

Connectivity and effective population size of east Australian grey nurse sharks

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Declaration

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All other research described in this report is my own original work.

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ABSTRACT

Knowledge of connectivity and effective population size (N_e) can be used to evaluate the impacts of various threatening processes. Estimating total population size or connectivity through direct field observation is often difficult and genetic techniques provide an alternative approach. This study uses thousands of single nucleotide polymorphisms to investigate connectivity and N_e of the critically endangered grey nurse shark (*Carcharias taurus*) across 1400 km of their east Australian range. This population has suffered a severe decline in numbers over the past few decades, has low genetic diversity, and is extremely susceptible to anthropogenic mortality. Genetic connectivity among aggregation sites in east Australia was previously unknown. This study revealed that east coast grey nurse sharks are genetically and demographically connected, with the population approximating genetic panmixia. Estimates of N_e were around 400 using two different estimation methodologies. Forward simulations that used current genetic variation to estimate the effect of genetic drift in the future, revealed that maintaining an N_e of 400 will lead to a loss of genetic diversity over the next 50 generations. This highlights the importance in effective cross-jurisdictional management of this critically endangered population of grey nurse sharks.

Key words: *Carcharias taurus*, genetic panmixia, conservation genetics, effective number of breeders

INTRODUCTION

Biodiversity loss

As human population growth rapidly continues, our impacts on the natural world and wild populations are increasing. Anthropogenic processes, including agricultural expansion, industrial development, overexploitation, pollution and climate change, continue to severely threaten wildlife populations through loss of suitable habitat (Mittermeier et al. 2011, Manel & Holderegger 2013). Currently, habitat degradation, fragmentation and loss are the key threats to global biodiversity (Bartlett et al. 2016). A reduction in viable habitat creates smaller and more isolated populations, making it more difficult for organisms to effectively disperse, and thereby reducing connectivity (Frankham 2015). This is accompanied by a loss of gene flow, and an increase in genetic drift and risk of inbreeding depression (Weeks et al. 2011, Frankham 2015). Subsequent decreases in genetic variation can have adverse effects on a populations ability to adapt to novel conditions, leading to an increased risk of extinction (Frankham 2016). This has been documented in many species, including winter flounder (*Pseudopleuronectes americanus*) (O'Leary et al. 2013), Swedish wolves (*Canis lupis*) (Jansson et al. 2015), prairie chickens (*Tympanuchus cupido pinnatus*) (Bouzat et al. 2008) and greater sage-grouse (*Centrocercus urophasianus*) (Gruber-Hadden et al. 2016).

We are likely undergoing the sixth mass extinction event in history (Ceballos et al. 2015), and in Australia alone there are over 440 animals listed as vulnerable, endangered or critically endangered under the Environment Protection and Biodiversity Act 1999 (EPBC Act). While marine species are also at risk, they are seriously underrepresented in threatened species lists, likely due to the difficulty in monitoring animals not visible at the sea surface (Edgar et al. 2005). In addition, for species that are not commercially exploited, often less resources are available to undertake scientific research (Edgar et al. 2005). This lack of representation is particularly evident through the EPBC Act, where only eight of the 180 shark species found in Australian waters are listed as threatened, despite global shark population declines (Baum et al. 2003, Dulvy et al. 2008). This highlights the necessity for more scientific research on connectivity, particularly for shark species in Australian waters, and where appropriate, the importance of estimating effective population size (N_e) to inform conservation management.

Defining connectivity

Definitions of connectivity in the literature are often confused, contradictory or absent altogether. Differences in definitions are problematic and can lead to variance in methods, analyses and

interpretations of genetic patterns and connectivity. This could have adverse impacts on conservation, through under or overrepresentation of connectivity, leading to incorrect management of threatened species.

Population connectivity refers to the dispersal of individuals among distinct populations, resulting in permanent or long-term settlement in the new population (Lowe & Allendorf 2010). Connectivity can be defined and measured in various ways, depending on the research context (Lowe & Allendorf 2010, Kool et al. 2012). For example, the criteria and consequences of connectivity for conservation purposes will be vastly different to those for harvesting purposes (Lowe & Allendorf 2010). This broad concept of connectivity, with multiple interrelated definitions, in turn leads to confusion, evident through non-existent or vague definitions (Kindlmann & Burel 2008) and incorrect use of connectivity measures, particularly genetic indices (Lowe & Allendorf 2010).

