleles per microsatellite locus ranged from 5 to 24 alleles with a mean of 11.3 alleles (SD = 5.6; Table S2.9). The 16 loci showed a mean observed heterozygosity (H_O) of 0.728 (SD = 0.151), expected heterozygosity (H_E) of 0.731 (SD = 0.144) and a polymorphic information content (PIC) of 0.702 (SD = 0.159; Table S2.9). No linkage was detected but one locus, Ev104, deviated significantly from Hardy-Weinberg equilibrium even after Bonferroni correction and was thus excluded from all further analyses (Table S10). Using the 12 least polymorphic loci, I calculated a conservative probability of identity (Paetkau *et al.*, 1995) and probability of identity among siblings (Waits, Luikart and Taberlet, 2001) of 6.4E-14 and 2.2E-05, respectively. Considering the population size estimate of the New Caledonian population (3,117 individuals; Zicos, Garrigue and Jackson, unpublished data; Table S2.13), the set of markers applied here offers sufficient resolution to differentiate individuals and their kin in this dataset.

Results of the rarefaction analysis showed that the informativeness of the five most powerful markers was sufficient to reach a confidence level of 100% to distinguish parent-offspring pairs from unrelated dyads. Using all 15 markers, parent-offspring pairs can be differentiated from half-sib pairs in 94% and full-sib pairs in 82% of cases. Correct distinction between half-sib pairs and full-sib pairs, and half-sib pairs and unrelated dyads is expected to be achieved in 70-75% of all cases. In all five relationship distinctions, the 12 most informative markers were nearly as powerful as the full set of markers, thus, supporting the minimum threshold of 12 loci required to accept genotypes for the paternity analysis (Figure S2.1). The

full results for the four informativeness measurements for each single genetic marker and multiple loci (provided by KinInfor) are provided in Table S2.10 and Table S2.11.

2.4.2 Paternity assignments

A total of 177 mother-offspring pairs were genotyped at a minimum of 12 loci and used in parentage analysis, representing 54.8% of all mother-offspring pairs observed (but not genotyped) since the start of the first survey in 1993 (N = 323). However, the actual proportion of sampled mother-offspring pairs might be slightly lower assuming that not all offspring born in New Caledonia were observed. Of those 177 mother-offspring pairs sampled, 173 consisted of mother and calf and four consisted of mother and yearling. A total of four mismatching alleles were found in five different mother-offspring pairs of which two pairs were from the same mother with two different offspring in different years. Electropherograms of these loci were inspected for possible genotyping errors and null alleles, and where necessary, loci were genotyped again to resolve the issue. All mismatches were resolved in favour of confirming maternity, with 1 dropout and 3 false alleles suspected of causing the original mismatches. Therefore, all 177 genotyped mother-offspring pairs were confirmed as having a parent-offspring relationship due to Mendelian inheritance patterns of microsatellite loci and sharing an mtDNA haplotype. The pool of candidate males used in the paternity analysis consisted of 936 genotyped adult males (males of unknown age or over the age of five in at least one survey year).

Using the ML method (CERVUS), 83 of 177 (47%) offspring were assigned paternities at the 80% confidence level, and 76 at the 95% confidence level from among the 936 sampled candidate males (Table 2.4). Using the FL approach (Colony), 55 of 177 (31%) offspring were assigned paternities with a posterior probability of > 0.8 (Table 2.4). For each paternity assignment (CERVUS and Colony), a minimum of 11 loci were compared among offspring, mother and father (mean = 13.3 loci, max. = 15 loci; Table S2.12). The probability of non-exclusion across all paternity assignments ranged from 3.10E-11 to 4.27E-05. Overall, the two methods produced similar results: 49 of 177 (27.7%) offspring were assigned the same father, while only 4 of 177 (2.3%) offspring were assigned a different father by the two methods (Table S2.12). In these four disagreements, paternity assignments of the FL method (Colony)

showed a higher number of mismatches than the ML method (CERVUS). All paternity assignments for these four offspring were excluded from further analyses.

To assess patterns of male reproductive success I created two paternity datasets based on different confidence criteria: (1) Relaxed: including paternity assignments assigned by the ML method (CERVUS) at the 80% confidence level and with a maximum of two mismatches (N = 79 paternity assignments of 66 fathers; Table 2.4, Figure 2.2), and (2) Conservative: including paternity assignments assigned by the ML method (CERVUS) at the 95% confidence level and with zero mismatches (N = 63 paternity assignments of 54 fathers; Table 2.4, Figure 2.2).

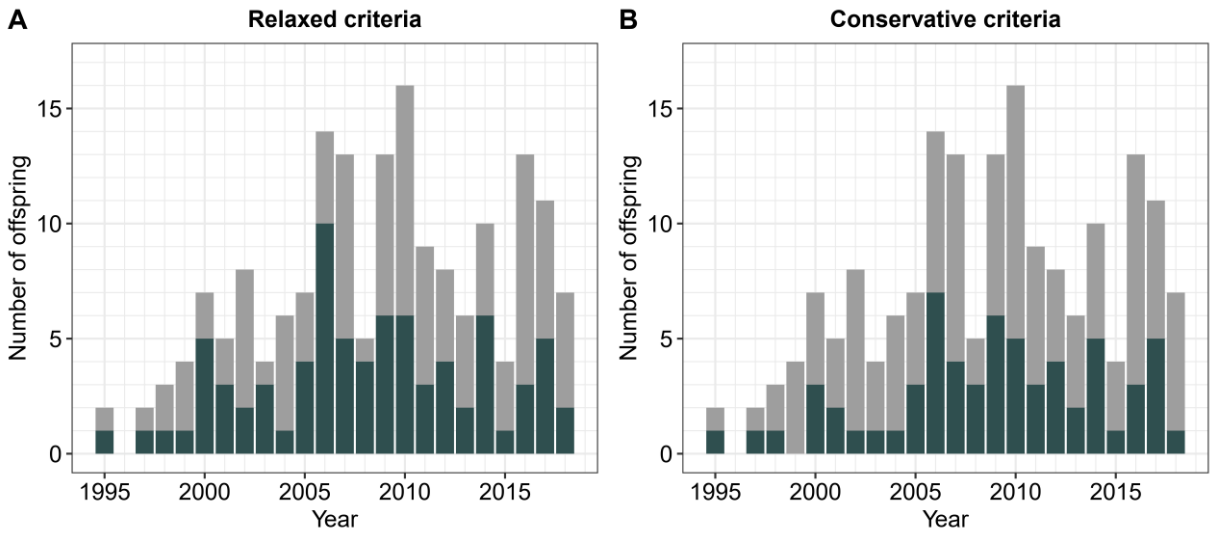


Figure 2.2. The yearly number of sampled mother-offspring pairs (light grey) and the yearly number of paternity assignments (green) for both paternity datasets (left: relaxed paternity; right: conservative) across the study period (1995-2018).

Table 2.4. Paternities assigned for sampled humpback whale mother-offspring pairs. Included are the year the offspring were sired (year of birth – 1), the number of sampled candidate male adults (at least 5 years old in the given year or unknown age), the number of sampled mother-offspring (M-O) pairs, the number of paternities assigned by each method (ML: maximum likelihood, CERVUS; FL: full likelihood, Colony) and the number of paternity assignments in the conservative and relaxed data set. No offspring were sampled for the year 1996 and the two study years before 1995.

Year	Candidate males	M-O pairs	Paternities assigned			Paternity dataset	
			ML (80%)	ML (95%)	FL (>0.8)	Relaxed	Conservative
1995	864	2	1	1	1	1	1
1996	864	0	0	0	0	0	0
1997	864	2	1	1	1	1	1
1998	864	3	1	1	1	1	1
1999	864	4	1	1	1	1	0
2000	864	7	5	4	4	5	3
2001	866	5	3	2	2	3	2
2002	866	8	2	2	1	2	1
2003	867	4	3	1	2	3	1
2004	867	6	1	1	1	1	1
2005	872	7	4	4	4	4	3
2006	874	14	10	10	5	10	7
2007	876	13	6	5	3	5	4
2008	880	5	4	4	3	4	3
2009	884	13	7	6	3	6	6
2010	889	16	7	6	6	6	5
2011	892	9	3	3	1	3	3
2012	900	8	4	4	3	4	4
2013	904	6	2	2	1	2	2
2014	906	10	6	6	4	6	5
2015	915	4	1	1	1	1	1
2016	926	13	3	3	3	3	3
2017	931	11	5	5	3	5	5
2018	936	7	3	3	1	2	1
Total	936	177	83	76	55	79	63

2.4.3 Observed male reproductive success

In the relaxed dataset, 870 (92.95%) males did not sire any of the 177 offspring sampled, 53 (5.66%) males sired one offspring, 13 (1.39%) sired two offspring, and no male sired three offspring (Figure 2.3A). In the conservative dataset, 882 (94.23%) males did not sire any of the offspring sampled, 45 (4.81%) males sired one, nine (0.96%) males sired two, and again no male sired three offspring (Figure 2.3B). In the relaxed paternity dataset only one father (NI0541) sired twice in the same year (2006), while in the conservative paternity dataset, no father sired more than once in the same year. Both the relaxed and conservative paternity datasets produced a similar average of 1.20 and 1.17 offspring/father, respectively.

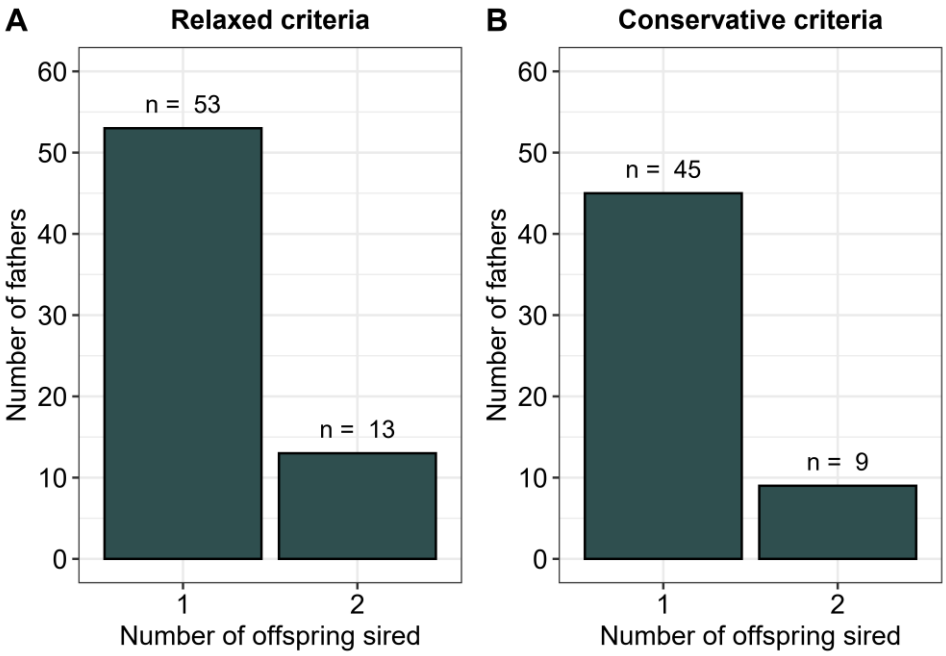


Figure 2.3. The number of males that sired one or two offspring in the (A) relaxed and (B) conservative paternity dataset. No male was found to sire more than two offspring in the study period. Note that 870 males were found not to have sired any of the sampled offspring under the relaxed criteria and 882 males were found not to have sired any of the sampled offspring under the conservative criteria.

2.4.4 Patterns of paternity in non-sampled fathers

In DadShare, the observed paternal relatedness values for offspring for which all sampled males were excluded as fathers (unassigned paternities: 0.33 and 0.36 under relaxed and conservative criteria, respectively) fell in between the range of expected values if each successful male sired one or two offspring (Figure 2.4). The simulation analysis in DadShare

yielded consistent observed paternal relatedness values across offspring with both assigned and unassigned paternities and in both paternity data sets (assigned paternities: 0.40 and 0.35 under relaxed and conservative criteria, respectively; Figure 2.4), as well as across all sampled offspring (0.38). These results indicate that the patterns of male reproductive success are similar for sampled and unsampled males. This further suggests that individuals have equal capture probabilities, an important assumption of GMR (section 2.3.6). Overall, the patterns of paternity in sampled and non-sampled fathers derived from the simulation analysis in DadShare further support the low male reproductive variation derived from the paternity analysis.

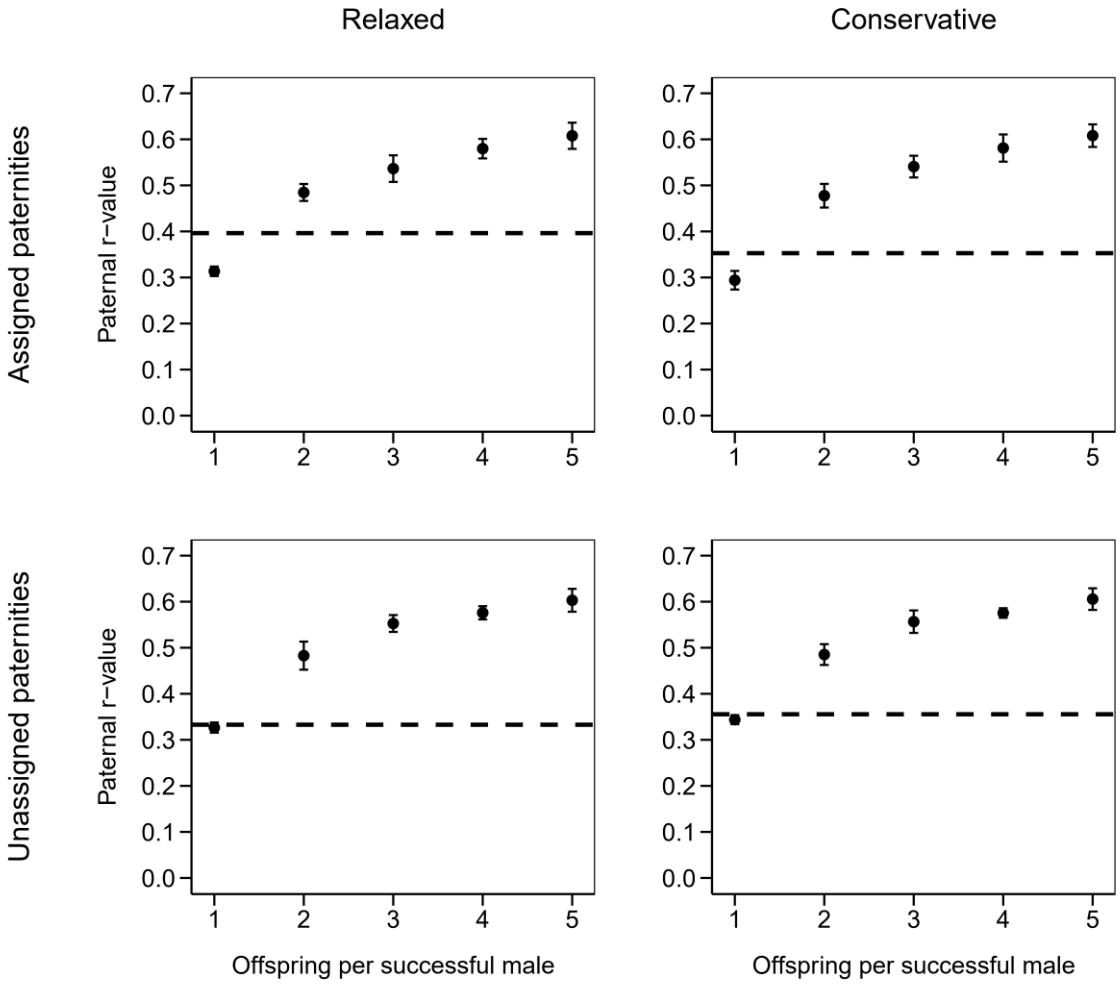


Figure 2.4. Observed paternal relatedness compared with expected values over a range of polygyny levels. Points are expected values of average paternal relatedness (r-values) between offspring if each successful male sired 1, 2, 3, 4, or 5 offspring. Error bars represent +/- one standard deviation (SD). Observed r-values for each set of sampled offspring are shown as horizontal dashed lines for both paternal data sets: relaxed and conservative. For each set of paternities, separate analyses were carried out for offspring that were assigned a father (assigned paternities) and offspring whose father could not be assigned (unassigned paternities). In all cases, the observed r-value falls between what would be expected if each successful male sired one or two offspring.

2.4.5 Test of equal reproductive success

The expected distribution of reproductive success derived from the random mating simulation differed significantly from the observed distribution of reproductive success based on the number of paternity assignments in the relaxed and conservative paternity datasets (Fisher's Exact Test (FET): relaxed: *p*-value < 0.01; conservative: *p*-value = 0.022, Figure 2.5 and Table 2.6). This was mainly due to fewer males than expected siring one offspring and an excess of males siring two offspring in the observed distribution compared to the expected distribution, in both datasets (Figure 2.5). Although the observed variance of male reproductive success was low, it was more than 3.5 times higher than expected under random mating for the sampled pool of candidate males and offspring (relaxed: observed variance = 0.161, expected variance = 0.046; conservative: observed variance = 0.142, expected variance = 0.036; Table 2.6). Despite the observed low skew and variation in male reproductive success (sections 2.4.3 and 2.4.4), overall, these simulation results suggest that variation in male reproductive success is higher than expected if mating was random.

Table 2.5. The number of single and multiple paternities assigned in the paternity analysis and determined in the simulation analysis based on the sampled 177 mother-offspring pairs and 936 mature males. The observed distribution is based on previously established paternities and the expected distribution under the assumption of equal reproductive success. Observed and expected distributions of male reproductive success were compared using the relaxed and conservative paternity datasets. For the expected distribution, the mean, minimum and maximum number of fathers in the 1,000 simulations are shown. The percentage of males from the total sampled mature males (N = 936) that sired zero, one, or more offspring was calculated. The last column shows the percentage of simulations in which the observed number of fathers was larger than the simulated (expected) number of fathers (and non-fathers for males that sired no offspring).

Paternity dataset	# Offspring sired	Observed distribution		Expected distribution			Obs>Exp (%)
		# Fathers	%	Mean simulated # Fathers	%	[min, max]	
Relaxed	0	870	92.95	860.40	91.92	[857, 869]	100
	1	53	5.66	72.28	7.72	[56, 79]	0
	2	13	1.39	3.23	0.35	[0, 10]	100
	3	0	0.00	0.08	0.01	[0, 3]	0
	4	0	0.00	0.00	0.00	[0, 0]	0
Conservative	0	882	94.23	875.13	93.50	[873, 880]	100
	1	45	4.81	58.79	6.28	[49, 63]	0
	2	9	0.96	2.04	0.22	[0, 7]	100
	3	0	0.00	0.04	0.00	[0, 1]	0
	4	0	0.00	0.00	0.00	[0, 0]	0

Table 2.6. Comparison of the observed and the simulation-derived expected mean, variance and standardised variance (SV, Equation 1; Coltman, Bowen and Wright, 1998) in male reproductive success for both paternity datasets.

Paternity dataset	Statistics	Observed	Expected
Relaxed	Mean	1.197	1.045
	Variance	0.161	0.046
	SV	0.134	0.044
Conservative	Mean	1.167	1.035
	Variance	0.142	0.036
	SV	0.121	0.034

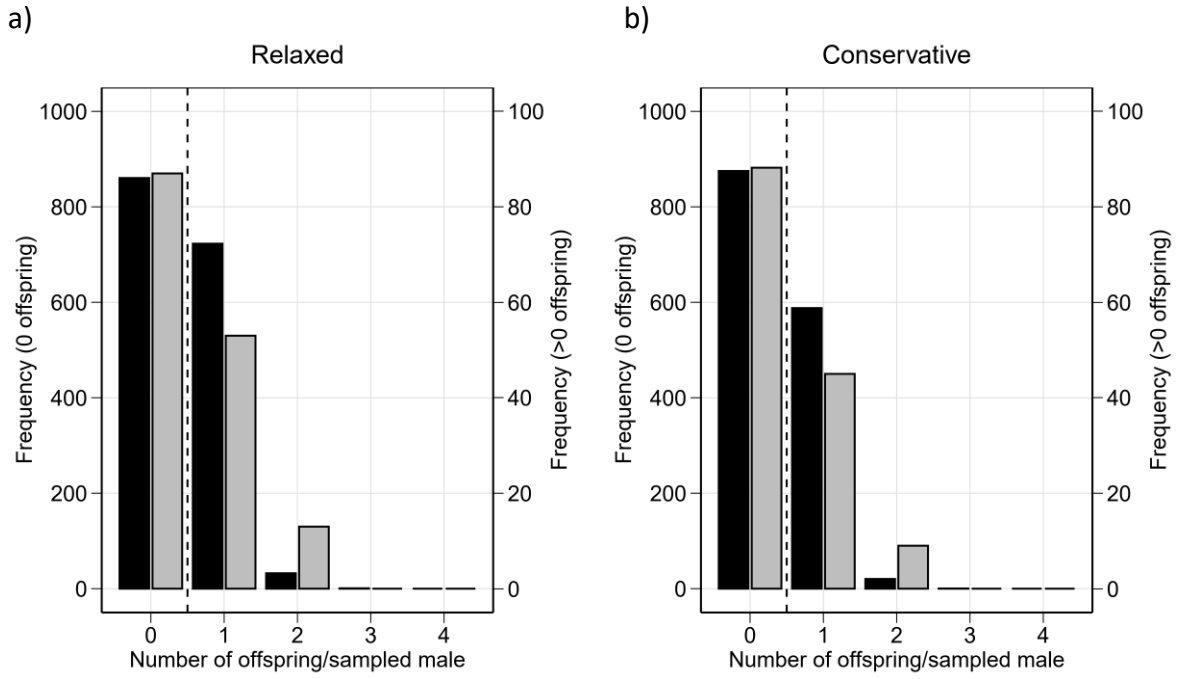


Figure 2.5. Distribution of male reproductive success in the New Caledonian humpback whale population across the entire study period (1995 – 2018) based on the 177 sampled mother-offspring pairs. The observed distribution (grey) of reproductive success was based on the relaxed (a) and conservative (b) paternity datasets, and the expected distribution (black) under the assumption of equal reproductive success was derived from 1,000 simulations. The left y-axis shows the frequency of males that were not assigned to any of the sampled offspring. The right y-axis shows the frequency of males that were assigned as fathers to one or more sampled offspring. The dashed vertical line indicates the switch from the left to the right y-axis given the differences in size.

2.4.6 Gametic mark-recapture (GMR) to estimate male abundance

The overall abundance estimates from the gametic capture-recapture analysis across the entire study period yielded a total of 2,058 males (95% CI = 1,732 - 2,384) for the relaxed, and a total of 2,564 males (95% CI = 2,071 – 3,057) for the conservative paternity datasets (Figure 2.6). GMR estimates derived from the conservative paternity dataset were higher overall, and had a greater uncertainty, compared to estimates derived from the relaxed paternity dataset.

2.4.7 Assessing the genetic interchange of the New Caledonian breeding population

To assess whether a substantial number of males from other populations were contributing to the paternity of New Caledonian calves, I assessed the degree of demographic interchange of the New Caledonian breeding population with other Oceanian breeding grounds by comparing the GMR and census estimates of male abundance within Oceania and New Caledonia. The overall abundance estimates from the GMR fell between previous estimates of the New Caledonian breeding population and estimates of the entire Oceanian metapopulation (Figure 2.6, Table S2.13).

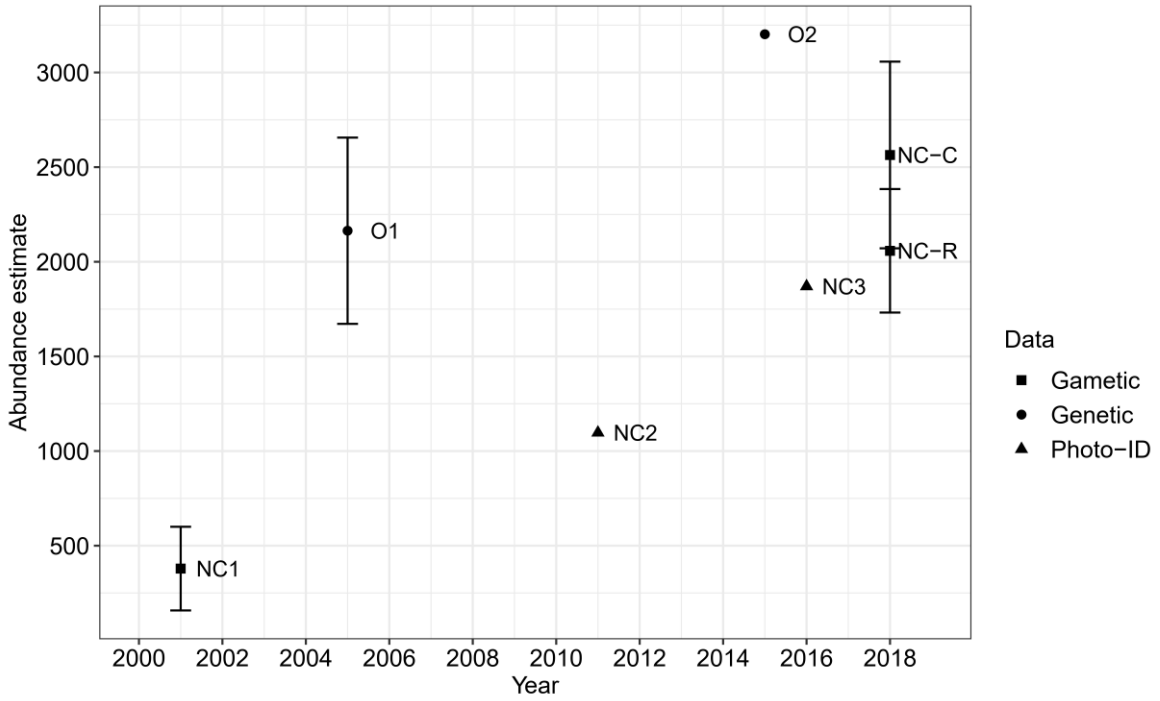


Figure 2.6. Comparison of male abundance estimates of the New Caledonian population and Oceanian metapopulation to assess the level of genetic interchange with the New Caledonian breeding ground across Oceania. Overall GMR estimates of the New Caledonian male breeding population derived from both paternity datasets (relaxed: NC-R; conservative: NC-C) fall between estimates of the Oceanian and the New Caledonian population. Male-specific estimates were derived from sex-unspecific estimates by division of a factor of two for estimates based on genetic data or based on the reported male-biased sex ratio of 1.5:1 (M:F) for estimates based on photo-ID, where necessary. O1: male-specific POPAN super-population estimate of Oceania between 1999 and 2005 by Constantine et al., 2012; O2: derived male-specific estimate from sex-unspecific median projected abundance of Oceania in 2015 based on genetic data by Jackson et al., 2015; NC1: male-specific gametic mark-recapture estimate of the New Caledonian breeding sub-stock between 1995 and 2001 based on paternity assignments by Garrigue et al., 2004; NC2: derived male-specific estimate from sex-unspecific POPAN super-population estimate of the New Caledonian population between 1996 and 2011 based on photo-ID by Garrigue et al., 2012; NC3: derived male-specific estimate from sex-unspecific (adult only) POPAN super-population estimate of the New Caledonian population between 1996 and 2016 based on photo-ID by (Zicos, Garrigue and Jackson, unpublished data).

2.5 Discussion

2.5.1 Patterns of male reproductive success

The results presented here show that variation in male reproductive success in New Caledonian humpback whales is lower than that of other polygynous species on land. Yet it falls within a similar range of other aquatically mating species (Figure 2.7), including conspecific populations (Cerchio et al., 2005; Nielson et al. 2001). Although sexual selection theory predicts high variation in reproductive success in polygynous species, the synchrony of female oestrus (Chittleborough, 1954), great dispersion of females across the breeding ground, and the 3D underwater habitat all reduce a male's ability to monopolise female mating access (Herman, 2017). These factors combined lower the degree of polygyny possible in this species compared to terrestrially-mating mammals with a polygynous mating system, and ultimately, reduce the variation in male reproductive success.

Although variation in male reproductive success was low, my results indicate that there was significantly more skew in male reproductive success than expected if mating was random. Fathers were at least 3.5 times more likely to sire more than one offspring than expected if mating was random (i.e., if each male had equal chances of siring), thus suggesting that some males are more successful than others in siring offspring.

Differences in reproductive success may be driven by behavioural or developmental differences. For example, there are a variety of mating behaviours displayed by male humpback whales on their breeding grounds, where males are frequently observed to physically compete to be closest to a female within competitive groups, to escort single females (with or without calf), and to sing elaborate songs showing high levels of complexity. Older males could be more successful in siring offspring due to experience or skill in these behaviours. While body size can be a determining factor in direct male-male competition either through increased strength via larger body size or increased agility via smaller size, older males might be more experienced irrespective of their size. For example, in a tropical butterfly (*Bicyclus anynana*), older males had considerably higher mating success than younger males (Fischer, Perlick and Galetz, 2008). Observations of small humpback whales, likely not yet sexually mature, suggest that young males might participate in competitive groups initially not to mate but to practice. In lekking societies, which the humpback whale mating system was

proposed to resemble (Clapham, 1996), male reproductive success is often strongly correlated with age across several taxa (reviewed in Fiske *et al.*, 1998). Older males are likely to be larger and/or more experienced in their behaviour than younger individuals, and thus, might be more successful in competing against other males. Additionally, yet non-mutually exclusive, certain males might also be more successful in siring offspring, not because they are more likely to win in direct male-male competition, but through female mate choice. If females prefer certain physical or behavioural traits, then males with these traits will be more successful in siring offspring than males without the trait or a less extreme manifestation of it. A study investigating the body size of male-female dyads in humpback whales in Hawaii using underwater videogrammetry found that mature-sized females showed a preference for larger mature-sized males (Pack *et al.*, 2012). This size-assortative pairing suggests that humpback whales discriminate amongst potential mates, thus allowing for female mate choice, where body size might play a role.

The highly complex songs of humpback whales, sung by males only, have been suggested to serve as a sexual display to attract females (Winn and Winn, 1978; Herman and Tavolga, 1980; Tyack, 1981; Frankel *et al.*, 1995; Herman, 2017) similar to birdsong (Searcy, 1992). In song sparrows (*Melospiza melodia*), females show a preference for males with more complex song (e.g., larger song repertoire: Searcy, 1984). If female humpback whales prefer to mate with males singing more complex songs, then song complexity might be correlated with male reproductive success. Given the humpback whale song's high complexity and the constant change of songs through cultural evolutions and revolutions, a higher song complexity might also reflect a greater learning capacity ('cognitive capacity hypothesis', Boogert, Giraldeau and Lefebvre, 2008). While this greater learning capacity could be due to genetic factors related to song development and/or general cognitive processing, older and thus more experience males might be more skilful singers and/or learners, which may or may not have an underlying genetic basis. The high structural variability found in humpback whale song appears ideal for conveying information on male quality, through superior genes or experience, thus allowing the possibility of female mate choice to be the driver of song complexity (Hebets and Papaj, 2005; Murray *et al.*, 2018). However, female preference for any humpback whale song characteristic and its possible link to male reproductive success is yet to be investigated.

The various male mating behaviours of singing, physically competing over single females, and escorting females, might represent alternative mating tactics. Although male humpback whales were found to engage in several mating behaviours across different years and/or within the same year (this study), males might nevertheless favour or be more successful adapting certain tactics over others. Males appear to adjust their mating tactic depending on their body size as smaller mature males were found to avoid the cost of competing for the highest-quality females of larger size but instead opted for smaller females that may or may not have been mature yet (Pack *et al.*, 2012). However, even if a male was to favour one tactic over another based on internal conditions or factors (e.g., body size), external factors might force him to adopt another, potentially less favourable, mating tactic. Males may thus adopt a specific behavioural tactic depending on their current condition, age, experience (e.g., singing skills), as well as external factors (e.g., number of receptive females, number of male competitors, dispersion of individuals). If a male can increase his chances of siring offspring by adopting one tactic rather than another depending on internal and/or external conditions, then the presence of alternative mating tactics could explain the only mild polygyny observed among male humpback whales as well as males adopting several tactics within a breeding season.

While the marine habitat and the wide dispersion of females across the breeding ground undoubtedly reduce the variation in male reproductive success, alternative male mating tactics and/or female mate choice might further lower it (see also Cerchio *et al.*, 2005). Yet, low variation in male reproductive success does not necessarily imply that sexual selection acting upon male behaviour is weak. The male-biased sex ratio and the scattered distribution of females still suggest intense male competition. However, male competition might be more about being able to reproduce at all rather than siring a large number of offspring. Although this does not lead to high variation in reproductive success, it still results in a highly skewed distribution of reproductive success. Male reproductive success in humpback whales, however, does not appear to be skewed towards few males siring a large proportion of the offspring but instead a large proportion of males siring no offspring. This highlights the importance of including the number of sexually mature males that did not sire any offspring in studies, in contrast to assessing the variation of reproductive success of only the males that were able to reproduce.

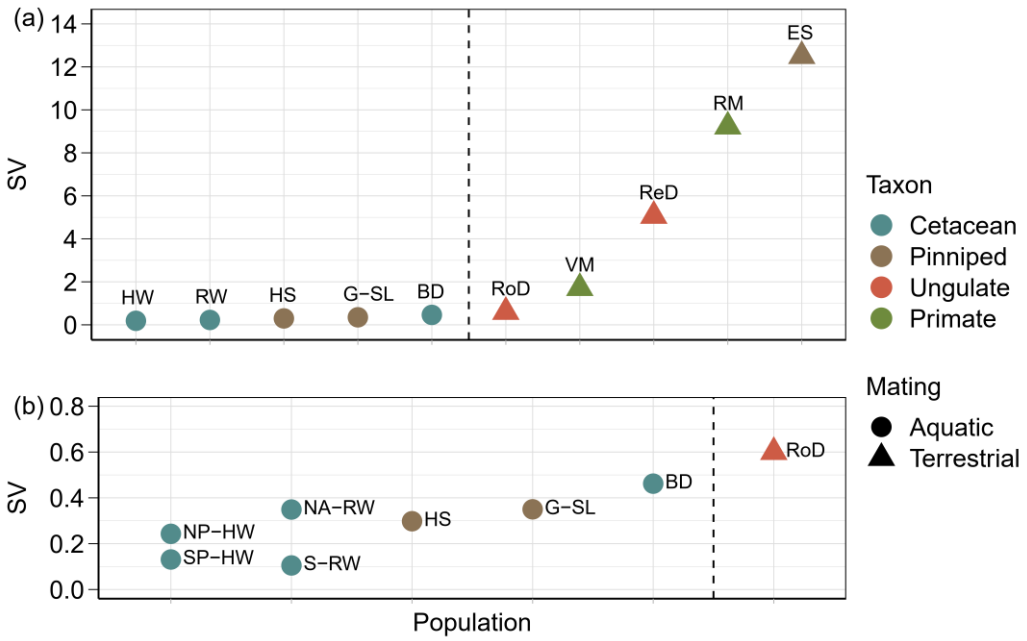


Figure 2.7. Standardized variance (SV = variance/mean) in male reproductive success across a range of species and taxa with (a) showing all populations, and (b)a close-up of populations with SV of less than 1. This figure has been adapted from Frasier et al. (2007). Abbreviations: SP-HW (South Pacific humpback whale, this study), NP-HW (North Pacfic humpback whale, Cerchio *et al.*, 2005), NA-RW (North Atlantic right whale, Frasier *et al.*, 2007), S-RW (Southern right whale, Carroll *et al.*, 2012), HS (harbour seals, Phoca vitulina, Hayes *et al.*, 2006), G-SL (Galpágos sea lion, Zalophus wollebaeki, Pörschmann *et al.*, 2010), BD (bottlenose dolphins, Tursiops sp., Krützen *et al.*, 2004), RoD (roe deer, Capreolus capreolus, Vanpé *et al.*, 2008), VM (vervet monkey, Chlorocebus pygerythrus, Minkner *et al.*, 2018), ReD (red deer, Cervus elaphus, Clutton-Brock, Guinness and Albon, 1982), RM (rhesus macaque, Macaca mulatta, Dubuc, Ruiz-Lambides and Widdig, 2014), ES (Southern elephant seals, Mirounga leonina, Fabiani *et al.*, 2004).

2.5.2 GMR estimate and gene flow among Oceanian breeding grounds

The overall GMR male abundance estimates (derived from both paternity datasets) for the New Caledonian population fell between previous estimates of the New Caledonian breeding population and estimates of the entire Oceanian metapopulation. This suggests that at least some males from other neighbouring Oceanian breeding grounds are contributing to the paternity of New Caledonian calves. Although the confidence intervals of the GMR male abundance estimates derived from the two paternity datasets (conservative and relaxed) overlap, their derived conclusions slightly differ. While the GMR estimate derived from the conservative paternity dataset lies in the middle between previous New Caledonian and Oceanian census estimates (Figure 2.6), and therefore, suggests considerable levels of gene flow, the abundance estimate derived from the relaxed paternity dataset falls very close to the most recent New Caledonian census estimate. However, considering that the effective population size is almost always smaller than the census population size, even the GMR estimate of the relaxed paternity dataset suggests at least some level of gene flow between

the New Caledonian population and other breeding grounds in the South Pacific. The larger GMR estimate in comparison to previous New Caledonian census estimates could also, at least partially, be due to population growth. It is, however, unlikely that population growth between the years 2016 and 2018 is the main factor contributing to the difference of 600 individuals between the two New Caledonian male abundance estimates for those years (NC3: 1,870 males; NC-C: 2,483 males; Figure 2.6). Thus, the larger GMR estimate compared to previous New Caledonian census estimates suggests that the population is not fully reproductively and demographically closed and that at least some males from neighbouring populations contribute to the paternity of New Caledonian calves.

The GMR estimates, especially the estimate derived from the conservative paternity dataset, hint towards a higher level of gene flow than previous differences between GMR and census estimates of the New Caledonian breeding population (Garrigue *et al.*, 2004) and previous comparisons of photo-ID and mtDNA haplotypes among Oceanian populations (Garrigue *et al.*, 2002; Olavarría *et al.*, 2007) suggest. However, previous studies were based on data collected during a time when the population was considerably smaller than it is today. There was an anomalous increase in the New Caledonian population after 2008 that was proposed to be due to an overspill of the eastern Australia population (Garrigue, Albertson and Jackson, 2012), which has been increasing at a greater rate than other areas in the South Pacific (Noad *et al.*, 2011). Population dynamics across humpback whale breeding populations in the South Pacific might be density-dependent, and as a result, the level of interchange (of whales and genes) between regions will increase with increasing population size. More recent studies have shown a substantial number of resights (Badhuge, 2022) and longitudinal movement (Derville *et al.*, 2020) between New Caledonia and eastern Australia. The reported increase in abundance of the New Caledonian population (Garrigue, Albertson and Jackson, 2012), the eastern Australia population (Noad *et al.*, 2011) and the wider Oceanian metapopulation (Jackson *et al.*, 2015) might have increased and expanded the level of gene flow among humpback whale breeding populations in the South Pacific.

Similar to patterns of genetic differentiation (see section 2.2), the dynamics of humpback whale song exchange across the South Pacific supports the differentiation of distinct breeding populations, yet also suggests movement of males between breeding grounds. Populations show a high level of conformity to the current arrangement and content of song (Winn and

Winn, 1978; Payne, Tyack and Payne, 1983) while populations that are geographically closer to each other show a higher level of similarity than those further away (Payne and Guinee, 1983). Song transmission is suggested to occur via male movement between breeding populations, song sharing along shared or partially shared migration routes and/or on shared summer feeding grounds (Payne and Guinee, 1983; Garland, Gedamke, *et al.*, 2013). As song evolves progressively through time and space (Payne and Payne, 1985; Garland *et al.*, 2011; Garland, Noad, *et al.*, 2013), the unidirectional transmission of song eastwards across the South Pacific might too be the result of differences in population sizes within breeding populations (Garland *et al.*, 2011). Changes in population size might thus influence the levels of interchange between populations through the transmission of both genes and song (Zandberg *et al.*, 2021).

Genetic differentiation between and high song conformity within breeding populations indicate that breeding site fidelity is higher compared to the degree of interchange, thus, reinforcing the reproductive autonomy and demographic closure of the New Caledonian population. My results suggest that New Caledonian females occasionally breed with males from neighbouring breeding grounds, however, the degree of interchange varies depending on the level of confidence applied to the paternity assignments used in the GMR. While the conservative GMR estimate could indicate a higher degree of genetic interchange than previously thought; based on the relaxed GMR estimate, fidelity to breeding grounds might still be more important than interchange between breeding grounds. Temporal population dynamics (e.g., abundance, age structure) could have changed the direction and degree of genetic interchange across breeding populations in recent years. More detailed analyses of the genetic structure and song dynamics across the South Pacific at a temporal scale are necessary to assess the level of gene flow and migratory movement between breeding populations and their dependence on population demography.

2.5.3 Assessment of applied methods and their limitations

Firstly, incorrect specification of parameters in paternity analyses using likelihood approaches can lead to incorrect estimates of confidence in paternity assignments (Marshall *et al.*, 1998; Nielsen *et al.*, 2001). The rate of genotyping error and the proportion of candidate parents sampled are two key parameters that can affect paternity assignments (Jones *et al.*, 2010).

Methods (and software) of paternity inference have often been developed and evaluated using well-studied and monitored populations (e.g., red deer (Cervus elaphus) on the Isle of Rum, Scotland: Kalinowski, Taper and Marshall, 2007) for which parameters relating to demography are much better known. Humpback whales, like many other marine mammals, are hard to observe and count, and as a result, estimates of abundance are much less precise and the proportion of individuals sampled is much lower compared to many group-living mammals on land. However, if genotypic data are informative enough and error rates are low, paternity analyses become less sensitive to the proportion of candidate parents sampled (Jones *et al.*, 2010). The sensitivity analysis showed that although altering values for two demographic parameters in CERVUS (number of candidate fathers and number of candidate fathers sampled) shift the threshold of confidence of paternity assignments (critical delta score: Table S2.14 and Figure S2.3), there was minimal change to the total number of paternity assignments (Figure S2.4). Further, altering the values of these input parameters (Table S2.14) did not change the rank order of compatible parents but merely changed the confidence in these assignments (Jones *et al.*, 2010). Considering the high informativeness of the microsatellite markers applied here (section 2.4.1, Figure S2.1) and the low genotyping error rate of this dataset (0.011, section 2.4.1), together with the robust results of the sensitivity analysis of input parameters, I conclude that the combination of parameters and genotyping data result in robust paternity assignments.