Two interrelated aspects of connectivity, genetic and demographic connectivity, are often unclear or used interchangeably. Genetic connectivity can be defined as the level to which gene flow affects evolutionary processes, and therefore refers to connectivity over many generations (Lowe & Allendorf 2010). It can be further broken up into three types; drift, inbreeding, and adaptive connectivity (Lowe & Allendorf 2010). Drift connectivity is the level of gene flow required to obtain similar allele frequencies between populations, i.e. genetic panmixia (Lowe & Allendorf 2010). This would require many more migrants than one per generation, and is associated with an F_{ST} of around 0.02 (Lowe & Allendorf 2010). Inbreeding connectivity is the level of gene flow that ensures the sharing of the same alleles among populations, yet not necessarily at the same frequencies, and prevents the effects of inbreeding (Lowe & Allendorf 2010). One migrant per generation is enough to maintain inbreeding connectivity, and is expected to be associated with an F_{ST} of around 0.2 (Lowe & Allendorf 2010). The third form of genetic connectivity is adaptive connectivity; the level of gene flow required to keep advantageous alleles in the population (Lowe & Allendorf 2010, Hawkins et al. 2016). In contrast to the other two measures of connectivity, it has been suggested that only one migrant per ten generations is enough to allow for the spread of useful alleles and maintain adaptive connectivity, and is associated with an F_{ST} of 0.35 (Lowe & Allendorf 2010).

Demographic connectivity is the extent to which dispersal affects population growth rates and other vital rates such as fecundity and survival rates (Lowe & Allendorf 2010). In contrast to genetic connectivity, demographic connectivity reflects a shorter temporal scale; within one or a few generations. Demographic connectivity is expected to increase population stability, as immigrants can

compensate for low local recruitment, i.e. low survival and birth rates of the resident population (Lowe & Allendorf 2010). Populations can be demographically dependent, relying heavily on immigration to maintain positive population growth, or demographically independent where they do not. Demographic connectivity is affected by traits of the dispersing individual, such as size, age and life stage (Lowe & Allendorf 2010). For example, some organisms disperse as larvae or juveniles, while others disperse as adults, and as a result, demographic connectivity will be sensitive to the life stage of the dispersing individuals. Demographic connectivity can also be defined as enough individuals moving from one population to another to be “demographically significant” (Leis 2006). This will vary depending on the research context; different numbers of migrants would be considered demographically significant for ecological, conservation and harvesting purposes (Leis 2006, Lowe & Allendorf 2010). Again, this highlights the importance for authors to explicitly state what type and scale of connectivity they are interested in, the research purpose, and intended outcomes, so that results can be practically applied in management and conservation.

Describing population connectivity is further complicated by the lack of consensus in what actually defines a population, despite being a central concept to many disciplines (Waples & Gaggiotti 2006). Groups of individuals at a particular location can be described as a population, and these are often used as units for conservation and management (Waples & Gaggiotti 2006). Yet, there are many questions that need to be considered when regarding the term ‘population’. What classifies a population? What geographical distance should be between groups of individuals for them to be deemed separate populations? When does a group of individuals become differentiated enough that they are considered multiple subpopulations or populations? While reviewed substantially, particularly for the ecological, evolutionary and statistical paradigms (Waples & Gaggiotti 2006), there does not yet seem to be a general consensus on the term ‘population’. The ecological paradigm defines a population as a group of individuals from the same species co-occurring spatially and temporally with the opportunity for interaction. Alternatively, the evolutionary paradigm defines a population as a group of individuals from the same species, close enough for the potential to mate with any other individual (Waples & Gaggiotti 2006). Therefore, it is likely there is no single definition for ‘population’, and that it is instead dependent upon the kinds of questions being asked and the overall context (Waples & Gaggiotti 2006). For example, if a researcher is interested in evolutionary processes such as migration and drift, the evolutionary definition may be favoured. Whereas, for conservation and management purposes, one may favour the ecological definition (Waples & Gaggiotti 2006).

Advances in genetic approaches have made clustering individuals, based on their genotypes, into distinct populations a popular method for describing groups of individuals. Yet, due to the unrealistic assumptions of these methods, which are often not met, these applications and interpretations should be undertaken with caution. For example, often genetic panmixia is assumed, yet this is often not reflected in the real world. Instead of using populations, we could be focusing on answering specific research questions and using defined units of study (such as a group of organisms at a sampled location) as our conservation units (Stow & Magnusson 2012). We could then be framing population connectivity as the amount of connectivity or gene flow between individuals at different locations, rather than between ‘populations’ (Stow & Magnusson 2012). This again highlights how critical it is to be explicit in definitions of ‘connectivity’ and ‘population’, as well as the temporal and spatial scale of interest dependent on the study organism or system, to avoid misapplication of methods and misinterpretation of results.

Measuring connectivity

Knowledge of connectivity is critical for conservation planning, as it informs decisions regarding restoration, reserve and corridor design, and the control of invasive species (Kool et al. 2012). Genetic variation can be investigated at different levels, for example, at two alleles within an individual, between individuals in a subpopulation, and between subpopulations within a population (Sunnucks 2000). As such, different approaches can uncover genetic variation at these various levels, reflecting different temporal scales of connectivity (Sunnucks 2000). Methods can be classified into two types; field observation and genetic approaches, with the combination of these covering a broad spatial and temporal range and resolution (Kool et al. 2012).

For estimates of population size and animal movement in the field, mark-recapture has been the most common method, where a sample of a population is captured, marked, released, and then recaptured (Kool et al. 2012). Other methods include radio tracking, telemetry and global positioning systems (GPS), yet with technological advancements, methods for measuring animal movement continue to improve (Kool et al. 2012). For example, geographic information systems (GIS), remote-sensing technologies, cameras, and environmental sensors, have allowed for more effective measurements and enormous quantities of data (Kool et al. 2012). We are now able to monitor fine-scale movement patterns over broad spatial and temporal scales, particularly at the individual level. For example, multi-modal tracking of highly mobile animals such as flying foxes, can capture information ranging from altitude and speed estimates, large-scale movements over days, to smaller scale movement such as

changes in posture (Sommer et al. 2016). These advancements have facilitated a major increase in the number of connectivity studies being carried out (Kool et al. 2012).

While tracking individuals through field observations is useful for detecting movement, these methods provide information on recent and current movement, i.e. demographic connectivity, and does not provide information on genetic connectivity, relatedness, and success of mating following dispersal. To bridge this gap, genetic data can be utilised to determine whether dispersal has resulted in reproduction (Hawkins et al. 2016). Rapid development in the detection and availability of genetic markers, such as next-generation sequencing and the ability to create high density single nucleotide polymorphism (SNP) data, has improved our ability to detect fine-scale genetic structure within and between populations across broad geographic ranges. For example, patterns of dispersal, fine-scale genetic structure and evidence of selection was found in the sea scallop (*Placopecten magellanicus*) through analysis of SNP data (Van Wyngaarden et al. 2017). A reduction in the cost and time of next-generation sequencing has allowed for whole genome sequencing of an endless number of species including humans, disease-causing bacteria (Kuroda et al. 2001), stickleback fish (Jones et al. 2012), the elephant shark (Venkatesh et al. 2014) and goats (Wang et al. 2016b). Coinciding with these technological advances are improvements in statistical methods and computing power. This has enabled more rigorous analysis of genetic data for more accurate inferences of genetic structure and connectivity.

Genetic approaches

The type of approach and genetic marker used in connectivity studies can uncover different levels of genetic structure over various temporal and spatial scales (Sunnucks 2000). These different approaches, which focus on gene genealogies, allele frequencies and genotypic arrays, allow us to infer demographic and genetic connectivity. It is therefore important that the correct markers and analytical approaches are used to answer specific questions (Sunnucks 2000, Wang 2005).

i) Gene genealogies

The study of gene genealogies covers the largest temporal scale of genetic approaches, as it can offer insight into connectivity over thousands of generations, within and between species (Sunnucks 2000). These techniques use mitochondrial DNA (mtDNA), microsatellites or SNPs to investigate relationships between novel alleles which have arisen through mutation (Sunnucks 2000). Knowledge of historical events and processes, such as speciation and phylogeographic events, can then be inferred

(Sunnucks 2000). For example, analysis of mtDNA of grey nurse sharks (*Carcharias taurus*) in Australia showed that the low genetic variation is likely a consequence of slow evolutionary processes rather than recent anthropogenic impacts (Stow et al. 2006).

ii) Allele frequencies

Studying allele frequencies of populations offers analysis of genetic variation and connectivity at a medium temporal scale, over multiple generations (Sunnucks 2000). Allele frequencies can change over time due to evolutionary processes such as genetic drift, founder effect, gene flow and natural selection (Sunnucks 2000). By using genetic markers that can be analysed at individual alleles rather than as genotypes, frequencies of alleles can be estimated (Sunnucks 2000). By comparing allele frequencies between groups of individuals, gene flow and population subdivision can be inferred, offering valuable information on genetic connectivity. There are three interrelated measures of genetic differentiation; F_{ST} , F_{IT} and F_{IS} . These are extremely useful indices of genetic differentiation as they are directly related to the variance in allele frequency between populations, and between individuals within a population (Holsinger & Weir 2009). The most common method for assessing gene flow between populations is F_{ST} (Holsinger & Weir 2009). F_{ST} describes the correlation between alleles within the subpopulation relative to the entire population, F_{IT} refers to the correlation between alleles within an individual relative to the entire population and F_{IS} describes the correlation between alleles within an individual relative to the subpopulation in which it belongs to (Holsinger & Weir 2009). For biallelic markers, F_{ST} lies between 0 and 1, where a small F_{ST} indicates little differentiation, and a high F_{ST} indicates high differentiation (Meirmans & Hedrick 2011). Therefore, by comparing allele frequencies between populations, we can gather information on connectivity over multiple generations and provide insights into demographic history, such as sex-biased dispersal (Holsinger & Weir 2009).

iii) Genotypic arrays

The shortest time frame and finest scale genetic structure can be uncovered through analysis of genotypic arrays (Sunnucks 2000). Genotypes are shuffled each sexual generation and therefore can be used to uncover connectivity within one, or a few generations (Sunnucks 2000). Genotypic analyses are very useful in population genetics as it allows for identification and tracking of individuals, analysis of relatedness, and inference of relationships between individuals, such as parentage and sibship (Ritland 2000). Similarly, assignment tests use genotype data to place individuals into populations in which they most likely belong to (Manel et al. 2005). These methods are powerful tools for uncovering demographic connectivity, especially when spatial data is incorporated into analyses. By looking at the

spatial distribution of related individuals, through spatial autocorrelation and Mantel tests, patterns of dispersal can be inferred within a single generation (Jones et al. 2010). Furthermore, sex-biased dispersal and geographic barriers to dispersal can be detected using spatial autocorrelation analyses (Diniz-Filho et al. 2016).