Secondly, although the two paternity methods (ML and FL) assigned the same father to 27.7% of all 177 sampled offspring and showed an overall agreement of 79.7% on whether an offspring's father was in the set of sampled males or not, the FL method implemented in Colony yielded 28 fewer paternity assignments than the ML method in CERVUS (ML-80%: 83; FL: 55; Table 2.4). The reason for the higher rate of paternities assigned by the ML method likely lies in the difference in their approaches. Unlike the ML method in CERVUS which follows a pairwise approach and only looks at the two most likely fathers, the FL approach implemented in Colony, partitions all sampled individuals into family clusters according to shared parentage or sibship relationships (Wang and Santure, 2009). In FL paternity methods, larger family size will increase the information on common parents, and thus, enable more accurate inference of parentage (Wang and Santure, 2009). Such methods are therefore more suitable on species with a large family or sibship size but considerably diminish prospects for successful reconstruction with fewer than 8 – 10 offspring in the progeny array, thus,

rendering the method less useful in species with small families (Jones *et al.*, 2010). While the accuracy of parentage inferences in FL methods decreases rapidly with sibship size, the accuracy of parentage inferences in pairwise methods is not affected by sibship size as only two individuals are ever considered at a time (Wang and Santure, 2009). Sibship size in humpback whales is low, and in our dataset, the largest known sibship size consisted of four maternal siblings (Figure S2.2). Thus, the FL approach based on the reconstruction of family clusters as implemented in Colony is not a suitable method for paternity assignments in humpback whales and explains the lower assignment rate in comparison to the ML method.

Thirdly, the GMR method relies on the assumption of random mating to provide equal capture probabilities amongst males, yet mating in wild populations is rarely entirely random. The test of equal reproductive success further indicated that mating in this population of humpback whales was not random (section 2.4.5). The violation of the assumption of random mating can lead to fewer males siring offspring than expected, which would decrease the number of gametic recaptures (Carroll *et al.*, 2012). This would result in an overestimation of male abundance, and in this study, would indicate a higher level of gene flow than might be the case. Further, although variation in male reproductive success was low, it was skewed towards males not siring any offspring. In humpback whales, males differ in the amount of time they spend on the breeding ground due to differences in migratory timing (Dawbin, 1966; Craig *et al.*, 2003) and some males may be entirely absent on the breeding ground in a given year (Van Opzeeland *et al.*, 2013; Magnúsdóttir and Lim, 2019). Males that spent less time on the breeding ground are less likely to be captured, yet might show equal chances to reproduce (be recaptured). Despite the reported site fidelity to breeding grounds, a considerable proportion of individuals moving through the New Caledonian breeding grounds are transients from other regions of Oceania (Constantine *et al.*, 2012; Madon *et al.*, 2013). Transients, by definition, pass through the area only once, and therefore, have zero capture probability (Pradel *et al.*, 1997). If transients contribute to the pool of offspring in New Caledonia, their reduced capture probability compared to residents could further inflate the GMR estimates of this study.

Fourthly, using paternity assignments derived from likelihood-based approaches, which require prior knowledge of the population size to estimate input parameters, to then estimate population size using GMR, creates a problem of circularity. Input parameters for the paternity

analysis (e.g., the proportion of candidate parents sampled in CERVUS) based on abundance estimates that are larger than the actual population, can lower the number of accepted paternity assignments, and thus, potentially increase the number of missed paternity assignments. In the GMR, a lower number of paternity assignments leads to higher abundance estimates. Thus, overestimating the population size in the paternity analysis can inflate GMR-derived abundance estimates, and vice versa (underestimation can lead to deflation). The relatively stable number of paternity assignments as shown in the sensitivity analysis (Figure S2.4) indicates that microsatellite markers were powerful enough and genotyping error rates low enough to result in robust paternity assignments making them less sensitive to altering input parameters.

Lastly, considering the long lifespan of humpback whales, or baleen whales in general, estimates of male reproductive success captured in this dataset represent only a snapshot of their lifetime reproductive success. Humpback whales on average are estimated to live for roughly 80 years and reach their sexual maturity at around 9-11 years (Best, 2011). The study period of this dataset covers only around a third of a male's reproductively active life or slightly more depending on the reproductive senescence of humpback whales. Thus, variation in male lifetime reproductive success could be larger than my results suggest.

2.6 Conclusion

This study provides further evidence of the low reproductive skew in male humpback whales. However, despite the low reproductive skew, sexual selection is nevertheless suggested to be strong. Male competition in humpback whales might be more about being able to reproduce at all rather than siring a large number of offspring. Despite the long-term dataset and the high number of individuals sampled, studying reproductive success in baleen whales remains challenging and offers only a glimpse into the reproductive life of humpback whales. How female mate choice shapes patterns of male reproductive success is an open question.

Based on the GMR estimates in this study, current levels of gene flow between New Caledonia with neighbouring populations in the South Pacific may be larger than estimated 20 years ago. Recent increases in the abundance of populations in Oceania (Garrigue, Albertson and Jackson, 2012; Jackson *et al.*, 2015) and eastern Australia (Noad *et al.*, 2011) could have

expanded and increased the level of gene flow across breeding populations in recent years. More detailed analyses to revise previous records of individual and genetic interchange, and genetic differentiation between breeding populations in the South Pacific are needed. My results provide insights into the reproductive skew of humpback whales and the population dynamics across Oceanian populations, two important factors affecting the recovery of endangered humpback whales in the South Pacific.

Chapter 3

Epigenetic ageing reveals changes in age-specific sexual selection in a recovering humpback whale population

3.1 Abstract

The evolutionary response to long-term exploitation results in substantial changes in the demography of a population and can drive traits away from their naturally selected evolutionary optima. Little is known about the evolutionary consequences of commercial whaling on the demography, mating behaviours and sexual selection of exploited populations of baleen whales. Here, I estimated the age of 485 male humpback whales (*Megaptera novaeangliae*) breeding in New Caledonia, South Pacific (24% of the estimated population size), using epigenetic ageing to quantify the presence and extent of age-related changes in their behaviour and reproductive success. I divide the 26-year (1995-2020) survey period into two, enabling assessment at two stages: recovering and stabilising. The male population over the first period was consistent with a recovering population (left-skewed) but became more balanced in the second half, consistent with a stabilisation of the age structure. Older males were more often observed to engage in certain mating behaviours (escorting and singing) and were more successful in siring offspring in the second half of the study period. This suggests that reproductive tactics and reproductive success in male humpback whales may be age-dependent and that commercial whaling changed not only the population dynamics but also patterns of sexual selection. This work provides critical insights into how sexual selection is currently acting on the complex male mating behaviours in a recovering population of humpback whales.

3.2 Introduction

Sexual selection is one of the central forces in evolution and explains a wide diversity of animal morphology and behaviour (Andersson, 1994). Importantly, the strength and form of sexual selection can vary across space and time within species (Siepielski, DiBattista and Carlson, 2009; Cornwallis and Uller, 2010). The temporal dynamic of sexual selection is shaped by environmental conditions (e.g., environmental quality, climatic fluctuations) and population demography (e.g., density, sex ratio, number of competitors) (Robinson *et al.*, 2008; Punzalan, Rodd and Locke Rowe, 2010; Martin *et al.*, 2016). Population size and the operational sex ratio (OSR) influence the density of competitors, and can thereby impact the strength of sexual selection by changing the frequency of mating opportunities, the possibility of mate choice, and the degree of competition between members of the same sex (Chapter 1). When mating opportunities are fewer, and/or the density of competitors and degree of mate choice is higher, variation in reproductive success, a necessary pre-condition for sexual selection to act upon a trait, is usually also higher.

An additional factor to consider in determining the strength and pattern of sexual selection is the age structure of a population. Reproduction in mammals is often age-dependent, as mating success typically increases with age and then declines with senescence (Festa-Bianchet, Jorgenson and Réale, 2000). The age or experience of an individual or its stage of development, can influence its competitive ability and, in turn, reproductive success. Alternative mating tactics may have evolved to overcome those differences by allowing individuals to incorporate information about their ability or status (relative to other competitors) to adopt a mating tactic that maximises their fitness (Gross, 1996). In bighorn sheep (*Ovis canadensis*) for example, males use different mating tactics depending on their social rank and age (Hogg and Forbes, 1997); horn size, a sexually selected trait, was found to influence mating success in older males (> 7 years) but not in younger ones (Coltman *et al.*, 2001). In a harvested population of bighorn sheep, the proportion of older competitors decreased, and consequently, the number of mate guarding (i.e., tending) rams. This allowed typically less competitive younger males to obtain an increasing proportion of mates and led to increased sexual selection on their body weight and horn length (Martin *et al.*, 2016). Little is known about age-specific selection and how changes in population density and age

structure shape patterns of sexual selection and the distribution of mating behaviours in species and populations.

Human activities can drive evolutionary change that often opposes natural and/or sexual selection. This can move traits away from their naturally-selected evolutionary optima allowing otherwise suboptimal phenotypes to increase in frequency (Coltman *et al.*, 2003; Hutchings and Rowe, 2008; Allendorf and Hard, 2009). The evolutionary response to long-term exploitation not only results in substantial changes in the demography of a population but may further cause changes in individual growth rate, breeding parameters (i.e., age at maturity, birth interval), body size and productivity of an individual or a population (H. Kato, 1995; Conover, Munch and Arnott, 2009). For example, the unrestricted hunting of bighorn sheep resulted in declines in body weight and horn size, traits that are positively correlated with male mating success in the absence of hunting (Coltman *et al.*, 2001, 2003). This evolutionary response to human-induced selection, therefore, opposes sexual selection and could reduce the variation in reproductive success observed in the population. This could be one mechanism by which human exploitation can cause significant changes to selective pressures, genetic diversity, and sustainability of wild populations (Allendorf and Hard, 2009). Thus, it is crucial to consider the long-term consequences of exploitation and their impact on the demography, mating behaviour and reproductive skew of affected populations when studying sexual selection.

There has been little research on the evolutionary consequences of a dramatic example of human exploitation, commercial whaling. Up until very recently, the great whales were exploited intensely across all oceans for several centuries (Clapham, 2016). Many baleen whale populations declined to 1% of their pre-exploitation size, among them the Southern Hemisphere populations of the humpback whale (*Megaptera novaeangliae*) (Baker and Clapham, 2002; Clapham, 2016). While many humpback whale populations have shown a recent increase in population size, the degree of recovery varies greatly and several populations currently remain at low levels relative to historical abundance (Thomas, Reeves and Brownell, 2016). The humpback whale subpopulations in the Arabian Sea and Oceania (Figure 2.1 in Chapter 2) are still considered Endangered under the IUCN Red List (Childerhouse *et al.*, 2008; Constantine *et al.*, 2012; Thomas, Reeves and Brownell, 2016). A comprehensive assessment of the Southern Hemisphere humpback whales estimated the

Oceanian metapopulation to have recovered to only 47% of its pre-exploitation size by 2015 (Jackson *et al.*, 2015). In contrast, its neighbouring population of East Australia is considered to have fully recovered (Noad, Kniest and Dunlop, 2019). The reasons for the slower recovery of the Oceania metapopulation are currently uncertain (Constantine *et al.*, 2012; Jackson *et al.*, 2015).

The New Caledonian breeding population (BSE2), part of the Oceanian breeding metapopulation, recently showed an anomalous increase in abundance after 2008 (Garrigue, Albertson and Jackson, 2012). While this recent population growth may partially be driven by immigration from the neighbouring East Australian population (Orgeret *et al.*, 2014), more recently, high reproductive capacity was suggested to be another non-exclusive driver (Chero *et al.*, 2020). The high estimated calving rate of the New Caledonian breeding population (Chero *et al.*, 2020), and the high pregnancy rates observed on the migratory corridor of the Kermadec Islands (Riekkola *et al.*, 2018) and humpback whales in general on their Antarctic feeding grounds (Pallin, Baker, et al., 2018b) further support this hypothesis (Chero *et al.*, 2020). However, these conclusions are based on female breeding parameters alone. Thus, it remains unclear how reproductive parameters and behaviours of males have changed in response to commercial whaling and how, or if, those changes are contributing to the high reproductive capacity in this recovering population. Recent observations show a highly male-biased sex ratio on the New Caledonian breeding grounds (1.5:1 [M:F], Chero *et al.*, 2020) and the observed mating behaviours (e.g., singing and intense contest competition, see Table 2.2 in Chapter 2) suggest the presence of multiple reproductive tactics. Given these observations, sexual selection theory would predict intense competition among males, leading to high male reproductive skew and strong sexual selection on males (Andersson, 1994; Gross, 1996). However, contrary to these predictions, male reproductive skew was found to be low in the New Caledonian breeding population (Chapter 2; Demastia 2016), and this is consistent with data from other populations of humpback whales (Cerchio *et al.*, 2005). Both the occurrence of alternative mating tactics and a left-skewed population age structure could explain the observed low reproductive skew and thus mild polygyny in this population (see Cerchio *et al.*, 2005). How demographic processes resulting from commercial whaling have shaped patterns of sexual selection in male humpback whales across time and how this might have impacted their recovery and mating system today remains unclear.

Here, I estimated the age of 485 male humpback whales (24% of the estimated male population size, see Chapter 2) using epigenetic ageing technique (e.g., Polanowski *et al.*, 2014; Horvath and Raj, 2018) to quantify the presence and extent of age-related changes in male behaviour and reproductive success. First, following the methods described in Polanowski et al. (2014), I calibrated their ageing model to the New Caledonian breeding population and assessed its accuracy and precision using 78 calibration samples of known age. Second, I applied the calibrated ageing model to assess the age structure of the male population across time. Third, I examined the likelihood of males engaging in certain behaviours across different age categories. Finally, using previously established paternity assignments consisting of 66 fathers and 79 offspring (Chapter 2), I investigated if males of all age categories are equally likely to sire offspring. This work provides critical insights into how sexual selection is currently acting on the complex male mating behaviours in a recovering population of humpback whales.

3.3 Methods

3.3.1 Study site and data collection

Samples for genetic analysis and photographs were collected at the breeding ground in New Caledonia from 1995 to 2020 during annual field surveys in the austral winter. During focal follows, whales were carefully approached to be photographed and their behaviour and any changes in group composition were recorded, following published methodology (Garrigue, Greaves and Chambellant, 2001; Derville *et al.*, 2019). Individual humpback whales were identified using photo-identification (Katona and Whitehead, 1981) and/or microsatellite genotypes (Garrigue *et al.*, 2004). Paternities were previously inferred for 66 fathers of 79 offspring. Further details on the data collection, genetic profiling, and paternity analysis are described in Chapter 2. Here, I additionally used 535 skin samples collected from 1996 to 2020 to estimate the age of 485 individuals using the Humpback Epigenetic Age Assay (HEAA) developed by Polanowski et al. (2014).

3.3.2 Molecular age biomarkers

Molecular age biomarkers measure age-related modifications to DNA or RNA to estimate an individual's chronological age (Jarman *et al.*, 2015). Some of these age-dependent modifications occur in the epigenome, such as the DNA methylation of specific genes, which cause changes in gene expression (e.g., Polanowski *et al.*, 2014). Recent advances in genetic aspects of age-dependent processes have brought forward a powerful and minimally-invasive tool to estimate the age of animals living in the wild, including those usually submerged underwater.

In an analysis of whales of known age, Polanowski et al. (2014) screened 37 CpG sites in eight different humpback whale genes for a relationship with age. They identified seven CpG sites on three different genes (CDKN2A, TET2, GRIA2) with a strong methylation-age relationship and developed an ageing model for the estimation of age in humpback whales (Humpback Epigenetic Age Assay (HEAA); Polanowski *et al.*, 2014).

Genomic DNA from a total of 535 samples was sent to the Australian Genome Research Facility (Perth, Australia) for analysis using the HEAA of Polanowski et al. (2014). DNA samples were treated with sodium bisulphite to convert unmethylated cytosines to the RNA base uracil allowing the differentiation and detection of unmethylated versus methylated cytosines. Cytosine methylation levels were measured at eight CpG sites in three different humpback whale genes (CDKN2A, TET2, GRIA2; Table 3.1) previously identified in Polanowski et al. (2014). Pyrosequencing was performed on a PyroMark Q24 system (Qiagen) following methods described in Polanowski et al. (2014).

Table 3.1. Comparison between %DNA methylation levels at the studied CpG sites of the 535 samples. The position of the 5' Cytosine of each CpG in the respective humpback whale gene is given relative to the gene's start codon. The values indicate the distance in base pairs to the 3' of the start codon.

Gene	CpG position	CpG site	Mean % DNA Methylation	Standard Deviation	Minimum	Maximum
CDK2NA	+297	CDK2NA_A	3.07	1.24	0.35	10.95
	+303	CDK2NA_B	5.59	1.60	0.71	13.24
	+309	CDK2NA_C	4.29	1.40	0.84	11.29
	+327	CDK2NA_D	8.66	3.11	2.04	59.58
TET2	+16	TET_A	16.05	3.73	4.92	31.71
	+21	TET_B	14.35	3.78	3.20	30.75
	+31	TET_C	16.70	4.52	4.75	35.33
GRIA2	+202	GRIA2	2.46	1.10	0.00	8.16

To calibrate and assess the ageing model, a dataset of known age individuals and methylation levels is first needed. I applied the HEAA using published data (Polanowski et al. 2014, n = 45 individuals, of which n = 40 from Gulf of Maine, n = 4 from western Australia and n = 1 from eastern Australia) combined with newly generated data (n = 33 samples from 23 males, from New Caledonia) to generate the calibration dataset (n = 78 samples from 68 males; Table 3.2). The chronological age of those individuals was determined by the resighting of males first seen as dependent calves and identified via photo-ID and/or using microsatellite genotypes (Garrigue *et al.*, 2004; Polanowski *et al.*, 2014).

The calibrated ageing model was then used to estimate the age of a total of 485 New Caledonian male humpback whales using 535 samples, including the 33 samples from the calibration dataset (Table 3.2). For age estimation, individuals were selected to include the fathers identified in the paternity analysis (n = 66 males, see Chapter 2) and to cover a wide range of male mating behaviours and group types. To assess if the age structure of these selected males was representative of the New Caledonian male population age structure, a random subset of individuals (n = 100 males) was randomly drawn without replacement from all biopsied males (n = 1,047 males) at the New Caledonian breeding ground throughout the entire study period.

Table 3.2. Description of the different datasets with the number of samples and individuals. The calibration dataset consists of two subsets of male humpback whale samples of known age: New Caledonia (NC) and the calibration data from Polanowski et al. (2014; Pol). The age of calves in the calibration dataset was set to 0.1 years. The mean age and age range are based on the known age of individuals in the calibration and the estimated ages of individuals in the full NC dataset. For the full NC dataset, information on the age range is given for the raw estimates (age of the individual in the year it was sampled), meaning that individuals with multiple samples are represented several times. The age for all individuals in the Year 2020 was calculated from the estimated Year of Birth. When there were multiple samples from the same individual, the mean estimate of all samples was used as the Year of Birth.

Dataset	Description	Samples	Individuals	Mean age	Min. age	Max. age
Calibration	NC	33	23	3.5	0.1	23
	Pol	45	45	11.5	0.1	30.3
	Combined (NC + Pol)	78	68	8.6	0.1	30.3
Full NC	Raw estimates	535	485	11.1	-9.5	37.2
	Year 2020		485	20.6	2	49

3.3.3 Calibration and assessment of the ageing model

Before combining the newly generated New Caledonian data with the previously published dataset (Polanowski *et al.*, 2014), a Pearson correlation coefficient was calculated for each of the eight previously identified age-associated CpG sites in each of the two calibration datasets. This was to identify the CpG sites that showed both a consistent age-methylation correlation in both datasets and a significant relationship with age, for further analysis. All analyses were performed using R Statistical Software (v4.0.4; R Core Team, 2021) and the code is available on github (https://github.com/francae/PhD-Thesis_FrancaEichenberger). A Fisher's z-Test using the *cocor* package (Diedenhofen and Musch, 2015) tested whether the age-methylation correlations at each of the CpG sites differed between the two calibration sub-datasets (Diedenhofen and Musch, 2015). To correct *p* values for multiple testing, a Benjamin-Hochberg (BH) correction was applied (Benjamini and Hochberg, 1995). Corrected *p* values < 0.05 were considered statistically significant in all analyses. I integrated all possible combinations of either two or three CpG sites of separate gene regions into multiple linear regression models and used the Akaike Information Criterion (AIC) to identify which combination of CpG sites had the best ability to predict known age.

Three individuals in the calibration dataset were represented by duplicate or triplicate samples. To decide whether these non-independent samples should be excluded, I assessed their influence in the ageing model by calculating the Cook's Distance and hat values using the *stats* package in R. The accuracy of the ageing model was assessed with a linear regression of the model's estimated age and the known age for each male. In that linear regression, R^2 represents the proportion of variation in known age that is explained by the HEAA as a proxy of the strength of the relationship, and the gradient of the regression line the rate of change in methylation with age (Polanowski *et al.*, 2014; Jarman *et al.*, 2015). The ageing model was further assessed with a Leave One Out Cross Validation (LOOCV with k = 1; *caret* package) and 5-fold Cross Validation (5fCV with k = 5; caret package: Kuhn, 2021). For the LOOCV, one sample was treated as a validation set on the model built using the remaining 77 samples. Model evaluation was conducted on the linear regression of the LOOCV estimated ages and known ages of the calibration dataset. The correlation between known and LOOCV-estimated ages was further assessed via the Spearman rank-correlation coefficient. For the 5fCV, 1/5 of the data was randomly selected for retention, the models were fitted to the remaining 4/5

and then the prediction error of the fitted model was measured against predictions for the retained 1/5.

3.3.4 Applying the ageing model to estimate the age of humpback whales

I applied the calibrated ageing model to 535 samples collected on the New Caledonian breeding ground between 1996 and 2020 to estimate the age of 485 male humpback whales. Based on the year the whale was sampled and the age estimate obtained from that sample, I calculated each individual's estimated year of birth. For example, if a whale was estimated to be 10 years old in 2004, its inferred birth year was 1994. The estimated year of birth was then used to calculate the age of individuals in different years throughout the study period for subsequent analyses on the age structure of the population and the behaviour and reproduction of male humpback whales.

To assess if the age structure of the specifically selected samples was representative of the New Caledonian male population age structure, I conducted a t-test to compare the mean age of the random subset of individuals (section 3.3.2) and the remaining selected individuals (n = 396 males). Under the assumption that this random subset of individuals represents the overall age structure of the male population in New Caledonia, a non-significant result would indicate that the selected males are also representative of the New Caledonian male breeding population given the underlying data collection it is derived from.

3.3.5 Assessing the population age structure across time

Using the estimated year of birth, I calculated the age of all sampled individuals for each year of the study period. To accommodate the uncertainty in the underlying age-methylation relationship of our ageing model I grouped age estimates into four age categories: 2 – 9 years, 9 – 16 years, 16 – 23 years, and 23+ years. Intervals of age categories are open on the right (and closed on the left), e.g., $2 \leq x < 9$ years. Calves and yearlings (< 2 years) were excluded from all further analyses. The size of the age categories was based on measures of accuracy from the LOOCV (section 3.4.1). The threshold of the first two categories (2 – 9 years and 9 – 16 years) were centred around the reported age of sexual maturity of 8 – 10 years in humpback whales, based on an annual accumulation rate of earplug laminations (Best, 2011).

Due to the unknown accuracy of age estimates outside the calibration range (Pol: 0.1 – 30 years; NC: 0.1 – 23 years; Table 3.2), the last age category (23+ years) was left-bounded (right unbounded) including any male 23 years or older.

First, I assessed the age structure of the male population for the last year of the study period (2020) by calculating the proportion of males in each age category. Then, I investigated the changes in the male population age structure across the study period. Since every male is represented in each year of the study period (unless he was estimated to not yet have been born, or was < 2 years of age), the population age structure in consecutive years is subject to temporal autocorrelation. To reduce this non-independency of the time series count data, I divided the study period into two time windows (2000 – 2009 and 2010 – 2018) and compared the mean number of males in each age category across those two time windows. The division of these two time windows was set to 2009, the year in which the New Caledonian breeding population showed an anomalous increase in abundance (Garrigue, Albertson and Jackson, 2012). This captures the observed turning point in population size and creates a contrast of lower abundance in the first time window versus higher abundance in the second time window, mirroring what has been observed. To compare the population age structure to the age structure of fathers, the first time window starts with the year 2000 and the last time window ends with the year 2018 which are the first and last year of the paternity dataset, respectively. The years 1996 – 1999 were thus excluded from the analysis of the population age structure across time, as there were no fathers sampled across these years. I tested if the mean number of males in each age category differed between the two time windows using a Fisher's Exact test (FET).

3.3.6 Does the likelihood of males engaging in certain behaviours differ among age categories?

To further assess the reproductive behaviour of males I compared the age structure of males engaging in seven different behaviours and social contexts: solitary male, male dyad (group of two males), a solitary male escort of a single female or a mother with a calf, a principle escort, secondary escort, or challenger in a competitive group, and a singer (see Tables Table 2.2Table 2.3 in Chapter 2). The contexts of escorting, being in a competitive group, and singer are suggested to be associated with mating opportunities. For each of the seven behaviours, I

compared the proportion of males in each age category to the age distribution of the underlying male population using a Chi-squared test over the entire study period, and across the two time windows (2000 – 2009 and 2010 – 2018), separately. Additionally, I performed a post hoc analysis on the Chi-squared residuals to identify which age category of males more often engaged in certain behaviours. I applied the Benjamin-Hochberg correction (Benjamini and Hochberg, 1995) on all *p* values to account for multiple testing.

3.3.7 Are males of all age categories equally likely to sire offspring?

Using the estimated year of birth, I calculated the age of putative fathers in the year they sired offspring and grouped age estimates into the four age categories previously described (section 3.3.5). Firstly, I tested whether the age structure of fathers changed across time by comparing the number of males in each age category across the two time windows (2000 – 2009 and 2010 – 2018) using a Fisher's Exact test (FET). Secondly, I compared the age distribution of inferred fathers to the underlying age distribution of the male population 1) across the entire study period and 2) within each time window, using a simulation approach to generate the expected age distribution of fathers assuming all males had equal chances of siring offspring. To determine the expected age structure of fathers, based on the underlying male population age structure, if males from all age categories were equally likely to sire, I ran simulations using the following steps: For each year from 2000 to 2018, males for the number of inferred fathers in the first year were selected (with replacement) from the male population with the number of males and the observed age structure that year. The number of simulated fathers in each age category was then summed across the entire study period and within each of the two time windows. This process was repeated 10,000 times to generate the expected age distribution of fathers assuming age-independent, random mating. Lastly, I calculated the percentage of simulations in which the observed number of fathers was as high or higher than the simulated number of fathers in each age category across the entire study period and within each of the two time windows to explore whether any age categories were more or less likely than expected to achieve paternity success. *P* values of < 0.05 were considered statistically significant.

3.4 Results

3.4.1 Calibration and assessment of the ageing model

Two CpG sites (CDK2NA_A and CDK2NA_C) showed a different age-methylation correlation between the two calibration datasets (the first from Polanowski et al. (2014) including the Gulf of Maine and Australia, and the second from New Caledonia) and were thus removed from further analysis, allowing the combining of the two calibration datasets (Table 3.3, Figure 3.1). Of the six remaining CpG sites, four showed a significant age-methylation relationship using the combined calibration dataset (TET_A, TET_B, TET_C, GRIA2; Table 3.3) and were integrated in three different combinations of CpG sites on separate gene regions in the multiple linear regression models (Table 3.4). The model with the best AIC score (delta AIC to the next best model was 3.12) contained the two CpG sites: TET_C and GRIA2 (Figure S3.1). The age-methylation regressions of the two CpG sites selected for the HEAA are shown in Figure 3.2. Seven individuals in the calibration dataset were represented by multiple samples. The Cook's Distance and hat values (leverage) associated with these data indicated that none of these samples had a large influence on the model parameter estimates, and thus, all samples were retained for model calibration (Table 3.5, Figure S3.2).

The accuracy of the HEAA was assessed from a linear regression of the estimated ages from the methylation levels at two CpG sites and the known age of individuals in the calibration data. The regression R^2 of 0.58 indicates that although a considerable proportion of the variation in methylation can be explained by age, there are additional factors affecting methylation levels at the selected CpG sites. The y-intercept and gradient of the HEAA regression show that while young whales will have their age slightly overestimated, the age of older males will be slightly underestimated (Figure 3.3A; Model output in Figure S3.3). LOOCV and 5fCV yielded similar results for all three metrics: root-mean-squared error (RMSE), R^2, and mean absolute error (MAE; Table 3.6). The LOOCV quantile-quantile plot shows heavy tails on both ends but overall residuals seem to follow a normal distribution (Figure S3.4). A Breusch-Pagan test for non-constant variance further shows that the assumption of homoscedasticity in the residuals is met (BP = 0.188, p = 0.664). The Cook's Distance for all data points in the LOOCV was below 0.5 (Figure S3.4). The LOOCV mean difference of known and estimated age (mean residuals) of 0.04 years shows that the model is unbiased, however, the mean absolute difference between known and estimated age (MAE: mean absolute error) was 4.48 years

(Figure 3.3B). The standard deviation of the residuals of the LOOCV was 5.82 years and the 95% confidence interval was 11.41 years. These values mean that although the model is unbiased, for any new case we can expect an error of +/- 4.48 years, and 95% of the time we can expect age estimates to be within ~11.41 years of an individual's true age. Overall, this means that our model is unbiased but slightly less precise than the previous implementation of this approach (Polanowski *et al.*, 2014). The Spearman's rank correlation coefficient between known and estimated ages in the combined calibration dataset was 0.79.

Table 3.3. Regressions of CpG methylation with age for the eight CpG sites on three different humpback whale genes. The Pearson correlation coefficient *r* with corresponding *p*-value is shown for each of the two calibration datasets (NC: New Caledonia, Pol: Polanowski et al. (2014)) separately, as well as combined (NC + Pol). Results of the Fisher's z-Test indicate whether the correlation coefficient in the two subsets NC and Pol were significantly different from each other. The proportion of variation in methylation levels (adjusted R^2) by each identified CpG site with a consistent age-methylation relationship in all calibration data (sub)sets (NC, Pol, and combined) was further assessed using linear regression. GRIA2 showed a positive correlation between %DNA methylation and age (hypermethylation) while TET_A, TET_B and TET_C showed a negative correlation between %DNA methylation and age (hypomethylation), consistent with Polanowski et al. (2014). All *p*-values were corrected for multiple testing using the Benjamin-Hochberg correction. The reason for excluding a particular CpG site from further analyses is highlighted in bold.

CpG sites	NC: Pearson correlation [*r*, *p*]	Pol: Pearson correlation [r, *p*]	Fisher's z-Test	NC + Pol: Pearson correlation [r, *p*]	adj. R^2	adj. p-value	Age relationship
CDK2NA_A	-0.109, ns	0.695, p<0.001	**p<0.001**	0.291, p=0.016			
CDK2NA_B	0.032, ns	0.123, ns	ns	**-0.053, ns**			
CDK2NA_C	0.087, ns	0.599, p<0.001	**p=0.044**	0.264, p=0.026			
CDK2NA_D	0.063, ns	0.119, ns	ns	**-0.206, ns**			
TET_A	-0.555, p=0.003	-0.439, p=0.003	ns	-0.552, p<0.001	0.295	p<0.001	Hypomethylation
TET_B	-0.54, p=0.003	-0.455, p=0.003	ns	-0.529, p<0.001	0.27	p<0.001	Hypomethylation
TET_C	-0.537, p=0.003	-0.684, p<0.001	ns	-0.636, p<0.001	0.397	p<0.001	Hypomethylation
GRIA2	0.417, p=0.032	0.76, p<0.001	ns	0.541, p<0.001	0.284	p<0.001	Hypermethylation

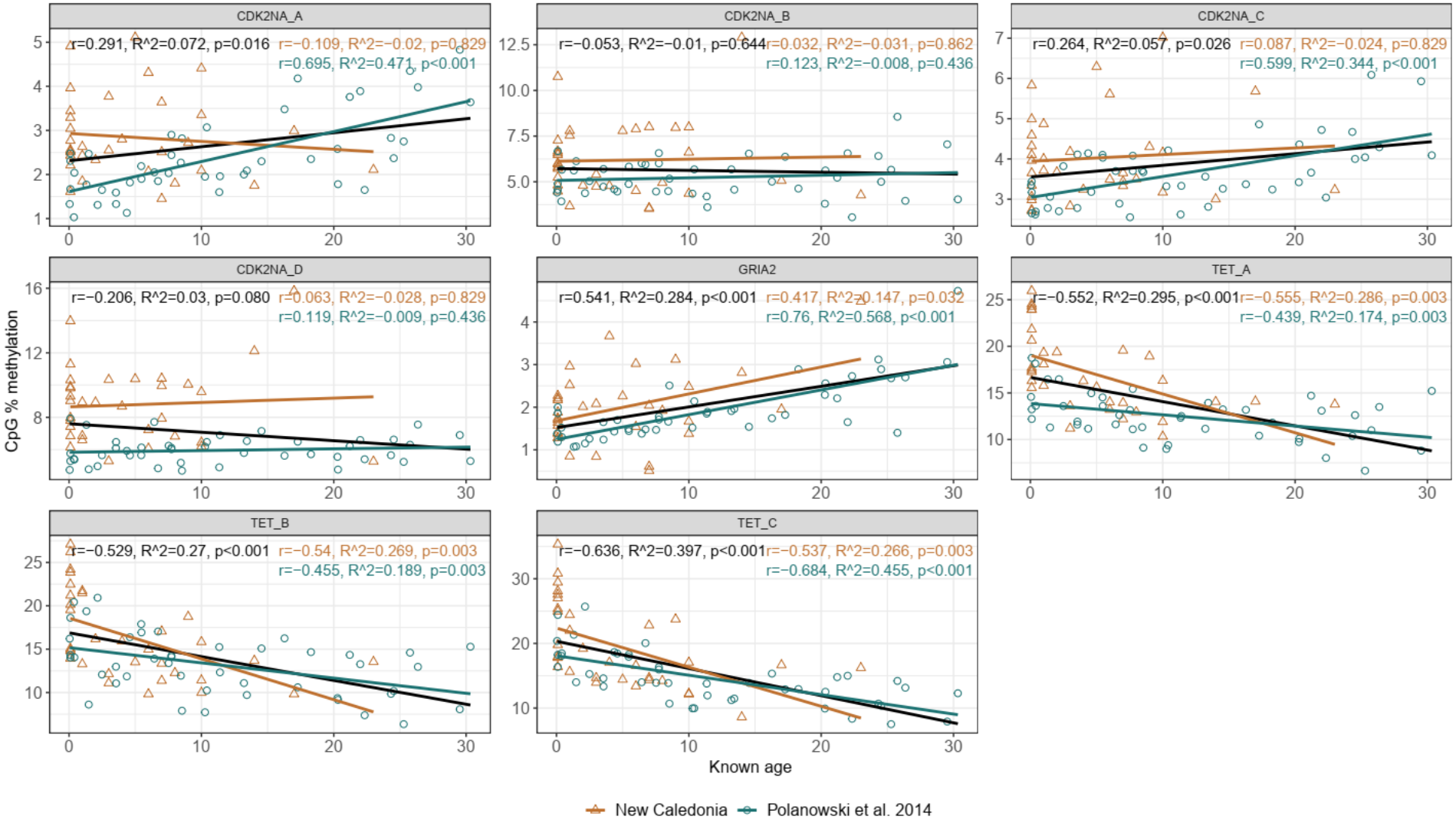


Figure 3.1. Linear regression between age and %DNA methylation for each CpG site with adj. R-squared (R^2), *p*-value and Pearson correlation coefficient (r) for each of the two calibration subsets (NC: New Caledonia, orange; Pol: Polanowski al (2014), blue) and the combined (NC + Pol, black) calibration dataset. All *p*-values were corrected for multiple testing using the Benjamin-Hochberg correction.

Table 3.4. Assessment of the multiple linear regression models with all three possible combinations of CpG sites and their adjusted R-squared and AIC scores. The combined calibration dataset and only CpG sites with a significant age-methylation correlation were used. The model with the best AIC score was selected for the HEAA and contained the following CpG sites: TET_C and GRIA2.

Combination of CpG sites	df	adj. R^2	AIC
TET_C + GRIA2	4	0.564	496.84
TET_A + GRIA2	4	0.539	501.22
TET_B + GRIA2	4	0.511	505.85

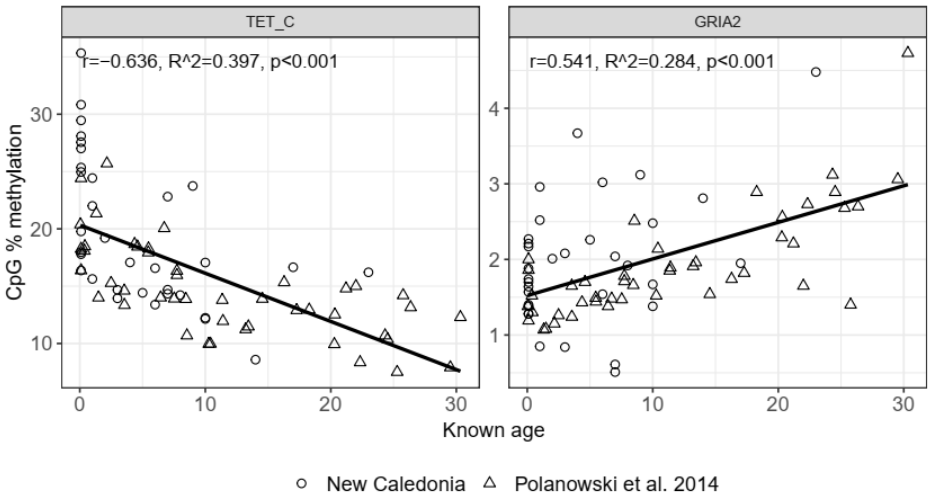


Figure 3.2. Age-methylation regression of the two selected CpG sites (TET_C and GRIA2) from the HEAA. The Pearson correlation coefficient *r* with the corresponding *p*-value is shown for the age-methylation correlation at each of the two CpG sites using the combined calibration data (New Caledonia + Polanowski et al. 2014), along with the adjusted R-squared (R^2) of the linear regression. Calibration samples from the New Caledonia and Polanowski et al. (2014) datasets are indicated with circles and triangles, respectively.

Table 3.5. The duplicate and triplicate samples of seven individuals in the calibration dataset with information on known age, estimated age and two measures of influence: Cook's Distance and Hat value (leverage). The low Cook's Distance and Hat values of these non-independent samples indicate that those samples only had a small influence on the model parameter estimates. Thus, all samples were retained for the model calibration.

Individual	Sample	Known age	Estimated age	Cook's distance	Hat value
HNC1054	NC16-034	6	16.5	0.035	0.014
HNC1054	NC17-324	7	4.3	0.004	0.013
HNC1054	NC18-095	8	10.6	0.002	0.013
HNC1054	NC20-045	10	11.1	0.001	0.013
HNC1059	NC08-111	0.1	0.8	0.006	0.026
HNC1059	NC14-016	6	6.8	0	0.014
HNC107	NC01-042	5	12.0	0.012	0.015
HNC107	NC06-137	10	9.7	0	0.013
HNC211	NC01-026	1	9.1	0.017	0.023
HNC211	NC07-081	7	-3.2	0.046	0.013
HNC533	NC13-116	9	8.4	0	0.013
HNC533	NC14-075	10	10.9	0.001	0.013
HNC533	NC18-178	14	19.4	0.031	0.018
HNC704	NC07-142	0.1	6.6	0.006	0.026
HNC704	NC10-152	3	5.7	0	0.018
NI11087	NC11-210	0.1	8.0	0.012	0.026
NI11087	NC18-130	7	10.8	0.003	0.013

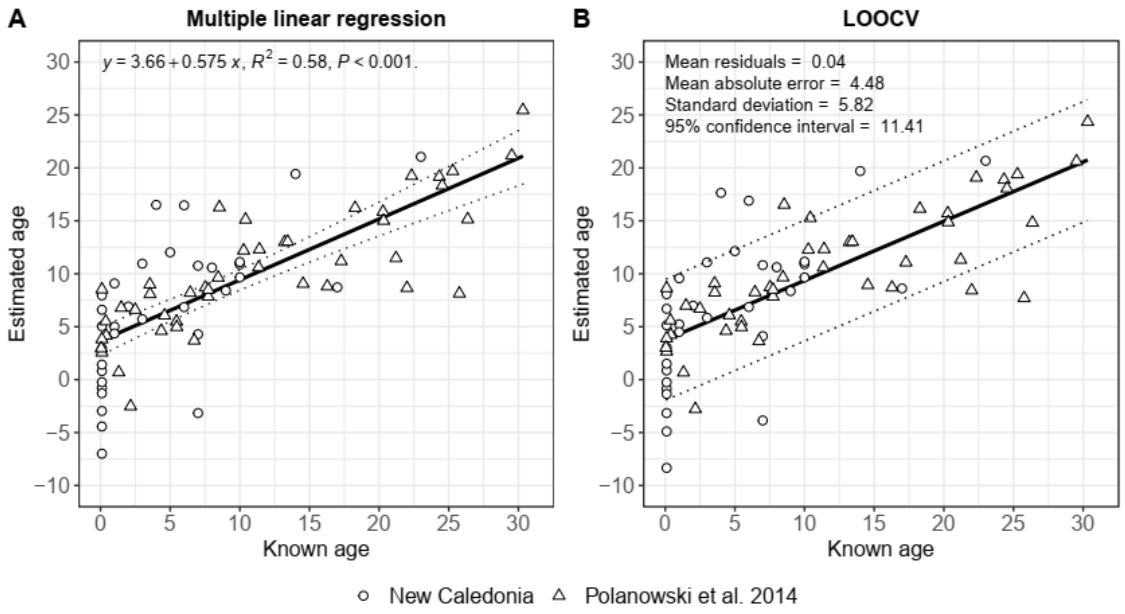


Figure 3.3. Accuracy and precision of the best-supported ageing model using the combined calibration data. A) Multiple linear regression for estimated ages of 78 samples from 68 whales from measurements of CpG methylation at two CpG sites (TET_C and GRIA2). The equation, R^2, and *p*-value of the fitted model are shown in the upper left corner. B) Results of the Leave One Out Cross Validation (LOOCV) analysis. The estimated ages of every sample in the calibration data when the model is based on the other 77 samples plotted against the known age. The mean residuals, mean absolute error (MAE), standard deviation, and 95% confidence interval of the difference between the known and estimated age of the LOOCV is shown in the upper left corner. In both plots, the 95% confidence interval of the regression (dotted line) is shown. The calibration samples from the New Caledonian and Polanowski et al. (2014; Gulf of Maine and Australia) datasets are indicated with circles and triangles, respectively.