Effective population size

Estimating census size (N), the total number of sexually mature males and females in a population (Ferchaud et al. 2016), through direct field observation can be difficult, particularly for endangered marine species that are not visible at the sea surface. On the other hand, effective population size (N_e) can be estimated from genetic data, and is defined as the number of individuals in an ideal Wright-Fisher population that would lose genetic variation at the same rate as the population under consideration (Holsinger & Weir 2009, Husemann et al. 2016). Consequently, N_e is a crucial parameter for many disciplines in biology, as it provides knowledge on evolutionary processes by quantifying the level of genetic drift and inbreeding in real populations (Husemann et al. 2016, Wang et al. 2016a). In an ideal population, N_e and N would be equal, yet in real populations the number of breeding individuals is usually only a small proportion of N (Waples 2005). This is due to many factors, including the presence of individuals that are pre- or post-reproductive, skewed sex-ratio, the number of reproductive individuals per generation, reproductive mode and varying population size (Hedrick 2000, Trask et al. 2017).

Despite its importance, definitions of effective size and the temporal scale that estimates apply to are often confused and unclear in the literature. While many types of N_e have been described (Luikart et al. 2010), this paper will only focus on inbreeding N_e and variance N_e . Inbreeding N_e predicts the rate of heterozygosity loss, and is influenced by the number of parents in a population (Luikart et al. 2010, Baalsrud et al. 2014). Variance N_e reflects the rate of change in allele frequencies between different generations, and is influenced by the number of offspring in a population (Luikart et al. 2010, Baalsrud et al. 2014). For example, if a population decreases rapidly from 300 to 5 individuals, the variance N_e would be close to 5, yet the inbreeding N_e would still be close to 300, reflecting the previous generation (Luikart et al. 2010). Therefore, variance N_e is more appropriate for early detection of population decline, while inbreeding N_e would take a generation or more to display the consequences (Luikart et al. 2010). Despite the differences in these estimates, if the rate of random drift and heterozygosity loss is equal, or the population is isolated and maintains a constant size, there is no difference between inbreeding and variance N_e (Waples 2005, Luikart et al. 2010).

Definitions and interpretations of effective population size are further complicated by the methodological approach and the type of biological system of the organism being studied, as these can impact the temporal scale and cohort of individuals that an estimate applies to (Waples 2005). The two approaches to estimating contemporary N_e using genetic data are temporal and single sample methods (Luikart et al. 2010). When these methods are applied to discrete, nonoverlapping generations, a genetic estimate of N_e for that generation is found (Waples 2005). When applied to iteroparous organisms with overlapping generations, N_e cannot be accurately inferred, as the sample potentially contains multiple generations (Waples 2005). Rather, an estimate of the effective number of breeders (N_b) for the previous year or breeding season is found (Waples 2005). That is, the number of individuals in the previous cohort that gave rise to the progeny cohort under consideration.

Temporal methods compare estimates of allele frequencies from the same population at two or more points in time to calculate N_e (Waples & Yokota 2007). The temporal method can be used to find variance N_e (i.e. the effective size of the offspring generation) and the harmonic mean effective size (\bar{N}_e) of the entire period that the samples cover (Luikart et al. 2010). Temporal methods assume discrete, nonoverlapping generations, although there have been some variations to this method to account for effects of age structure and iteroparity (Waples & Yokota 2007). While this method is popular, it can be difficult and expensive to gather genetic data at multiple time points, particularly for species with long generation time and intervals (Ferchaud et al. 2016), such as the grey nurse shark. As genetic data is often acquired opportunistically, single sample methods are becoming increasingly common for finding contemporary N_e .

Single sample methods require sampling individuals from a population at just one point in time, offering information on the inbreeding N_e of a population (Wang 2016). These methods have been reviewed extensively (Wang 2016), and this study will only focus on the linkage disequilibrium (LD) and sibship frequency method. The LD method is based on the premises that large, random mating populations have alleles that are independent within and between loci (Wang 2016). As a population decreases in size, the rate of genetic drift increases, leading to associations between alleles at a locus (heterozygote excess) or between alleles at different loci (LD) (Wang 2016). For a population with random mating, no migration and no selection, LD must be a consequence of genetic drift and thus can be used to estimate N_e (Wang 2016). This method assumes unlinked loci, yet with genetic markers like single nucleotide polymorphisms (SNPs) which can number in the thousands, it is unlikely that all physically linked loci are removed through filtering of the dataset (Waples 2016). This can

downwardly bias estimates of N_e , yet this method has been corrected for the presence of physically linked loci (Waples 2006). Estimates using the LD method can also be downwardly biased in organisms that have overlapping generations, yet an empirical correction factor that removes this bias and estimates N_e from N_b is available (Waples et al. 2014). While this method is used to calculate contemporary N_e , LD is not a function of one generation of random mating, but is built up over many generations. As such, the time period that LD estimates of N_e apply to are not clear. It is possible that this method reflects a short-term harmonic mean N_e of the previous few generations, but more research needs to be carried out to clarify this.