Table 3.6. Results of the Leave One Out Cross Validation (LOOCV) and the 5-fold Cross Validation (5fCV). Both Cross Validations yielded similar results for all three metrics: root-mean-squared error (RMSE), R^2, and mean absolute error (MAE).

	LOOCV	5fCV
RMSE	5.78	5.63
R^2	0.54	0.56
MAE	4.48	4.30

3.4.2 Applying the ageing model to estimate the age of male humpback whales

Age estimates of the 535 samples collected between 1996 and 2020 ranged from -9.5 to 37.2 years and were centred around 11.1 years (Figure 3.4A-B; Table 3.2). Several samples (n = 20) yielded negative age estimates (Figure 3.4B), illustrating the errors that still remain in the estimation. The negative age estimates were set to zero to derive each individual's estimated year of birth. For individuals with multiple samples (n = 91 samples of 41 individuals), the mean estimated year of birth was calculated. The oldest male in this dataset was estimated to have been born in 1971 and the youngest in 2018 (Figure 3.4C). The size of the age categories (7 years) was set larger than the mean absolute error from the LOOCV (4.48 years).

Only 89/100 randomly selected samples had sufficient DNA available and/or were successfully aged. The mean estimated age of the random subset of individuals did not significantly differ from the mean estimated age of the selected (non-random) individuals (t-test: t = -0.943, df = 123.96, p = 0.347; mean estimated year of birth: non-random subset = 1999, random subset = 2000; Figure 3.5). Therefore, I concluded that the non-random dataset is as representative of the New Caledonian male breeding population as possible given the underlying data collection it is derived from, and the two subsets (random and non-random) were thus pooled for all further analysis.

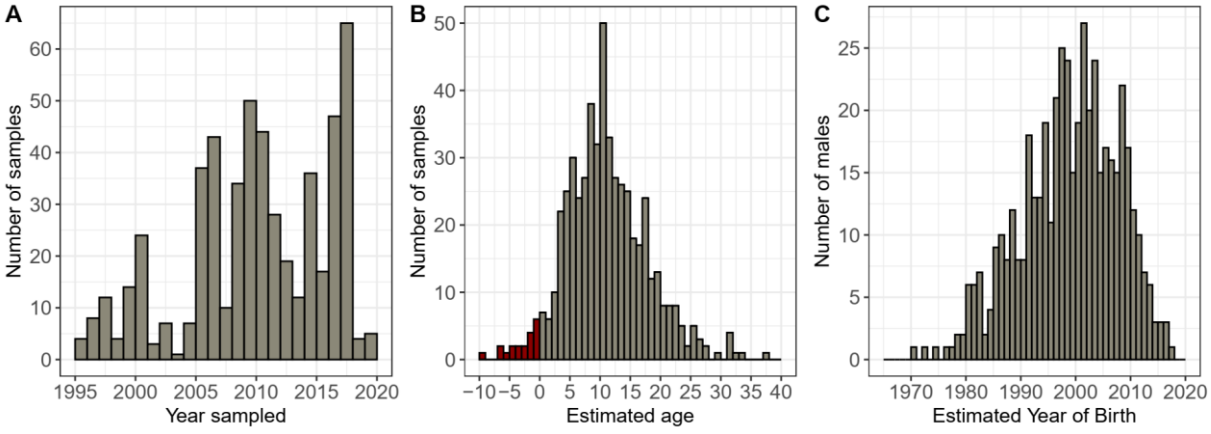


Figure 3.4. Overview of all samples (N = 535) and their estimated age. A) The number of samples collected in each of the study years (1996 – 2020). B) The distribution of age estimates of all analysed samples. Samples with negative age estimates (n = 20) are coloured in red. C) The distribution of the estimated Year of Birth of all sampled individuals.

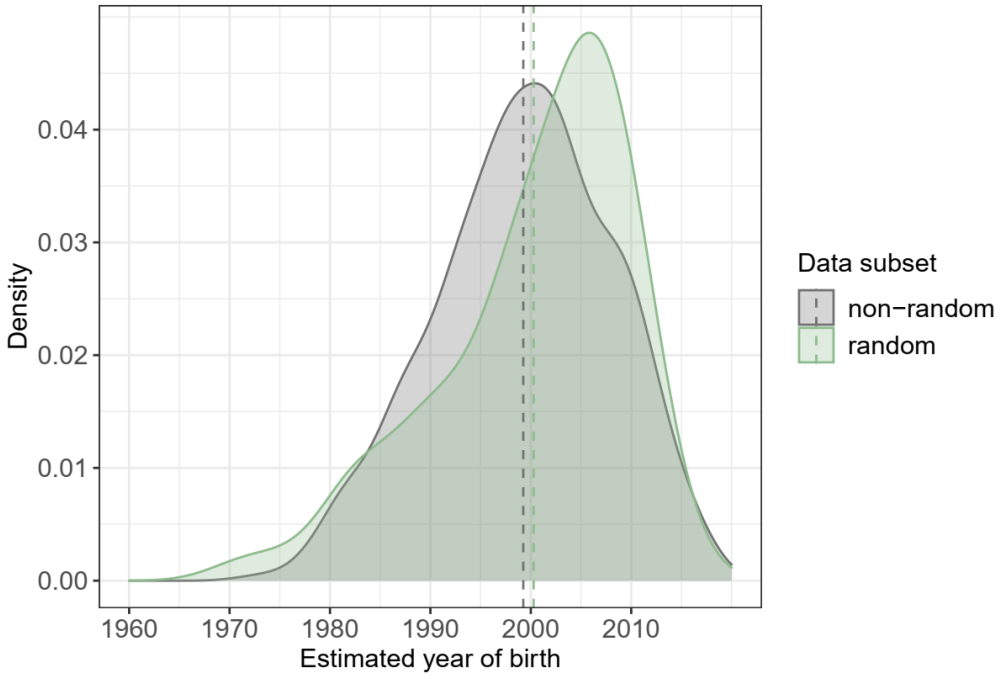


Figure 3.5. The age distribution for the subset of non-randomly selected individuals (n = 396; grey) compared to the random subset of individuals (n = 89; green) with overlying density lines (solid lines) and mean estimated year of birth (dashed line). The mean estimated year of birth for the random and non-random subset of individuals was 2000 and 1999, respectively.

3.4.3 Assessing the population age structure across time

The New Caledonian male breeding population in 2020 shows a mean age of 20.6 years with the oldest sampled male estimated to be 49 years old (Figure 3.6A; Table 3.2). In the year 2020, 6.8% were 2 – 9 years old, 23.5% were 9 – 16 years old, 31.8% were 16 – 23 years old, and 37.9% were 23+ years old (Figure 3.6B). Despite most males being in the oldest age category, only 15% were over 30 years old (N = 73 males), and less than 1.5% were older than 40 years (N = 7 males). The age structure of the male population in the first time window (2000 – 2009) was significantly different from the age structure in the second time window (2010 - 2018; FET: $p < 0.001$; Figure 3.7A2). The age structure of the male population at the beginning of the study period was predominantly comprised of young individuals in the first two age categories (2 – 9 years and 9 – 16 years). As individuals grew older and recruitment continued throughout the study period, the male age structure became more evenly distributed toward the end of the study period (Figure 3.8).

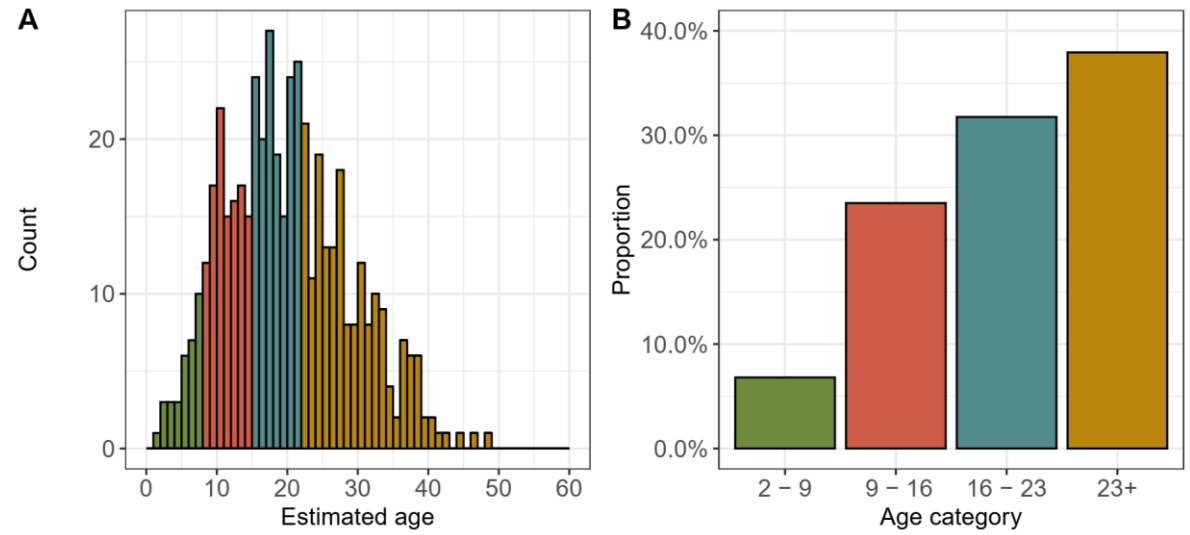


Figure 3.6. Age estimates of all sampled New Caledonian male humpback whales in the year 2020. A) Distribution of the raw age estimates of the sampled male population in the year 2020. The mean age was 20.6 years, and the oldest sampled male was estimated to be 49 years old in the year 2020. The different coloured bars indicate the different age categories also shown in the right panel. B) The proportion of aged males in each of the four age categories (green: 2 – 9 years, red: 9 – 16 years, blue: 16 – 23 years, yellow: 23+ years). Males below the age of 2 years (calves and yearlings) were excluded from all further analyses.

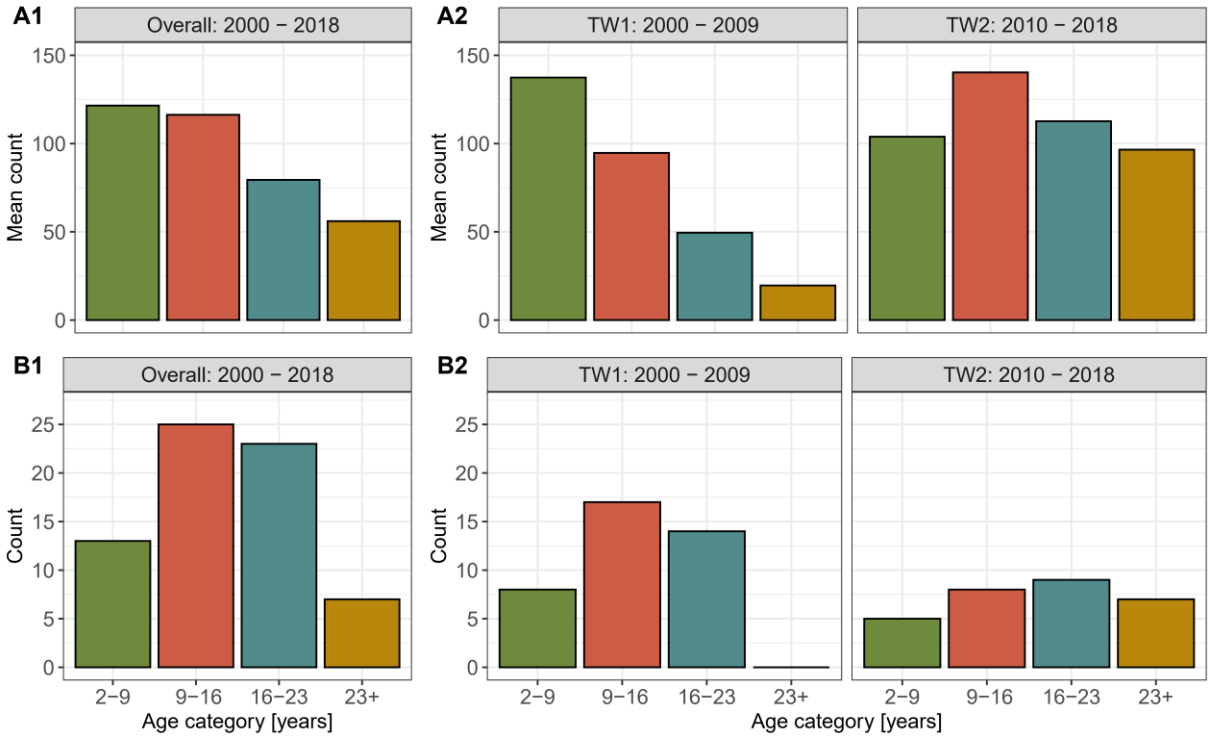


Figure 3.7. A) Age structure of the male population: the mean number of males in each of the four age categories (2 – 9 years, 9 – 16 years, 16 – 23 years, 23+ years) over the entire study period (A1: Overall) and in the two different time windows (A2; TW1: 2000 - 2009 and TW2: 2010 - 2018) in the male population. B) Age structure of fathers: the number of fathers in each age category over the entire study period (B1: Overall) and in the time window they sired offspring (B2).

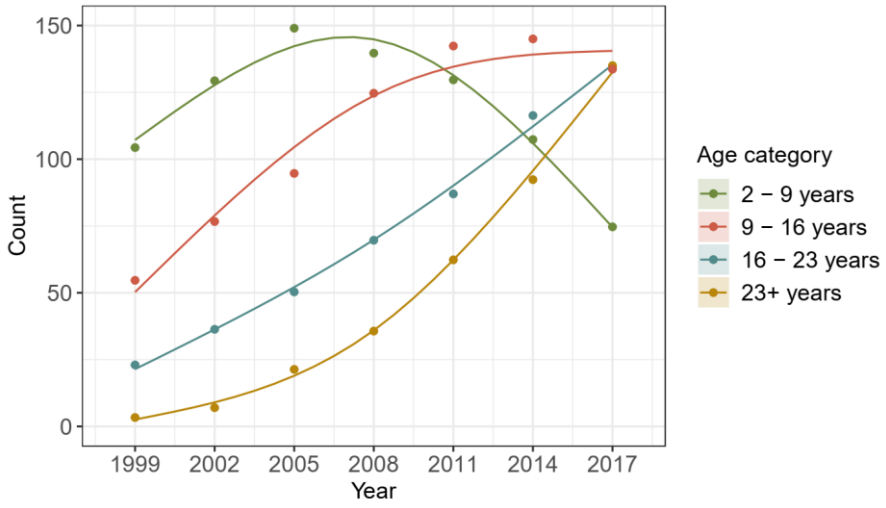


Figure 3.8. The changes in the male population age structure across the study period were descriptively explored by applying a Generalised additive model (GAM) with age category and the smoothed interaction term of time period and age category as explanatory variables using a cubic regression spline and assuming a Gaussian error distribution. I used the mean number of males (count) in each age category across seven consecutive three-year time periods between 1998 and 2018 (1998-2000, 2001-2003, 2004-2006, 2007-2009, 2010-2012, 2013-2015, 2016-2018) as a response variable to reduce the autocorrelation in model residuals. The GAM model output (Figure S3.5) and diagnostic plots (Figure S3.6) are shown in the Supplementary Material.

3.4.4 Does the likelihood of males engaging in certain behaviours differ among age groups?

The 460 aged male humpback whales (i.e., males at least 2 years old) were sighted in a total of 1,849 observations in 1,101 different groups between 2000 and 2018. Of those, 390 males engaged in one of the seven behaviours of interest in 1,084 observations of 743 different groups (Figure 3.9, Table 3.7). Only the age distribution of solitary escorts (of a female or mother with calf) and singers were significantly different from the underlying age distribution of the male population both overall (2000 - 2018) and in the second time window period (2010 - 2018) (overall: SolE: $\chi^2(df = 4, N = 212) = 43.4$, $p_{adj.} < 0.001$; Si: $\chi^2(df = 4, N = 119) = 35.0$, $p_{adj.} < 0.001$; TW2: SolE: $\chi^2(df = 4, N = 121) = 36.48$, $p < 0.001$; Si-TW2: $\chi^2(df = 4, N = 56) = 45.03$, $p < 0.001$), however, not in the first time window (2000 - 2009)(Table 3.8, Figure 3.10). Solitary escorts and singers were observably older on average than expected (Figure 3.9). The post-hoc analysis revealed that while younger males (2 – 9 years) were less often observed as solitary escorts and singers based on their distribution in the male population, older males were more frequently found as singers (23+ years and 16 – 23 years) and solitary escorts (23+

years) than expected based on the underlying age structure of the population (Figure 3.10, Table 3.8).

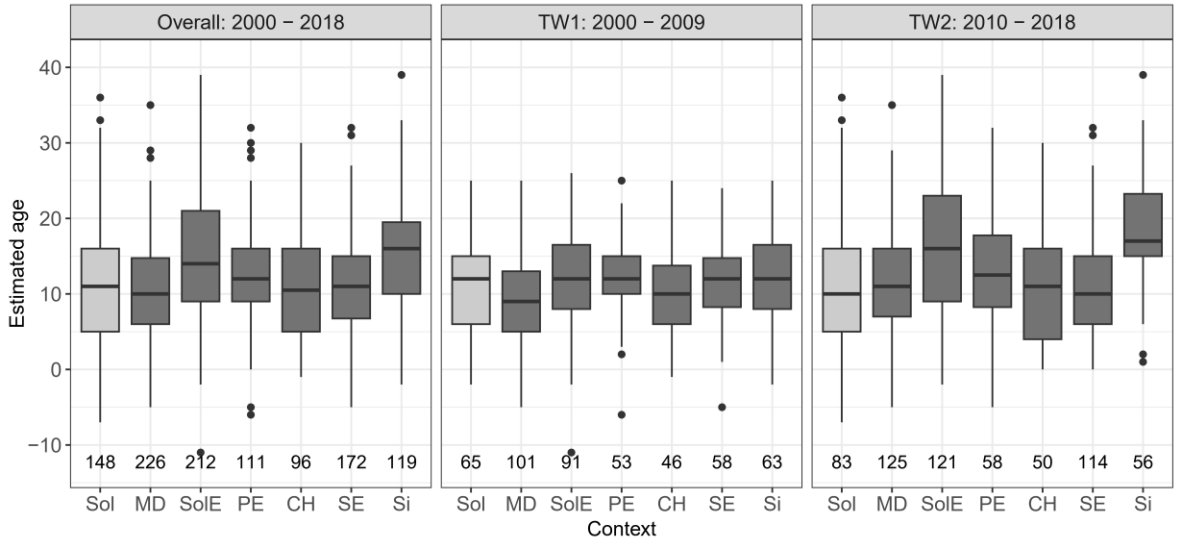


Figure 3.9. Boxplot of the estimated age of males engaging in different behaviours including mating behaviours A) over the entire study period and B) across the two time windows (2000 – 2009 and 2010 – 2018). The number of observations for each behaviour is indicated at the bottom of each boxplot. Abbreviations: Sol: Solitary male, MD: Male dyad, SolE: Solitary escort of a female or mother with calf, PE: Principle escort, CH: Challenger, SE: Secondary escort, Si: Singer

Figure 3.10. Chi-square of the observed age distribution of males engaging in different behaviours compared to the age distribution of the aged male population (expected distribution) A) over the entire study period and B) across the two different time windows (TW1: 2000 - 2009 and TW2: 2010 - 2018). Significant results of the chi-squared test are indicated with an asterisk. Abbreviations: Pop: Population age structure, Sol: Solitary male, MD: Male dyad, SolE: Solitary escort of a female or mother with calf, PE: Principle escort, CH: Challenger, SE: Secondary escort, Si: Singer

Table 3.7. The number of observations and mean estimated age in years of all sampled males in the population (section 3.4.3), for males that were observed to engage in specific mating behaviours (section 3.4.4), and fathers that sired offspring (section 3.4.5) over the entire study period (Overall) and within the two time windows (TW1: 2000 - 2009 and TW2: 2010 - 2018). Abbreviations: Total number of observations for Solitary male (Sol), Male dyad (MD), Solitary escort of a female or mother with calf (SolE), Principle escort (PE), Challenger (CH), Secondary escort (SE), Singer (Si), total number of observations across all mating behaviours (Total). Total number of unique individuals across all mating behaviours for Males and Fathers.

		Number of observations			**Mean estimated age**		
		Overall	TW1	TW2	Overall	TW1	TW2
Population	Total	1,849	743	1,106			
	Males	460	212	344	11.7	11.1	12.1
Mating behaviours	Sol	148	65	83	11.5	11.2	11.7
	MD	226	101	125	10.6	8.9	12.0
	SolE	212	91	121	14.7	12.7	16.2
	PE	111	53	58	12.7	12.2	13.0
	CH	96	46	50	10.9	10.8	11.0
	SE	172	58	114	10.8	11.0	10.8
	Si	119	63	56	15.2	12.2	18.6
	Total	1,084	477	607			
	Males	390	183	275	12.1	11.2	13.2
Reproduction	Fathers	68	39	29	15.3	13.5	17.3

Table 3.8. Chi-squared test and post-hoc analysis on the age distribution of males engaging in the different mating behaviours over the entire study period (Overall) and within the two time windows (TW1: 2000 - 2009 and TW2: 2010 - 2018) compared to the underlying age structure of the population. Abbreviations: Sol: Solitary male, MD: Male dyad, SolE: Solitary escort of a female or mother with calf, PE: Principle escort, CH: Challenger, SE: Secondary escort, Si: Singer, ns: non-significant ($p > 0.05$).

Mating behaviour	Time period	X^2	*df*	$p_{adj.}$	Cramer's V	< 2 yrs	2-9 yrs	9-16 yrs	16-23 yrs	23+ yrs
Sol	Overall	5.98	4	0.23	0.10	ns	ns	ns	ns	ns
Sol	TW1	7.72	4	0.29	0.17	ns	ns	ns	ns	ns
Sol	TW2	2.36	4	0.72	0.08	ns	ns	ns	ns	ns
MD	Overall	6.75	4	0.23	0.09	ns	ns	ns	ns	ns
MD	TW1	14.61	4	0.026	0.19	ns	ns	ns	ns	ns
MD	TW2	4.06	4	0.51	0.09	ns	ns	ns	ns	ns
SolE	Overall	43.40	4	<0.001	0.23	ns	ns	ns	ns	<0.001
SolE	TW1	12.02	4	0.06	0.18	ns	ns	ns	ns	0.034
SolE	TW2	36.48	4	<0.001	0.27	ns	0.039	ns	ns	<0.001
PE	Overall	8.75	4	0.16	0.14	ns	ns	ns	ns	ns
PE	TW1	6.67	4	0.36	0.18	ns	ns	ns	ns	ns
PE	TW2	3.10	4	0.63	0.12	ns	ns	ns	ns	ns
CH	Overall	3.40	4	0.49	0.09	ns	ns	ns	ns	ns
CH	TW1	5.12	4	0.48	0.17	ns	ns	ns	ns	ns
CH	TW2	2.08	4	0.72	0.10	ns	ns	ns	ns	ns
SE	Overall	6.21	4	0.23	0.10	ns	ns	ns	ns	ns
SE	TW1	4.04	4	0.51	0.13	ns	ns	ns	ns	ns
SE	TW2	5.64	4	0.46	0.11	ns	ns	ns	ns	ns
Si	Overall	35.00	4	<0.001	0.27	ns	0.012	ns	<0.001	0.019
Si	TW1	4.40	4	0.51	0.13	ns	ns	ns	ns	ns
Si	TW2	45.03	4	<0.001	0.45	ns	<0.001	ns	<0.001	0.002

3.4.5 Are males of all age categories equally likely to sire offspring?

From the 66 fathers of the 79 paternity assignments derived in Chapter 2, a total of 61 fathers of 73 paternity assignments were successfully aged (Figure 3.11A). Four putative fathers were unrealistically estimated to have sired offspring before the age of 2 (Table S3.1, Figure 3.11C) and were thus excluded from all further analyses. The mean age of males siring their first sampled offspring was 14.7 years, and 17.2 years for males siring their second sampled offspring (Figure 3.11B). Most aged males that sired offspring over the entire study period of the paternity analysis (1996 – 2018; see Chapter 2) were between 9 to 23 years old (Figure 3.11D). The youngest estimated age at reproduction was 3 years in the relaxed paternity dataset (later rejected in Chapter 5 section 5.4.3), or 4 years in the conservative paternity dataset (see Chapter 2 section 2.4.2). However, considering the mean difference between the estimated and known age of 4.48 years (MAE in LOOCV), this male could be up to 7 – 9 years old (see Figure 3.12 for an overview of the yearly number of inferred fathers in each age category).

The distribution of fathers in the first time window (2000 – 2009) was significantly different from the age distribution of fathers in the second time window (2010 – 2018; FET: $p = 0.01$; Figure 3.7B2). In the first half of the study period (TW1), it was predominantly males between 9 – 23 years that sired offspring, while no father above the age of 23 years was sampled (Figure 3.7B2-TW1). In the second half of the study (TW2), the age structure of fathers, like the overall population age structure, was more evenly distributed (Figure 3.7B2-TW2). However, even though males from 9-16 years of age are most frequent in the population in TW2 (Figure 3.7A2-TW2), it was males in the age category of 16-23 years that sired the highest number of offspring (Figure 3.7B2-TW2). In both time windows, males of the youngest age category (2-9 years) sired, as expected, the least number of offspring. Throughout the study period, younger males were progressively overtaken by older males in siring offspring (Figure 3.12, Figure 3.13). Overall, this indicates that not only the age structure, but the distribution of father's ages changed over the study period.

The simulation analysis revealed that males of the youngest age category (2 – 9 years) were less successful in siring offspring than expected based on their underlying distribution in the population. In less than 1% of simulations was the observed number of fathers larger than the simulated number of fathers in this age category (2 – 9 years) over the entire study period

or within the first time window, and it was less than 15% of the simulations in the second time window (Figure 3.14, Table 3.9). Males of the second age category (9 – 16 years) were considerably more successful in siring offspring based on the underlying population age structure over the entire study period (in 80% of simulations) and in the first time window (in 93% of simulations), but sired substantially less offspring than chance in the second time window (26% of simulations; Figure 3.14, Table 3.9). Males of the second oldest age category (16 – 23 years) were more successful in siring offspring based on the underlying population age structure over the entire study period (>99% of simulations) and within both time windows (in >99% and 74% of simulations, respectively). However, males of the oldest age category (23+ years) were only more successful in siring offspring based on the underlying population age structure in the second time window (in 63% of simulations; Figure 3.14, Table 3.9).

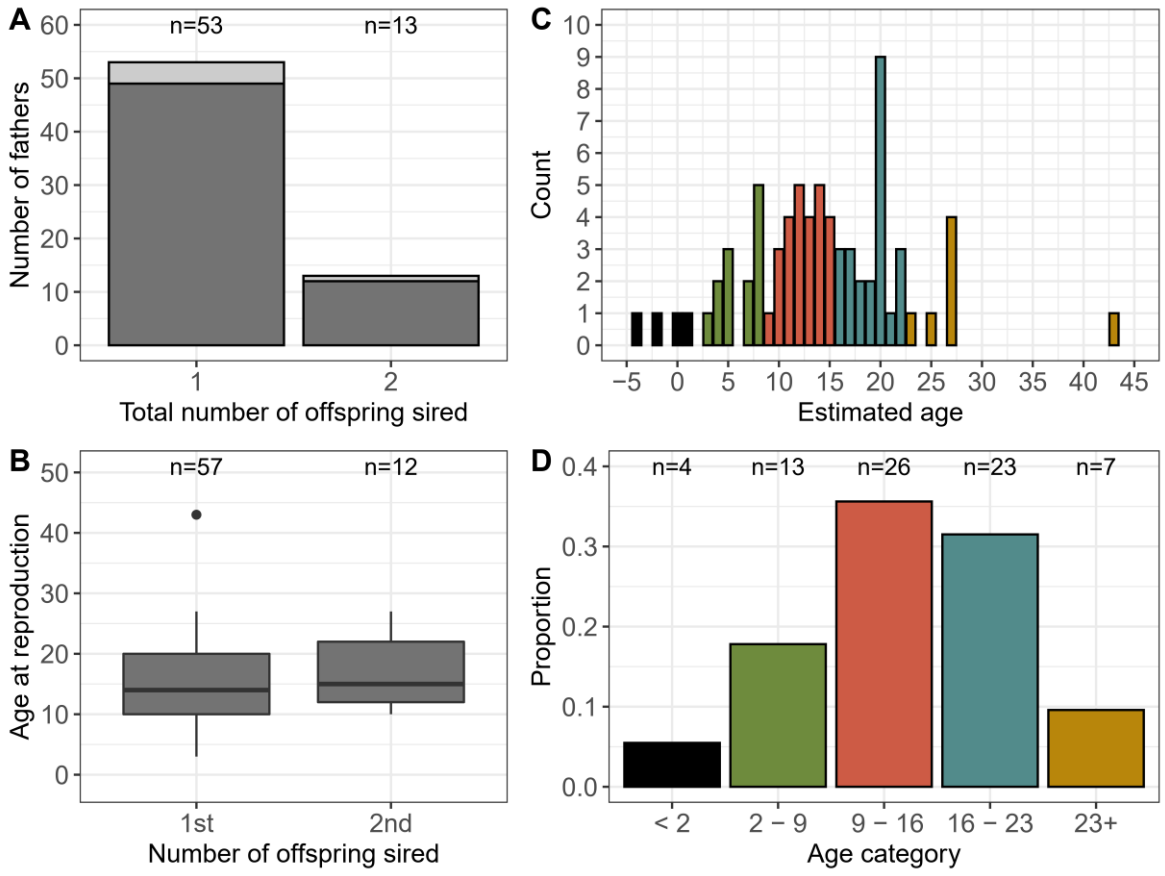


Figure 3.11. Age at reproduction. A) The number of fathers with (dark grey) and without (light grey) age estimates that sired one or two offspring. B) The mean age at reproduction of males siring their first and second sampled offspring. C) Raw estimated age of males in the year they sired offspring. Four fathers were estimated to be less than 2 years old in the year they sired offspring and were thus excluded from all further analyses. D) The proportion of fathers in each of the four age categories (green: 2 – 9 years, red: 9 – 16 years, blue: 16 – 23 years, yellow: 23+ years). Males below the age of 2 years (calves and yearlings) were excluded from the analysis and are coloured in black.

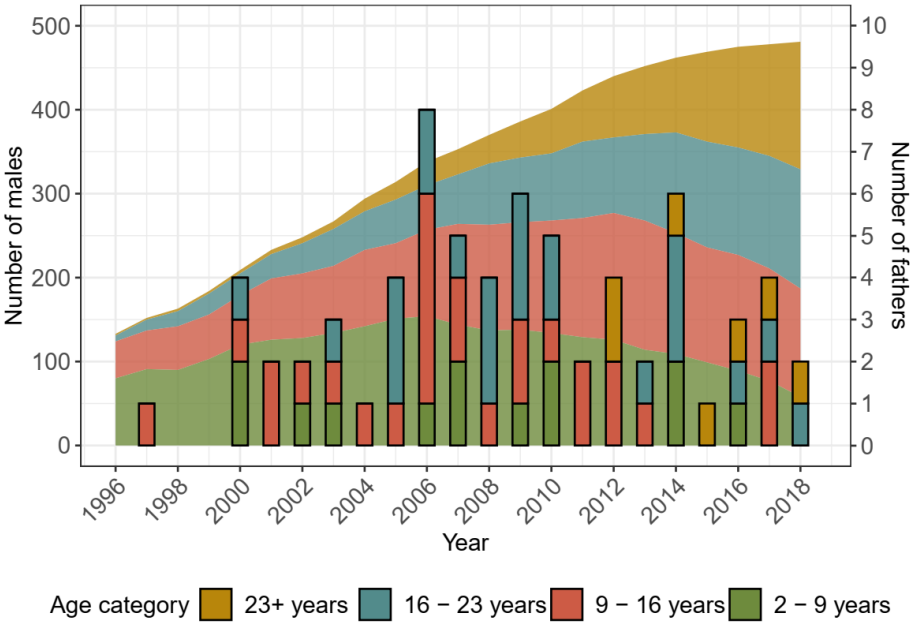


Figure 3.12. The number of males in the population (left-hand y-axis) in each of the four age categories (2 – 9 years, 9 – 16 years, 16 – 23 years, 23+ years) across the entire study period (1996 - 2018) and the yearly number of fathers (right-hand y-axis) in each of the four age categories (bars).

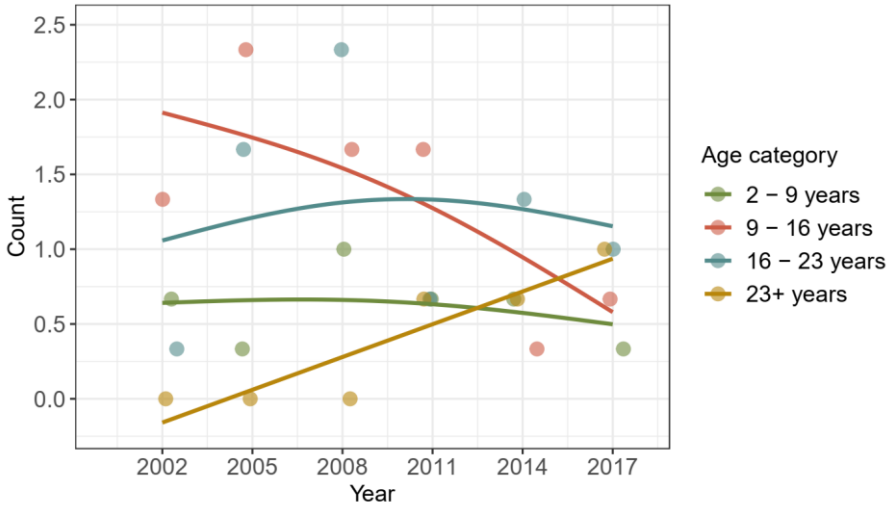


Figure 3.13. The changes in the age structure of fathers across the study period were descriptively explored by applying a Generalised additive model (GAM) with age category and the smoothed interaction term of time period and age category as explanatory variables using a cubic regression spline and assuming a Gaussian error distribution. I used the mean number of fathers in each age category across six consecutive three-year time periods (2001-2003, 2004-2006, 2007-2009, 2010-2012, 2013-2015, 2016-2018) as a response variable to reduce the autocorrelation in model residuals. Points were jittered horizontally (width = 0.5) to avoid overplotting. The GAM model output (Figure S3.7) and diagnostic plots (Figure S3.8) are shown in the Supplementary Material.

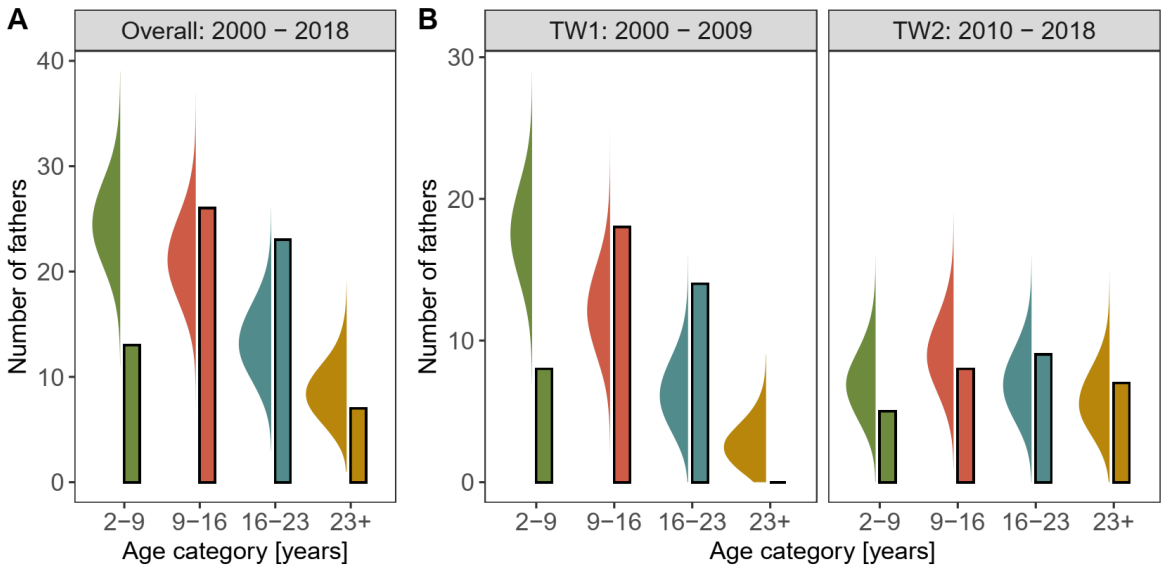


Figure 3.14. Distribution of the simulated number of fathers based on the underlying age structure of the male population (half-violin) compared to the observed number of fathers (bars) for each age category A) across the entire study period and B) within the two time windows (TW1: 2000 - 2009 and TW2: 2010 - 2018). Note the departure from random expectations of males aged 2-9 during the first part of the study (TW1).

Table 3.9. Results from the simulation analysis across the entire study period and within the two time windows (TW1: 2000 - 2009 and TW2: 2010 - 2018) for each of the age categories with the observed number of fathers and the average simulated number of fathers. The far-right column shows the percentage of simulations in which the observed number of fathers was larger than the simulated number of fathers.

Time period	Age category	Observed # fathers	Average simulated # fathers	Obs. > Simu. [%]
Full	2 - 9 years	13	25	0.05
Full	9 - 16 years	25	21	80.09
Full	16 - 23 years	23	13	99.61
Full	23+ years	7	9	22.06
TW1	2 - 9 years	8	18	0.04
TW1	9 - 16 years	18	12	92.91
TW1	16 - 23 years	14	6	99.72
TW1	23+ years	0	3	0.00
TW2	2 - 9 years	5	7	13.14
TW2	9 - 16 years	8	9	26.19
TW2	16 - 23 years	9	7	74.37
TW2	23+ years	7	6	63.14

3.5 Discussion

Here, I calibrated a previously developed epigenetic ageing model for humpback whales (Polanowski *et al.*, 2014) to a population of humpback whales breeding off the coast of New Caledonia in the South Pacific. The population age structure was left-skewed before a reported population increase but then became more balanced indicating a stabilisation of the population. This shift in the age structure of the male population further caused changes in the age distribution of fathers and patterns of sexual selection. This study demonstrates epigenetic age estimation to be a powerful tool in aiding the assessment of endangered populations and improving our understanding of population dynamics and reproductive behaviours of animals in the wild.

3.5.1 Population age structure reveals signs of recovery

Age estimates of 485 male humpback whales revealed a left-skewed population age structure with a mean age of 20.6 years in the last year of the study period in 2020. Other studies estimating age structure in the region reported an average age of 13.8 years in 2015 in the Kermadec Islands (Riekkola *et al.*, 2018), and 10.01 years in 2009 in East Australia (Polanowski *et al.*, 2014). Projected to the year 2020, those age estimates fall within a similar range to the estimated average age of the New Caledonian population and further highlight the rarity of individuals older than 30 years. Considering the adjacency of these areas, and that a considerable proportion (~49% in 2015) of the whales migrating through the Kermadec Islands are from New Caledonia (Riekkola *et al.*, 2018), these findings are consistent with the New Caledonian population being exposed to similar timings of whaling pressure with other populations in the region. In comparison to the highly left-skewed population age structure at the beginning of the study period (TW1: 2000 – 2009), the age distribution became more balanced in the second half of the study period (TW2: 2010 – 2018). While legal commercial whaling ended in 1963 in the Southern Hemisphere, illegal whaling continued into the 1970s (Yablokov *et al.*, 1998; Clapham *et al.*, 2009). Thus, we cannot be precise about the timescale of these obvious exploitation impacts on the age structure. However, the changes in the male population age structure throughout the study period together with recent population estimates (Figure 2.6 Chapter 2) provide evidence that the New Caledonian breeding population is likely recovering, especially after the reported anomalous increase (Garrigue, Albertson and Jackson, 2012), and appears to be reaching a more stable equilibrium. The long-

term dataset spanning over 26 years has provided a unique opportunity to assess the potential changes in male reproductive tactics, male reproduction, and sexual selection during this process of recovery and reported population growth.

3.5.2 Age-dependent reproductive tactics

As reproduction in mammals is age-dependent, individuals might start to engage in reproductive tactics around the time, or even slightly before, they reach sexual maturity. If certain reproductive tactics require skills that need to be learned or socially acquired (e.g., simulated oestrus in African elephants, Loxodonta africana: Bates et al., 2010), older males might exhibit such behaviours more often, or more successfully, than younger individuals. Yet, younger individuals might engage in these behaviours to practice and hone their skills before they start reproducing. A male's age, or stage of development, might thus influence how likely he is to employ a certain reproductive tactic and/or how likely a tactic renders him successful in siring offspring. For example, juvenile male Java sparrows (*Lonchura oryzivora*) repeatedly practise their courtship dance well before sexual maturation (Soma *et al.*, 2019). By doing so, they increased their motor performance and gradually became capable of singing and dancing simultaneously (Soma *et al.*, 2019). Further, this early-life dance practising was suggested to influence their future reproductive success (Soma *et al.*, 2019).