Contemporary N_e can also be estimated through sibship frequency analyses. As a population decreases in size, the chance that two randomly selected individuals share a mother or father increases (Wang 2016). It is therefore possible to calculate the N_e of the parental generation, from the frequency of half- and full-sibling dyads within a sample, in relation to the total population size. This method is based on individual genotypes that are shuffled each sexual generation and so it reflects the N_e of the previous generation (or N_b of the parental cohort).

Marine protected areas

Marine protected areas (MPAs) are regions where anthropogenic activity, such as fishing, boating and diving, are regulated or totally excluded, in an effort to protect and conserve the organisms and habitat in that area. The level of human restrictions in an MPA will depend on the overall conservation goal. For example, no-take fishing zones may be implemented in an MPA where there are recovering fish populations, yet tourism activities such as diving may be permitted. Recently there has been much debate over whether MPAs are effective in protecting species, particularly highly mobile species such as sharks (Graham et al. 2016).

Ideally, MPAs are designed to protect the highest number of species and heterogeneous habitats at the lowest cost possible (Almany et al.). Many factors, including size, location and spacing, need to be considered when designing, implementing and monitoring MPAs (Almany et al.). In the past, MPAs have been spaced in a way so that if an environmental event occurs (such as a cyclone or oil spill) near one MPA, species that are affected may still be represented elsewhere in another MPA (Almany et al.). Unfortunately, this method does not take connectivity into account, and critical areas for dispersal may not be protected.

Connectivity is increasingly being recognised as a key objective in conservation planning due to the relationship between connectivity and effective population size, and population recovery and species persistence (Almany et al. 2009). Knowledge of species connectivity and the processes that affect dispersal is therefore critical for designing successful MPAs. For example, if an MPA is protecting a shark aggregation site, yet failing to encompass an important breeding or nursery area, the shark species may not be benefiting from the reserve. Similarly, if aggregation sites are protected, but migration corridors are not, numbers may decline despite their protection at aggregation sites. By gaining knowledge on the focus species dispersal capabilities, conservation plans and MPAs can better protect critical habitat, and ensure valuable conservation resources are used effectively and in the right places.

Grey nurse sharks

Shark populations have been declining globally, and although there are over 400 described species, the behaviour of sharks remains poorly understood (Chapman et al. 2015). Sharks play a significant role in marine ecosystems through top down effects, which cascade to lower trophic levels (Chapman et al. 2015). Sharks do not have a larval stage, and often individuals remain at their natal site for a substantial amount of time before actively dispersing to other sites (Chapman et al. 2015, Momigliano et al. 2015). Therefore, it is these active movements, usually at older life stages, that tend to determine the population structure of shark species (Chapman et al. 2015). Often, this is characterised by male-biased dispersal and female philopatry, where females return to or remain in their natal sites (Schultz et al. 2008, Chapman et al. 2015), as seen in the tiger shark (*Galeocerdo cuvier*) (Holmes et al. 2017) and great white shark (*Carcharodon carcharias*) (Pardini et al. 2001).

The grey nurse shark (*Carcharias taurus*) (Rafinesque, 1810), also known as the sand tiger shark or ragged-tooth shark (Smith et al. 2015), has a global yet disjunct distribution, with records off the coast of North America, South America, South Africa, Japan, eastern Indonesia and Australia (Ahonen et al. 2009, Momigliano & Jaiteh 2015). The large, coastal-dwelling shark inhabits sub-tropical to temperate waters, and typically aggregates in caves or sandy channels close to land (Stow et al. 2006, Hoschke & Whisson 2016). Grey nurse sharks are strongly K-selected, with late onset of sexual maturity at 6-7 years for males and 9-12 years for females (Goldman et al. 2006, Smith et al. 2015), and estimated lifespans of over 34 years and 40 years for males and females respectively (Passerotti et al. 2014). They also exhibit low fecundity, with females giving birth to a maximum of two pups every two years (Ahonen et al. 2009). The reproductive mode is characterised by embryonic cannibalism and oophagy,

where the first pups born in each of the two uteri attack and kill the siblings, and consume the unfertilised ova (Chapman et al. 2013).

A combination of the inshore distribution and life history traits of grey nurse sharks make them extremely susceptible to human processes and vulnerable to declines (Lynch et al. 2013). Globally, the grey nurse shark is classified as vulnerable under the World Conservation Union (Cavanagh et al. 2003b, Stow et al. 2006). The eastern Australian population of *C. taurus* is classified as critically endangered, and the western Australian population is listed as vulnerable under the EPBC Act (Cavanagh et al. 2003a). In many parts of their distribution grey nurse shark numbers are declining (Bowden et al. 2015), with the greatest threats being recreational and commercial fishing for their oil and meat (Lynch et al. 2013, Robbins et al. 2013). Since European settlement in Australia, grey nurse sharks have suffered severe population declines from fishing and culling due to their aggressive appearance (Parker & Bucher 2000, Robbins et al. 2013). Despite conservation efforts, Australian populations have continued to decline (Lynch et al. 2013). This is a result of targeted fishing and accidental bycatch, as well as mortalities from bather protection programs which utilise mesh netting and baited drumlines (Lynch et al. 2013). In one year alone, between 2015-16, 19 grey nurse sharks were entangled in the NSW mesh nets (Department of Primary Industries 2017).

Microsatellite analysis of Australian grey nurse sharks has identified strong genetic partitioning between the west and east coasts (Stow et al. 2006, Ahonen et al. 2009). Microsatellite analysis has shown that the eastern Australian population is characterised by relatively low genetic variation, evident through a single shared mitochondrial haplotype (Stow et al. 2006, Ahonen et al. 2009). As well as low genetic diversity, the most recent effective population size estimate, based on six microsatellite loci is relatively low, at 126.31(95% CI: 67.73 - 474.11) (Ahonen & Stow). As small, isolated populations are at a higher risk of genetic drift, the eastern Australian grey nurse shark population is prone to extinction if human-related mortality continues (Otway et al. 2004a)

In eastern Australia, several grey nurse shark aggregation sites have been identified along the species range, which spans central Queensland to southern New South Wales (Otway & Ellis 2011). While individuals have been found to move freely up and down the east coast, covering distances of up to 1550 km, the level of genetic connectivity of the population was unknown prior to this study (Otway & Ellis 2011). Movement patterns appear to differ depending on the maturity and sex of individuals, as well as whether the female is pregnant or not (Bansemer & Bennett 2011). While immature sharks show no obvious movement patterns, mature males and females have been shown to migrate northward

to mate in late spring to early summer. Pregnant females tend to aggregate in southern Queensland, before migrating south to pup in late winter to late spring (Bansemer & Bennett 2011). Along the species range in east Australia, 26 marine protected areas (MPAs) ranging in size from 6.76 ha to 6,579 ha, have been put in place to protect grey nurse sharks (Lynch et al. 2013). Due to the grey nurse sharks' broad range, this network of reserves is cross-jurisdictional, leading to disparities in legislations and the level of protection offered (Lynch et al. 2013).

This study uses single nucleotide polymorphisms (SNPs) to investigate the connectivity and effective population size of grey nurse sharks in eastern Australia. Specifically, these data are used to investigate (1) if the east coast population deviates from genetic panmixia; (2) if the population displays sex-biased dispersal; (3) the effective population size; and (4) the potential effects of future genetic drift on current levels of genetic variation.

METHODS

SNP genotype-by-sequencing and filtering

A total of 63 grey nurse shark DNA samples were collected between 1999 and 2007 from nine locations along the east coast of Australia between Flat Rock, Queensland and Wallagoot Lake, New South Wales (Fig. 1, Table S1). These samples were collected by NSW fisheries as part of autopsy and tagging programs, and sex was determined at the time of sampling. Five individuals were sampled in NSW but did not have location data, and therefore were removed from the map and spatial analyses. Library preparation, sequencing and single nucleotide polymorphism (SNP) discovery was carried out at Diversity Arrays Technology Pty. Ltd (DArT, Canberra, Australia), following the standard DArTSeq protocol (Jaccoud et al. 2001, Momigliano et al. 2017, Thompson et al. 2017).