Here, older males were more likely to be observed as solitary escorts and singers, and were more successful in siring offspring than younger males. Yet, this was more evident in the second half of the study period after a known increase in population abundance. The similar age structure of males observed as solitary escorts and singers, and fathers could indicate that these two behaviours are more strongly associated with mating success than other behaviours seen on the breeding ground. It is unclear whether escorting a single female results in copulation or whether it reflects mate guarding following earlier copulation (Clapham, 1996). While under the first scenario, a solitary escort might be the successful winner and previous principal escort of a competitive group; under the second scenario, the solitary escort might try to defend the female from mating with other males, and if he is challenged, might lead to the formation of a competitive group. Younger individuals (2 – 9 years) were less often observed as solitary escorts or singers than older males, yet there was no apparent age-dependent pattern detected among males of different age categories within all other

behaviours, including the three behavioural roles in competitive groups (principle escort, secondary escort, and challenger). This suggests that males of all ages regularly participate in competitive groups. Although older males were not found more likely to participate in competitive groups than younger ones, principles escorts were on average 1 – 2 years older than secondary escorts and challengers over the entire study period and within each of the two time windows (Table 3.7). While this is well within the error window of age estimates, it could indicate that males of all ages participate in competitive groups but that older, more sexually mature, or more experienced males are more likely to secure the superior position as a principle escort closest to the female than younger males. Younger males might nevertheless participate in competitive groups to learn and practice until they become experienced enough to successfully defend a female from other male challengers as a principle or solitary escort.

Similarly, and just like songbirds (Catchpole and Slater, 2008), male humpback whales might start practising their singing before the onset of reproduction. The underlying function(s) of humpback whale song remains debated but male-only production during the breeding season (Herman, 2017) means it is broadly recognized as a male mating behaviour (Glockner 1983; Baker and Herman 1984; Darling et al. 2006; Smith et al. 2008). Humpback songs change progressively each year and sometimes are rapidly replaced by a novel song (Noad *et al.*, 2000; Garland *et al.*, 2011). This indicates that despite the song's high structural complexity, males are able to learn entirely novel songs very quickly (i.e., within a breeding season). Song learning in humpback whales, thus, is a constant and crucial component throughout a male's life; from when young males first begin to sing (developmental ontogeny) and seasonally at the onset of the breeding season (seasonal development) (Kowarski *et al.*, 2022). On the New Caledonian breeding ground, males were found singing from an early age (<9 years), however, most singers were considerably older (section 3.4.4). Males might begin to practice their singing and/or song learning skills at or even before the onset of sexual maturity. The singing and song learning skills of young singers (beginners) are presumably less advanced compared to older, thus likely more experienced or skilled, singers. However, whether older males sing more complex song, and whether they learn their songs quicker than younger individuals, remains to be investigated.

3.5.3 Age-dependent reproduction

There was age-dependent male reproduction in the New Caledonian population suggesting older, potentially more experienced males may be more likely to sire offspring. The random mating simulations based on the underlying population age structure and the number of sampled offspring suggest that three males of the oldest age category (23+ years) were expected to have sired offspring. Yet no male older than 23 years sired offspring in the first time window in this dataset. While this could indicate that older males (23+ years) are less likely to sire offspring, the fact that they are more likely to sire offspring than expected based on the underlying, and by then more balanced, population age structure in the second time window, suggests otherwise. It might thus be, that due to the skewed population age structure towards younger individuals, older males, and thus older fathers, were scarce, and their (and their offspring's) probability to be sampled was much lower compared to fathers of the younger age categories. These results may indicate that while the age distribution of fathers across time partially reflects the temporal changes in the population age structure, overall, older males might be more likely to sire offspring considering their lower density in the population compared to younger males.

Younger males were, as expected based on the species' estimated age of sexual maturity, less successful in siring offspring than older males. However, mating success was not solely restricted to older males as occasionally males of the youngest age category (2 – 9 years) were found to sire offspring (20% and 17% of paternities in TW1 and TW2, respectively). In light of the species' estimated mean age of sexual maturity of 9 - 11 years (Best, 2011) and assuming some individual variation around the age at sexual maturity, some males are expected to reach their sexual maturity before the age of 9 years (and some after the age of 11 years). Female humpback whales in Alaska had their first calf at a mean age of 11.8 years, yet showed considerable variation as some were found to be as young as 8 years and some up to 16 years old when sighted with their first calf (Gabriele, Straley and Neilson, 2007). In New Caledonia, the youngest female observed with a calf was known to be 8 years old (Chero *et al.*, 2020). While some of these young fathers in our dataset might indeed be males who reached their sexual maturity earlier than the estimated mean age at sexual maturity, the imprecision of the ageing model might have underestimated the age of others (see section 3.4.1; Figure 3.3). Nevertheless, considering the high proportion of young individuals in the population, young males (2 – 9 years) were only rarely able to successfully sire offspring. Male humpback whales

in general may be physiologically able to reproduce at a young age, yet because of their lack of experience and undeveloped skills in reproductive tactics compared to older males, they are not particularly successful in siring offspring. Another possible non-mutually exclusive explanation is that the physiology of male humpback whales has changed in response to the anthropogenic pressure caused by commercial whaling (e.g., Minke whales, Balaenoptera bonaerensis: Hidehiro Kato, 1995). Similar to the decreased birth interval of female humpback whales in New Caledonia, hypothesized to be a sign of phenotypic plasticity (Chero *et al.*, 2020), males may have become sexually mature at a younger age as a result of commercial whaling. More research and long-term population monitoring is required to address these questions. Nevertheless, young males did reproduce, and thus, aided the recovery of this population.

Altogether, these findings suggest that although male humpback whales start to engage in mating behaviours from an early age, and potentially before their sexual maturity, it may require experience and time for them to become skilful enough to successfully outcompete their older male conspecifics. Older males might achieve mating success by engaging in reproductive tactics that require experience or a particular set of skills, such as direct contest competition in competitive groups or singing complex song. Younger males might by contrast try to achieve mating access through alternative mating tactics that are less dependent on age or experience. A male humpback whale's reproductive tactics, his ability to compete over mating access and successfully reproduce are therefore likely to be age-dependent.

3.5.4 Patterns of age-specific sexual selection

Age-related patterns in reproductive tactics and reproductive success were only evident in the second half of the study period after a reported increase in population abundance (Garrigue, Albertson and Jackson, 2012) and once the age structure became more balanced. Even if older males are more likely to sire offspring if they are proportionally less abundant in the population than younger individuals, their age-related reproductive advantage may be overridden by their disadvantage in number. In turn, young individuals may benefit from having fewer competitively superior males in the population.

In bighorn sheep, larger horn size was correlated with increased mating success in older rams. However, younger or smaller rams achieved mating success through alternative mating tactics that were less dependent on horn length and body size (Coltman *et al.*, 2001). Unrestricted selective harvesting for rams with large horns led to a decrease in horn length over time and decreased the number of competitors in the population (Pigeon *et al.*, 2016). As a result, young males obtained an increasing proportion of mates and experienced increased sexual selection on horn length and body mass (Martin *et al.*, 2016). Similarly, young male humpback whales may experience stronger sexual selection through increased male-male competition among young conspecifics as they account for a greater proportion of all males. I hypothesize that under the left-skewed age structure of the New Caledonian population post-whaling, young male humpback whales experienced increased sexual selection through increased male-male competition compared to young males in the same population under a more balanced age structure. Populations that experienced a shift in their age structure towards younger individuals as a result of population exploitation might thus further be subject to changes in the patterns of sexual selection. Together with the observed differences in the age-related patterns of reproductive tactics and reproductive success in the two time windows, these findings highlight the importance of accounting for the changes in the population age structure across time. More research is needed to better understand how demographic processes shape patterns of sexual selection in wild populations.

3.5.5 Assessment of epigenetic ageing and its limitations

Emerging technologies for measuring DNA methylation are increasing the potential for developing biomarkers for estimating chronological age for a wider range of species (e.g., Jarman *et al.*, 2015; De Paoli-Iseppi *et al.*, 2017). Several factors influence the performance of epigenetic ageing models including the age distribution of the calibration dataset, the number of samples in the calibration dataset, the number of screened and identified CpG sites, and other biological and/or external factors affecting the variation in measured methylation levels (García-Vernet *et al.*, 2021; e.g., Mayne, Berry and Jarman, 2021; Robeck *et al.*, 2021). Here, I applied a previously developed epigenetic ageing model for humpback whales by Polanowski et al. (2014) and adapted it to the New Caledonian humpback whale breeding population with a sample size of 68 individuals, close to the recommended 70 minimum (Mayne, Berry and

Jarman, 2021). The New Caledonian humpback whale epigenetic ageing model was able to predict the age of individuals with an accuracy of +/- 4.5 years (mean absolute error, MAE = 4.48). This was similar or better compared to methylation clocks of other cetacean studies on fin whales (MAE = 4.87; García-Vernet *et al.*, 2021), Indo-Pacific bottlenose dolphins (MAE = 6.01; Scheu, 2021), and belugas (MAE = 3.65; Bors *et al.*, 2021) but worse than what was found in Robeck et al. (2021; Pacific white-sided dolphin: MAE = 1.7; killer whale: MAE = 3.2) using skin samples. The standard deviation of the mean difference between known and predicted ages (sd = 5.97) was larger compared to some studies (sd = 2.99, Polanowski *et al.*, 2014; sd = 2.94, García-Vernet *et al.*, 2021) but smaller compared to others (sd = 8.87, Goto, Kitakado and Pastene, 2020). Further, similar to other epigenetic age studies (Polanowski *et al.*, 2014; Goto, Kitakado and Pastene, 2020; García-Vernet *et al.*, 2021), the Y-intercept and slope of the regression indicated that the New Caledonian ageing model overestimated the age of young individuals and underestimated the age of older whales. Overall, this suggests that the accuracy and precision of the New Caledonian humpback whale epigenetic ageing model was comparable to that of others from wild cetaceans, and are subject to similar limitations. Further, the estimated accuracy of my ageing model (MAE = 4.45 years) covers less than 5% of the estimated maximum lifespan of humpback whales (ca. 90 years; ear plug laminations: Chittleborough, 1959; see also: Gabriele et al., 2010; molecular biomarker: Mayne et al., 2019; Mayne and Jarman, unpublished; see also: Carroll et al., 2023). Thus, in relation to the longevity of humpback whales, I was able to estimate the age of New Caledonian humpback whales with good accuracy.

3.5.5.1 Age distribution of the calibration dataset

A skewed age distribution in the calibration dataset reduces the performance of epigenetic ageing models (Mayne, Berry and Jarman, 2021). There was a lack of older individuals of known age in both calibration data subsets (NC + Pol). The oldest male of known age in the combined calibration dataset was ~30 years old; roughly a third of a humpback whale's estimated max. lifespan (ca. 90 years; Chittleborough, 1959; Gabriele et al., 2010; Mayne et al., 2019; Mayne and Jarman, unpublished; Carroll et al., 2023). Further, the combined calibration dataset was highly skewed towards younger individuals with an overall mean known age of only 8.6 years. A more uniform age distribution and a wider age range in the calibration data likely would improve the accuracy of my New Caledonian ageing model.

However, the sampling of old individuals in wild populations, especially in previously exploited populations of long-lived species such as the humpback whale, is extremely challenging. Here, less than 1.5% of the aged male population was estimated to be older than 40 years old (section 3.4.3).

3.5.5.2 Number of identified CpG sites

The accuracy of the ageing model is influenced by the total number of methylation sites identified (e.g., Zhang *et al.*, 2019). To develop an epigenetic DNA methylation clock for Indo-Pacific bottlenose dolphins (*Tursiops aduncus*), Peters et al. (2022) measured methylation levels at over 37,000 CpG sites which allowed them to identify a total of 43 CpG sites with an age-methylation relationship to calibrate their model. Their ageing model was highly accurate showing a median absolute age error (MAE) of only 2.1 years (Peters *et al.*, 2022). Further, by screening a similarly large number of CpG sites, Barratclough et al. (2021) more than tenfold increased the number of identified CpG sites associated with chronological age which greatly improved the accuracy of age estimates for bottlenose dolphins (*Tursiops truncatus*) compared to a previous study (Beal *et al.*, 2019).

Although screening a large number of CpG sites (> 30,000) undoubtedly improves the accuracy of epigenetic age estimates, it also renders the development of epigenetic ageing models computationally, statistically and financially more challenging. More recent epigenetic ageing studies, often rely on elastic net regression models that are suitable for scenarios in which predictor variables exceed the number of observations and where some predictor variables are expected to be correlated (Barratclough *et al.*, 2021; e.g., Bors *et al.*, 2021; Robeck *et al.*, 2021; Peters *et al.*, 2022; Lu *et al.*, 2023).

Here, a total of eight CpG sites were screened for a potential association with age in New Caledonian humpback whales, however, only two CpG sites with a significant age-methylation relationship were retained in the final ageing model (section 3.4.1). Thus, I expect that screening a larger number of CpG sites would also improve the accuracy of age estimates in New Caledonian humpback whales.

3.5.5.3 Biological and environmental factors affecting DNA methylation levels

There are a variety of factors affecting methylation levels at CpG sites that can result in variation across individuals, populations and species. The variation in DNA methylation levels across very young individuals (i.e., calves) in this dataset was extremely large (Figure 3.3). Individual variation in methylation levels was larger in the youngest as well as the oldest individuals in the calibration dataset (Figure 3.3). This likely further added to the elevated standard deviation of the mean difference between estimated and known age, and ultimately, reduced the accuracy in age estimates of especially the younger individuals. This may further explain the occurrence of negative age estimates (Figure 3.4B) yielded by our model and observed in other studies (e.g., Polanowski *et al.*, 2014; Scheu, 2021). Despite the large variation in their methylation levels, we retained the calves in the calibration data as their mean methylation level anchored the intercept of the multiple regression analysis and yielded a model with a better fit (Figure S3.9). Overall, this suggests that individual variation in DNA methylation levels may not be constant across age groups and that, for certain age groups, individual variation in DNA methylation levels can be as large, or larger, than the variation across age groups. Biological and environmental factors, such as sun exposure (Grönniger *et al.*, 2010), dietary changes (Jacobsen *et al.*, 2012), early life stress (Naumova *et al.*, 2012), and chemical pollutants can affect methylation patterns over time (Fraga *et al.*, 2005; Feil and Fraga, 2012) which can lead to differences between species, or even populations. Although epigenetic DNA methylation clocks between closely related species can be very similar, species-specific clocks are generally more accurate than multi-species clocks (Peters *et al.*, 2022). Further, methylation patterns can differ even between populations of the same species, as was found for two populations of North Atlantic fin whales (*Balaenoptera physalus*) which were suggested to be the result of genetic differences and/or dissimilar environments affecting both populations (García-Vernet *et al.*, 2021). However, implementing data from related populations and species to widen the age range or increase the number of individuals of known age offers a way to generate broad epigenetic clocks for species that are more challenging to sample (Peters *et al.*, 2022; Lu *et al.*, 2023; Parsons *et al.*, 2023).

The New Caledonian humpback epigenetic ageing model showed a coefficient of determination (R^2) of only 58% (section 3.4.1) indicating that a considerable part of the variation in methylation levels across individuals could not be explained by differences in their chronological age. This means DNA methylation levels at selected CpG sites are influenced by

other unknown factors. The New Caledonian humpback epigenetic ageing model was calibrated using a combined dataset of 23 individuals sampled in New Caledonia and 45 individuals sampled in the Gulf of Maine and Australia (Polanowski *et al.*, 2014). Despite using the same set of CpG sites, adding the New Caledonian samples resulted in a different combination of CpG sites being the most informative (TET_C + GRIA2) compared to those from the original calibration in Polanowski et al. (2014; CDKN2A_A + TET_C + GRIA2). Further, although several CpG sites showed a significant methylation-age correlation using the combined calibration dataset, these correlations were less consistent across and explained less of the variation in methylation levels (smaller R^2 values) than those in Polanowski et al. (2014). At two CpG sites (CDK2NA_A and CDK2NA_C), methylation patterns differed between the two calibration subsets (NC + Pol; Table 3.3) and were thus not retained for the final selection of CpG sites in the age model (section 3.4.1). There are three possible non-exclusive explanations for the reduced accuracy and reduced age-related correlation at CpG sites in the New Caledonian humpback epigenetic ageing model compared to the HEAA of Polanowski et al. (2014). Firstly, there are differences in the sample size of the two calibration data subsets (NC: N = 23 males, Pol: N = 45 males). The larger calibration subset (Pol) might thus have had more weight in the calibration of the model using the combined calibration dataset (Pol + NC). Secondly, the samples of the Pol calibration subset showed a wider age range and a larger proportion of older individuals than the NC subset which included a larger number of very young individuals (Pol: mean =11.5 yrs, max = 30.3 yrs; NC: mean = 3.5 yrs, max = 23 yrs). Lastly, methylation patterns between the sampled populations of humpback whales might be slightly different due to genetic differences and/or dissimilar environments. In conclusion, the New Caledonian humpback epigenetic age assay (NC-HEAA) was unbiased yet not as precise as that produced by Polanowski et al. (2014). A larger population-specific calibration dataset with a more uniform age distribution (i.e., a larger proportion of older males) and a larger set of CpG sites (as suggested by García-Vernet et al., 2021; Mayne, Berry and Jarman, 2021; Robeck et al., 2021) would likely increase the accuracy of the NC-HEAA. Epigenetic ageing models of studies that identified a large number of age-dependent CpG sites were able to explain a much larger proportion of the variation in methylation levels (e.g., R^2 = 94%, Bors *et al.*, 2021; R^2 = 86%, Peters *et al.*, 2022). This suggests that even though CpG sites with a methylation-age relationship are affected by other biological or extrinsic factors, increasing

the number of age-dependent CpG sites can reduce the age-unrelated noise in methylation levels, and thus, may improve the accuracy of age estimates.

While the cost of lab analyses may often constrain the number of screened CpG sites in epigenetic ageing studies, the size and age distribution of calibration datasets are often limited by data collection and the life history of the species. Sampling a large number of individuals of known age, and especially of older individuals, needed to calibrate the ageing clock, is extremely challenging in wild populations and especially in species with a long lifespan. This renders large calibration datasets with a uniform age distribution extremely rare, and the use of multi-species or species-wide epigenetic ageing clocks, thus, becomes tempting. However, the possible differences in age-methylation relationships at CpG sites across species and/or populations require further investigation and should not be ignored. Applying epigenetic clocks across species or populations, as well as the pooling of calibration datasets, as done in this study, should always be thoroughly assessed and interpreted with caution. Despite its underlying limitations, epigenetic ageing is a tool of great potential in aiding the study of populations in the wild. Recent studies provide valuable insights and guidance on the use of epigenetic age clocks to improve the accuracy of age estimates, thus, promising to increase our understanding of the life history trends and population dynamics of wild populations.

3.6 Conclusion and future directions

Here, I have demonstrated how age-related changes in the population can impact sexual selection. The male New Caledonian humpback whale population was consistent with a recovering population over the first period but became more balanced in the second half, consistent with a stabilisation of the age structure. Older males were more often observed to engage in certain mating behaviours (escorting and singing) and were more successful in siring offspring in the second half of the study period. This suggests that reproductive tactics and reproductive success in male humpback whales may be age-dependent and that commercial whaling changed not only the population dynamics but also patterns of sexual selection. Such differences in reproductive success across the different time windows highlight the importance of accounting for the changes in the underlying population age structure across time, especially in previously exploited populations. Long-term monitoring is thus crucial to

assess population dynamics and their consequences on the recovery of exploited populations in the wild.

Chapter 4

Seasonal and age-related changes in male humpback whale testosterone on a breeding ground

4.1 Abstract

Hormones help to regulate and coordinate the physiology and behaviour of an animal. Many marine mammals are seasonal breeders with breeding activity regulated by the seasonal production of hormones. However, the elusive nature, high mobility, and underwater habitat make endocrine studies on marine species challenging. Here, I investigated seasonal and age-related changes in the levels of testosterone in male humpback whales on their breeding ground in New Caledonia, South Pacific, to gain insight into their reproductive physiology. Testosterone was measured in 457 blubber samples from 209 males collected over a 25-year-long study period, using previously validated steroid hormone extraction and testosterone enzyme immunoassay (EIA) methods. Results showed that blubber testosterone levels slowly decrease over the breeding season in the male population. However, the seasonal trend in blubber testosterone observed in this dataset could be driven by differences in the migratory timing of individuals with differing hormone levels, rather than a decrease in blubber testosterone in individual males on the breeding ground. Further, blubber testosterone levels of male humpback whales appear to be highest during puberty, then decrease and level off at the onset of maturity, with some evidence that it increases again in males maturing into their late 20s and early 30s. Furthermore, blubber testosterone in males was highly variable at any point during the breeding season and across males of all ages. This chapter demonstrated that the integration of endocrine and molecular age markers in long-term datasets is a powerful tool for understanding a species' life-history trends, ontogenetic changes, and mating systems.

4.2 Introduction

The physiology and behaviour of an animal is strongly affected by the endogenous expression of hormones. Studying how these affects occur and their outcomes can provide important insight into the mechanisms and evolution of behaviour. Hormones do not cause behavioural changes directly, but affect the likelihood of a specific behaviour occurring in the presence of the appropriate stimuli in the appropriate context (Nelson and Kriegsfeld, 2017; Gruchalla Russart and Nelson, 2019). While hormones affect behaviour, behaviour and external stimuli can also feed back and affect hormone concentrations. For example, while elevated levels of testosterone can lead to more aggressive behaviour (Wingfield *et al.*, 1990; Klukowski and Nelson, 1998; Gould and Ziegler, 2007), losing an aggressive encounter can, in turn, decrease circulating testosterone (Archer, 1991; Huhman *et al.*, 1991). This bidirectionality considerably complicates the study of hormone-behaviour interactions, especially in wild populations.

The extent to which behaviour is mediated by hormones differs among species. While sexual behaviour in rodents is highly dependent on hormones, in primates, sexual behaviour is less dependent on hormones and more on social interactions and learning (Nelson and Kriegsfeld, 2017; Bakker, 2019). Both environmental factors and social cues can influence hormone-behaviour interactions (e.g., time of day, perceived food availability, social interactions, population density, presence of females; Wilsterman *et al.*, 2019). This allows an individual to regulate its endocrine system and, consequently, its behavioural output, both according to, and also adjusted to, its immediate social and ecological environment.

Androgens (i.e., male sex hormones), such as testosterone, play an important role in the stimulation of spermatogenesis, the development of primary and secondary sex characters and the mediation of reproductive behaviours (Slater, 1978; Nelson and Kriegsfeld, 2017). Androgens are known to mediate courtship displays (e.g., birdsong in male canaries, Serinus canaria; Alward, Balthazart and Ball, 2017), aggressive behaviour (e.g., male red deer, Cervus elaphus; Nelson and Kriegsfeld, 2017), and migration (e.g., female Japanese eel, Anguilla japonica; Sudo and Tsukamoto, 2015) in many vertebrate species. The seasonal changes in androgen levels, therefore, correlate with the reproductive pattern and behaviour of many seasonal breeders.

Many marine mammals are also seasonal breeders, but their elusive nature, high mobility, and underwater habitat make endocrine studies on them challenging. In wild cetaceans,

hormones are commonly measured from blubber biopsy samples (e.g., Kellar et al., 2006a; Kershaw et al., 2017; Mello et al., 2017; Pallin, Robbins, et al., 2018; Atkinson et al., 2020, 2023; Mingramm et al., 2020), to a lesser extent, from non-invasive respiratory vapour ("blow") samples collected from living animals (e.g., Mingramm et al. 2019), or from baleen plates for the retrospective and longitudinal assessment of hormones in dead animals (Hunt *et al.*, 2014, 2016; Lowe *et al.*, 2022). Although hormone levels in these tissue types are less dynamic than hormone levels in blood serum, they are often used as an approximation of circulating blood hormone levels. However, detailed information on the perfusion rate of hormones from blood to other tissues is often not available (e.g., blood to blubber: hours to weeks) and can further differ between species (Kellar *et al.*, 2013; Champagne *et al.*, 2017, 2018).

Blubber testosterone shows annual cyclicity in several large baleen whales where it has been studied, including male blue whales, *Balaenoptera musculus* (Melica, Atkinson, Gendron, *et al.*, 2021), fin whales, *Balaenoptera physalus* (Carone *et al.*, 2019), grey whales, *Eschrichtius robustus* (Melica, Atkinson, Calambokidis, *et al.*, 2021), and humpback whales, *Megaptera novaeangliae* (Vu *et al.*, 2015; Cates *et al.*, 2019). In all these cases, males experience higher levels of testosterone during the breeding compared to the feeding season, as is expected for seasonal breeders. Although blubber testosterone in male humpback whales peaks during the breeding season, testosterone levels already start to increase during autumn prior to the onset of the breeding season, then decrease towards the end of the breeding season and migration back to the feeding grounds (Cates *et al.*, 2019). Further, males show higher levels of testosterone on their migration towards the breeding grounds compared to levels on their migration back to the feeding grounds (Mingramm et al., 2020). While increased testosterone levels before the onset of the breeding season likely indicate physiological preparation for reproduction through spermatogenesis, as the migration to breeding grounds approaches (Vu *et al.*, 2015), testosterone may further play a role in mediating the migration to and from the breeding ground (Cates *et al.*, 2019), and the reproductive behaviours of male humpback whales during the whole period.

Despite these general patterns, it is also a robust finding that there is considerable individual variation in male testosterone levels (Cates *et al.*, 2019), the timing of migration (Dawbin, 1956; Baker and Herman, 1981; Corkeron *et al.*, 1994), and the observed

reproductive behaviours on humpback whale breeding grounds among males ('alternative mating tactics': Cerchio, 2003). Other environmental and biological factors, such as prey availability on the feeding ground, photoperiod, age, reproductive state, and body condition are likely to influence male testosterone levels, which in turn may promote the initiation of migration from the feeding grounds to the breeding grounds (Baker *et al.*, 1985; Craig *et al.*, 2003; Cates *et al.*, 2019) and a male's reproductive behaviour on passage and arrival. Humpback whale song production by males, thought to be a courtship display at least partially analogous to birdsong (Garland and McGregor, 2020), correlates with the annual cycles of male testosterone levels (Cates et al., 2019). Specifically, both song and testosterone are mainly produced on breeding grounds and solely by males. Song has also regularly been recorded on migratory routes (Winn and Winn, 1978; Clapham and Mattila, 1990; Smith *et al.*, 2008), and to a lesser extent, on feeding grounds (Clark and Clapham, 2004; Stimpert et al., 2012; Vu et al., 2012; Garland, Gedamke, et al., 2013). Along with seasonality, age is another factor influencing the testosterone level of males. A previous study of blubber testosterone samples from 24 individuals in Hawaii indicated that male humpback whales may experience peak testosterone concentrations from 8 to 25 years on the breeding grounds (Cates et al., 2019). However, the degree to which variation in blubber testosterone and reproductive behaviours among males that is mediated by other factors such as seasonality, age and behaviour (their own and/or that of others) remains unclear.

Here, I investigated seasonal and age-related changes in the reproductive physiology of male humpback whales on their breeding ground in New Caledonia, South Pacific. Using steroid hormone extraction and testosterone enzyme immunoassay (EIA) methods previously validated for humpback whale blubber samples (e.g., Cates et al., 2019; Mingramm et al., 2020), testosterone was measured in 457 blubber samples from 209 males collected over a 25 year-long study period. First, I assessed the seasonal trend and variation in male testosterone levels within the breeding season at two levels: (a) the mean population level across the breeding season using all samples and (b) the individual level using multiple samples of the same individual within the same breeding season. Second, I explored how testosterone levels vary between males of different age categories, based on epigenetic age estimates derived in Chapter 3. This included multiple samples from the same individual across different years. Based on previous studies, I expected to find a high level of individual level variation, but also consistent patterns of temporal variation over the breeding season

and between individuals of different age classes. I took advantage of a uniquely powerful long-term dataset to conduct an exploratory analysis to understand if testosterone patterns matched findings in other seasonally-breeding taxa and generate hypotheses for understanding humpback mating behaviour and its relationship to song.

4.3 Methods

4.3.1 Study site and sample collection

Samples for the analysis of blubber testosterone were collected at the breeding ground in New Caledonia from 1996 to 2020 during the austral winter (July to September). On annual surveys, whales were carefully approached to be photographed and biopsied using a crossbow with a specially adapted bolt (Lambertsen *et al.*, 1994) or a modified veterinary rifle (Krützen, 2002). Blubber samples were separated from the skin using a sterile razor blade, wrapped in sterilised aluminium foil, and temporarily stored in a standard freezer at the end of the fieldwork day before being transferred to -20˚C for long-term storage at the end of the field season. Here, I selected 457 samples from 209 male humpback whales to address the main research questions of investigating changes in male testosterone levels 1) within the breeding season and 2) with age. Further details on the data collection and age estimation are described in Chapters 2 and 3, respectively.

4.3.2 A note on collaborations and contributions

Testosterone was measured from blubber samples that were stored at the French National Research Institute for Sustainable Development (IRD) in Nouméa, New Caledonia. Samples were shipped to the University of Queensland Moreton Bay Research Station located on North Stradbroke Island, Queensland, Australia, for lab analyses. Due to travel restrictions during Covid-19, I was unable to travel to New Caledonia and Australia to prepare the samples for their shipment and to conduct the lab analyses myself. Thus, research assistants were hired on-site. Hugo Bourgogne prepared the blubber samples for shipment (e.g., transferring blubber samples from the aluminium foil into 5 mL polystyrene tubes) under the supervision of Dr Claire Garrigue (IRD, Noumea, New Caledonia). Jose Daniel Gonzalez Jaramillo conducted

the testosterone lab analyses (steroid hormone extraction and testosterone EIA) under the supervision of Assoc. Prof. Rebecca Dunlop (Cetacean Ecology and Acoustics Laboratory (CEAL), University of Queensland) at Moreton Bay Research Station on North Stradbroke Island, Queensland (Australia). All further calculations and analyses were conducted by myself.

4.3.3 Steroid hormone extraction

Blubber steroid hormones were isolated using an organic solvent extraction following standard protocols (Kellar *et al.*, 2006b; Trego, Kellar and Danil, 2013) with slight modifications (see detailed extraction protocol in supplementary S4.1.1). In brief, 0.1 ± 0.05 g (wet weight) of blubber was dissected into small pieces (~ $2mm^3$), placed into 1 ml ethanol and homogenized using 1 mm silica carbide beads (Daintree Scientific, Australia) for six 45-second cycles (Mini-Beadbeater-16, BioSpec). Samples were centrifuged (3000 rpm for 10 min at 4°C) before the homogenate was transferred into a 5 ml polypropylene tube (LBS504N, ThermoFisher, Australia). In a second wash step, another 1 ml of ethanol was added to the empty homogenization tube, vortexed for 5 min and centrifuged for 30 seconds to remove any remaining blubber residue. Both fractions were combined and then dried under nitrogen gas. To the dried extract, 2 ml of acetonitrile were added, and after vortexing (10 min) and centrifuging (3500 rpm for 10 min at 4°C), the supernatant was transferred to a new 6 ml glass tube. Next, 4 ml of hexane were added, vortexed for 5 min, and centrifuged (3500 rpm for 5 min at 4°C) to separate acetonitrile and hexane layers. In a final step, the hexane layer was discarded, and the acetonitrile layer was transferred to a new 2 ml Eppendorf tube and evaporated under airflow. The final dried residue was stored at -20°C. Extractions were carried out in batches of 12 samples (i.e., 11 samples + 1 extraction control). Extraction controls were prepared for every 11 samples analysed, and were prepared the same way as samples but with no blubber being added. The 457 blubber samples were analysed in a total of 42 extraction rounds. Extracts were stored frozen at -20˚C until assayed.

4.3.4 Testosterone EIA

The concentration of testosterone (T) in blubber steroid hormone extracts was measured using an enzyme immunoassay (EIA) with a double antibody system previously used and validated for several cetacean species (e.g., Hunt et al., 2017), including humpback whales (Vu *et al.*, 2015; Cates *et al.*, 2019) by the CEAL lab group (Mingramm et al., 2019; Mingramm et al., 2020). In this system, the primary antibody is the hormone-specific anti-body (here: rabbit anti-testosterone) binding to the antigen, while the second antibody (here: goat anti-rabbit gamma globulin, GARG) targets the primary antibody (Brown, 2008). Immunoassays are based upon the competition between unlabelled antigen (i.e., testosterone in the sample) and labelled antigen ('tracer', here: HRP) in binding to an antibody (see EIA protocol in supplementary S4.1.1). The amount of unlabelled antigen in the sample is thus inversely proportional to the signal (i.e., intensity of colour) generated by the labelled antigen (Brown, 2008). The lower the signal, the more testosterone there is in the sample.

Samples were analysed across 14 testosterone EIA plates. Each microtitre plate was pre-coated by hand with GARG solution (Arbor Assays A0009-25MG). Prior to analysis, hormone extracts (samples) and extraction controls were re-suspended in 0.1 ml ethanol and 0.4 ml assay buffer (Arbor Assay #X065), vortexed for 2 min and incubated at room temperature for 5 min (vortex and incubation were repeated twice more) to solubilise the hormones (see coating protocol in supplementary S4.1.1).

Optical density (OD) on all EIAs was evaluated using a Biotek Reader Elx808 (*Gen5*™ software; Biotek, Winoowski, VT, USA; with read and reference wavelengths of 405 and 540 nm). All reads (i.e., OD scores) underwent a background correction by subtracting the mean non-specific binding (NSB; i.e., amount of binding due to component others that the antibody) run in duplicate on each plate. The percentage binding (%B/B0) was then calculated by dividing these corrected plate readings by the average total binding (B0; i.e., binding in the absence of competition for binding sites) on each assay before multiplying by 100. Testosterone concentrations were then derived from the percentage binding using the standard curves.

A Four Parameter Logistic (4PL) regression model was used to obtain the standard curves. A 4PL model allows for a non-linear curve fitting, and therefore, provides a better fit than a linear regression model as immunoassays of biological systems seldom follow a linear

response. The 4PL models were fitted to the known concentration (dose) and percentage binding (response) of seven testosterone standard concentrations (standards) ranging from 16.38 pg/ml to 4,000 pg/ml run on each assay, using the function 'dr4pl' (Package: dr4pl; Landis *et al.*, 2021) in R (v4.2.2, R Core Team, 2022). From these assay-specific standard curves, fitted parameter estimates were obtained (Equation 1, function 'dr4pl') and used to calculate the testosterone concentration (Equation 2).

Equation 1
$$y = d + \frac{a - d}{[1 + \left(\frac{x}{c}\right)^{b}]}$$

Equation 2
$$x = c \times \left(\frac{a - d}{y - d} - 1\right)^{\frac{1}{b}}$$

a = lower limit (minimum)
b = slope factor
c = inflection point at mid-range concentration
d = upper limit (maximum)
y = dependent variable (here: binding)
x = independent variable (here: concentration)

The accuracy of measured standard concentrations was assessed by the percentage of testosterone recovered by the EIA (% recovery = (concentration observed / concentration expected) x 100). Raw concentrations (pg/ml) of samples were corrected for dilution factor (0.5 ml) and blubber mass (wet weight, g), and expressed as ng/g.

All samples were run blind and in duplicate. Intra-assay variation was assessed by calculating the coefficient of variation (CV = standard deviation/mean) between duplicates on each EIA. Only samples with an intra-assay CV < 15% were accepted. Inter-assay variation is commonly monitored using 2 – 3 internal control samples (assay controls), assayed in duplicated and treated as unknown but run in every assay. Assay controls ideally provide an estimate of variability over the range of the standard curve. Here, EIAs were not run using the same set of assay controls across all assays. Instead, each EIA set up with 1, 2 or 3 assay controls (low dose: ~100 pg/ml, medium dose: ~500 pg/ml; high dose: ~700 pg/ml) run in duplicate. This is less ideal as it doesn't allow for a full comparison across all 14 assays over the range of the standard curve.

Apart from the inconsistent arrangement of assay controls, assays (P5 – P14) were run following general EIA guidelines with the exception of four assay plates (P1 - P4). While assay plate P1 was run with eight standards instead of the seven used on all other assay plates (one additional at 10,000 pg/ml), and is therefore unproblematic, assay plates P2, P3, and P4 were

each run with only two standards. Standard curves are commonly calculated separately for each assay. However, without a full set of seven standards, I was not able to establish assay-specific standard curves for assays P2 to P4. Instead, the standard curve obtained from the standards on assay P1 (using only the same seven standard concentrations also on assays P5 – P14 to be as consistent as possible) was used to calculate the testosterone concentrations of samples on all four assays (P1 – P4) as all four assays were prepared together in the lab. The two standards on assay P2 – P4 were then treated as assay controls to assess the inter-assay variation as no additional internal assay controls were run on these plates. Further, there were no binding parameters (NSB and B0) on assay plates P2 – P4. Thus, binding parameter wells on assay P1 were used for the NSB-correction and the calculation of percentage binding on plates P2 – P4. Essentially, assay plates P1-P4 (all run on the same day) were analysed as a group (P1-4). All other assay plates (P5-P14) were analysed separately. Plate settings of all 14 assays are provided in Figure S4.2.

Biological validations of testosterone EIAs for humpback whales have yet to be completed. Assay validation tests are commonly carried out for each species, tissue type, hormone extraction method and type of immuno-assay or hormone, and ideally for each study. There are three important assay validation tests: 1) parallelism to test if the assay is actually measuring what it should be measuring, 2) recovery-accuracy check to assess the degree to which the measured concentration corresponds to the true concentration of a hormone (here: testosterone) as substances contained within the biological sample (here: blubber) may interfere, and 3) spike-recovery test to assess and monitor the efficiency of the hormone extraction method for a specific matrix (here: blubber). All three assay validation tests require serial dilutions and/or spiked samples of a pool of samples that need to be run in addition to all other samples, standards, and assay controls. As this was not done for this specific study, I was not able to carry out any assay validation tests. However, assay validations have been carried out on humpback whale blubber and testosterone EIA as part of another study conducted in the same laboratory, and these indicated good accuracy of blubber testosterone measurements (Mingramm et al., 2019). Although the analysis was similar, slight modifications in the hormone extraction protocol and expected inter-individual differences in lab techniques (e.g., accuracy in pipetting) could result in differences in the accuracy of testosterone measurements. Whilst the inconsistent distribution of assay controls across plates, the absence of assay-specific standard curves on three assays, and the lack of study-

specific assay validation tests are not ideal, they were beyond my control (see section 4.3.2). Assay validation tests can still be carried out in future, however, not within the time frame of this thesis.

4.3.5 Statistical analyses

The role of male testosterone on the humpback whale breeding ground was investigated by assessing the changes in male testosterone (1) over the breeding season and (2) with age. Changes in male testosterone across the breeding season were analysed by tracking the mean level across all available data in a given period (1a) and at the individual level using multiple samples of the same individual over a season (1b).

4.3.5.1 Breeding season: male population (1a)

I fitted a linear mixed effects model (LMM) to all male samples that passed quality control (Table 4.1; n = 331 samples, N = 178 males) with testosterone concentration (ng/g) as the response variable and time within the breeding season (in Julian calendar days) as the explanatory variable. Accounting for a possible effect of the amount of blubber used during the hormone extraction on measured testosterone levels, blubber weight (in grams) was added as an additional explanatory variable. The year of sample collection was added as a random intercept effect to allow for baseline testosterone levels to vary between years, and to partition out the potential effect of sample storage time, or other year-specific factors, on measured testosterone levels. I expected a significant effect of calendar day if testosterone concentration monotonically increased or decreased as the breeding season progressed, but note that the LMM framework could no accommodate non-linear trends (such as a mid-season peak and subsequent decline).

4.3.5.2 Breeding season: individual males (1b)

Changes in testosterone levels of individually identified males that were sampled multiple times in a season were assessed in a second LMM, using only data from males that were sampled multiple times within the same breeding season (Table 4.1; n = 87 samples, N = 41

males, each 2 – 3 times sampled in a season between 1996 and 2020). To account for a possible effect of sample storage time on measured testosterone levels or differences between years, only one breeding season per male was selected for this analysis (the one in which it was most frequently sampled or, if even, at random). Testosterone concentration (ng/g) was used as a response variable, calendar day and blubber weight (in grams) were used as explanatory variables, and individual was used as a random effect so that trends over time could be separated from individual variation.

4.3.5.3 Age (2)

Lastly, changes in male testosterone with age were explored by assessing how blubber testosterone varies between males of different age classes. Epigenetic age estimates derived in Chapter 3 were available for all but five males that passed the quality control. All males estimated to be at least two years old were included in this analysis, including 45 that were sampled multiple times across years (Table 4.1; 316 samples, N = 169 males). A third LMM was fitted using testosterone concentration (ng/g) as a response variable, and the four age categories ($2 \leq x < 9$ years, $9 \leq x < 16$ years, $16 \leq x < 23$ years, and $x > 23$ years; Chapter 3) as an ordered categorical explanatory variable, together with calendar day, blubber weight (in grams) and storage time (in years) as continuous explanatory variables, and individual as a random effect.

All statistical analyses were conducted using the software R (v4.2.2, R Core Team, 2022). The three linear mixed regression models were fitted with a Gaussian error distribution using the function 'lmer' (package: lme4, Bates *et al.*, 2015). A logarithmic transformation of the testosterone data was additionally applied for all three models (in supplementary S4.3) to check for any artefacts deriving from the weak positive, right-skewed response data (Figure S4.11) but these models returned the same conclusions. *P* values < 0.05 were considered statistically significant. Performance measures for all mixed effects models were inspected as marginal R-squared, conditional R-squared and interclass correlation coefficient (ICC). The marginal and conditional R-squared indicate how much of the variance is explained by the fixed effects only and by the complete model (fixed + random + residuals), respectively. The interclass correlation coefficient gives a sense of how much variance is explained by the random effect. Performance measures for mixed-effects models were estimated by division

of the corresponding variance components of mixed models: marginal R-squared as fixed effects variance divided by the total variance; conditional R-squared as the fixed and random effects variance divided by the total variance; ICC: random effects variance divided by the random and residual variance. The different variance components of mixed models were calculated using the function 'get_variance' (package: insight, Lüdecke, Waggoner and Makowski, 2019) in R based on Nakagawa et al. (2017) and Johnson (2014).

Table 4.1. Humpback whale testosterone data included in each analysis. Not all samples analysed in the lab (steroid hormone extraction and testosterone EIA) passed the quality control (Figure 4.2). Epigenetic age estimates derived in Chapter 3 were available for all but five males that passed the quality control. A different set of samples and individuals was used for each of the different study questions.

Data	**Samples**	**Individuals**	**Study period**
All samples	457	209	1996 - 2020
QC data: samples passing quality control (QC)	331	178	1996 - 2020
Aged males in QC data	326	173	1996 - 2020
1. Changes in male testosterone within the breeding season			
a) Male population (all QC data)	331	178	1996 - 2020
b) Adult males sampled multiple times within a season	87	41	1999 - 2020
2. Changes in male testosterone with age			
a) Aged male individuals (>= 2 years)	316	169	1996 - 2020

4.4 Results

4.4.1 Testosterone EIA

EIA binding parameters (NSB and B0) remained fairly consistent across all assay plates except for assay plates P1 and P13. Average OD scores for NSB wells on assay plates P1 and P13 were extremely high, resulting in very low readings after the NSB-correction, and thus, also percentage binding (Figure S4.3A). However, standards, assay controls and samples on plates P1 and P13 were all within range of all other assay plates, and intra-assay variation was below 10% (Tables S4.2 and S4.3). Thus, for plates P1 (and thus also P2-P4) and P13, NSB was derived from the averaged NSB values across all other plates (Figure S4.3B). NSB-correction on all other assay plates was done using plate-specific NSB values.

Standard curves were established by fitting 4PL regression models to the percentage binding and known concentration of standards run on each assay plate (Figure S4.6), apart from plates P2 – P4 (see section 4.3.4). The derived fitted parameter estimates of the regression were used to calculate the testosterone concentration (Table S4.4). Across all plates and standards, between 84.9 to 121.2 % of the testosterone was recovered by the EIA (Figure S4.8). Lower and higher concentrated standards showed more variation in their recovery between assay plates, while medium concentrated standards had more consistent recoveries across plates.

Intra-assay variation was assessed as mean CV (%) between sample duplicates on each plate which ranged between 3.63% and 36.47% with a mean of 10.22% (Table S4.1 and Figure S4.4). Two assay plates (P11 and P12) showed a mean CV higher than 15% and showed a high proportion (> 20%) of sample pairs with a CV above 15% (Table S4.4). On assay P12, only 2 out of the 36 sample pairs (including extraction controls) that were analysed on the plate worked. One of these two sample pairs showed a CV of >60%, which lead to the observed high mean CV between sample pairs and the high proportion of sample pairs with a CV above 15% on assay P12. All samples with CV >15% (n = 50 samples) were removed from all further analyses (Figure S4.4).

Inter-assay variation was monitored using three assay controls with different doses of testosterone (low, medium, and high) across plates (Table S4.2). The mean CV across plates and controls was 7.0% (sd = 1.5%) using percentage binding and 21.3% (sd = 19.5%) using

testosterone concentration (ng/g), as estimated using equations 1 and 2. As there were no assay controls on plates P1-4, inter-assay variation on these plates was assessed between the eight standards on plate P1 and the two standards on plates P2, P3 and P4 (Table S4.3). The mean CV in percentage binding across plates P1-P4 was 8.0% (sd = 6.9%) using percentage binding and 21.6% (sd = 25.4%) using testosterone concentration (ng/g), similar to the mean inter-assay CV for assay plates P5 - P14. Monitoring inter-assay variation using standard rather than specifically selected control dosages is less ideal, however, given the plate setup of assays P1-P4, it was the only way possible to assess the variation between plates P1–P4. Inter-assay variation between all plates (P1-4 and P5-P14) was considerably higher using measured testosterone concentrations compared to percentage binding as testosterone concentrations are derived from plate-specific standard curves (apart from plates P2-4), which themselves slightly vary between plates. The inter-assay variation in both percentage binding and testosterone concentration was higher at lower testosterone concentrations (Tables S4.2 and S4.3). Only the low-dosage assay controls (~100pg/ml) on plates P5-P14 and low-dosage standards on plates P1-P4 (16.38 pg/ml, 40.96pg/ml, and 102.4pg/ml) showed an inter-assay CV above 15% (Tables S4.2 and S4.3). The testosterone concentration of these low-dosage assay controls and standards is lower than the majority of measured humpback whale blubber samples (Figure S4.7).

Extraction controls, treated as samples but without any blubber added, measured a 0.14±0.19 ng/ml (mean± sd) of testosterone across all 42 extraction rounds. All but four extraction controls (from extraction rounds 17 - 20) measured less than 0.25 ng/ml of testosterone and measured less or as much as the lowest concentrated blubber samples extracted within the same extraction round (Figure S4.1).

4.4.2 Humpback whale sample quality control

Testosterone was quantified in all 457 blubber samples of 209 male humpback whales. The measured concentrations were corrected for the blubber mass (in grams) used in the steroid hormone extraction (Section 4.3.3). Blubber-corrected concentrations ranged from 0.26 – 16.87 ng/g with a mean of 2.07 ng/g of testosterone. There was considerable variation in blubber mass used for the steroid hormone extractions (Figure S4.12A-B), which even after correcting for blubber weight, resulted in a significant negative correlation between the

measured testosterone concentration and blubber weight (coefficient of determination (R^2) = 0.18, P < 0.001; Figure 4.1A). Further, there was a weak effect between measured testosterone levels and sample storage time (R^2 < 0.01, P = 0.033; Figure 4.1B). Albeit both correlations are significant, based on the low coefficient of determination (R^2) in both regression models, only a small proportion (vanishingly small in the case of storage time) of the variation in measured testosterone levels was explained by either blubber weight or storage time alone. Nevertheless, to reduce the impact of potentially confounding variables on the analysis of the temporal patterns of male testosterone in humpback whales, I excluded samples with a blubber weight <0.075g, a CV >15%, and a binding (%) that lies outside the range of 20-80% of maximum binding (Figure 4.2). A total of 331 samples from 178 individuals passed this quality control filter (Table 4.1) and their testosterone levels ranged from 0.26 to 4.41 ng/g (mean = 1.82 ng/g).

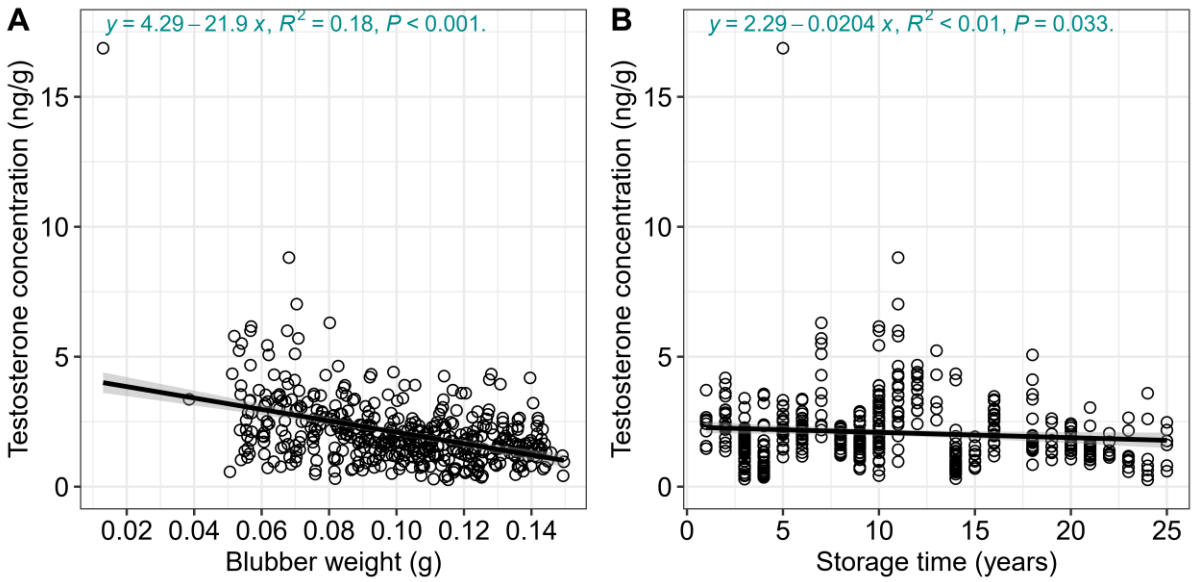


Figure 4.1. Relationship between blubber testosterone (ng/g) with A) blubber weight (g) and B) storage time (years) on all analysed humpback whale samples (n = 475 samples, N = 209 individuals). Linear regression analyses indicate a slight but significant correlation between measured testosterone levels and blubber weight, as well as testosterone and storage time. Albeit significant, the goodness of fit (R^2) for both regressions was low (blubber weight: R^2 = 0.18; storage time: R^2 < 0.01). See Figure S4.13 for the relationship between blubber testosterone with blubber weight and storage time after sample quality control.

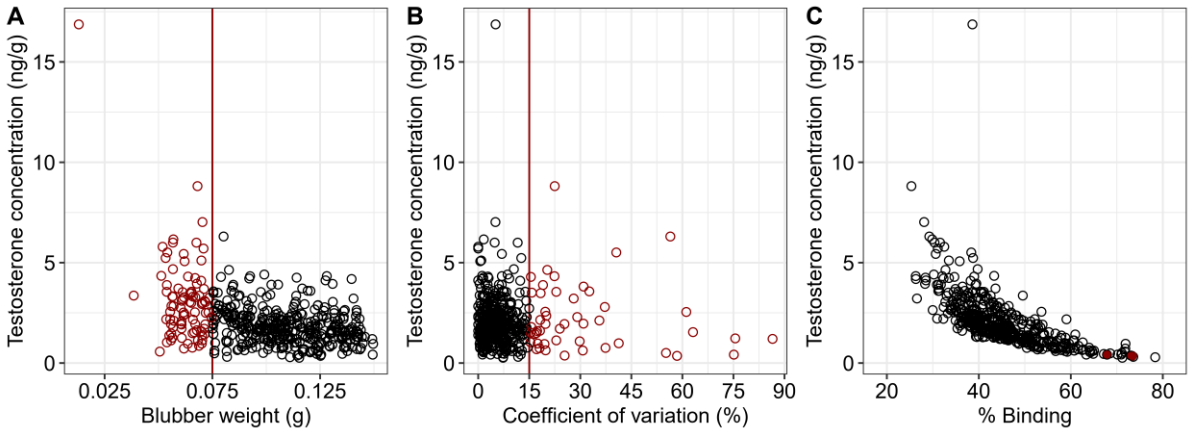


Figure 4.2. Samples and quality control based on blubber weight and coefficient of variation (CV). A) Blubber weight (g) against concentrations of testosterone (ng/g). Samples for which less than 0.075g of blubber was used for the steroid hormone extraction were excluded from all further analyses (red circles, blubber weight <= 0.075g). B) Coefficient of variation (CV) against testosterone concentration (g) where samples above CV 15% were excluded (red circles, CV > 15). Red lines: indicate the quality threshold set for blubber weight and CV. C) Mean % binding across sample duplicates. Sample (even if just one of the two samples of each duplicate pair) that lie outside the 20% - 80% range of plate-specific maximum binding were excluded from all further analyses (red circles).

4.4.3 Changes in male testosterone across the breeding season

4.4.3.1 Breeding season: male population (1a)

Blubber testosterone concentrations of 178 males (n = 331 samples) of all age classes were analysed to assess changes in testosterone in the male population on the breeding ground across the season. The first model tested whether calendar day within the breeding season, predicted male testosterone concentration, whilst accounting for variation in blubber weight. Male testosterone levels showed a significant decline over the course of the breeding season (β = -0.006, p = 0.005; Table 4.2, Figure 4.3A). As previously shown in section 4.4.2, there was also a significant negative relationship between measured levels of testosterone and blubber weight (β = -10.021, p > 0.001; Table 4.2, Figure 4.3B). Only a small proportion of the observed variance in male testosterone was explained by the two predictor variables (calendar day and blubber weight; marginal R^2 = 0.064), whereas the full model including the random effect (year) accounts for more than half of the observed variance in male testosterone (conditional R^2 = 0.533). This, along with the interclass correlation coefficient (ICC = 0.501) and the standard deviation of the random effect (year: sd = 0.586) suggest that there are important inter-annual differences in male blubber testosterone levels in our dataset (Figure S4.14). Overall, blubber testosterone concentrations of males on the breeding ground were highly variable at any point during the breeding season, yet, on average, slowly decreased (Figure 4.3A).

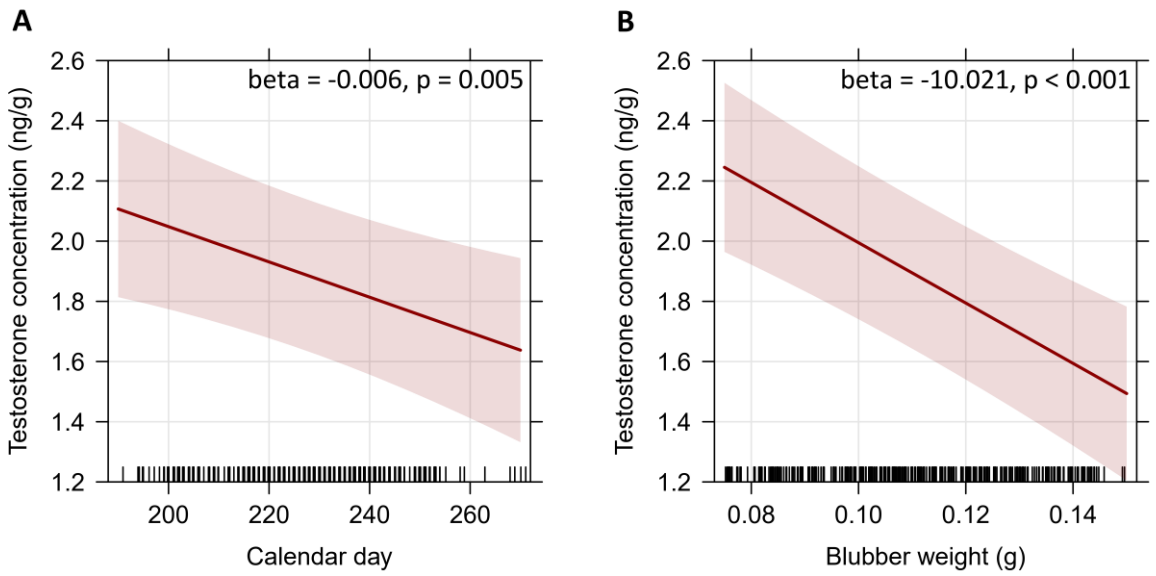


Figure 4.3. Male blubber testosterone (n = 331 samples) of 178 male humpback whales sampled on their breeding ground in New Caledonia. A) Blubber testosterone in the male population declined over the course of the breeding season. B) Blubber testosterone was also negatively correlated with blubber weight used during the steroid hormone extraction.

4.4.3.2 Breeding season: individual males (1b)

Changes in blubber testosterone of individual males across the breeding season were assessed in a second LMM on 41 males that were sampled multiple times within the same breeding season (n = 87 samples; Figure 4.4), with individual set as a random effect. For one male (HNC462), multiple samples within the season were available for two different years (2012 and 2015). I randomly selected one of these two years so that each male was only represented with samples collected from one breeding season to keep the effect of sample storage time on testosterone constant within individuals (section 4.4.2). Testosterone levels of individual males did not significantly change with the progression of the breeding season (β = -0.003, p = 0.365; Table 4.2, Figure 4.5A). Male blubber testosterone was still negatively correlated with blubber weight (β = -8.96, p = 0.012; Table 4.2, Figure 4.5B). Only a small proportion of the variance in male testosterone was explained by the two predictor variables (calendar day and blubber weight, marginal R^2 = 0.041). When including individual as a random effect (full model), the model accounts for a much higher proportion of the observed variance in male testosterone (conditional R^2 = 0.724). Further, both the interclass correlation coefficient (ICC = 0.712) and the standard deviation of the random effect (individual: sd = 0.705) were relatively large. Altogether, this suggests that there is considerable inter-individual variation in the temporal changes of male testosterone on this humpback whale breeding ground (Figure 4.4).

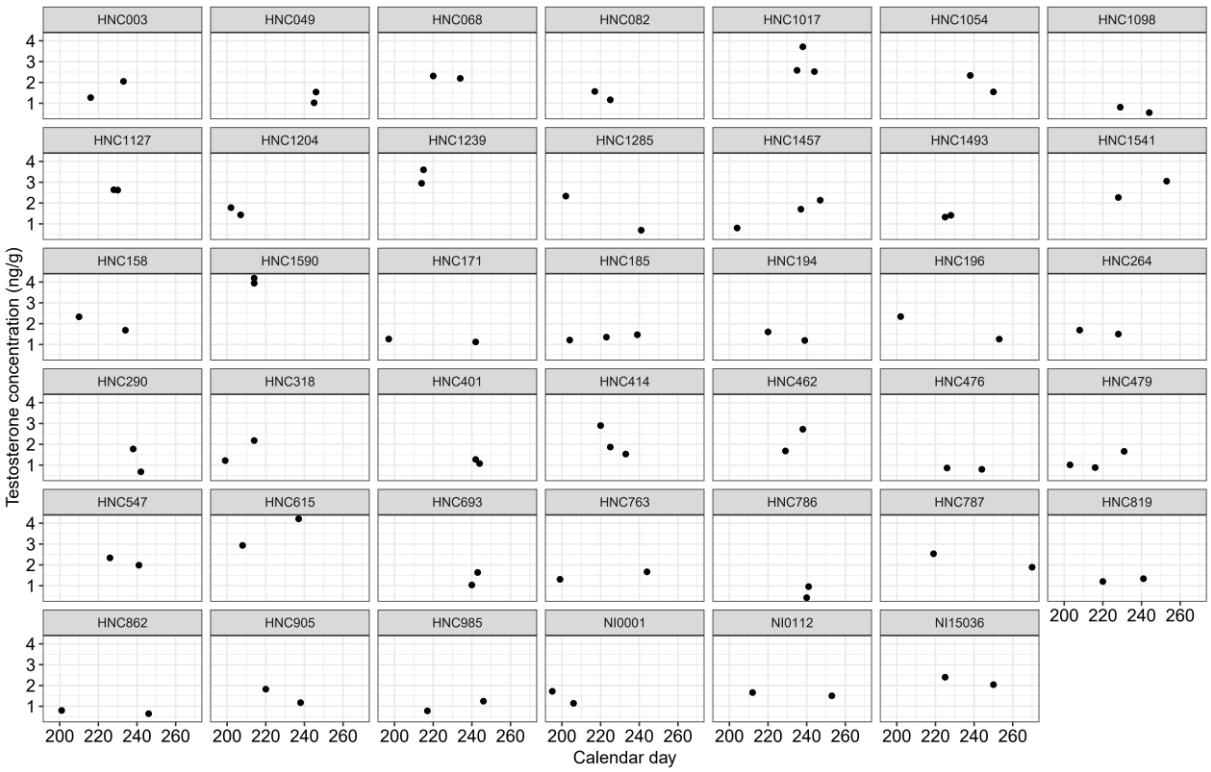


Figure 4.4. Testosterone concentration (ng/g) of males sampled multiple times within the same breeding season.

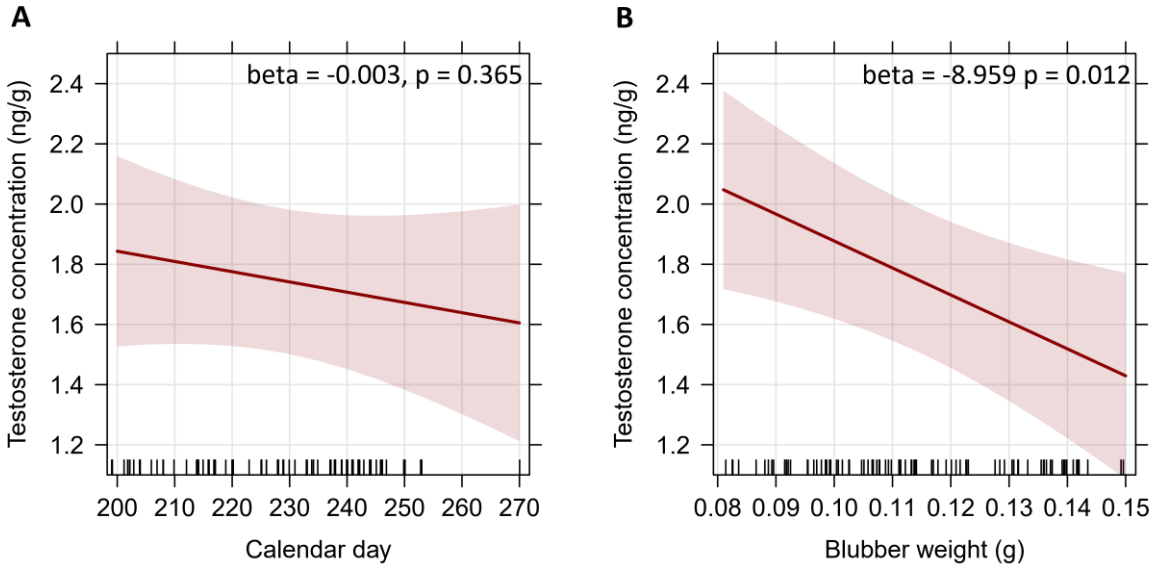


Figure 4.5. Blubber testosterone (n = 87 samples) of 41 male humpback whales sampled multiple times within the same breeding season. A) Blubber testosterone levels did not significantly decrease across multiple samples of the same male over the breeding season. B) Blubber testosterone was still negatively correlated with blubber weight used during the steroid hormone extraction.

Table 4.2. Model parameters and performance measures for regression models assessing the patterns of male blubber testosterone 1) across the breeding season a) in the male population and b) in individual males (section 4.4.3), and 2) with age (section 4.4.3.3). The coefficient estimates of the predictor variables (Estimates), their 95% confidence interval (95% CI), and p-value are shown for all three models (p). Performance measures for the mixed effects models are shown as marginal R-squared, conditional R-squared and interclass correlation coefficient (ICC). The marginal and conditional R-squared indicate how much of the variance is explained by the fixed effects only and by the complete model (fixed + random + residuals), respectively. The interclass correlation coefficient gives a sense of how much variance is explained by the random effect (year in 1a, and individual male in 1b and 2). Performance measures for mixed-effects models were estimated by division of the corresponding variance components of mixed models: marginal R-squared as fixed effects variance divided by the total variance; conditional R-squared as the fixed and random effects variance divided by the total variance; ICC: random effects variance divided by the random and residual variance. The different variance components of mixed models were calculated using the function 'get_variance' (package: insight, Lüdecke, Waggoner and Makowski, 2019) in R based on Nakagawa et al. (2017) and Johnson (2014). P-values < 0.05 were considered statistically significant. The full model outputs and diagnostic plots are provided in the supplementary material (Figures S4.15, S4.17, and S4.20).

	1a) Breeding season: male population			**1b) Breeding season: individual males**			**2) Age**		
Predictors	*Estimates*	*95% CI*	*p*	*Estimates*	*95% CI*	*p*	*Estimates*	*95% CI*	*p*
(Intercept)	4.334	[3.300, 5.368]	<0.001	3.549	[1.69, 5.416]	<0.001	4.617	[3.370, 5.867]	<0.001
Calendar day	-0.006	[-0.010, -0.002]	0.005	-0.003	[-0.011, 0.004]	0.365	-0.005	[-0.010, 0.000]	0.037
Age 9 – 16 yrs							-0.298	[-0.518, -0.079]	0.009
Age 16 – 23 yrs							-0.300	[-0.555, 0.044]	0.022
Age 23+ yrs							-0.081	[-0.423, 0.262]	0.645
Blubber weight	-10.021	[-13.570, -6.488]	<0.001	-8.959	[-15.668, -2.060]	0.012	-10.340	[-14.635, -6.006]	<0.001
Storage time							-0.023	[-0.036, -0.010]	<0.001
Marginal R^2	0.064			0.041			0.127		
Conditional R^2	0.533			0.724			0.302		
ICC	0.501			0.712			0.200		

4.4.3.3 Changes in male testosterone with age (2)

Testosterone levels were significantly higher in males of the youngest age category (2 – 9 years) compared to the males of the next two age categories (9 – 16 years: β = -0.298, 95% CI = [-0.518, -0.079], p = 0.009; 16 – 23 years: β = -0.300, 95% CI = [-0.555, -0.044], p = 0.022; Table 4.2). No significant difference was found between testosterone levels of males in the youngest (2 – 9 years) and oldest (23+ years) age categories. Although blubber testosterone levels seemed, on average, to increase again for males in the oldest age category (23+ years), there was considerable variation in testosterone levels across males within this age category (Figure 4.7A), possibly due to the lower sample size for older males (Table 4.3). Overall, blubber testosterone levels were highest in young males, then decreased and levelled off in more mature males (Figure 4.7A).

Blubber testosterone concentration was negatively correlated with calendar day, blubber weight and storage time (Table 4.2; Figure 4.7B-D). Overall, only a small proportion of the observed variance in male testosterone was explained by the three predictor variables (age category, calendar day, blubber weight, and storage time; marginal R^2 = 0.127). The full model including the random effect (individual) accounts for twice as much of the observed variance in male testosterone (conditional R^2 = 0.302). This suggests that there may be considerable variation between individuals of similar ages, which was further supported by the interclass correlation coefficient (ICC = 0.201) and the standard deviation of the random effect (individuals: sd = 0.35).

Testosterone concentrations of samples from aged males sampled multiple times across different years (n = 45 males), which were included in this analysis, are shown in figure S4.19. Altogether, and when controlling for differences in blubber weight, storage time and time within the breeding season, testosterone levels were highest in males approaching sexual maturity (8 - 10 years; Best, 2011), then decreased and levelled off in mature males (9 – 23 years). Yet, testosterone concentrations were highly variable across males of all ages.

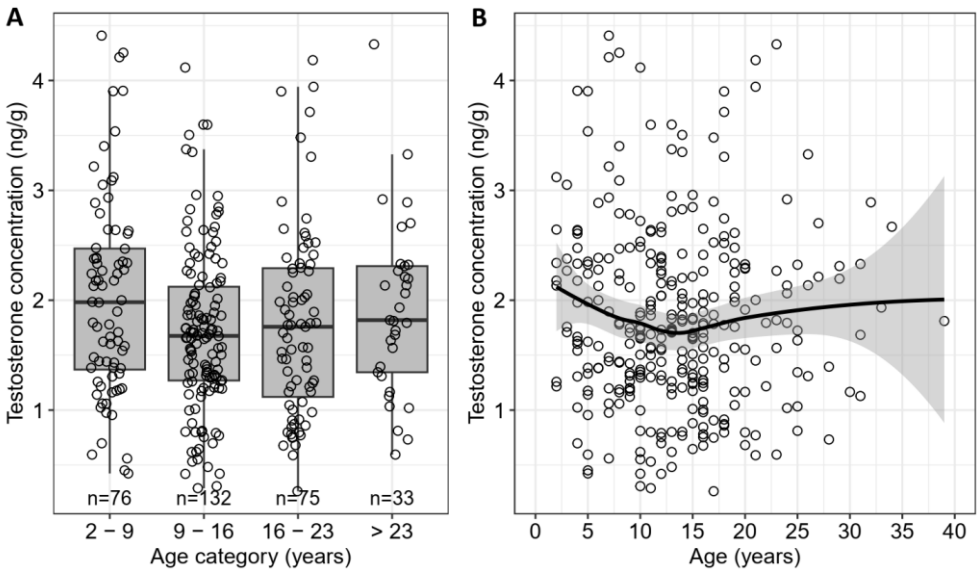


Figure 4.6. Male blubber testosterone across age (categories). A) Boxplot of measured male testosterone concentrations (ng/g) across the different age categories derived from epigenetic ageing in Chapter 3 (see also Table 4.3). B) Measured male testosterone concentration (ng/g) against age point estimates derived from epigenetic ageing in Chapter 3. A smoothing line was added using Local Polynomial Regression Fitting ('loess') with 95% confidence intervals shaded in grey.

Table 4.3. The number of analysed samples and individuals in each age category. Males below the age of 2 years were excluded from the analysis. Summary statistics with mean, standard deviation (sd), and range (min, max) of measured testosterone (T) concentration (ng/g) in each age category.

Age category	Samples	Males	mean T	sd T	min T	max T
2 – 9 years	76	60	2.02	0.91	0.42	4.41
9 – 16 years	132	88	1.72	0.74	0.29	4.12
16 – 23 years	75	45	1.78	0.86	0.26	4.19
23+ years	33	17	1.92	0.80	0.60	4.33

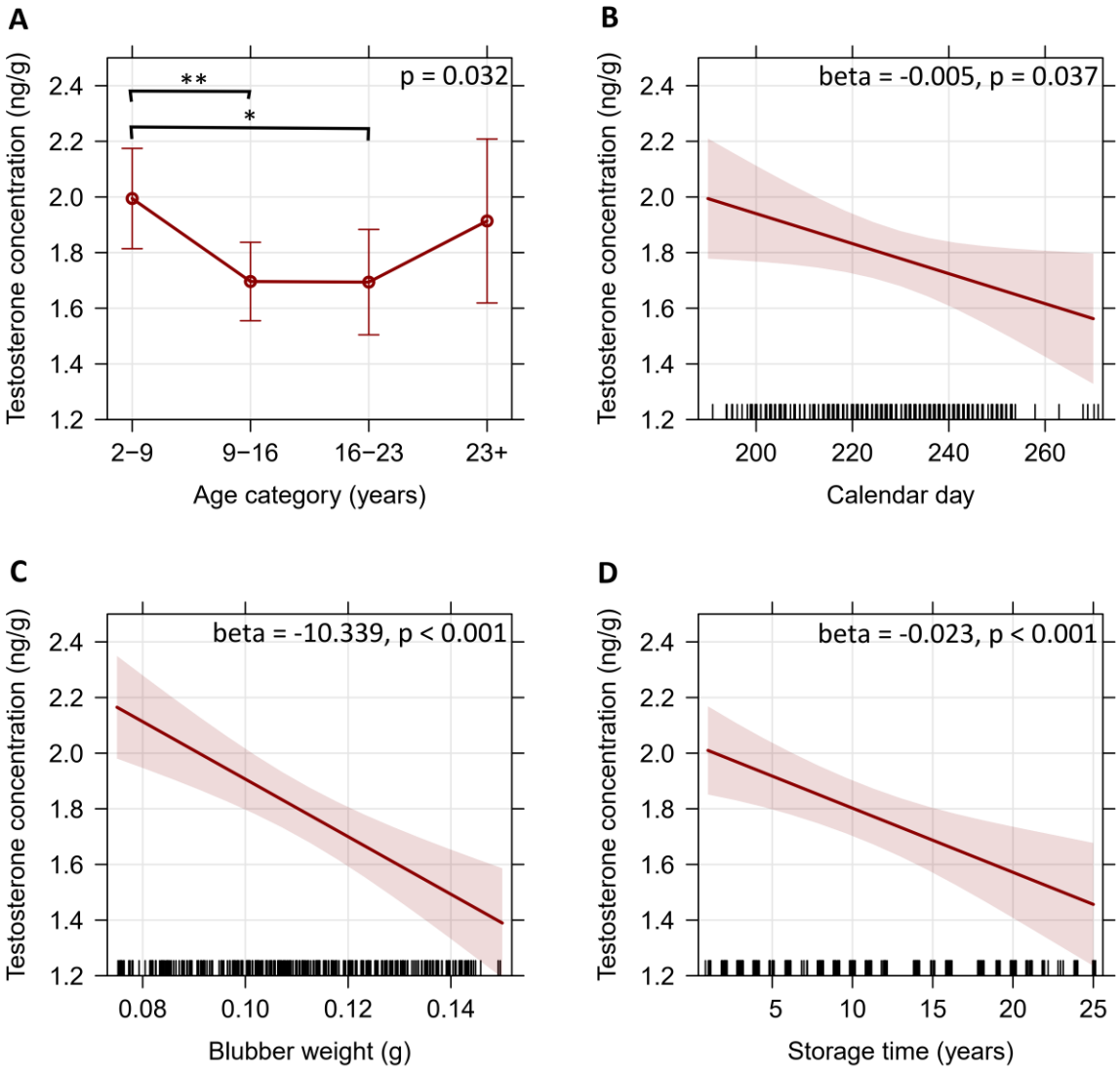


Figure 4.7. Age model: Marginal effect of each variable on male testosterone while all other predictor variables are held constant. Plot A) – D) corresponds to the four predictor variables: A) age category, B) calendar day, C) blubber weight, and D) storage time. The error bars and shaded areas show the confidence band for fitted values based on standard errors of the regression coefficients. The rug plot at the bottom of graphs B) and D) shows the location of the respective samples.

4.5 Discussion

Here, I assessed seasonal and age-related changes in the reproductive physiology of 178 individual male humpback whales on their breeding ground. Measured levels of male blubber testosterone (0.26 – 4.41 ng/g) fell within a similar range to previous studies of male blubber testosterone on humpback whale breeding grounds (Cates et al., 2019; F. M.J. Mingramm et al., 2020). Testosterone decreased across the season in the overall male population but did not differ significantly within an individual across the season. Testosterone was highest in younger individuals approaching puberty. Yet, despite these seasonal and age-related changes

in testosterone levels, male blubber testosterone was highly variable at any point during the breeding season and across males of all ages.

4.5.1 Seasonal changes in male testosterone

Male blubber testosterone slowly decreased over the course of the winter breeding season in the New Caledonian population, mirroring the reported seasonal decline in male blubber testosterone in humpback whales on their Hawaiian breeding ground (Cates *et al.*, 2019). This decrease in blubber testosterone during the breeding season further aligns with the reported higher levels of male testosterone on their migration towards the breeding grounds compared to levels on their migration back to the feeding ground (Mingramm et al., 2020). However, the observed decrease in male blubber testosterone on the breeding ground in this study was smaller and less clear compared to differences observed between opposite migratory directions (Mingramm et al., 2020), as well as between feeding and breeding grounds (Vu *et al.*, 2015; Cates *et al.*, 2019). However, the timeframe over which these seasonal changes in testosterone occur is much shorter at the breeding ground (breeding season: 2 – 3 months) compared to the time interval between opposite migratory directions (4 - 6 months between northbound and southbound migration in eastern Australia; Mingramm, T. Keeley, et al., 2020). Any seasonal changes in testosterone at the breeding ground are thus expected to be less pronounced than changes between opposite migratory directions and between feeding and breeding grounds.

Elevated levels of testosterone during the autumn have been suggested to indicate reproductive conditioning before the onset of the breeding season (Vu et al. 2015), and may also initiate migration from feeding grounds to breeding grounds (Baker *et al.*, 1985; Craig *et al.*, 2003; Cates *et al.*, 2019). In turn, decreasing levels of testosterone towards the end of the breeding season and during migration back towards the feeding ground may initiate a decrease in breeding activity at this time (Mingramm et al., 2020). Testosterone may not directly cause individuals to leave their breeding ground, yet it likely plays a role in the coordination of their reproductive physiology and behaviour. Changes in blubber testosterone levels, migratory behaviour and breeding activity of humpback whales appear to be tightly correlated and may be regulated by the same proximate mechanism. While it remains unclear what environmental and/or endocrine cues initiate humpback whale migration in either

direction, all animals on the breeding ground presumably received the initial 'urge' to migrate to the breeding grounds, however, the individuals in the current study have not yet received the presumed second 'urge' initiating their return to the feeding grounds. Differences in blubber testosterone between males on the different migratory directions are therefore expected to be larger, not only because of the potential increased time interval between sample collection, but also because of the proximate mechanism that made individuals migrate, and increase/decrease their breeding activity, in the first place.

Despite the observed decrease in blubber testosterone in the male population, I was unable to detect a clear seasonal decline in testosterone levels in individual males that were sampled multiple times over the breeding season. This inconsistency in the temporal pattern of blubber testosterone across the breeding season in the male population compared to individual males in this dataset could result from four non-mutually exclusive explanations: 1) differences in samples size (1a - male population: n = 331 samples, N = 178 males; 1b - individual males: n = 87 samples, N = 40 males), 2) longer time interval between the first sampled individual and the last sampled individual in the population (1a – male population: max. 71 days, mean = 48 days) than between first and last sample of the same individual (1b – individual males: max. 51 days, mean = 18 days) within the same breeding season, 3) the limited number of resights within a season (1 – 3 samples of the same individual) in the analysis of intra-individual seasonal changes, and 4) differences in migratory timing of individuals that is associated with testosterone levels in some way. If blubber testosterone correlates with a male's reproductive status and/or age, then differences in migratory timing between individuals of different ages will lead to changes in the age structure of the male population across the breeding season. Humpback whale migration in both directions is characterised by a staggering of age and reproductive status with immature animals arriving and departing earlier than mature males (Nishiwaki, 1959; Chittleborough, 1965; Dawbin, 1966; Craig *et al.*, 2003). The age structure of the male population on the breeding ground is thus expected to shift towards more older and fewer immature individuals as the season progresses. This would result in an overall decline in blubber testosterone in the male population even if testosterone in individual males does not mirror the seasonal decline in testosterone observed at the population level. However, differences in the migratory timing of individuals, irrespective of maturational class, result in differences in the duration

individuals spend on the breeding ground which may further contribute to the observed individual variation in blubber testosterone.

4.5.2 Age-related changes in male testosterone

Blubber testosterone levels were highest in young males (2 – 9 years), then decreased and levelled off in older males (9 – 23 years). A previous study suggested peak lifetime blubber testosterone concentrations are seen on the breeding ground in animals around age 8 – 25 years (Cates *et al.*, 2019). However, the sample size was small (5 males of known age; 19 males with estimated minimum age). Although the authors reported a very broad peak of male blubber testosterone, their data based on estimated minimum age derived from behavioural observations (19 males), suggests a decline in male blubber testosterone after the average age of sexual maturity (8 – 10 years; Best, 2011) in humpback whales (Figure 10, p.8 in Cates et al., 2019). The current study refines age-related trends of testosterone and estimates of peak testosterone with a substantially larger dataset (n = 316 samples, N = 169 males).

Here, blubber testosterone levels were highest in young males that presumably have not yet reached their sexual maturity, then decreased and levelled off in more mature males (Figure 4.7A). Male humpback whales might experience an increase in testosterone during puberty similar to male adolescence in humans (Nottelmann *et al.*, 1987) and rodents (Bell, 2018). In male humans, testosterone plays an important role in several pubertal processes such as genital growth (Huang *et al.*, 2012), change in body composition (Hansen *et al.*, 1999), and maturation of the brain (Peper *et al.*, 2009; see also: Khairullah *et al.*, 2014). Thus, young male humpback whales might experience higher levels of testosterone because they are still undergoing the physiological development required to reproduce, in contrast to already sexually mature males. Apart from this peak in testosterone during puberty, data here suggest a potential second peak in testosterone as males mature into their late 20s and early 30s (Figure 4.7A). This second increase in testosterone could be linked to a change in reproductive tactics and/or increased reproductive output, as older males (23+ years) were more often observed as singers, solitary escorts and successful sires than expected based on the underlying population age structure (see Chapter 3). However, the sample size of the oldest age category (23+ years) was smaller (N = 17 males) than all other age categories (Table 4.3), and more samples of older males are needed to confirm this potential second peak in male

blubber testosterone. With the observed effect of age on male mating tactics (Chapter 3) and the age-related pattern in male blubber testosterone, a male's level of testosterone may thus also affect his mating tactic (or the other way around, see section 5.2). Future studies may explore how testosterone levels in mature males influence their likelihood to engage in singing or physical competition.

4.5.3 Variation in male blubber testosterone on the breeding ground

Despite the observed seasonal decline in the population and the age-related pattern in male blubber testosterone, testosterone levels across individuals varied considerably at any point within the breeding season and across males of all ages. The dataset of 331 samples of 179 males collected over 25 years allowed me to assess seasonal and age-related trends in male testosterone while controlling for several possibly confounding variables (e.g., sample storage time, blubber weight, time within the breeding season). Sample storage time and blubber weight both showed a significant negative correlation with blubber testosterone levels (Table 4.2). While any confounding effects of sample storage time were not an issue for the first two models (1a – male population and 1b – individual males) as changes in blubber testosterone during the breeding season were analysed within years, it was a significant predictor in the third model (2 – Age) with a coefficient roughly four times larger than calendar day within the breeding season (Table 4.2). However, the effects of sample storage time were considerably smaller than the observed age-related changes in male blubber testosterone. Blubber weight showed a significant effect on measured levels of blubber testosterone according to all three models. The blubber wet weight of samples used in analyses ranged between 0.08 – 0.14 g of blubber with a maximum possible difference of 0.06 g of blubber between the lightest and the heaviest sample. Based on the coefficient estimates for blubber weight in the third model (2 – Age), differences in the blubber weight in this dataset would result in a maximum difference of ~0.62 ng/g between the lightest and heaviest samples. This effect is larger than observed seasonal and age-related changes in male blubber testosterone, yet, the full set of predictor variables (incl. blubber weight) in all three models accounts only for a small proportion of the observed variation in male blubber testosterone levels (marginal R^2, Table 4.2). Further, conditional R^2 and ICC, indicating the amount of variance explained by the random effects of a model, suggest that both differences across individuals (Figure 4.4) and years (Figure S4.14)

account for a considerable proportion of the observed variation in male blubber testosterone. Thus, although variable blubber weight measured during hormone extraction does introduce variation in measured blubber testosterone data, this effect appears to be smaller in proportion to the variation due to individual and inter-annual differences (Figure 4.8). Other biological, social and/or environmental factors likely influence male testosterone levels on the breeding ground.

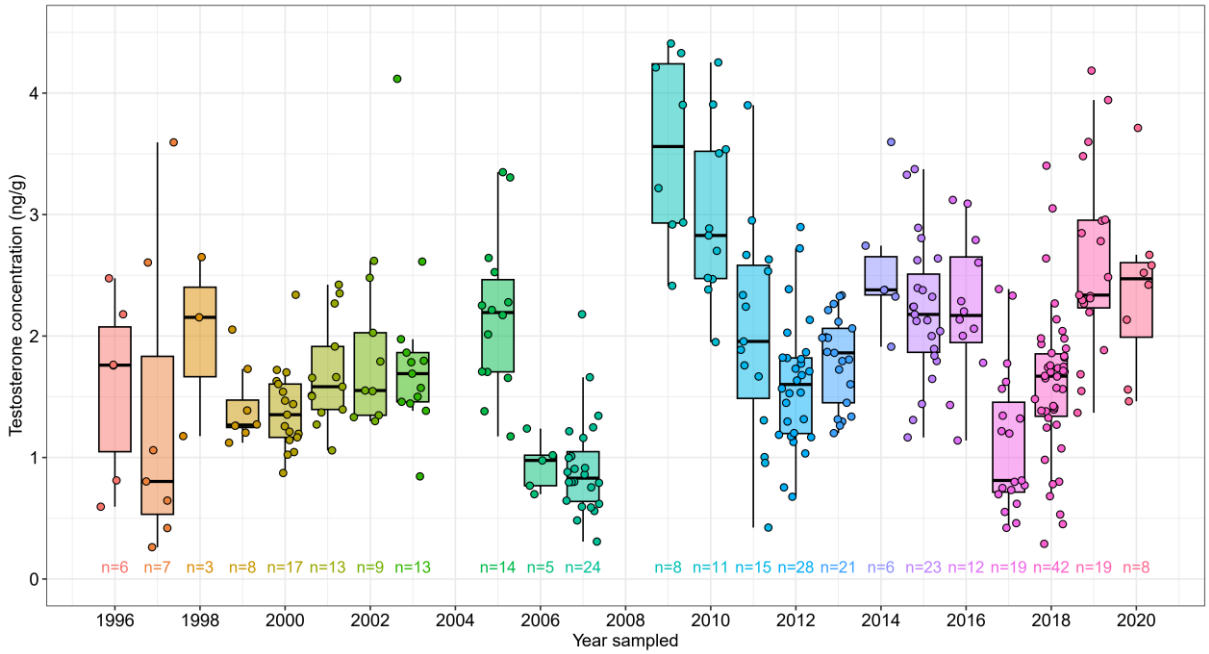


Figure 4.8. Inter-annual differences in blubber testosterone across 25 years of 178 male humpback whales (n = 331 samples).