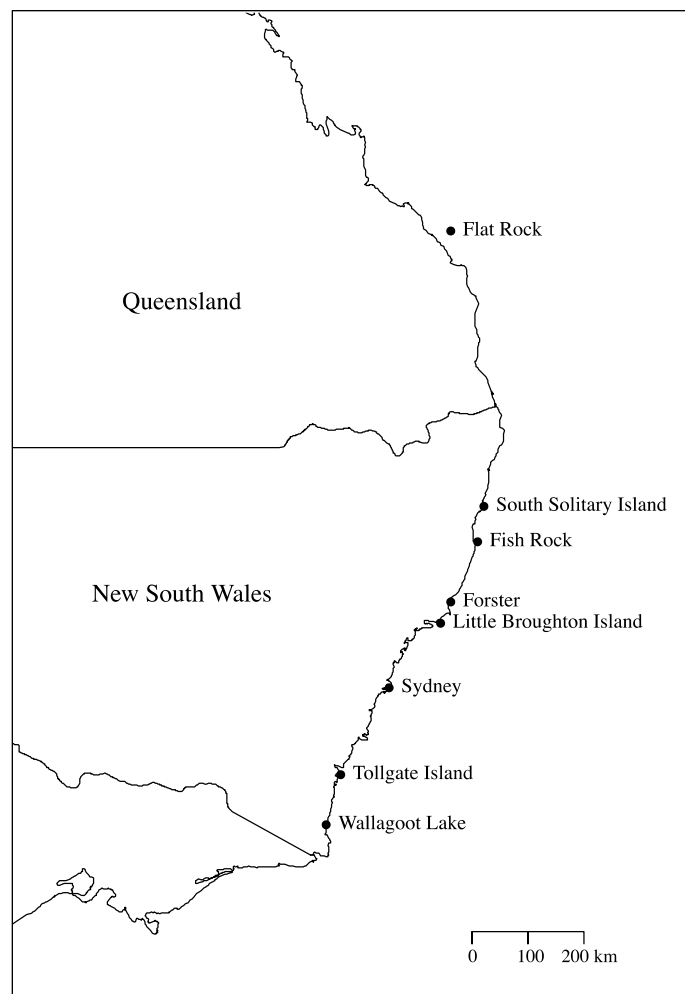


Fig. 1. Map of aggregation sites along the east coast of Australia where grey nurse sharks (*Carcharias taurus*) were sampled.

Grey nurse shark DNA was extracted using the commercially available GenCatch™ Blood & Tissue Genomic Mini-Prep Kit (Epoch Biolabs), following the manufacturer's protocol. Extracted DNA was then stored in Multi-Core™ 1X restriction enzyme buffer (Promega). For confirmation that samples contained high-molecular-weight DNA and were not contaminated with nucleases, all DNA samples were electrophoresed on a 0.8% agarose gel, pre-stained with GelRed™. A combination of *Pst*I and *Sph*I restriction enzymes were then used to digest 100 ng of each DNA sample, and adapters that were complementary to cut sites were then ligated to each DNA fragment. An Illumina flow cell attachment sequence, a sequencing primer and a barcode sequence unique to each individual DNA sample was contained within the *Pst*I adaptor.

Following digestion and ligation, all samples were purified using a spin-column PCR cleanup kit (Qiagen) and amplified by PCR, using primers, and barcode sequences specific to the adaptor. The PCR conditions involved 1 min at 90 °C for initial denaturation, 30 cycles of 20 sec denaturation at 94 °C, 30 sec annealing at 58 °C, 45 sec extension at 72 °C, followed by a final extension of 7 min at 72 °C. Following PCR, equimolar amounts of all samples were pooled together, diluted and denatured using NaOH. To sequence the library, an Illumina HiSeq2500 single read platform was used. This process involved 77 cycles, resulting in equal fragment lengths of 69 bp. A set of technical replicates, created by running 15% of the samples back through the whole library preparation protocol and downstream analysis, were used to assess the reproducibility of SNP calls.

Illumina HiSeq2500 software converted the raw sequence data to fastq files, and individuals were separated based on the unique ligated barcodes. The quality of each read was assessed, and any containing a Phred (Ewing et al. 1998) quality score of <25 were removed. Potential contaminants were identified by checking all reads against Genbank bacterial and viral sequences, and the DArT database (Diversity Arrays Technology). SNPs were then identified and called, following the standard procedure in DArT pipeline DArTSoft14™ (Diversity Arrays Technology). This pipeline is very similar to the STACKS pipeline (Catchen et al. 2013), yet differing in that DArTSoft14™ first calls the sequence clusters for the pooled sample, prior to each individual. As part of the DArT pipeline, all monomorphic clusters were removed and only SNPs that were present in both homozygous and heterozygous forms were called. Loci with very high read depths were removed, leaving SNPs with reproducibility of >95%, read depths ≥ 5 , and an average ratio read depth of 0.72 (range: 0.30-2.98) between alleles.

Following the DArT pipeline, the SNP dataset was further filtered for SNPs with 100% reproducibility and a call rate of >90%, leaving less than 10% missing data over the entire dataset. To reduce the

number of false heterozygotes due to sequencing errors, the dataset was filtered for a maximum read depth equal to $d + 3 * \sigma(d)$, whereby d is the average read depth and σ is standard deviation. Any locus with read depth <10 was removed from the dataset. To avoid tightly linked loci, only the first SNP in each fragment was retained. Finally, SNPs that had minor allele frequencies <0.01 were considered rare and removed (Mdladla et al. 2016). Some of the following analyses required the removal of certain individuals, whereby monomorphic loci in the new dataset were also removed. See Table S2 for details on the number of samples and sites used in each analysis.