The coordination of physiology and behaviour through hormones allows individuals to adjust to changes in their environment. Reproductive rates of female humpback whales have been shown to be positively correlated with prey availability in the previous year (Kershaw *et al.*, 2021; Pallin *et al.*, 2023). During years of low food availability, females may not have been able to accumulate the energy reserves necessary to successfully complete pregnancy and/or lactation due to the high energetic demands during these life-history changes (Kershaw *et al.*, 2021). As capital breeders, humpback whales rely on abundant prey resources at their high-latitude feeding grounds to store energy reserves needed for their migration to and from low-latitude breeding grounds and reproduction (Lockyer, 1981; Baker *et al.*, 1986). Compared to females, male baleen whales bear none of the costs of gestation and lactation, yet it is

reasonable to assume that male reproduction in humpback whales too is, at least to some extent, affected by prey availability on feeding grounds. In years of low food availability, individuals may not have been able to build up sufficient energy storage to attend to their seasonal migration and go through an elongated fasting period. Under such circumstances, individuals may adjust their migratory timing and spend less time on their breeding grounds, or not migrate at all and remain on their feeding grounds; a life-history trade-off between the investment in survival versus reproduction. While differences between individuals in the time spent on their breeding grounds could explain the individual variation in male blubber testosterone, environmental fluctuations (e.g., El Niño, climate change) may lead to observed inter-annual differences in testosterone levels.

Variation in male blubber testosterone on humpback whale breeding grounds could also be due to or may result in behavioural differences. Singing correlates with the annual cycle of male testosterone (Cates *et al.*, 2019) on breeding grounds and migratory routes. Further, higher blubber testosterone in male humpback whales has been linked to more aggressive reproductive behaviours (i.e. competitive groups) as principal escorts (considered dominant) showed higher levels of testosterone than secondary escorts (considered subordinate) (Mingramm et al., 2020). While testosterone may stimulate male singing and/or aggression during male-male competition, social interactions between individuals on the breeding grounds and/or outcomes during aggressive encounters within competitive groups could, in turn, affect testosterone levels. Both the behaviour of an individual and its interactions with other individuals can feed back on the individual's hormone levels (Oliveira, 2004). Thus, testosterone levels may be correlated with an individual's reproductive tactic (e.g., singing, direct competition) and with its social environment.

Individual variation in testosterone levels may also be due to genetic differences among individuals (Kempenaers, Peters and Foerster, 2008). Maintaining high levels of testosterone may come at the cost of reduced immunocompetence ('immunocompetence handicap hypothesis'; Folstad and Karter, 1992). The promoting effects of testosterone on the development of secondary sexual characteristics, and thus potential mating success, may simultaneously suppress the immune system and decrease disease and parasite resistance (Folstad and Karter, 1992; Martin, Weil and Nelson, 2008). If only the highest quality individuals can 'sustain' the highest testosterone levels, then individual variation in

testosterone levels may reflect variation in quality (Kempenaers, Peters and Foerster, 2008). Within this physiological trade-off between the reproductive and immune system, the testosterone-dependent development and maintenance of morphological and/or behavioural traits (e.g., song) serve as an honest signal of quality. In red grouse (*Lagopus lagopus scoticus*), male comb size, a testosterone-dependent trait, honestly indicates a males' immunocompetence as males with larger combs had lower T-cell mediated immunity (Mougeot *et al.*, 2004). Although evidence in favour of the 'immunocompetence handicap hypothesis' is far from conclusive across vertebrate taxa (Roberts, Buchanan and Evans, 2004), the tight connection between the endocrine and immune systems is apparent, and androgens most likely play a role in it (Martin, Weil and Nelson, 2008).

4.5.4 Limitations

While the sample size of 331 samples of 178 individuals collected over 25 years offers a unique opportunity to assess seasonal and age-related trends in male testosterone of humpback whales, there are some limitations in the interpretation of measured testosterone levels. Firstly, I was not able to calculate plate-specific standard curves for three assays which likely reduced the accuracy of calculated testosterone levels of samples on these assays (section 4.3.4). Secondly, binding parameters (i.e. NSB) on two plates were extremely high, thus, the background correction on these plates was likely less accurate (section 4.4.1). Thirdly, the use of consistent inter-assay controls comprised of a low, medium, and high dose on each assay plate would have led to a more accurate estimate of inter-plate variation spanning a wider range of testosterone levels (section 4.3.4). Fourthly, no assay validation tests have been carried out for this specific study (section 4.3.4), but can still be carried out retrospectively. Fifthly, the amount of blubber used for the extraction of steroid hormones was highly variable. As blubber weight was negatively correlated with blubber testosterone level (Figure 4.1A), more consistent measuring of blubber mass could have reduced the amount of variation observed in male blubber testosterone levels. Lastly, sample degradation, as indicated by the negative correlation between testosterone levels and sample storage, may have lowered the testosterone levels of older samples (Figure 4.1B). Overall, however, with the post hoc quality controls I imposed, measured levels of male blubber testosterone (0.26 – 4.41 ng/g) did fall within a similar range to previous studies of male blubber testosterone on humpback whale

breeding grounds (Cates et al., 2019; F. M.J. Mingramm et al., 2020) indicating the results were representative.

4.6 Conclusions

This study integrates insights into the physiology and age of male humpback whales. The results indicate that blubber testosterone levels slowly decrease over the breeding season in the male population. However, population-level patterns of blubber testosterone levels did not mirror seasonal changes in blubber testosterone at the individual level. The seasonal trend in blubber testosterone observed in this dataset could be driven by a link between individual testosterone levels and migratory timing, rather than a decrease in blubber testosterone in individual males on the breeding ground. Further, blubber testosterone levels of male humpback whales appear to be highest during puberty, then decrease and level off in mature males before potentially increasing again in males maturing into their late 20s and early 30s. Yet, blubber testosterone in males was highly variable at any point during the breeding season and across males of all ages. While future studies are left to explore additional environmental and biological factors contributing to this variation, this chapter demonstrated that the integration of endocrine and molecular age markers in long-term datasets is a powerful tool for understanding a species' life-history trends, ontogenetic changes, and mating systems.

Chapter 5

Influence of MHC diversity on patterns of reproductive success in humpback whales

5.1 Abstract

Genetic diversity is an important factor determining the fitness and viability of wild populations. Genetic diversity at the major histocompatibility complex (MHC) is linked to immune defence against disease and parasites. Maintaining a high MHC diversity is important in upholding an effective immune defence, and thus, may affect the long-term survival rate of populations. Both pathogen-mediated natural selection and MHC-mediated sexual selection are possible non-mutually exclusive forces shaping patterns of MHC diversity. Here, I integrated 25 years (1996-2020) of photo-ID and genetic data to assess the MHC diversity and dissimilarity in an endangered breeding population of humpback whales and its influence on patterns of male reproductive success. MHC diversity based on the phylogenetic distance and composition of alleles and haplotypes was considerably higher at MHC class I than at class IIa genes (DQB and DRB-a), indicating higher natural selection pressure at class I for the protection and resistance against viruses as compared to bacteria and parasites. Further, MHC dissimilarity between individuals (incl. mother-father pairs) was higher based on the composition of alleles (i.e., allele sharing) rather than based on their phylogenetic distance. Validated mating pairs (n= 58) shared less alleles than expected under random mating in more than 90% of simulations (n = 1,000) at MHC class I, supporting the possibility of an MHC-mediated mate choice in humpback whales. This study provides novel insights into the natural selection pressures of the marine environment acting on MHC in cetaceans, and how female humpback whales may shape patterns of male reproductive success.

5.2 Introduction

High genetic diversity positively influences the fitness and viability of a population, including its adaptation to environmental changes (Reed and Frankham, 2003; Chapman et al., 2009). A major goal of conservation biology is to maintain viable populations of wild animals that have levels of genetic diversity capable of facilitating natural selection and avoiding inbreeding (e.g., Schwartz, Luikart and Waples, 2007). However, not all genetic variation is equally suitable for identifying the adaptive potential and fitness of a population. The genetic diversity of wild animal populations has typically been assessed using neutral genetic markers (i.e., microsatellites and SNPs) that, while correlating with genome-wide diversity metrics, offer little insight into their adaptive potential (Hoelzel, Bruford and Fleischer, 2019). Functional genetic diversity, measured using markers linked to ecologically important traits, is argued to be more relevant to the conservation of populations than neutral genetic diversity (Petchey and Gaston, 2006; Petchey, J. O'Gorman and Flynn, 2009).

A commonly assessed functional genetic markers is the major histocompatibility complex (MHC; reviewed in Piertney & Oliver, 2005). The MHC plays a fundamental role in the adaptive immune response of vertebrates, thus making it an ideal measure of functional genetic diversity. In bottlenose dolphins (*Tursiops aduncus*), for example, functional genetic diversity at the MHC better reflects the viability of the population than neutral genetic diversity based on mitochondrial and microsatellite markers (Manlik *et al.*, 2019). The MHC recognises foreign proteins, presents them to specialist immune cells and so initiates an immune response (Klein, 1986). The binding of foreign proteins takes place at receptors called antigen-recognizing sites (ARS) located in peptide binding regions (PBR). MHC genes can be divided into two major groups (class I and II) depending on the type of pathogens they target. MHC class I genes are responsible for the immune defence against intracellular pathogens (e.g., viruses, intracellular bacteria), while MHC class II genes are involved in the immune defence against pathogens coming from the extracellular environment (e.g., bacteria, nematodes) (Sommer, 2005). MHC polymorphism is associated with variation in receptors determining disease and parasite resistance (Hedrick, Kim and Parker, 2001). High MHC diversity may therefore be crucial in maintaining an effective immune defence (Janeway *et al.*, 2001) and thus may affect the long-term survival rate of populations (Paterson, Wilson and Pemberton, 1998; Hedrick, Kim and Parker, 2001).

MHC genes are some of the most polymorphic loci known in vertebrates (Hedrick, 1994). It is generally thought that balancing selection, a form of natural selection, is the determining force shaping patterns of diversity of MHC genes (Hughes and Nei, 1989; Bernatchez and Landry, 2003). Natural (balancing) selection may favour rare alleles through an evolutionary arms race with pathogens (frequency-dependent selection), or heterozygotes due to their greater fitness relative to homozygotes (overdominance) (Hughes and Nei, 1988). Although the extraordinary polymorphism at MHC is thought to be mainly driven by pathogen-mediated balancing selection, MHC-mediated sexual selection could offer an additional (non-exclusive) mechanism for maintaining MHC diversity (Tregenza and Wedell, 2000; Janeway et al., 2001; Piertney and Oliver, 2006; Winternitz et al., 2013). In artifically bred salmon where animals were deprived from the potential benefits of mate choice, offspring had higher parasite loads and were less MHC dissimilar than offspring of wild salmon (Consuegra and Garcia de Leaniz, 2008).

Sexual selection shapes patterns of reproductive success, and so can affect the genetic diversity, and thus fitness, of offspring via pre- or post-copulatory tactics (see Chapter 1). MHC-mediated sexual selection may occur pre-copulation through disassortative mating (reviewed in Kamiya *et al.*, 2014), or post-copulation through biased fertilization (Wedekind et al., 2004; Løvlie et al., 2013) or biased mortality of zygotes (Alberts and Ober, 1993). Through a preference for mates (or gametes) with (i) dissimilar MHC genotypes (compatibility), (ii) higher absolute MHC diversity, or (iii) particular MHC alleles with advantageous effects, sexual selection can enhance the disease and parasite resistance in the offspring through increased MHC diversity (reviewed in Piertney and Oliver, 2006). Individuals may signal their MHC profile to prospective mates through (a) chemical signalling as MHC molecules are bound to volatile chemicals that are excreted via the skin, urine or faeces (Penn, 2002; Ruff et al., 2012; Overath, Sturm and Rammensee, 2014), or (b) condition-dependent traits as only males with high immunocompetence (as determined by their MHC genotype) can bear the cost of elaborate sexual displays (Hamilton and Zuk, 1982; Folstad and Karter, 1992). If a particular trait is an honest indicator of a male's genetic quality (e.g., immunocompetence), then females might use that information to assess and choose their potential mates. In song sparrows, song complexity was found to advertise optimal MHC diversity, thus, by choosing mates with complex song, females may enhance the immunocompetence and disease resistance of their offspring (Slade, Watson and MacDougall-

Shackleton, 2017). MHC-mediated sexual selection can facilitate inbreeding avoidance (Potts, Manning and Wakeland, 1994), enrich genome-wide diversity, and thus, can allow small populations to mitigate the loss of genetic diversity over time.

MHC-diversity is typically studied in easy access terrestrial species. With their evolutionary transition from land to sea fifty million years ago (Mya), cetaceans are faced with a different range of pathogens associated with the marine environment compared to their terrestrial taxonomic relatives. Early findings of reduced MHC class II diversity in marine mammals compared to terrestrial mammals were suggested to be the result of a diminished selective pressure for maintaining MHC polymorphism stemming from the relatively low prevalence of infectious disease in the marine environment (Trowsdale, Groves and Arnason, 1989). However, later studies revealed considerable sequence variation at MHC class II genes in some species of baleen whales (mysticetes) and toothed whales (odontocetes) (Flores-Ramirez, Urban-Ramirez and Miller, 2000; Yang et al., 2005; Baker et al., 2006). Further, the higher nonsynonymous divergence at the exon 2 of the MHC class II DQB locus (especially at BPR) in several mysticetes suggests that polymorphism at this gene is under positive (overdominance) selection (humpback whales and southern right whales, Eubalaena australis: Baker *et al.*, 2006; blue whales, Balaenoptera musculus: Moreno-Santillán *et al.*, 2016). These findings argue against a reduction in the selection pressure of the marine environment (Yang, Chou and Hu, 2012). Moreover, with MHC polymorphism arising from several loci and gene classes, the MHC diversity at one locus cannot holistically represent the adaptive immune response and functional genetic diversity of a species (Yang, Chou and Hu, 2012). The limited research on MHC diversity in cetaceans, so far, has mainly been focused on MHC class II genes, thus, neglecting immune response against viruses coming from class I genes. Further, the influence of MHC diversity on patterns of reproductive success and the influence of sexual selection on MHC diversity have remained largely unexplored in cetaceans.

Here, I integrate 25 years of photo-ID and genetic data (1996-2020) to assess the influence of MHC diversity on patterns of male reproductive success in an endangered breeding population of humpback whales in New Caledonia, South Pacific. First, by applying a recently developed and validated humpback whale MHC amplicon sequencing panel (Heimeier et al., in press), I assessed the MHC diversity of the New Caledonian population (n = 329 individuals) at three MHC genes (class I, class IIa DQB and class IIa DRB). Second, I tested the hypothesis

of an MHC-mediated mate choice as a pre-copulatory strategy in humpback whales. I predicted that MHC genotypes between mating pairs (n = 58 paternities, see Chapter 2) should be more dissimilar than MHC genotypes from random pairs. MHC diversity and dissimilarity were estimated via both phylogenetic distance-based and composition-based metrics (see Methods) at four levels of analysis: nucleotides, amino acids, alleles and haplotypes. Applying a multi-level and multi-metric analysis allowed me to capture MHC diversity and dissimilarity resulting from both evolutionary processes at the sequence level (nucleotide and amino acids) and variable gene copy number level (alleles and haplotypes). This study provides novel insights into how female humpback whales may shape observed patterns of male reproductive success.

5.3 Methods

5.3.1 Study site and data collection

Humpback whale skin samples for the MHC analysis and photographs for identification were collected at the New Caledonian breeding ground from 1996 to 2020 during the austral winter (July to September). On annual surveys, whales were carefully approached to be photographed and biopsied using a crossbow with a specially adapted bolt (Lambertsen *et al.*, 1994) or a modified veterinary rifle (Krützen, 2002). Skin samples were stored in 70% ethanol at -20˚C. Individual humpback whales were identified based on photo-identification from unique markings on the ventral surface of their tail flukes (Katona and Whitehead, 1981) and/or their genotypes (see Chapter 2 section 2.3). Paternities were previously inferred for 66 fathers of 79 offspring (Chapter 2). Further details on the data collection, genetic profiling, and paternity analysis are described in Chapter 2. Here, I selected 335 individuals, including 58 paternity trios (mother, father, offspring), to assess the MHC diversity in New Caledonian humpback whales.

5.3.2 A note on collaborations and contributions

After I selected samples for this analysis, Dr Doro Heimeier performed all MHC-related lab analyses (amplification and sequencing) at the University of Auckland, including the

development of the amplification panel, called alleles (genotyping) and inferred haplotypes. I then received a list of identified alleles and haplotypes for each individual and their allele sequences. I conducted all further analyses myself (sections 5.3.3 onwards), which included the phylogenetic analysis, the validation of paternity trios, the calculation of genetic diversity and dissimilarity, and assessing MHC-mediated mate choice. During two lab visits to the University of Auckland and many video calls, Dr Heimeier explained lab procedures and analyses including iterative mapping and clustering steps performed during calling alleles (genotyping) in Geneious.

5.3.3 Analysis overview

The nucleotide sequence of a gene has a higher information content than the amino acid sequence of the corresponding protein under the assumptions of the neutral theory of molecular evolution as most genetic mutations are neutral with respect to gene function (Kimura, 1968). However, some genetic mutations can lead to synonymous (silent mutations that result in the same amino acid) and non-synonymous (mutations that result in a different amino acid) changes in the amino acid sequence that can result in functionally, and ultimately evolutionarily relevant, differences between MHC alleles. To capture both these aspects of genetic and functional diversity, we assessed MHC diversity and dissimilarity via both phylogenetic distance-based (sequence) and composition-based (presence, absence and sharing) metrics at four different levels: 1) nucleotides, 2) amino acids, 3) alleles and 4) haplotypes (Figure 5.1, Table 5.1). The two lower levels (nucleotides and amino acids) were analysed using phylogenetic distance-based metrics from phylogenetic trees built using sequences of nucleotides and amino acids, respectively (section 5.3.5).

The higher two levels (alleles and haplotypes), which I term genotypic diversity hereafter, were analysed using composition-based metrics to assess the composition of alleles and haplotypes of an individual based on a presence/absence (within individual diversity; section 5.3.6) or shared/non-shared (between individual dissimilarity, i.e. allele or haplotype sharing; section 5.3.7) basis. Alleles and haplotypes represent units of inheritance, and their diversity thus is a pre-requisite for selection to act upon.

Assessing sequence variation (nucleotide and amino acid) through phylogenetic distance-based measures alongside genotypic variation through allelic and haplotype richness captures a greater spectrum of genetic diversity within and between individuals than either metric alone. The multi-level and multi-metric analysis applied here allowed me to capture the genetic diversity resulting from both evolutionary processes at the sequence level (nucleotide and amino acids) and variable gene copy number level (alleles and haplotypes). Further, while phylogenetic distance-based metrics reflect evolutionary (genomic) diversity, genotypic diversity (in alleles or haplotypes composition) arguably is a better estimate of phenotypic trait diversity (here, immune response; see section 5.2) which ultimately may result in fitness variation among individuals.

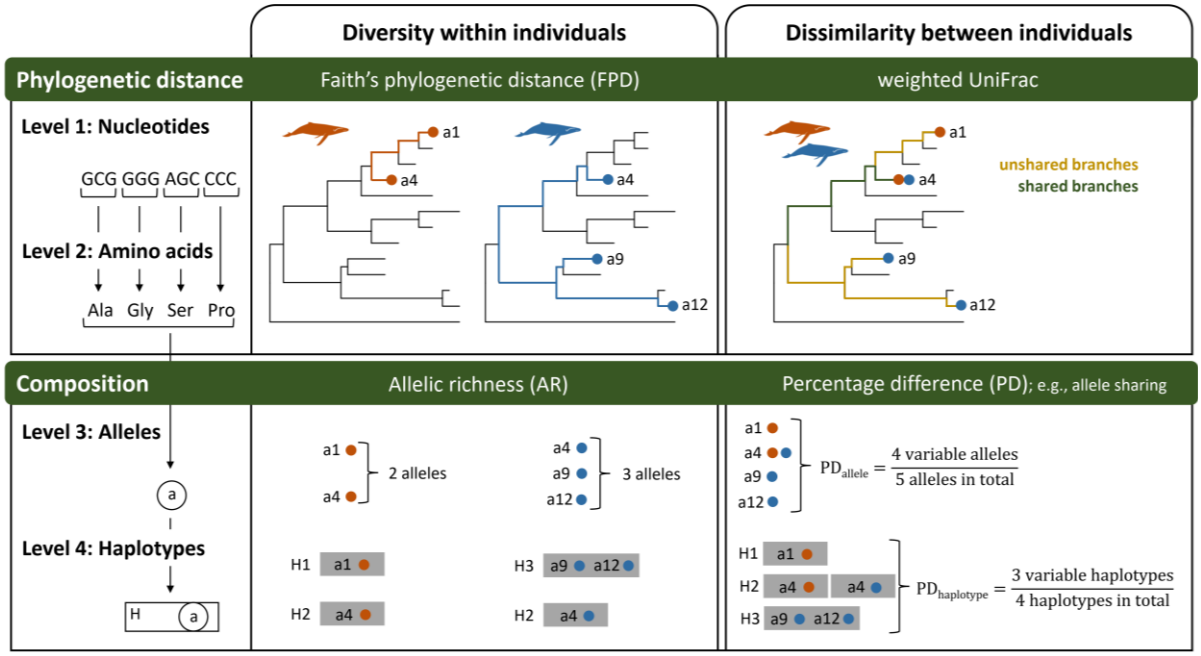


Figure 5.1. Overview of the multi-level multi-metric analysis of individual humpback whales. Phylogenetic distance-based and composition-based metrics were used to measure genetic diversity within individuals and genetic dissimilarity between individuals at the level of 1) nucleotides, 2) amino acids, 3) alleles and 4) haplotypes. The phylogenetic distance of nucleotide and amino acid sequences was measured using Faith's phylogenetic distance (FDP) and weighted UniFrac. The composition-based metric was applied at the level of alleles and haplotypes to assess allelic richness (AR) and the percentage difference (PD) in allele and haplotype composition (e.g., allele sharing). Haplotype richness was only measured at the level of the population (see section 5.4.2), and not the individual.

Table 5.1. Overview of the diversity and dissimilarity measures at four different levels (nucleotide, amino acid, allele, and haplotype) using both phylogenetic-based and composition-based metrics at three MHC genes (class I, class IIa DQB and class IIa DRB). For the phylogenetic-based metric all three MHC genes were analysed separately, but for the composition-based metric the two MHC class II genes (DQB and DRB) were analysed together. MHC diversity within individuals was estimated via Faith's phylogenetic distance (FPD) and allelic richness (AR) in section 5.3.6. MHC dissimilarity between individuals was estimated using the weighted unique fraction metric (UniFrac) and percentage difference (PD) in allele and haplotype composition (i.e., allele and haplotype sharing) in section 5.3.7.

Level	Diversity	Dissimilarity	MHC class I	MHC class IIa	
				DQB	DRB
Phylogenetic distance					
1) Nucleotides	FPD	UniFrac	x	x	x
2) Amino acids	FPD	UniFrac	x	x	
Composition					
3) Alleles	AR	PD	x	x	
4) Haplotypes		PD	x		

5.3.4 MHC sequencing and genotyping

To assess MHC diversity in the New Caledonian humpback whale population, 341- to 391-bp fragments of exon 2 of the MHC class I, class IIa DQB, and class IIa DRB-a (hereafter called DRB) molecules were sequenced using MiSeq amplicon sequencing (Heimeier et al., in press). Amplicon sequencing is a targeted sequencing method enabling the analysis of genetic variation in specific genomic regions (see Heimeier et al., in press). Individual samples are attached with an adapter allowing the formation of indexed amplicons (DNA products of a polymerase chain reaction (PCR)) before sequencing. Thus, multiple samples can be sequenced on a single sequencing run.

Genomic DNA was extracted from skin samples as described in Chapter 2 section 2.3.2. Gene-specific primers for amplification of variable regions (exon 2) of the MHC class I and class IIa DQB and DRB genes in cetaceans were designed in conserved flanking regions (between framework genes DDx39B and TRIM26) by Heimeier et al. (in press) (Table S5.1). MHC class IIa DQA and DRA genes are monomorphic in humpback whales (Heimeier et al., in press) and were thus not included in the current analysis. PCR amplification for each gene was performed separately with a final volume of 20 µl containing 0.6 µl dNTPs, 0.8 µl of each primer, 4 µl Phsion Plus GC enhancer, 0.2 µl PhusionPlus^{TM} Polymerase (5 U/µl), 4 µl 5x reaction buffer and

3 µl genomic DNA (at 50 ng/µl). Cycling programs consisted of 30 s initial denaturation at 98˚C, followed by 28 cycles at 98˚C for 5 s, 62˚C for 10 s, 72˚C for 30 s final extension at 72˚C for 5 min. Amplicon concentration was estimated on 1.5% agarose gels, pooled for each of the 335 individuals using 5 µl of each amplicon and finally purified with 25 µl of Ampure beads according to the manufacturer's protocol with slight modifications (see Heimeier et al., in press). The concentration of the elute was measured on Qubit and samples were diluted to 5 ng/µl. Resulting libraries were sequenced on an Illumina MiSeq platform by Auckland Genomics (University of Auckland) and individuals were tagged with Nextera indexes supplied by IDT (San Diego, USA).

MHC genotyping was conducted in Geneious 10.0.9 (https://www.geneious.com) following published protocols (Lighten, Van Oosterhout and Bentzen, 2014; Sebastian et al., 2016; Roved et al., 2022) and as described in full detail in Heimeier et al. (in press)(Figure 5.2). After initial quality control (remove trimmed bases with >0.01 error probability), paired-end reads were merged with BBMerge in Geneious using default settings (Bushnell, Rood and Singer, 2017). Merged reads were then mapped to individual full-length MHC class I and class IIa genes sourced from a fully annotated NCBI genome assembly of the blue whale, *Balaenoptera musculus* (accession number: NC_045795) using the inbuilt Geneious mapper (minimum overlap identity of 85% and allowing gaps) to separate reads into the three amplicons (class I, class IIa DQB and DRB).

After mapping the respective blue whale reference genes, primer sequences were trimmed from both ends. Merged reads from each gene were then mapped to already identified MHC class I and class IIa alleles of humpback whales by Heimeier et al. (in press) with 100% identity and 300 bp overlap. Unmapped reads were then mapped again with 99% identity and 300 bp overlaps. Reads that mapped with 99% but not 100% identity, if found in many individuals, could indicate new alleles with just a few bp differences (e.g., indicated as N03v01 and N03v02 in allele names). Any reads that still remained unmapped were *de novo* assembled to form clusters of highly similar reads (100% identity) to identify potential new alleles while retaining read number information (read depth). Allele calling was based on the 'degree of change' (DOC) method by Lighten et al. (2014). For that, clusters were then sorted in descending order of their read depth. The threshold dividing true alleles from artefacts is then determined by ordering clusters by their read depth and finding where the drop in

cumulative percentage of reads is the highest. Consensus sequences were created for each cluster above the determined DOC threshold, representing a potential allele. Sample IDs, cluster number, and read depth of the cluster from which the consensus sequence originated were retained. Consensus sequences for each gene were aligned across all individuals in MAFFT (Katoh *et al.*, 2002). The potential new alleles were reconciled with previously identified alleles to give a list of unique alleles per gene. Alleles were named following Heimeier et al. (in press) with a four-letter species abbreviation, followed by the gene name and consecutive two-digit numbers (xx). Alleles that differed by just a few bp, identified through mapping to previously identified alleles by Heimeier et al (in press) with 99% identity, were indicated with a second set of two-digit numbers (yy). Since all alleles were derived from humpback whales, allele names in this study are reported without their four-letter species abbreviation (*Meno* for humpback whales) in the following format: gene-name[xx]v[yy] with DQB for class IIa DQB, DRB for class IIa DRB-a, and N for class I as number of loci of class I were unknown. The functionality of alleles was assumed if predicted CDS, inferred from the blue whale reference sequence, was in reading frame with no stop codons or frameshift mutations. Alleles that were only found in one individual, but at high read numbers, were termed singletons. As MHC genes follow a Mendelian inheritance pattern, the inferred paternity trios (see Chapter 2) were used to confirm identified alleles and to infer haplotypes (see Heimeier et al., in press). Similarly, Mendelian inheritance of alleles and haplotypes was used to validate paternity trios (section 5.4.3). As some alleles at MHC class IIa DRB were presumed to be non-functional (section 5.4.1), haplotypes were only inferred for MHC class I.

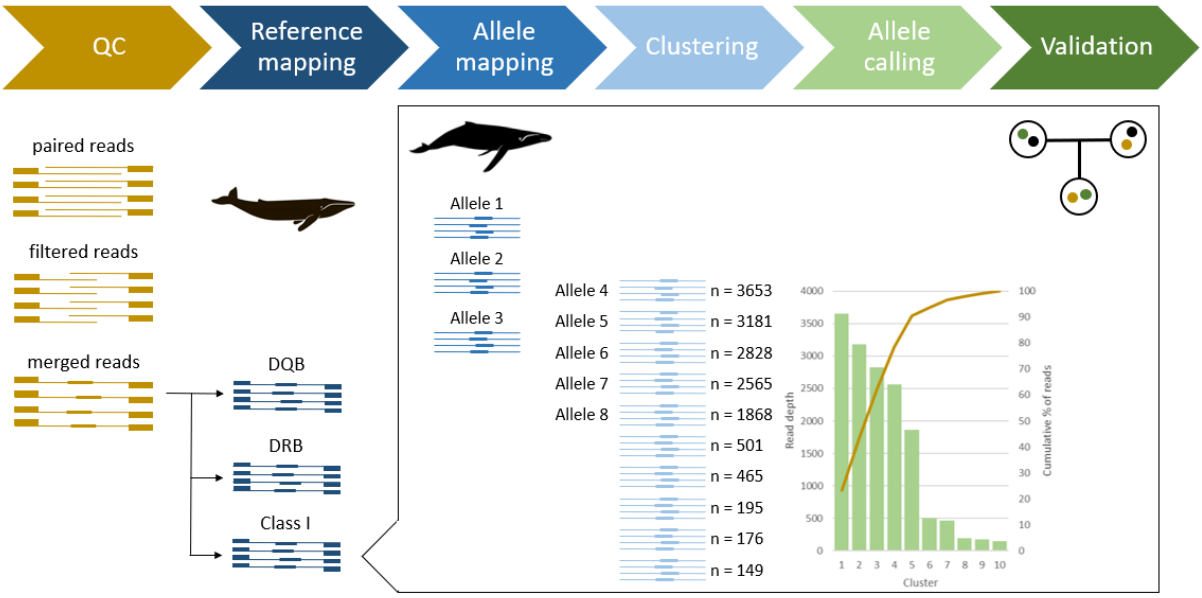


Figure 5.2. Schematic workflow from quality control (QC), mapping to the blue whale reference for each gene, mapping to previously identified alleles in humpback whales by Heimeier et al. (in press), clustering unmapped reads to allele assignment and validation using Mendelian inheritance on previously inferred paternity trios (Chapter 2). The read numbers are arbitrary and for illustrative purposes only. The degree of change (DoC) method was used to distinguish true alleles from artefacts. Coloured circles at the top right illustrate the Mendelian inheritance of alleles within a paternity trios. This graph has been derived from the schematic workflow in Heimeier et al. (in press) and adjusted to the applied methods of this study.

5.3.5 Phylogenetic analysis on sequency diversity

To estimate the phylogenetic distance-based measures of MHC diversity and dissimilarity, I aligned allele sequences and constructed phylogenetic trees for each of the three analysed MHC regions (class I, class IIa DQB, class IIa DRB) at the level of 1) nucleotides and 2) amino acids (predicted CDS from blue whale reference only) in the software R (v4.2.2.; R Core Team, 2022). MHC class I and class IIa were analysed separately due to the different types of pathogens they target (section 5.2). Further, MHC class IIa genes were analysed separately as DRB could only be analysed at the nucleotide level due to stop codons interrupting the reading frame in some DRB alleles (section 5.4.1). For each gene, sequences were aligned using the function 'AlignSeqs' (package: DECIPHER; Wright, 2016) with the blue whale reference sequence. Pairwise distance between allele sequences was computed ('dist.ml', package: phangorn; Schliep, 2011) to perform the neighbour-joining (NJ) tree estimation of Saitou and Nei (1987; 'NJ', package: phagorn; Schliep, 2011). The NJ algorithm allows for unequal rates of evolution so that branch lengths are proportional to the amount of change. The NJ tree estimation in phangorn produces an unrooted tree. Thus, NJ trees were rooted with the blue whale reference sequence as an outgroup in a separate step ('root', package: ape; Paradis and

Schliep, 2019) for further analyses. All phylogenetic methods are based on assumptions about the process of nucleotide and amino acid substitution (Felsenstein, 1988). The construction of phylogenetic trees therefore depends on the underlying model of evolution used in its phylogenetic inference. Different nucleotide or amino acid substitution models were tested for each tree ('modelTest', package: phangorn; Schliep, 2011) to find the best fitting model of evolution for each MHC gene. The best model based on Bayesian information criterion (BIC) was selected for each MHC gene and incorporated in the previously constructed NJ tree ('pml', package: phangorn; Schliep, 2011). Edge length (i.e., genetic distance between alleles) and tree topology (using nearest neighbour interchange) of the updated phylogenetic tree were optimized ('optim.pml', package: phangorn; Schliep, 2011). A subsequent non-parametric bootstrap analysis (100 iterations) was performed ('bootstrap.pml', package: phangorn; Schliep, 2011) to assess the confidence of internal edges. As bootstrapping unroots the trees, all final trees were rooted again with the blue whale reference sequence as an outgroup in a separate step ('root', package: ape; Paradis and Schliep, 2019) for the calculation of phylogenetic distances.

Next, a Binary Operational Taxonomic Unit (OTU) table was constructed based on the presence/absence of a particular allele in a genotype (i.e., individual). Information from the OTU table and the constructed phylogenetic tree were combined ('phyloseq', package: phyloseq; McMurdie and Holmes, 2013) to calculate the phylogenetic distance between alleles (based on nucleotide and amino acid sequences) within an individual (MHC diversity, section 5.3.6) and between individuals (MHC dissimilarity, section 5.3.7).

5.3.6 Genetic diversity within individuals

Within individual genetic diversity at each MHC gene (class I, class IIa DQB and class IIa DRB) was estimated via a phylogenetic distance-based metric at the level of 1) nucleotides and 2) amino acids, and via an composition-based metric at the level of 3) alleles (Table 5.1). As the phylogenetic distance-based metric, I calculated Faith's phylogenetic diversity (FPD) using the NJ trees built in section 5.3.5 based on nucleotide and amino acid sequences. FPD was calculated as the sum of all branch lengths within a phylogenetic tree separating taxa (here: alleles) in a community (here: individual) (Faith 1992). A higher FPD means more and/or longer

branches, thus, more diversity (i.e., larger phylogenetic distance). Additionally, I assessed whether FDP across MHC genes is correlated to see whether individuals who were more diverse at one MHC gene were also more diverse at other MHC genes. As the composition-based metric at the level of alleles, I used allelic richness (AR). AR is the total number of different alleles an individual carries. Both metrics (FPD and AR) were calculated using the function 'pd' (package: picante; Kembel *et al.*, 2010).

5.3.7 Genetic dissimilarity between individuals

The genetic dissimilarity between individuals was estimated via a phylogenetic distance-based metric at the level of 1) nucleotides and 2) amino acids, and via a composition-based metric at the level of 3) alleles and 4) haplotypes. For the phylogenetic distance-based metric, I used the unique fraction metric (UniFrac). UniFrac is commonly used for computing differences between microbial communities based on phylogenetic information (Lozupone and Knight, 2005). Here, I used weighted UniFrac to calculate the phylogenetic distance between two individuals in the NJ phylogenetic tree generated in section 5.3.5, as a fraction of the branch length of the tree that leads to either individual but not both (Lozupone and Knight, 2005). All alleles found in one or both individuals are placed on a phylogenetic tree. Branches leading to alleles from both individuals are marked shared, while branches leading to alleles that appear in only one individual are marked as unshared. UniFrac then calculates the fraction of unshared branch lengths between the two individuals. To account for the relative abundance of alleles shared between individuals rather than just presence/absence, I applied the weighted UniFrac. I opted for weighted UniFrac as it accounts for the relative abundance of alleles shared between individuals rather than just presence/absence of alleles in unweighted UniFrac. In weighted UniFrac, the length of a branch is weighted by the difference of the fractions of sequences (nucleotides or amino acids) belonging to the branch for the two individuals (Lozupone et al., 2007). Weighted UniFrac distance was calculated using 'unifrac' (package: phyloseq; McMurdie and Holmes, 2013) as developed by Lozupone et al. (2007). Values range from 0 (identical) to 1 (nothing shared).

As a composition-based metric, I used the percentage difference (PD) in allele and haplotype composition (i.e., allele and haplotype sharing). PD is calculated purely on a

shared/non-shared basis at the level of 3) alleles for class I and class IIa (DQB and DRB combined), and 4) haplotypes for class I as described in equation 1 and following Strandh et al. (2012). This dissimilarity metric works for MHC regions with variable gene copy number, as observed in MHC class I of humpback whales (Heimeier et al, in press, section 5.4.2 this study). As both class IIa genes (DQB and DRB) carry up to two alleles each, they were combined for the calculation of PD to allow for more possible differences in allele composition and maximise information content. PD in haplotype composition was only calculated for MHC class I as haplotypes for class II were not inferred.

$$\mathrm{PD} = \frac{V_{ab}}{F_a + F_b}$$

V_{ab} = total number of variable alleles/haplotypes present in individuals a and b (shared alleles/haplotypes count as 1)

F = number of alleles/haplotypes within an individual

equation 1

5.3.8 Assessing MHC-mediated mating choice in humpback whales

To test if mating pairs are more genetically dissimilar at their MHC than expected under random mating from the sampled gene pool, I compared the genetic dissimilarity of observed mating pairs (based on the paternity analysis in Chapter 2) with the genetic dissimilarity of randomly sampled pairs of mothers and mature males. This was accomplished by a simulation test that randomly paired a mother from the previously validated paternity trios (n = 58, see section 5.4.3) with a male presumed to be old enough to reproduce ('candidate fathers') in the year she sired her offspring. This step was repeated to reach a total of 1,000 permutations with each permutation consisting of 58 simulated mating pairs.

The presence of an MHC-mediated mating preference was assessed at all four levels (nucleotides, amino acids, alleles and haplotypes) at all three MHC genes (Table 5.1). For that I used two previously calculated pairwise genetic dissimilarity metrics (see section 5.3.7): pairwise phylogenetic distance (weighted UniFrac) at the level of 1) nucleotides, and 2) amino acids, and the percentage difference (PD) in 3) allele, and 4) haplotype composition. Note that at the haplotype level, the analysis was based on 56 mating pairs instead of the 58 mating pairs at the allele level (see section 5.4.2).

To assess significance, I first calculated the percentage of permutations for which the mean genetic dissimilarity of observed mating pairs for each metric (observed dissimilarity) was larger (more dissimilar) than that of simulated mating pairs (simulated dissimilarity). In the presence of an MHC-mediated mating preference I expect a larger percentage of permutations for which observed dissimilarities are higher than the simulated dissimilarities.

5.4 Results

5.4.1 MHC genes and phylogenies

A total of 44 class IIa (DQB: 23; DRB: 21) and 45 class I alleles were found in 335 individual humpback whales (Table 5.2). Among identified alleles, 8 alleles (termed singletons) were found in only one individual but at high read numbers. All 8 singletons were on class IIa, of which 3 were on DQB (DQB21s-DQB23s) and 5 on DRB (DRB06s, DRB18s-DRB21s)(Table S5.3). All but one singleton of the 9 identified in Heimeier et al. (in press) across MHC class IIa and MHC I genes from the initial 30 humpback whale individuals were found in more individuals when including an additional 305 individuals in this study. I thus expect the singletons identified in the current study to also be found in more individuals (and therefore lose their singleton status) with a larger sample size. Of the 45 class I alleles, 5 were presumed to be non-classical by Heimeier et al. (in press), as they were found in almost all analysed individuals (Table S5.3). Non-classical alleles are more conserved and less variable than classical alleles, but their role in immune defence is not as well understood. Since the aim of this study was to assess genetic diversity, I only included the 40 classical alleles for MHC class I. All DQA and class I alleles were in reading frame and assumed to be functional. However, when using the same reading frame as predicted for the blue whale, 10/21 DRB alleles were predicted to be non-functional with multiple stop codons (Table S5.3). While these 10 DRB alleles may be functional with an alternative reading frame (see Heimeier et al., in press), this was not assessed within this study. DRB alleles were thus only analysed using their nucleotide sequences (Table 5.1). Phylogenetic trees of identified alleles at all three MHC regions are shown in Figure 5.3.

Table 5.2. Overview of the number of MHC alleles identified. Columns show the total number of reads that mapped to each MHC gene on the blue whale reference sequence, the number of individuals successfully sequenced (after quality control), the number of alleles that were found across all individuals at each MHC gene, the number of alleles that were presumed to be functional based on the predicted CDS, and the mean number of different MHC alleles each individual (i.e., genotype) carries. Some individuals were not successfully sequenced at class IIa or were excluded due to unresolved genotypes (section 5.4.2). Abbreviations: Ave.: average, var: variation, min: minimum, max: maximum.

MHC		Mapped reads	# individuals	# alleles	# functional alleles	Ave. alleles per individual (±var) [min-max]
Class I	Classical	1,172,775	335	40	40	3.6±1.1 [1-6]
	Non-classical	913,947	335	5	5	3.7±0.8 [1-5]
	Total	2,086,722	335	45	45	7.3±1.9 [4-10]
Class IIa	DQB	1,745,849	334	23	23	1.9±0.1 [1-2]
	DRB	979,919	330	21	11	1.5±0.2 [1-2]
	Total	2,725,768	329	44	34	3.4±0.3 [2-4]
Total		4,812,490	335	89	79	10.7±2.3 [7 -14]

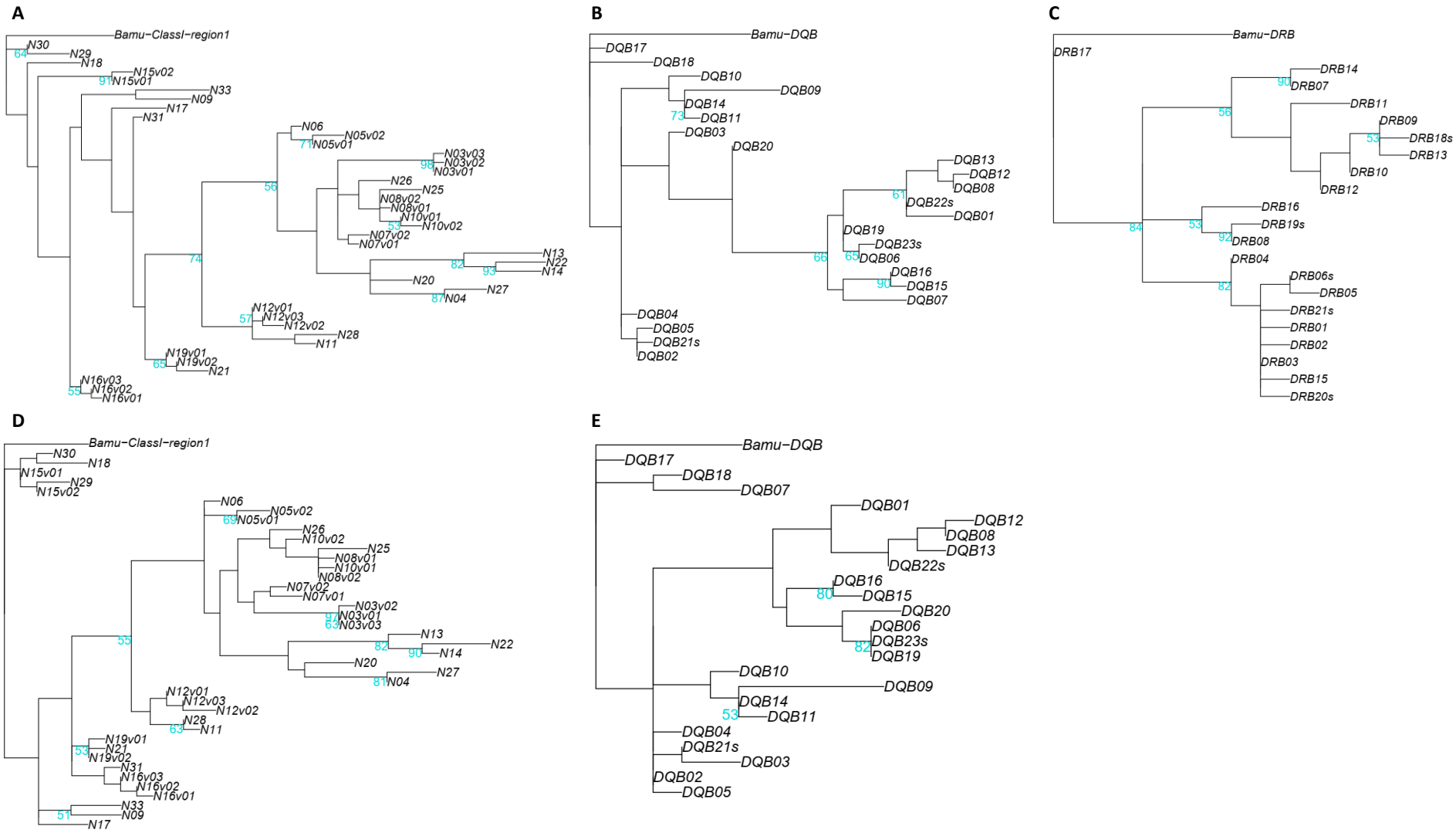


Figure 5.3. Phylogenetic trees of MHC class I (A and D), class IIa DQB (B and E) and DRB (C) based on nucleotide sequences (A-C) and amino acid sequences (D-E). All phylogenetic trees were rooted using the respective blue whale reference sequence. MHC class IIa DQB and DRB cluster separately in a combined MHC class IIa phylogenetic tree (Figure S5.1).

5.4.2 MHC genotypes and haplotypes

A total of 335 individuals were typed at MHC class I and class IIa (DQB and DRB). Four individuals failed to be typed at DRB (individuals – sample: HNC402 – NC99-029; HNC770 – NC11-071; NI13038 – NC13-125; NI16026 – NC16-092). Two individuals were removed from the analyses due to unresolved genotypes at DQB (NI0819 - NC08-128 at DQB) and DRB (HNC335 – NC05-053 at DRB) as read numbers for both indicated four alleles instead of the maximum of two alleles that were expected for MHC class IIa DQB and DRB each. All other 329 individuals carried 1 – 2 unique alleles at each class IIa region (DQB and DRB). All individuals were successfully typed at class I with 1 - 6 alleles. Only the 329 individuals typed at all three MHC regions (class I, class IIa DQB and DRB) were used for further analyses.

Classical MHC class I alleles were further resolved into 44 haplotypes (Figure 5.4). These haplotypes consisted of 1 – 3 different alleles, thus showing variable gene copy numbers (Table S5.4). Of the 40 identified classical MHC class I alleles (section 5.4.1), all but three alleles (N15v02, N17, and N22) were confidently assigned to at least one haplotype. Several alleles were found in more than one haplotype; e.g., allele N10v01 occurred in 7 different haplotypes (Table S5.4). Most haplotypes (39/44) were confirmed as inherited in the paternity trios (thus with a high level of confidence). Only 5 haplotypes (H06, H26, H40, H43, H44) were not confirmed as inherited in the paternity trios yet were inferred from parent-offspring relationships (medium confidence). Most individuals were assigned one or two different haplotypes except for 21 individuals (including individuals from two paternity trios) for which the assignment of haplotypes was unclear based on the alleles they carried. Thus, for analyses at the haplotype level, the dataset was further reduced to a total of 308 individuals.

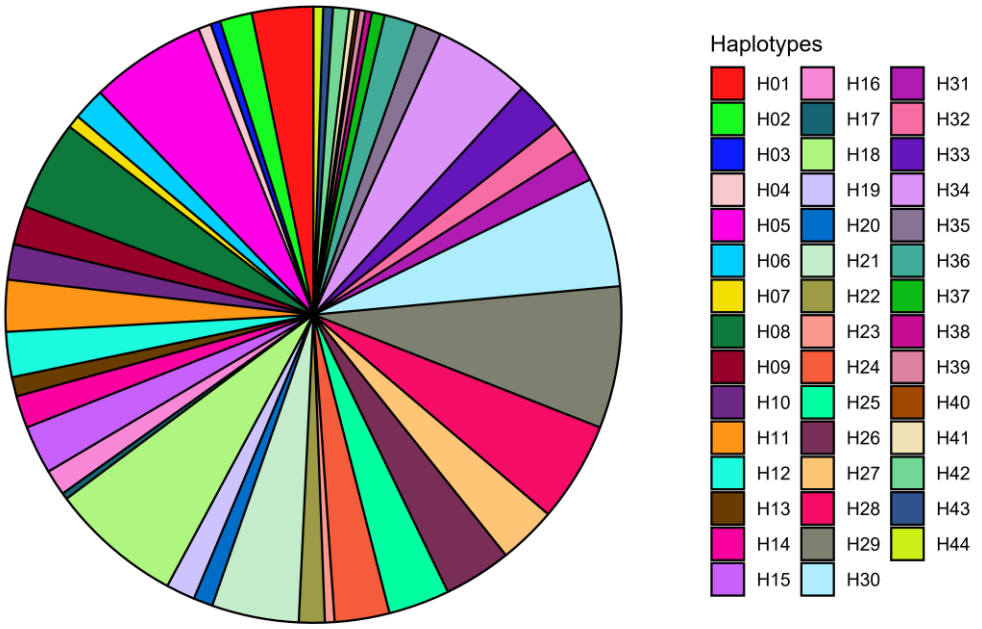


Figure 5.4. The proportion of MHC class I haplotypes (n = 44) in the sampled population.

5.4.3 Validation of paternity through Mendelian inheritance at MHC

Alleles at assessed MHC genes (class I, class IIa DQB and DRB) are passed on from parents to offspring following a Mendelian inheritance pattern (Heimeier et al, in press). Microsatellite inferred paternities (Chapter 2) were considered validated if alleles of mother-offspring pairs (maternity) and father-mother-offspring trios (paternity) matched a Mendelian inheritance pattern at class I and class IIa genes (DQB and DRB). Maternity was confirmed in all 63 mother-offspring pairs (Table S5.5). Paternity was validated based on the inheritance of alleles at all three MHC genes for 58/63 (92.0 %), of which all but two (Trio 32 and 47) were also validated based on the inheritance of class I haplotypes (Table S5.5). Two paternity trios (Trio 32 and 47) showed incomplete inheritance of the haplotype (i.e., not all alleles within a haplotype were passed on from parent to offspring) from mother to offspring (Trio 47) and from putative father to offspring (Trio 32) due to possible allelic dropout, incomplete sequencing, or mutation. Paternity (father-offspring) was rejected in 4/63 (6.4 %) trios (Trio 8, 9, 10, 13) which were then excluded from all further analyses. All four rejected paternity trios were part of the relaxed paternity dataset comprised of paternities assigned at a lower confidence compared to the conservative paternity dataset (see Chapter 2). In 1/63 (1.6 %) paternity trios (Trio 19), the genotypes of mother and putative father at all three MHC genes were very similar, thus, suggesting high relatedness between individuals in this trio. Although paternity could not be rejected for this trio based on Mendelian inheritance, it is unclear whether this is due to a true

parental relationship or shared ancestry. This paternity trio (19) was thus also excluded, leaving a total of 58 paternity trios for all further analyses at the level of nucleotides, amino acids and alleles. For analyses at the level of haplotypes, only the 56 paternity trios that were validated at the level of both alleles and haplotypes were included.

5.4.4 Genetic diversity within individuals

Within individual genetic diversity at each MHC region was estimated at the nucleotide and amino acid level using Faith's phylogenetic diversity (FPD) and at the allele level using allelic richness (AR) within the sample population (n = 329 individuals). FDP based on nucleotide and amino acids sequences between the alleles an individual carries were considerably higher and more variable at class I than class II genes (Figure 5.5; Table 5.3). Within class IIa, FDP was higher and more variable at DQB than DRB (nucleotide sequences only) (Figure 5.5, Table 5.3). Further, FPD derived from the amino acid sequences of alleles were much higher compared to nucleotide sequences in general. FPD was not correlated across MHC regions using both nucleotide and amino acid sequences; individuals that showed a higher diversity at class I did not also show a higher diversity at any of the two class IIa genes, or vice versa (Figure 5.6).

The number of different alleles individuals carried (here: AR) was considerably higher overall and more variable across individuals at class I than class IIa. The number of different class I alleles ranged between 1 to 6 alleles per individual (Table 5.3), matching previous findings of variable gene copy number at the MHC class I gene in humpback whales (Heimeier et al., in press). Most individuals carried 3 – 4 different alleles at class I and between 1 – 2 different alleles per locus at class IIa (Figure 5.7). While most individuals appear to be heterozygous at DQB, almost half of the population appears to be homozygous at DRB (Figure 5.7). Overall, MHC diversity was consistently higher at class I compared to class II across both phylogenetically-based and composition-based metrics.

Table 5.3. Summary statistics on MHC diversity metrics: Faith's phylogenetic distance (FPD) at the level of 1) nucleotides and 2) amino acids, and allelic richness (AR) at the level of 3) alleles. This table complements Figure 5.5 and Figure 5.7. Abbreviations: sd: standard deviation; var: variance; [min, max]: range of values (minimum, maximum).

MHC region	Level	Diversity metrics	mean	sd	var	[min, max]
Class I	nucleotides	FPD	0.17	0.04	0.001	[0.05, 0.26]
DQB	nucleotides	FPD	0.05	0.02	<0.001	[0.01, 0.09]
DRB	nucleotides	FPD	0.02	0.01	<0.001	[0.02, 0.04]
Class I	amino acids	FPD	0.40	0.10	0.010	[0.08, 0.70]
DQB	amino acids	FPD	0.14	0.04	0.002	[0.02, 0.25]
Class I	alleles	AR	3.59	1.04	1.085	[1.00, 6.00]
DQB	alleles	AR	1.86	0.35	0.121	[1.00, 2.00]
DRB	alleles	AR	1.56	0.50	0.247	[1.00, 2.00]

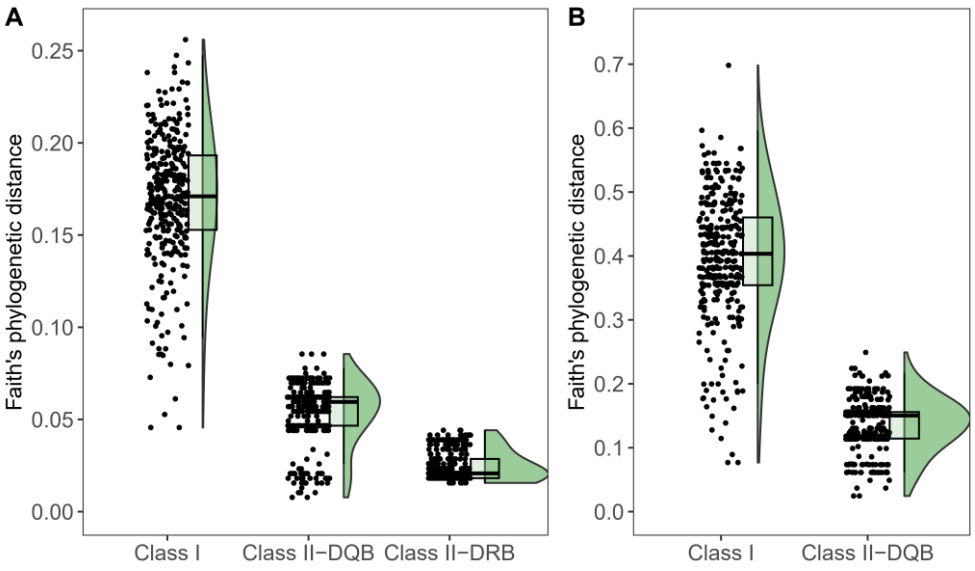


Figure 5.5. The genetic diversity of individuals in the population was estimated using Faith's phylogenetic distance (PD) at the level of A) nucleotide and B) amino acid sequences of alleles at the three MHC genes (class I, class IIa DQB and class IIa DRB). Note the different scales of the y-axes in A) and B).

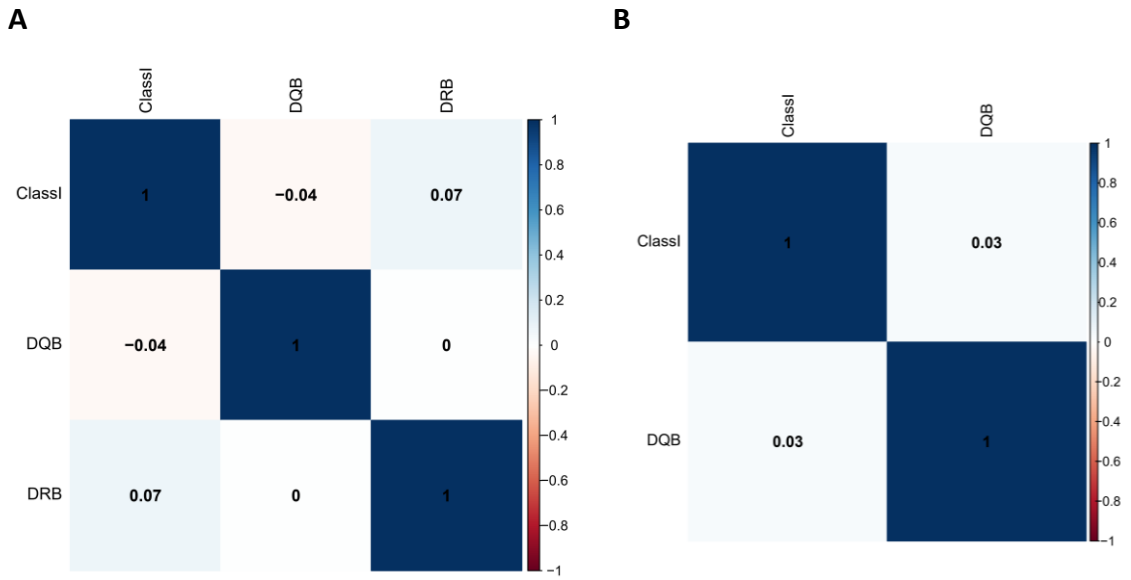


Figure 5.6. Correlation of Faith's phylogenetic distance calculated at the level of A) nucleotides and B) amino acids, between the three MHC genes (class I, class IIa DQB and class IIa DRB). Graphs were generated via the 'corrplot' function (package: corrplot, Wei and Simko, 2021) using the Pearson correlation coefficient. This shows that there is little correlation between high levels of diversity in one gene being present in another (all correlation were non-significant using a significance threshold of 0.05).

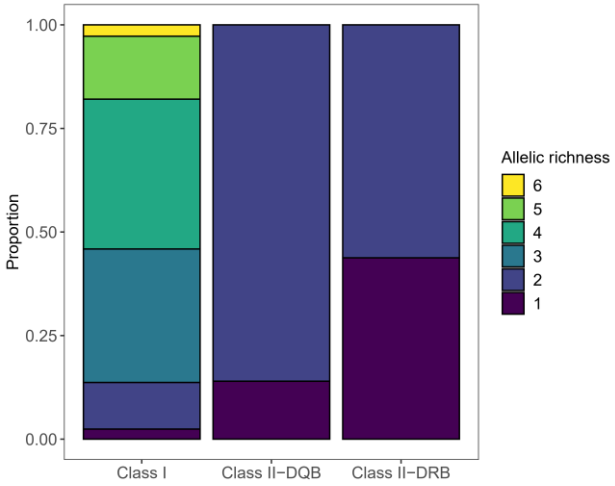


Figure 5.7. The number of alleles individuals in the population carry (allelic richness) at each MHC gene.

5.4.5 Genetic dissimilarity between individuals

The genetic dissimilarity between individuals at each MHC region was estimated using weighted UniFrac for pairwise phylogenetic distance and percentage difference (PD) for pairwise differences in allele and haplotype composition in the population (n = 329 individuals). Weighted UniFrac between pairs of individuals in the sampled population (n = 329) was similar across all analysed MHC genes at the allele level using both nucleotide sequences and amino acid sequences (Figure 5.8, Table 5.4). There was a large variation in the phylogenetic distance between pairs of individuals spanning almost the entire range of possible UniFrac values (0 to 1) (Table 5.4). Distributions of the pairwise phylogenetic distances at the three MHC genes at the nucleotide and amino acid level are largely unimodal, however, with the exception of a slightly bimodal and even tri-modal distribution at the nucleotide level at DQB and DRB, respectively. This is likely due to the lower number of different alleles individuals carry at each of the two class IIa genes (up to 2 alleles) compared to class I (up to 6 alleles) (Table 5.2).

Pairwise differences in the allele composition (PD) were less variable than pairwise phylogenetic distances (weighted UniFrac) at class I (Figure 5.9, Table 5.4). Most pairs of individuals had an entirely different set of alleles at class I, and to a lesser extent at class IIa (Table 5.4). Overall, individuals were more genetically dissimilar based on the composition of the alleles rather than based on their phylogenetic distance.

Table 5.4. Summary statistics on two MHC dissimilarity metrics: pairwise phylogenetic distance (weighted UniFrac) at the level of 1) nucleotides and 2) amino acids, and percentage difference (PD) in 3) allele and 4) haplotype composition. This table complements Figure 5.8 and Figure 5.9. Abbreviations: sd: standard deviation; var: variance; [min, max]: range of values (minimum, maximum).

MHC region	Level	Dissimilarity metrics	mean	sd	var	[min, max]
Class I	nucleotides	UniFrac	0.33	0.14	0.019	[0, 0.81]
DQB	nucleotides	UniFrac	0.39	0.24	0.055	[0, 0.92]
DRB	nucleotides	UniFrac	0.25	0.21	0.045	[0, 0.80]
Class I	amino acids	UniFrac	0.41	0.16	0.024	[0, 0.89]
DQB	amino acids	UniFrac	0.44	0.23	0.052	[0, 1.16]
Class I	alleles	PD	0.94	0.10	0.011	[0.50, 1.00]
Class II	alleles	PD	0.73	0.13	0.017	[0.25, 1.00]
Class I	haplotypes	PD	0.96	0.09	0.008	[0.50, 1.00]

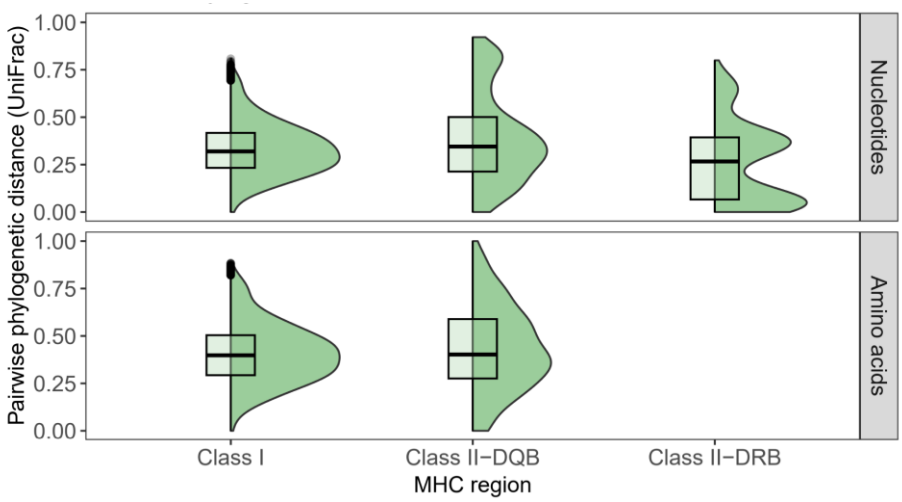


Figure 5.8. Pairwise phylogenetic distance (UniFrac) in the population (n = 329 individuals) at the level of 1) nucleotides and 2) amino acids at three different MHC regions.

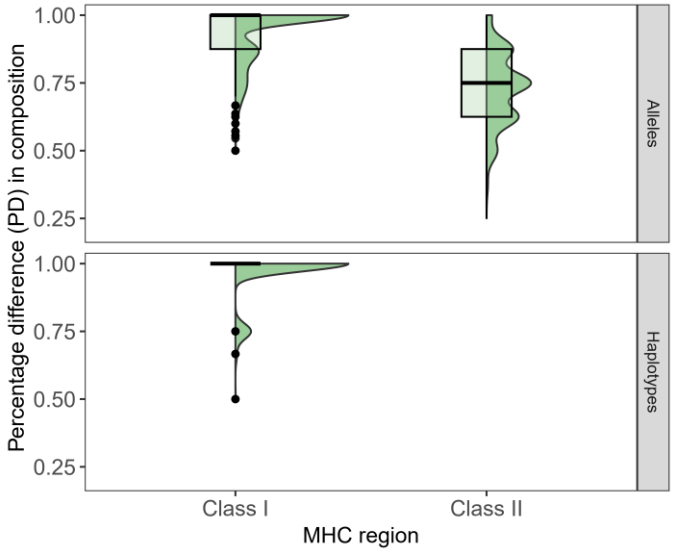


Figure 5.9. Percentage difference (PD) in 3) allele and 4) haplotype composition in the population (n = 329 individuals) at MHC class I and class IIa.

5.4.6 Assessing MHC-mediated mating choice in humpback whales

To test the hypothesis of MHC-mediated mate choice in humpback whales I compared the genetic dissimilarity of validated mating pairs (see section 5.4.3; observed dissimilarity) with the genetic dissimilarity of other male-female pairs in the population (expected dissimilarity). The genetic dissimilarity, using both phylogenetically-based (here: weighted UniFrac) and composition-based (here: PD) metrics, of mother-father pairs at all three MHC genes was very similar to male-female pairs in the sampled population at all four levels (nucleotides and amino acids in Figure 5.10; alleles and haplotypes in Figure 5.11). However, some male-female pairs were genetically very similar and comparable to several mother-offspring and father-offspring pairs, suggesting that some of these male-female pairs were related. Within paternity trios, mother-offspring pairs and father-offspring pairs were, as expected, less genetically dissimilar than mother-father pairs (Figure 5.10 and Figure 5.11).

To test if validated mating pairs (n = 58) were more genetically dissimilar at MHC than under random mating, 1,000 simulations were run with randomly selected pairs of mothers and mature males. There was no clear pattern for an MHC-mediated mating choice in either direction (dissimilarity/similarity) based on the phylogenetical distance metric (weighted UniFrac) at all assessed MHC genes (Figure 5.12). The percentage of simulations for which the mean genetic dissimilarity of validated mating pairs was larger (more dissimilar) than that of simulated pairs ranged between 21% at DQB to 75% at class I (Table 5.5). However, the percentage of simulations for which the observed mating pairs were more dissimilar than simulated mating pairs was much larger when genetic dissimilarity was based on the percentage difference (PD) in the composition of alleles and haplotypes compared to their phylogenetic distance. In more than 90% of all simulations, observed mating pairs were more dissimilar than randomly selected mother and mature male pairs at MHC class I based on both allele and haplotype composition (Figure 5.13, Table 5.5). At class II this was slightly lower (allele composition: 85%) but higher compared to the simulations on the genetic dissimilarity of mating pairs based on phylogenetic distance. Although not in the range of statistical significance (α = 5%), there is a pattern of observed mating pairs being more dissimilar than expected under random mating when MHC dissimilarity is based on allele and haplotype composition.

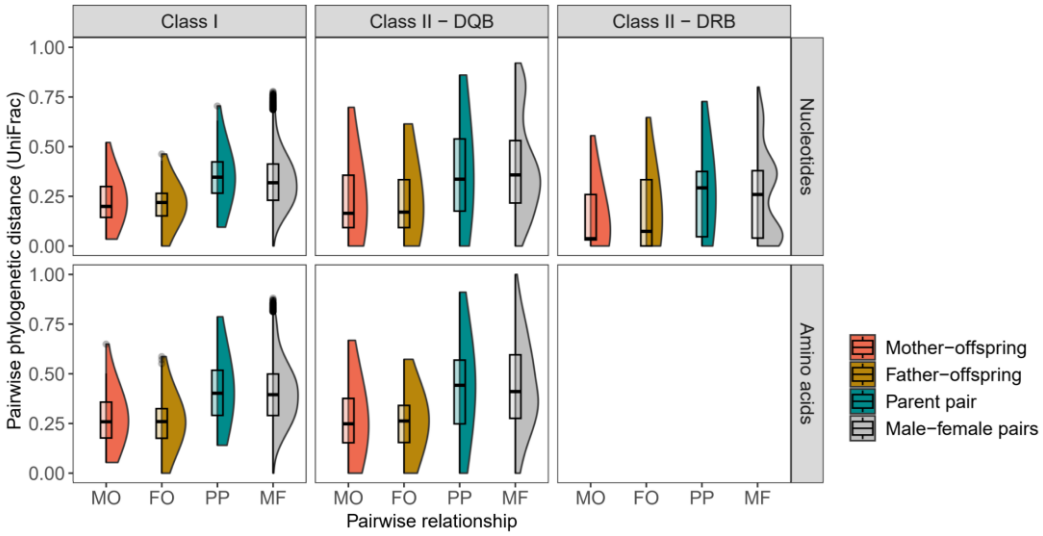


Figure 5.10. Genetic dissimilarity based on a pairwise genetic distance metric (UniFrac) between mother-offspring (MO), father-offspring (FO) and parent pairs (PP) of validated paternity trios (n = 58 trios), contrasted with all other possible pairwise combinations of male-female pairs (MF) in the sampled population.

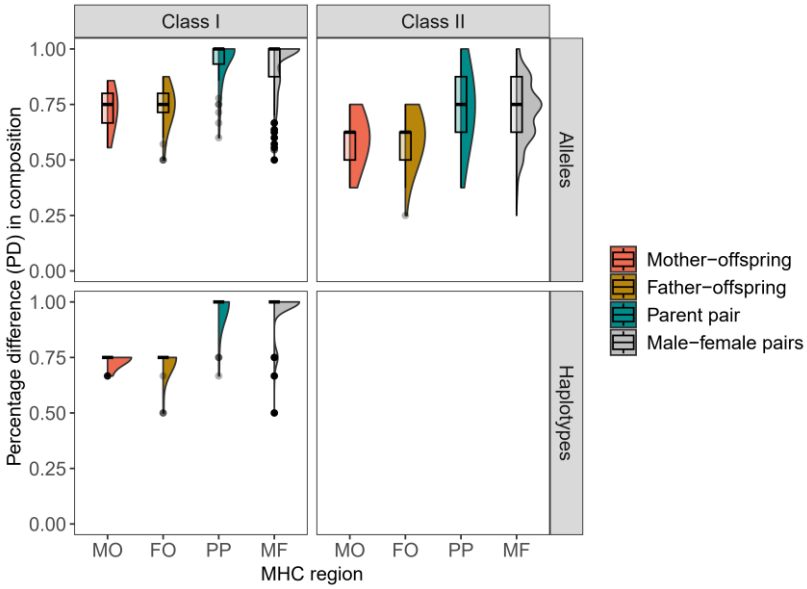


Figure 5.11. Percentage difference (PD) in allele and haplotype composition between mother-offspring (MO), father-offspring (FO) and parent pairs (PP) of validated paternity trios (n = 58 trios), contrasted with all other possible pairwise combinations of male-female pairs (MF) in the sampled population.

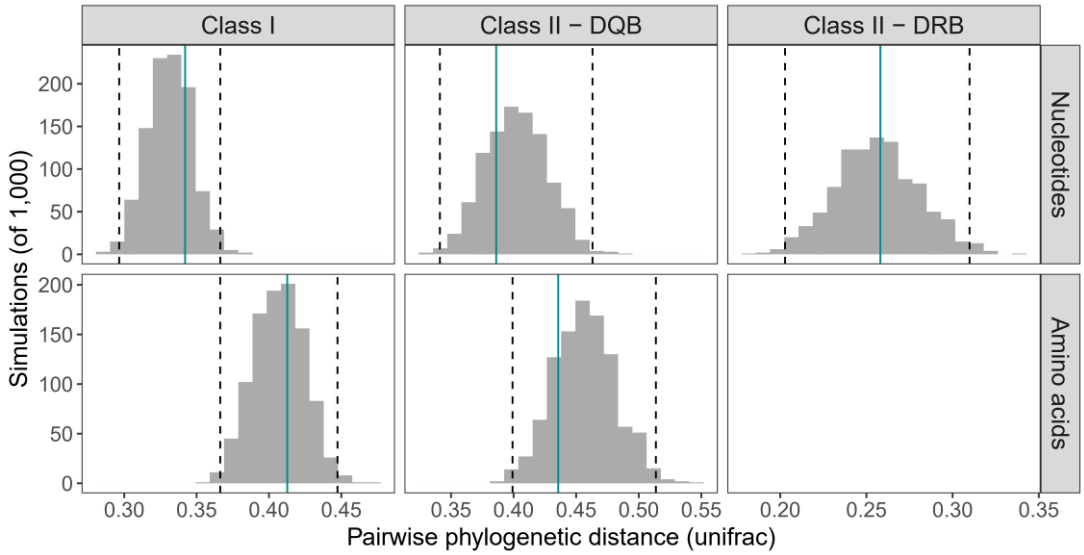


Figure 5.12. Mean pairwise genetic distance (UniFrac) of observed mating pairs (blue vertical line) compared to the distribution of the mean UniFrac of simulated mating pairs for each permutation (n = 1,000 simulations, grey histogram). The dashed lines indicate the 95% confidence interval across all simulated mating pairs.

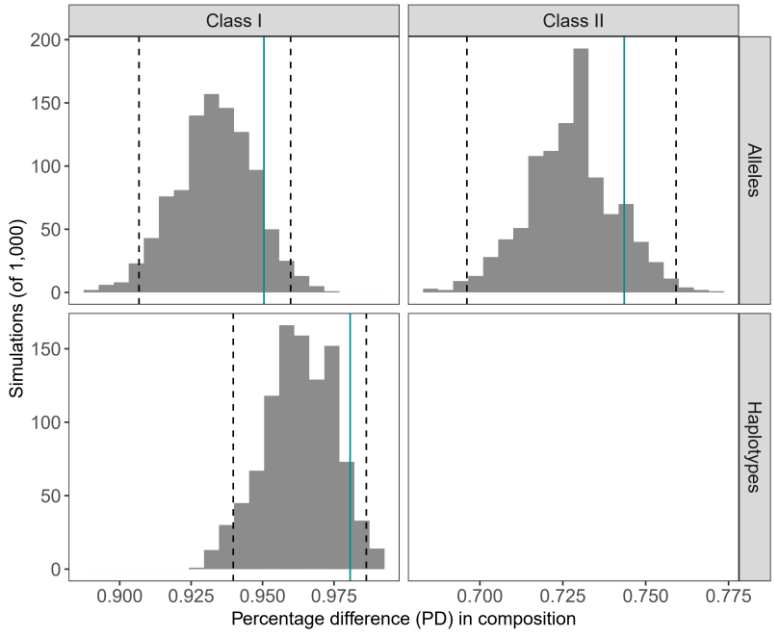


Figure 5.13. Mean percentage difference (PD) in allele and haplotype composition of observed mating pairs (blue vertical line) compared to the distribution of the mean PD of simulated mating pairs for each permutation (n = 1,000 simulations, grey histogram). The dashed lines indicate the 95% confidence interval across all simulated mating pairs.

Table 5.5. Percentage of permutations for which the mean genetic dissimilarity of observed mating pairs for each metric (observed dissimilarity) was larger (more dissimilar) than that of simulated mating pairs (simulated dissimilarity). The genetic dissimilarity between pairs was estimated using two different metrics: pairwise phylogenetic distance (UniFrac) at the level of nucleotides and amino acids and percentage difference (PD) in allele and haplotype composition.

MHC	Level	Dissimilarity metrics	Simulation mean	Observation mean	Obs > Simu (%)
Class I	nucleotides	UniFrac	0.331	0.342	75.1
Class II - DQB	nucleotides	UniFrac	0.402	0.386	27.0
Class II - DRB	nucleotides	UniFrac	0.256	0.258	53.8
Class I	amino acids	UniFrac	0.407	0.413	62.4
Class II - DQB	amino acids	UniFrac	0.456	0.436	21.2
Class I	alleles	PD	0.933	0.950	90.5
Class II	alleles	PD	0.728	0.744	84.8
Class I	haplotypes	PD	0.963	0.981	93.2

5.5 Discussion

5.5.1 The role of MHC-mediated mating choice in humpback whales

MHC-mediated mate choice where an individual shows a preference for mating partners that are more genetically dissimilar to themselves (MHC compatibility) serves as one potential mechanism to maintain and increase the genetic diversity and immunocompetence of a population (Piertney and Oliver, 2005). My results from the phylogenetic analysis show no clear signs that humpback whales prefer to mate with individuals that are more dissimilar at MHC based on nucleotide and amino acid sequences. However, when using a non-sequence composition-based dissimilarity metric (PD), most observed mating pairs were more dissimilar at MHC class I and class II (i.e., shared less alleles and haplotypes with each other) than randomly selected mating pairs. This contrasting pattern of MHC dissimilarity based on phylogenetic distance and allele/haplotype sharing could reflect the different components of genetic diversity measured by the two dissimilarity metrics. The phylogenetic distance-based metric (weighted UniFrac) measures genetic variation resulting from differences between the nucleotide and amino acid sequences of alleles between individuals. The composition-based metric (percentage difference (PD) in allele and haplotype composition) takes into account the identity and number of alleles individuals share irrespective of how different these alleles are based on their sequence. This means that while UniFrac captures sequence variation and

evolutionary distance, PD measures the structural variation, including variation in gene copy number.

There was variable gene content of classical class I MHC genes on haplotypes (section 5.4.2, Heimeier et al, in press) which was further supported by the inheritance analysis of paternity trios (section 5.4.3). MHC class I haplotypes had one to three classical class I genes that resulted in individuals carrying one to six different classical class I alleles. This is similar to MHC of cattle where haplotypes contain between one to four classical class I genes (Codner *et al.*, 2012; Hammond *et al.*, 2012). Structural variation, including copy number variation (CNV), is one predominant source of genetic variation in humans (Zhang *et al.*, 2009). Further, in a simulation analysis investigating the evolution of the number of MHC variants, the number of unique MHC variations (i.e., alleles) carried by individuals was positively correlated with host immunocompetence (proportion of pathogens recognised) (Bentkowski and Radwan, 2019). CNV likely has functional significance and may explain some phenotypic variation not captured by sequence-based studies (Manolio *et al.*, 2009). It may thus be that in the current study, the composition-based metric PD at the level of alleles and haplotypes captured genetic variation that is more functionally relevant than the sequence variation at the level of nucleotides and amino acids measured by UniFrac. Although this serves as a possible explanation for the contrasting patterns of MHC dissimilarity of mating pairs derived from the two different dissimilarity metrics, it does not conclusively confirm the presence of an MHC-mediated mate choice in humpback whales.

There are three possible explanations for the higher dissimilarity (composition-based metric PD, i.e., allele and haplotype sharing) at MHC class I and class II of the observed mating pairs compared to randomly selected mating pairs. MHC-mediated mate choice could occur where individuals choose genetically dissimilar mating partners as a way (1) to increase the genetic benefits in terms of pathogen resistance of their offspring, and/or (2) to avoid mating with closely related individuals (inbreeding avoidance), or (3) MHC-unrelated mating preference as a mechanism of inbreeding avoidance based on a non-MHC-related signal. If individuals avoid mating with closely related individuals, then regardless of the sensory and genetic mechanism underlying mate recognition, we would expect a non-random pattern of (neutral and functional) genetic diversity with unrelated individuals being more genetically dissimilar than closely related individuals. Thus, while the first two non-mutually exclusive

explanations of MHC-mediated mate choice require individuals to signal their own and assess other's MHC genotype, MHC-unrelated mate choice would not.

MHC signalling is wide-spread across vertebrate taxa and plays an important role in mate choice (Santos *et al.*, 2016). The signalling of MHC genotypes can occur directly through molecular mechanisms (e.g., MHC-mediated odours) or indirectly by influencing signals of quality (Ruff *et al.*, 2012). Studies have shown that mice can discriminate MHC genotypes by chemical cues (e.g., urinary peptides) detected by the olfactory system (Yamaguchi et al., 1981; Singh, Brown and Roser, 1987; Sturm et al., 2013). Olfactory cues may be key mediators in mammalian MHC-dependent mate choice (Santos *et al.*, 2016), however, their function and importance in cetaceans remains unclear. Compared to their toothed phylogenetic relatives (odontocetes), baleen whales are suggested to have a functional olfactory system (Berta, Ekdale and Cranford, 2014). For example, bowhead whales (*Balaena mysticetus*) have large, well-developed olfactory bulbs (Thewissen *et al.*, 2011). Further, genetic studies have demonstrated mysticetes carry a high proportion of functional genes coding for olfactory receptors (OR) (McGowen, Clark and Gatesy, 2008; Thewissen et al., 2011; Kishida et al., 2015), some of which show signs of adaptation to a novel (marine) environment (Jauhal, 2023). Humpback whales were also found to respond to powdered krill extract and DMS (Bouchard *et al.*, 2019), indicating they have a functioning olfactory system. Anatomical, genetic and behavioural studies combined support the functional role for olfaction in mysticetes and the notion that baleen whales may be able to smell (Jauhal, 2023). It thus remains an open question whether mysticetes are able to discriminate MHC genotypes based on chemical cues.

MHC genotype information can not only be conveyed directly through MHC-mediated chemical signals but also indirectly by influencing the expression of secondary sexual characters (Ruff *et al.*, 2012). According to the 'handicap hypothesis', only disease-resistant individuals are able to invest in costly, sexually selected displays (Zahavi, 1975). This creates a correlation between MHC genotypes and these condition-dependent morphological or behavioural displays through which individuals signal their quality (Hamilton and Zuk, 1982; Folstad and Karter, 1992). For example, in male song sparrows (*Melospiza melodia*), larger song repertoires (i.e., number of song types) were related to intermediate MHC diversity, thus suggesting that song complexity may signal optimal MHC diversity (Slade, Watson and

MacDougall-Shackleton, 2017). The relationship between song and MHC diversity in humpback whales, to date, remains unexplored. The complex songs and large vocal repertoire of male humpback whales allow for large signal variability between individuals, thus, holding the potential to convey individual quality (Murray *et al.*, 2018). Future studies may assess whether male humpback whale song conveys genetic quality and whether females use that information to assess and choose their potential mates.

Mate choice, MHC-related or not, shapes patterns of reproductive success and so can affect the genetic diversity and recovery of a population. The impact of such reproductive tactics (e.g., mate choice) is expected to become larger with decreasing population size (Frasier *et al.*, 2013). In a large population, most mates chosen at random will meet the criteria of dissimilar/unrelated. However, as the population decreases, so thus the number of dissimilar/unrelated mates. The impact of reproductive tactics on patterns of reproductive success may thus be higher, and thus easier to detect, in smaller populations (Frasier *et al.*, 2013). In the endangered North Atlantic right whale (*Eubalaena glacialis*), post-copulatory selection for dissimilar gametes was found to maintain genetic diversity with offspring having higher levels of microsatellite heterozygosity than expected under random mating (Frasier *et al.*, 2013). The study highlights that reproductive tactics can play an important role in mitigating the loss of genetic diversity of small populations over time (Frasier *et al.*, 2013).

Many baleen whales were exploited intensely across all oceans for several centuries during commercial whaling (Clapham, 2016). However, compared to North Atlantic right whales, humpback whale populations have shown recent increases in abundance, albeit their degree of recovery varies greatly (Jackson et al., 2015; Thomas, Reeves and Brownell, 2016; Noad, Kniest and Dunlop, 2019). The larger population size of New Caledonian humpback whales (Chapter 2), together with at least some level of gene flow among breeding grounds in the South Pacific, might render it more difficult to detect signals of MHC-mediated mate choice. Gene flow between humpback whale breeding grounds may introduce genetic variation of magnitudes greater than any selection acting within populations, including mate choice. Further, the high diversity at MHC, especially at class I, together with the range of natural selection pressures acting on MHC, could further mask or weaken signals of mate choice. Regardless, there is a pattern of female's choosing males that are more dissimilar supporting the possibility of an MHC-mediated mate choice in humpback whales.

5.5.2 MHC diversity in humpback whales

Within-individual genetic diversity of individuals from the New Caledonian humpback whale population (n = 329 individuals) was estimated at all three MHC genes (class I, class IIa DQB and class IIa DRB) at the level of nucleotides and amino acids using Faith's phylogenetic diversity (FPD), and at the level of alleles using allelic richness (AR). MHC diversity was consistently higher at class I compared to class II across all levels and metrics. Individuals also carried up to three times the number of different alleles at class I than either of the class II loci (Table 5.2). This mirrors previous findings for mammalian MHC, as class I is generally more variable compared to class IIa based on its faster evolutionary rate and higher gene copy number (Takahashi, Rooney and Nei, 2000). MHC class I genes are responsible for the immune defence against intracellular pathogens (e.g., viruses), while MHC class II genes are involved in the immune defence against pathogens coming from the extracellular environment (e.g., bacteria) (Sommer, 2005). The contrasting mating patterns in blue petrels (*Halobaena caerulea*) where mating partners were functionally more dissimilar at MHC class II but not class I was suggested to be an effect of the different targets of MHC class I and class II (Strandh *et al.*, 2012). Viruses are the most abundant life in the oceans (Suttle, 2005, 2007), and marine mammals are thus in intimate contact with a vast diversity of viruses throughout their lives (Wellehan and Cortes-Hinojosa, 2019). The prevalence and severity of emerging infectious diseases in cetaceans are expected to increase even further under climate change (van Bressem et al., 2009; Kebke, Samarra and Derous, 2022). The higher diversity at MHC class I compared to class II in humpback whales may indicate higher (natural) selection pressure at class I for the protection and resistance against viruses as compared to bacteria and parasites. This may add to the growing evidence against the view of reduced MHC diversity in marine mammals resulting from diminished selective pressure for mainting MHC polymorphism in the marine environment (Trowsdale, Groves and Arnason, 1989).

Individuals were considerably more genetically diverse based on phylogenetic distance (FPD) of amino acid sequences compared to nucleotide sequences (Figure 5.5, Table 5.3), suggesting that nonsynonymous changes may contribute considerably to the MHC diversity. Nonsynonymous changes are genetic mutations that alter the amino acid sequence which then may result in functional differences between MHC alleles. While this could potentially indicate that MHC class I and II in humpback whales is under positive selection, it may also be the result of the different sequence lengths analysed in the phylogenetic distance-based

analysis (nucleotides: full-length sequence; amino acids: CDS only). The longer sequences used at the nucleotide level result in more bases being shared by alleles of the same individual compared to the shorter sequences used at the amino acid level. To understand more about the selection pressure acting on the analysed MHC class I and class IIa genes, the nonsynonymous/synonymous mutation ratio (Ka/Ks) needs to be analysed.

5.5.3 Limitations and future directions

The phylogenetic-distance based dissimilarity metric UniFrac assigns more weight to either rare lineages (unweighted UniFrac) or to the most abundance lineages (weighted UniFrac, applied here). In the instance of a change in the composition of moderately abundant lineages, this can lead to a loss power (Chen *et al.*, 2012). Other metrics, such as generalized UniFrac detect a much wider range of biologically relevant changes (Chen *et al.*, 2012), and may offer a better estimation of the sequence variation at MHC in humpback whales. Further, sequence variation at the level of the amino acid in this study was measured from the predicted coding sequence (CDS) inferred from the blue whale reference sequence. However, most of the polymorphic variation of MHC genes is in the PBR (peptide binding region) region. It is thus expected to be more functionally meaningful to measure amino acid sequence variation in PBRs rather than the entire predicted CDS. Information on PBRs are not yet available for baleen whales. Future studies may infer MHC class I PBR using characterised physiochemical descriptor variables of amino acids (e.g., Sandberg *et al.*, 1998). Future studies on the genetic diversity in humpback whales may 1) assess MHC diversity/dissimilarity at structurally and functionally important regions (e.g., PBR), 2) screen a wider fraction of MHC (e.g., more exons) for better indicators of parasite resistance or genetic relatedness, 3) compare estimates of neutral and functional genetic diversity, and 4) investigate signs of selection by calculating the ratio of non-synonymous to synonymous nucleotide substitutions (dN/dS). Finally, in order to further assess the role of (MHC-mediated) mate choice in humpback whales, future studies may investigate 1) whether individuals show a mating preference for mates with high MHC diversity (e.g., allelic richness) or with particular alleles/haplotypes (in addition to the here analysed MHC dissimilarity), 2) if levels of genetic diversity (i.e., heterozygosity) in offspring deviate from expectations of random mating, and 3) whether humpback whale song conveys genetic quality (e.g., MHC diversity). MHC focused studies in baleen whales and cetaceans (in

general) are in their infancy; this is an exciting new avenue of research for which the current study provides some tantalising indications of mate choice.

5.6 Conclusions

Here, I assessed MHC diversity and dissimilarity of 329 humpback whales, including 58 validated mating pairs. The high diversity observed at MHC, especially class I, indicated that an inter-species comparison of MHC diversity at class I and class II genes may be appropriate to update views of reduced MHC diversity in marine mammals compared to terrestrial mammals. Functional genetic diversity, such as MHC diversity, is an important factor determining the fitness and viability of wild populations. Both natural and sexual selection are likely non-mutually exclusive drivers of MHC diversity in humpback whales. My study supports the possibility of MHC-mediated mate choice in humpback whales where females could shape patterns of male reproductive success. Whether MHC-mediated mate choice serves as a potential driver for song complexity in humpback whales remains unclear, yet, offers a question ripe to explore.

Chapter 6

General Discussion

6.1 Thesis synthesis

This thesis focused on patterns of male reproduction and sexual selection in humpback whales, aiming to improve our understanding of the complex mating behaviours of humpback whales and their role within the species' proposed polygynandrous mating system. The 26-year-long dataset on the humpback whale breeding population in New Caledonia, South Pacific, allowed for the unique opportunity to explore male reproductive success and reproductive tactics at the level of the individual for almost one third of the species' lifespan. With an integrative analysis of behavioural, (epi)genetic and endocrine data, I explored the interactions between reproductive success, age, physiology, and population dynamics across time. This further allowed for a broader view of the possible forces of selection acting on this endangered population of humpback whales. Combining analyses at the level of both the population and the individual further offered valuable insights into the interplay of (meta-)population dynamics, mating system, and reproduction. In this chapter, I discuss the implications of my results for humpback whales, how it impacts our understanding of sexual selection theory, and conclude by suggesting future research avenues. I bring together the various strands from each chapter into a coherent synthesis of the current status of recovery of the New Caledonian breeding population, our understanding of the reproductive tactics of humpback whales, and discuss insights on sexual selection in cetaceans and beyond.

In chapter 2, I investigated the strength of sexual selection and reproductive autonomy of male humpback whales on their breeding ground in New Caledonia. A paternity analysis of known mother-offspring pairs revealed low variation in male reproductive success. However, the high reproductive skew towards males that did not sire offspring and the male-biased sex ratio on the breeding ground still suggested intense male-male competition. Using gametic mark-recapture, the male population was estimated to range between 2,000 and 2,500 males, and suggested some level of gene flow between the New Caledonian breeding population and neighbouring Oceanian populations may be occurring. The results of this chapter provided

insights into the reproductive skew of humpback whales and the population dynamics across Oceanian breeding populations, two important factors affecting the recovery of humpback whales in the South Pacific.

In chapter 3, I explored age-specific changes in sexual selection in light of the population's recovery from commercial whaling. The left-skewed age structure of the male population in the first half of the study period compared to the more balanced age distribution in the second half of the study period was consistent with the stabilisation of the age structure. Building on the results from chapter 2, older males were more often observed to engage in certain reproductive tactics (singing and escorting) and were more successful in siring offspring once the population age structure was more even. The findings of this chapter suggest that reproductive tactics and reproductive success in male humpback whales may be age-dependent, and that commercial whaling changed not only the population dynamics but also patterns of sexual selection. This work provides novel, critical insights into how sexual selection is currently acting on the complex male mating behaviours in this recovering population of humpback whales.

In chapter 4, I assessed the seasonal and age-related changes in levels of testosterone in male humpback whales to gain insights into their reproductive physiology. Using enzyme immunoassays, testosterone was measured in 457 blubber samples, and combined with age estimates derived in chapter 3. Male blubber testosterone slowly decreased over the breeding season in the male population. However, the observed seasonal trend in blubber testosterone could be driven by differences in the migratory timing of individuals with differing hormone levels, rather than a decrease in blubber testosterone within individual males on the breeding ground. Blubber testosterone levels appeared highest during puberty, then decreased and levelled off at the onset of maturity (males in their teens), with some evidence that it increases again in males maturing into their late 20s and early 30s. Furthermore, male blubber testosterone was highly variable at any point during the breeding season and across males of all ages. This chapter demonstrated that the integration of endocrine and molecular age markers in long-term datasets is a powerful tool for understanding a species' life-history trends, ontogenetic changes, and mating systems.

In chapter 5, I investigated the influence of the major histocompatibility complex (MHC) diversity on patterns of male reproductive success in humpback whales. Applying a recently

developed and validated cetacean MHC amplicon sequencing panel, I assessed the MHC diversity and dissimilarity of 329 humpback whales at three MHC genes (class I, class IIa DQB and class IIa DRB). By building upon the results of chapter 2, this chapter tested the hypothesis of an MHC-mediated mate choice in humpback whales. Individuals were more genetically dissimilar based on their composition on a purely shared/non-shared basis of alleles and haplotypes than based on the phylogenetic distance of nucleotide and amino acid sequences. Further, mating pairs shared less alleles than expected under random mating in more than 90% of simulations (n = 1,000) at MHC class I. This chapter showed the first evidence of an underlying preference in female humpback whales for (MHC) genetically dissimilar mates thus shaping male reproductive success through pre-copulatory reproductive strategies.

6.2 New Caledonian humpback whales - then and now

Humpback whales across all oceans were heavily exploited during commercial and illegal whaling deep into the 20th century (Clapham, 2016). In the Southern Hemisphere, their populations were decimated to only 1% of their pre-exploitation population size (Baker and Clapham, 2002). While many humpback whale populations have since shown an increase in abundance, their degree of recovery varies considerably (Thomas, Reeves and Brownell, 2016). The Oceanian metapopulation was estimated to be the least abundant in the South Pacific by 2005 despite the wide range it covers (Constantine et al. 2012). The New Caledonian humpback whale breeding population is part of this wider Oceanian metapopulation and neighbours the Eastern Australian population to its west (Figure 2.1a in Chapter 2). A previous gametic mark-recapture (GMR) analysis using data collected on the New Caledonian breeding ground between 1995 and 2001 rendered an abundance estimate of 329 males (Garrigue *et al.*, 2004). The close agreement of this GMR estimate with a sex-specific estimate based on organismal recapture using photo-identification and genotyping data (382 males), suggested that the New Caledonian breeding ground was relatively demographically and reproductively autonomous at that time (Garrigue et al., 2004; see also: Palsbøll et al., 2005; Baker et al., 2005). However, almost two decades and an anomalous population increase later (Garrigue, Albertson and Jackson, 2012), patterns of movement and gene flow across humpback whale breeding grounds in the South Pacific, consequently, may have changed too.

My GMR estimates derived in chapter 2 using data collected between 2000 and 2018 (2,058 males or 2,564 males, depending on paternity confidence level) are five to six times higher than the previous GMR estimate of male abundance. This almost six-fold difference in male abundance suggests that there was a considerable increase in the New Caledonian breeding population over the last two decades. It is worth noting that the almost three times longer study period used in my GMR analysis (19 years) may have, at least to some extent, contributed to this much higher male abundance estimate compared to the GMR estimate of Garrigue et al. (2004; 7 years). Nevertheless, this substantial increase in the male population size between 2001 and 2018 is in line with another study assessing the abundance of the New Caledonian breeding population using data on photo-identification of individuals collected from 1996 to 2011 (see Garrigue, Albertson and Jackson, 2012). According to the yearly estimates derived from their POPAN model (considering capture and recaptures in an open population matrix), there was a two-fold increase in the New Caledonian population size from the year 2008 (562 individuals) to the year 2009 (1,291 individuals) (Garrigue, Albertson and Jackson, 2012). For such anomalous increase to occur within just one year due to population growth alone appears biologically implausible. Influx from the neighbouring Eastern Australian population into the New Caledonian population was suggested to be a possible driver of this observed increase in abundance after 2008 (Garrigue, Albertson and Jackson, 2012; Orgeret et al., 2014). The Eastern Australian population has increased at a much faster rate (similar to Brazil and Western Australia; Wedekin *et al.*, 2017), than other populations in the South Pacific (Noad *et al.*, 2011), and is considered to have fully recovered today (Noad, Kniest and Dunlop, 2019). If a "spillover" from the Eastern Australian population caused the sudden increase in the New Caledonian population more than a decade ago, then it is only reasonable to assume that at least some whales from the booming Eastern Australian population still move to/through the New Caledonian breeding ground today. Occasional genetic interchange among breeding populations in the South Pacific has been observed prior to the reported increase of the New Caledonian population size after 2008 (Steel *et al.*, 2018). This has since been further supported by a substantial number of resights (Badhuge, 2022), longitudinal movements (Derville *et al.*, 2020) and migratory interchange on the Chesterfield-Bellona archipelago in the Coral Sea (Garrigue et al., 2020; see Figure 2.1 of Chapter 2), located between New Caledonia and Eastern Australia. The GMR estimates in chapter 2 further hint towards some level of gene flow between the New Caledonian population and its easterly

neighbours, the wider Oceanian meta-population it is designated to be part of. The New Caledonian breeding population may thus receive organismal, and potentially genetic interchange from both the Eastern Australian and other Oceanian populations, then and now.

A high reproductive capacity was suggested to be another non-exclusive driver of the reported increase in the New Caledonian humpback whale population (Chero *et al.*, 2020). This was supported by several female breeding parameters: the high estimated calving rate of the New Caledonian breeding population (Chero *et al.*, 2020), and the high pregnancy rates observed on the migratory corridor of the Kermadec Islands (Riekkola *et al.*, 2018) and humpback whales in general on their Antarctic feeding grounds (Pallin, Baker, et al., 2018a). My results in chapter 3 showed that some males sired offspring at a very young age (2 – 9 years) and before the species' estimated age of sexual maturity (9 - 11 years; Best, 2011). There are two non-mutually exclusive explanations for the occurrence of such young fathers: 1) male humpback whales in general are physiologically able to reproduce at a young age, yet because of their lack of experience and undeveloped skills in reproductive tactics compared to older males they are not typically successful in siring offspring in a healthy population, or 2) males have become sexually mature at a younger age as a result of commercial whaling. While more research and continued long-term population monitoring are required to further address these questions, a younger age of sexual maturity in males would align with the reported high calving and pregnancy rate in females. An increased reproductive capacity resulting from changes in both female and male breeding parameters could have contributed to the recovery of the New Caledonian humpback whale population.

Estimating demographic parameters provides invaluable information on the recovery of previously exploited (meta)populations such as the Southern Hemisphere humpback whales. The change in age-structure of the New Caledonian male population from early to later years, demonstrated in chapter 3, resulted in a more balanced and stable age structure where older males sired more offspring. The recovery of a population from past exploitation not only influences population demography, and potentially important life history traits, but may further result in changes in movement and distribution patterns through time. Humpback whales are a highly mobile and migratory species. This renders both the assessment of their recovery and the definition of units of management extremely challenging. The New Caledonian breeding population is one of the most well-studied populations of humpback

whales in Oceania and worldwide. The 26-year long (and ongoing) monitoring of individually recognised humpback whales on their breeding ground in New Caledonian offers the unique opportunity to assess any changes at the level of the population and the individual whale across time. There is much we have already learned about their demography, reproduction, and behaviour. Yet, without more information on its neighbouring populations (e.g., Tonga, Vanuatu), it remains difficult to gain a holistic picture of their recovery and genetic interchange. Population dynamics across humpback whale breeding grounds in Oceania, Eastern Australia, and elsewhere, could be density-dependent. The level of interchange (of whales and genes) between regions may have increased with increasing population size. Thus, the increased abundance of the New Caledonian and Eastern Australian population, together with their reported signs of recovery, call for a careful examination of previous records of individual interchange, gene flow, and genetic differentiation among humpback whales in the western and central South Pacific. Yet, the observed changes in the populations of both New Caledonia and Eastern Australia from the past to the present lends itself to a positive outlook for humpback whales ocean wide.

6.3 The humpback whale mating system

The mating system of humpback whales was initially postulated as promiscuous (Clapham and Palsboll, 1997) which, by its definition (i.e., indiscriminate mating with multiple partners), indicates a lack of partner choice (see: Elgar, Jones and McNamara, 2013; Garcia-Gonzalez, 2017). However, truly 'promiscuous' mating in sexually reproducing animals is rare, as sexual interactions are often governed by at least some mate selection criteria (Elgar, Jones and McNamara, 2013; Garcia-Gonzalez, 2017). More recently, the humpback whale mating system, like that of most other baleen whale species, has been described as polygynous (males mate with multiple females) (e.g., Cerchio *et al.*, 2005), or potentially even polygynandrous (multi-male and multi-female) (see also Eichenberger, Garland and Carroll, 2023). Indeed, a growing number of studies hints towards a mating system of humpback whales where males and females mate with multiple partners and where both sexes may exhibit a certain level of choice or preference (e.g., assortative mating, mate choice).

In chapter 2, I identified 79 paternity trios (mother-offspring-father) by conducting a paternity analysis on 177 known mother-offspring pairs sampled over 25 years. All 79 trios

comprised of unique mating pairs indicating that males and females do not repeatedly pair with the same mate across years. Only one male was found to have sired twice within the same year. While this confirms that both males and females mate with multiple partners across years, the lack of any direct observation of mating in humpback whales renders any assessment of mating patterns within a single breeding season (e.g., polyandry) challenging. However, female humpback whales may mate with more than just one male within a given breeding season 1) to ensure successful fertilization, especially considering the cost of their elongated fasting period and long-distance migration, 2) to receive indirect benefits of mate guarding to avoid male harassment (see also: Cartwright and Sullivan, 2009), and/or 3) because the cost of refusing another mating may be higher than accepting it considering the often aggressive behaviour of male humpback whales towards females and their calf (see also: Boulton and Shuker, 2013). Unless oestrous females leave the breeding ground soon after their first mating, they likely benefit from mating with further males. Thus, considering both genetic and behavioural indications of polyandry, I hypothesize the mating system of humpback whales to be polygynandrous. Yet, without any direct observations of mating in humpback whales, this may never be more than a hypothesis.

6.3.1 Reproductive strategy

Based on behavioural observations on their breeding ground, the humpback whale mating system was further suggested to resemble a lek (Herman & Tavolga, 1980; reviewed in Herman, 2017). A lek is defined as a communal male display area that females visit primarily for mating (Emlen and Oring, 1977; Bradbury, 1981). Males may defend individual territories and engage in sexual displays to entice females that may choose among the males present based on certain perceived physical, behavioural, or vocal characteristics (Höglund and Alatalo, 1995). For example, male black grouse (*Lyrurus tetrix*) aggregate on territories where they perform acoustic and behavioural displays and compete with other males over females. During combats, males tear feathers from each other's tail ornaments. Intact ornaments indicate a male's superior fighting ability, and ultimately, his superior viability (Alatalo, Höglund and Lundberg, 1991). Female black grouse preferred larger leks (i.e., higher number of males) (Alatalo *et al.*, 1992) and victorious males (Alatalo, Höglund and Lundberg, 1991), which may provide them with benefits of 'good genes' for their offspring and/or reduced risk

of disease transmission (reviewed in Kirkpatrick & Ryan, 1991). Consequently, male tail length is correlated with mating success and thus under strong sexual selection (Rintamäki *et al.*, 2001).

Classical lek characteristics also observed on humpback whale breeding grounds are the absence of significant resources for females apart from males at the display site, the lack of male parental care, the complex vocal display of males, and the opportunity for female mate choice (Herman & Tavolga, 1980; reviewed in Herman, 2017). However, the wide-ranging distribution and longevity of baleen whales makes them distinct from many other terrestrial mammals and birds. Contrary to classical leks, humpback whales do not show male territoriality and instead move freely about (Craig & Herman, 2000; Helweg & Herman, 2010), and consequently their mating system has been described as a 'floating lek' (Clapham, 1996). The presence of maternal females on the breeding ground and the relatively low reproductive skew (Cerchio *et al.*, 2005; chapter 2, this thesis), are additional lek-atypical features observed in humpback whales. However, considering the strong breeding seasonality, the synchrony of female oestrus (Chittleborough, 1954), the unpredictability of female arrival, the great dispersion of individuals across the breeding grounds and the 3-dimensional underwater habitat, male monopolisation potential is reduced, and subsequently reproductive skew is expected to be lower (Herman, 2017). These lek-atypical features, therefore, do not disqualify the species' breeding aggregation from functioning as a lek (Herman, 2017). The 'floating lek' behaviour of humpback whales may thus be a reproductive strategy adjusted to their marine habitat, and similar to the lekking black grouse, there is scope for direct male-male competition, alternative reproductive tactics (section 6.3.2), and female mate choice (section 6.3.3).

6.3.2 Male reproductive tactics and the lingering question of the function(s) of song

Male humpback whales engage in a variety of different behaviours on their breeding ground, such as singing, physically competing over a female, and escorting of females, which have been described as alternative mating tactics (e.g., Cerchio *et al.*, 2005). Alternative mating tactics are observed among males and females and across a wide variety of taxa (Pagel, 2002). Perhaps the most famous example is that of the sneaky copulations by subordinate male hamadryas baboons (*Papio hamadryas*) to counter the monopolization of females by

dominant males (Kummer, 1968). Behavioural observations and photo-identification of humpback whales have long revealed that there is no single predominant tactic among males, and males frequently engage in more than one tactic even within a single breeding season (e.g., Cerchio, 2003; this dataset). Thus, perhaps, 'multiple reproductive tactics' is a more suitable term to describe mating behaviours of male humpback whales (e.g., singing, escorting, and participating in competitive groups) as the word 'alternative' comes with its attached meaning of 'one or the other'. Males may sing to attract females to a common, aggregated breeding area (e.g., a lek) and/or attract individual females (reviewed in Herman, 2017). Once a male finds a female, he becomes her escort which may result in copulation or the formation of a competitive group where each male attempts to outcompete all others and become/remain her sole escort. Escorting may also reflect mate guarding following earlier copulation (Clapham, 1996) to prevent sperm competition in response to polyandry. Escorting could thus represent both the start and/or the end of a competitive group rather than an alternative reproductive tactic. Similarly, a male that opted to sing may nevertheless end up in a competitive group physically competing over a female (i) if singing, so far, has rendered him unsuccessful in finding a female and he actively decides to abort the singing tactic and instead join a competitive group to physically compete over a female, or (ii) if he successfully found a female, but unfortunately also attracted other males (due to his broadcast song during escorting; Smith *et al.*, 2008) which ultimately may lead to the formation of a competitive group where males physically compete over the female. Nevertheless, a male may favour one tactic over another (e.g., Cerchio, 2003) depending on his age, status, experience, quality, or that of any possible female mating partner or male competitor (e.g., audience effects; see Dunlop, 2016), or on the density of both females and male competitors in the area.

My results in chapter 3 showed that older males (>23 years) were more often observed as singers and solitary escorts and were more likely to sire offspring under a balanced age structure than younger males (2 – 9 years). This suggests that a male's age or experience may influence which mating tactics he employs or how successful he performs this tactic to secure mating access. Even if older/more experience males tend to be more successful in securing mating access compared to younger/less experience males, the latter may nevertheless engage in the same behaviours and practice until they are themselves experienced enough to outcompete (or "out-sing") other competitors. Further, in a study assessing the body size of humpback whale dyads on their breeding ground in Hawaii (USA), males that associated with

immature-sized females tended to be either immature themselves or mature but smaller than males that associated with mature-sized females (Pack *et al.*, 2012). This size-assortative pairing indicates that humpback whales discriminate among potential mates and that males may adopt different reproductive tactics depending on their own body size, and that of a possible female mating partner, to avoid the costs of competing for the highest-quality females (Pack *et al.*, 2012). In a different study on the same breeding ground, males preferred to associate and competed more intensely for females with high reproductive potential (no calf vs with calf) but became progressively less choosy over the course of the breeding season as the number of females without a calf decreased (Craig, Herman and Pack, 2002). Altogether, these findings indicate that male humpback whales may adjust their reproductive investment and choices depending on their own age, status, experience, body size, as well as that of their possible female mating partner.

The intensity of or effort in competition over a female or against other males is not the only factor influencing male reproductive investment. In a lekking system, time spent on the lek (i.e., lek attendance) is another relevant factor affecting the mating success of males (Fiske, Rintamäki and Karvonen, 1998). In black grouse, heavier males had a higher level of lek attendance and fighting rate than lighter males (Nieminen *et al.*, 2016), yet yearling males showed an increase in lek attendance and fighting rate once adult male effort declined (Nieminen *et al.*, 2016). Such a dynamic may allow males with a lower competitive ability to gain some access to reproduction as they respond to the decline in the condition of dominant males throughout the breeding season (Mason *et al.*, 2012). Humpback whale migration in both directions is characterised by a staggering of sex, age and reproductive status with immature males and females without a calf arriving and departing earlier than mature males and females with calf (Nishiwaki, 1959; Chittleborough, 1965; Dawbin, 1966; Craig et al., 2003). Such differences in migratory timing of immature and mature males could result in differences in their time spent on the breeding ground. The seasonal decline in mean male blubber testosterone assessed in chapter 4 could be driven by the observed age-related changes in male testosterone together with differences in the migratory timing of individuals of different ages or reproductive status. Despite the observed seasonal decline in the population and the age-related pattern in male blubber testosterone, testosterone levels across individuals varied considerably at any point within the breeding season and across males of all ages. Differences in the time spent on the breeding ground, behavioural

differences, and/or individual quality could contribute to the observed variation in male blubber testosterone levels (see Chapter 4 section 4.5.3). However, if testosterone plays a role in the coordination of breeding activity and migratory behaviour, as hypothesised in chapter 4, then perhaps the duration of elevated testosterone levels (relative to baseline levels) is more influential on the time spent on the breeding ground than the magnitude of individual testosterone levels during a particular time period (see Kempenaers, Peters and Foerster, 2008).

The frequency and success of a male reproductive tactic may also depend on population density. For example, as the Eastern Australian humpback whale population increased over 18 years, males appeared to engage more often in physical competition over singing (Dunlop and Frere, 2023). Dunlop and Frere (2023) suggested that changes in the population-level male density resulted in a shift in the frequency, and fitness pay-off, of 'alternative' mating tactics of male humpback whales. This is, however, at odds with observations of singing at high density on seamounts that are part of the wider New Caledonian breeding ground (this dataset; pers. comms. C Garrigue and E Garland), indicating it may be population specific. While the likelihood of finding a female depends on the density of females, a male's chances of bumping into other male competitors which then potentially results in the formation of a competitive group depends on the density of males on the breeding ground.

Central to understanding male reproductive tactics is the lingering issue regarding the function of humpback whale song. Is singing a male reproductive tactic performed by all (or most) males to aggregate individuals to a common breeding ground, or is it an alternative reproductive tactic, directed at males or females, that some males favour over the tactic of physical competition, or perhaps both? Further, is the 'original' function it once evolved for still applicable or is its current function(s) an entirely different one? Whether or not it is the song's original, current or main function, or simply a by-product, humpback whale song serves in longer-distance communication thus aiding individuals in aggregating on a common breeding area and, ultimately, facilitating males and females to find each other. (Nunn, 2000)(termed male prospecting; see Smith et al., 2008). However, it does not explain the evolution of the high level of complexity present in humpback whale song. The long-distance communication of song (e.g., aggregating or attraction of females to an area) is, thus, likely not its primary function but brings further direct benefits received solely by the singer. This

would be the case if song serves a role in male-male competition and/or female mate choice where mates and/or males assess the singer's quality or status by his song (e.g., complexity, accuracy, novelty), such as in the European sedge warbler (Acrocephalus schoenobaenus; Catchpole and Slater, 2008). Even if song originally evolved as a signal to aggregate individuals to a common breeding ground, females or males, nevertheless, may since have evolved a way to assess a singer's status or quality by his song. Further, female mate choice serves as a likely driver for the high complexity and potentially runaway evolution of humpback whale song (Noad et al., 2000; Cerchio, Jacobsen and Norris, 2001; Herman, 2017; Garland and McGregor, 2020). If females were to prefer males with superior singing skills (i.e., more complex song), which is potentially dependent on experience, older males might experience a reproductive advantage, yet they might fall behind in endurance and stamina during physical contest competition compared to younger competitors (Cerchio *et al.*, 2005). Unravelling the contribution of each tactic to successful reproduction is a clear next step but may rely on direct observations of mating.

6.3.3 The role of female mate choice within the humpback whale mating system

To understand the contribution of inter-sexual drivers on the function(s) of elaborate sexual displays, the potential benefits to a female that exhibits a preference need to be evaluated. If a certain song characteristic reliably indicates the quality of the signaller, then the signal (song characteristics) may be used by females to identify high quality mates (Andersson, 1994). For a signal to be a reliable indicator of male quality it must carry underlying costs, such as time and energy expended during signalling or costs associated with the development of the trait (Zahavi, 1975; Vehrencamp, 2000). For example, in zebra finches (*Taeniopygia guttata castanotis*) song complexity is suggested to indicate a male's learning ability (Airey and DeVoogd, 2000; Airey et al., 2000; see also: 'cognitive capacity hypothesis', Boogert, Giraldeau and Lefebvre, 2008). Song complexity and learning ability may also be linked to early rearing conditions (Bangalese finches, Lonchura striata, Soma *et al.*, 2006) and stress responsiveness (Templeton, Laland and Boogert, 2014), and consequently signal greater male quality ('developmental stress hypothesis', Nowicki and Searcy, 2004, 2005). Further, song complexity may signal genetic quality such as optimal MHC diversity (Slade, Watson and MacDougall-Shackleton, 2017), which is linked to the immune defence against disease and

parasites (Klein, 1986; Piertney and Oliver, 2006). For example, in male song sparrows (*Melospiza melodia*), larger song repertoires (i.e., number of song types) were related to intermediate MHC diversity. Humpback whale song is highly complex and subject to constant evolution (Payne and Payne, 1985; Garland et al., 2011; Allen et al., 2018), making song learning a continuous part of a male's life. The complexity and novelty of humpback whale songs allows for large signal variability among individuals, thus, holding the potential to convey individual quality (Murray *et al.*, 2018).

However, for song to be driven by female mate choice it does not need to reliably indicate male quality. A female preference for a particular song characteristic (e.g., song complexity) could have evolved through a pre-existing sensory or perceptual bias of females ('sensory bias hypothesis'; Ryan, 1990, 1998; Ryan and Cummings, 2013). In this case, the preference may have nothing to do with how costly the signal is or what it means, but rather how it grasps and holds the receiver's attention (Rosenthal and Ryan, 2022). Choosers are more likely to prefer a signal that is easier to detect and results in greater sensory stimulation (Ryan and Keddy-Hector, 1992; Andersson, 1994). The sensory system commonly habituates to a repetitive stimulus, whereupon the stimulus might lose its function ('anti-habituation hypothesis'; Krebs, 1977; Searcy, 1992). A song that deviates from last year's song and/or from the average chorus (e.g., through higher complexity or greater novelty) within a female's hearable surroundings may stick out more, and thus, may be more likely to attract her attention. This is likely the case in the South Pacific, where novel song types sweep through the region and males rapidly adopt these novel songs while concurrently abandoning old songs (Noad et al., 2000; Noad, 2002; Garland et al., 2011). A female preference for a particular song characteristic, thus, could have arisen not only because of mating decisions but in response to ecological selection on sensory tuning and other preference mechanisms (Rosenthal and Ryan, 2022).

The 3D underwater habitat, the slightly larger body size of females relative to males (Ralls, 1976), and the highly male-biased sex ratio on the breeding ground, all promote female behavioural freedom, and thus allow for the evolution of female mate choice. Further, considering the high reproductive costs to females (see Chapter 1), it seems reasonable to assume that female humpback whales are unlikely to choose their mates at random. There are several findings that support this hypothesis. Mature female humpback whales showed a preference for large mature-sized males (Pack et al., 2012), indicating that maturity status

and/or body size may play a role in mate choice. Signal frequency decreases with increasing body mass (e.g., Mikula *et al.*, 2021), and song may thus convey information on the singer's body size. Moreover, my results from chapter 5 hint towards an MHC-mediated mate choice in humpback whales where females prefer males that are more genetically dissimilar (share less alleles), possible to improve the immune competence of their offspring (see also Piertney and Oliver, 2006). In order to unravel the role of female mate choice within the humpback whale mating system future studies may explore whether any behavioural, acoustic, or morphological male trait conveys male quality, and ultimately, whether they correlate with male reproductive success.

6.4 Of reproduction and recovery in cetaceans and beyond

Baleen whales differ from most other mammals due to their large body size, long life span, wide-ranging distribution, and high mobility. They also share a unique history of human exploitation. Baleen whales worldwide were exploited intensely across all oceans for several centuries during commercial whaling; many populations remain critically endangered and are still recovering from their past exploitation today (Clapham, 2016). How changes in the density and age-structure from, and in response to, commercial whaling (i.e., exploitation and recovery) of their populations shapes their life history and reproduction today is unclear. Yet, this represents a crucial aspect in the assessment of conservation status at a population and species level.

For example, in the endangered North Atlantic right whale (*Eubalaena glacialis*), reproductive success was biased towards older males, with most males not reproducing until they reached an age almost twofold the average age at reproduction of females (males: ~15 years; females: ~8 years; Frasier et al., 2007). Young males nevertheless participate in surface active groups (SAGs), perhaps to practice mating behaviours, yet compared to adult males, they were never seen copulating with a female (Kraus and Hatch, 2001). Young males may not have the skills or strength to outcompete their adult conspecifics (Kraus & Hatch, 2001; see also Ham et al., 2023), which results in a lower effective population size (Frasier *et al.*, 2007). Thus, age-dependent reproduction seemed to negatively impact the species' recovery from commercial whaling. On the other hand, post-copulatory selection for dissimilar gametes, was found to maintain genetic diversity, with offspring having higher levels of microsatellite

heterozygosity than expected under random fertilization (Frasier *et al.*, 2013). Female choice might therefore play an important role in mitigating the loss of genetic diversity of this small population over time (Frasier *et al.*, 2013).

Baleen whales are not the only marine mammals that were impacted by decades of commercial whaling (Clapham, 2016). Sperm whales were intensely hunted worldwide for their valuable high-quality oil (Whitehead and Shin, 2022) and many populations have still not recovered (Carroll et al., 2014; Gero and Whitehead, 2016; Whitehead and Shin, 2022). With mature males being up to 40% larger and three times heavier than females, sperm whales are the most sexually dimorphic of all cetaceans (Rice, 1989). Male sperm whales invest in slow and continued growth and delay reproduction until they are large enough to be competitive (Whitehead, 2003). Their slow recovery could be due to the intrinsically low reproductive potential of sperm whales and the lingering demographic effects of the removal of large adult males on reproduction (Whitehead, 2003; Eguiguren, Konrad Clarke and Cantor, 2023). Both these cetacean examples plus the findings of this thesis thus demonstrate that both life history trade-offs and reproductive tactics influence the recovery of populations through changes in the effective population size and genetic diversity.

The largest mammal on land, the African savanna elephant (*Loxodonta africana*) offers further insights into the balancing of energetic investment in growth and reproduction of mammals at the long-lived and slow growing end of the mammalian life history continuum. Male reproductive success in elephants increases with age through increased inter-sexual selection by females for older males (Moss, 1983; Poole, 1989) and decreased intra-sexual competition (Joyce H. Poole, 1989); it consequently peaks relatively late in life (Andersson, 1994). As males grow older, they allocate more energy into locating receptive females (Poole *et al.*, 2011; Taylor *et al.*, 2020). Yet, despite the higher competitive ability and reproductive dominance of older males, younger males seem to undertake opportunistic reproductive tactics, where they do not engage exclusively in energetically expensive searching behaviour, and thus, still contribute to the gene pool (Rasmussen *et al.*, 2008; Taylor *et al.*, 2020). Contrary to predictions from behaviour and life-history traits, male African elephants reproductive skew was lower compared to many mammals with a similar mating system (Rasmussen *et al.*, 2008). This indicates that trophy hunting and ivory poaching of elephants, which targets older bulls, can have substantial behavioural and genetic effects on populations

(Rasmussen *et al.*, 2008), mirroring the scenario we see in the current study of male New Caledonian humpback whale.

In a population with a left-skewed age structure, there may be less reproductive skew as the population comprises males of a similar age and experience, but fewer competitively superior males. The low reproductive skew observed in the New Caledonian humpback whale population (Chapter 2), and other previously exploited populations, might thus be the result of such changes in the pattern of sexual selection in response to the shift in the population age structure towards younger individuals. However, such a skewed structure might not represent the conditions under which the mating behaviours we observe today have evolved. My analysis on the variation in reproductive success and patterns of sexual selection in male humpback whales, provides only a small glimpse into their reproductive life and impacts from human exploitation of these animals. In relation to their elongated life span, lifetime reproductive success in male humpback whales might be more skewed and variable than suggested (see Chapter 2). Yet, by combining photo-identification, genetic, and ageing data from 26 years, just over a third of the species' life span, I was nonetheless able to detect signs of age-dependent reproduction in males and female mate choice. As humpback whale populations continue recovering from commercial whaling over the coming decades, male reproductive skew and variation in reproductive success may also increase reflecting this change in demographics. Assessing the changes and variation in reproductive success over time in highly elusive species with long life spans and slow life histories is challenging, especially so if they inhabit remote habitats like many baleen whales and cetaceans more widely do.

Similarities across baleen whales, cetaceans, and mammals indicate that human exploitation may have long-lasting impacts on patterns of sexual selection and life history trade-offs in animals with slow life history. The same traits that make baleen whales so unique, might also oppress their recovery from commercial whaling. The suggested role of female mate choice and the possible shifts in male and female breeding parameters may have allowed humpback whales to mitigate some of the impacts of commercial whaling. Further, the complex male song facilitating connectivity between population (i.e., horizontally transmitted song patterns; Garland et al., 2011) and aggregation to common breeding grounds may reduce the costs of their wide-ranging distribution and long-distance migration. Combined, these

behavioural, acoustic and physiological traits, shaped by sexual selection, may contribute to their growing numbers worldwide.

6.5 Conclusions and future directions

There is an intricate interplay among sexual selection, reproductive tactics, and population recovery. While changes in the population demography shape patterns of reproductive success and sexual selection, reproductive tactics, in turn, affect effective population size and genetic diversity, and thus, population recovery. This thesis focused on patterns of male reproduction and sexual selection in humpback whales, which has improved our understanding of the complex reproductive tactics of humpback whales and their role within the species' proposed polygynandrous mating system. I demonstrated how age, population structure, testosterone and genetic differences (MHC) may all impact male reproductive success, and provided strong evidence for the polygynandrous mating system and impacts of demography.

Future studies should re-assess the level of gene flow across humpback whales in the South Pacific in light of the recent population increases and investigate how patterns of reproduction and reproductive tactics change as populations continue recovering from commercial whaling. Such work can only be undertaken by continuing long-term data collection, which is expensive, time consuming and has historically been undervalued (and funded). Further, only through long-term population monitoring can we continue to assess the changing patterns of reproduction and population dynamics as humpback whales keep recovering from commercial whaling over the coming decades. Investigating how population growth and demographic changes influence male reproductive skew may further increase our understanding of male reproductive tactics and patterns of sexual selection in humpback whales.

Humpback whales, like many other baleen whales, are capital breeders; they are reliant on abundant prey resources at their high-latitude feeding grounds to store energy reserves required for migration and reproduction (Lockyer, 1981; Baker et al., 1986). Future studies should assess how environmental fluctuations (e.g., El Niño, climate change) and prey availability on Polar feeding grounds influence the timing of migration, testosterone levels and

reproduction (e.g., breeding parameters, reproductive tactics, time spent on breeding ground).

A particularly understudied area in cetaceans and marine mammals in general is the MHC. Early findings of reduced MHC class II diversity in marine mammals compared to terrestrial mammals has led to the now outdated view that cetaceans experience reduced selective pressure on maintaining MHC polymorphism stemming from the relatively low prevalence of infectious disease in the marine environment (Trowsdale, Groves and Arnason, 1989). A new inter-species comparison of MHC diversity at class I and II genes is thus appropriate to explore the selective pressures cetaceans face(d) after their evolutionary transition from land to sea fifty million years ago (Mya). This may further provide crucial insights on the health status of populations and their vulnerability to the increasing prevalence and severity of emerging infectious diseases, especially viruses, under climate change (van Bressem et al., 2009; Kebke, Samarra and Derous, 2022).

Finally, the enigma of humpback whale song is ripe for further exploration. This highly complex, cultural, and sexually selected display has both intrigued and frustrated scientists for over 50 years. Only through integrative analyses that combine acoustic, (epi)genetic, and photogrammetry will we be able to explore if song conveys information on male genetic quality or body size, and ultimately, to finally unravel its underlying function(s).

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