Preliminary analyses

Observed heterozygosity (H_O), expected heterozygosity (H_E), and the fixation index $F = 1 - (H_O/H_E)$ with standard errors for the total dataset were calculated in the Microsoft Excel add-in package GenAlEx v. 6.5 (Peakall & Smouse 2012). Deviation from Hardy-Weinberg equilibrium (HWE) for each locus was calculated using exact test methods of Guo & Thompson (Guo & Thompson 1992) in GENEPOP v. 4.6 (Rousset 2008), and corrected for multiple testing using Bonferroni correction. There was no significant difference between results when non-HWE loci were included or removed from analyses, and so these loci were retained in the dataset. To explore genetic structure, a Principal Coordinate Analysis (PCoA) using pairwise genetic distance among all individuals was run in GenAlEx v.6.5.

Allelic differentiation

Allelic differentiation between locations was used as a proxy for gene flow. Locations that had five or more individuals were used in this analysis, as a minimum of two individuals per location is necessary to measure F_{ST} (Nazareno et al. 2017). Weir & Cockerham's (Weir & Cockerham 1984) method for estimating pairwise F_{ST} was carried out using the 'diffCalc' function in the R package diveRsity (Keenan et al. 2013). Default parameters and 500 bootstraps were used to calculate the 95% confidence intervals.

Spatial genetic structure

To investigate spatial genotypic structure, pairwise geographic and genetic distance matrices were created in GenAlEx v. 6.5 for the 58 individuals that had geographic data. A Mantel test using 999 permutations was then performed in GenAlEx v. 6.5 to test for patterns of isolation-by-distance. To further investigate genetic structure at different spatial scales, a spatial autocorrelation analysis was performed on 7 distance class bins (0 km, 250 km, 750 km, 1000 km, 1250 km and 1500 km) in

GenAlEx v. 6.5. These bins were chosen to represent the various distances between sampled aggregation sites, which range between 0 km (within the same aggregation site) to 1386 km. Significance was assessed using 999 permutations to estimate the 95% confidence interval around zero (no autocorrelation) and 1000 bootstraps to estimate the 95% confidence intervals around the autocorrelation coefficient r . Heterogeneity testing for spatial autocorrelation was used to test whether results were statistically credible (Smouse et al. 2008).

To explore the possibility of sex-biased dispersal, spatial autocorrelation analyses were then conducted under the same conditions for adult males and adult females separately in GenAlEx v. 6.5. While males and females reach sexual maturity at around 2 m and 2.2 m respectively (Bansemer & Bennett 2011), individuals longer than 1.8 m are considered subadults (Lynch et al. 2013). For the purpose of this study, individuals ≥ 1.8 m in total length were classified as adults and individuals < 1.8 m were classified as juveniles. Individuals that did not have length data recorded ($n = 12$) were assumed to be adults, as pups were usually noted.

Relatedness

Relatedness was calculated at five locations with sufficient numbers of individuals (≥ 5), as high pairwise relatedness among individuals at an aggregation site most likely indicates intergenerational fidelity to that location. Pairwise relatedness was calculated in COANCESTRY v. 1.0.1.7 (Wang 2011) using the moment estimator from Queller & Goodnight (Queller & Goodnight 1989). Then, using the geographic distance and relatedness matrices, mean relatedness for each location was plotted in GenAlEx v. 6.5.

The software program Colony2 v. 2.0.6.3 (Jones & Wang 2010) was used to reconstruct first and second degree relationships for the entire grey nurse shark dataset. Colony2 uses multilocus genotypes to infer parentage and sibship; whether two individuals share one parent (half siblings) or two parents (full siblings). The program then clusters individuals into groups according to these relationships (Jones & Wang 2010). As there can be multiple configurations, the program repeats this for the dataset until the best configuration with the highest likelihood is chosen. Unlike many other parentage and sibship methods, Colony2 can accommodate for genotyping error and polygamous mating systems, and does not require information on the parental genotypes (Jones & Wang 2010, Ackerman et al. 2017). As the grey nurse shark generations overlap, and there was no information on age of the sharks, all individuals were classified as offspring. Therefore, in this study, rather than identifying full and half-sibling dyads,

the program identifies first and second-degree relatives. The analysis can be tailored to the study organism, accomplished through the many parameters that are specified by the user.

Because the SNP dataset contained >2000 loci, the analysis was run in non-GUI mode. A comma-delimited input data file was created according to the Colony User Guide, incorporating the following parameters: analysis method = full-likelihood; likelihood precision = medium; length of run = medium; update allele frequency = no; sibship scaling = yes; number of runs = 1; random number seed = 1234; sibship prior = no prior; marker type = codominant; allelic dropout rate = 0.0000; dioecious; diploid. A conservative error rate of 0.01 was chosen, which is the equivalent of one error per 100 genotypes. Error rate has little effect on accuracy of results (Ackerman et al. 2017) so this error rate was chosen according to previous studies (Mourier et al. 2013, Pirog et al. 2017). As grey nurse sharks have been shown to have multiple paternity (Chapman et al. 2013), polygamy was chosen as the mating system for both males and females. Preliminary analysis showed no significant difference in results if inbreeding was accounted for or not, so all simulations were run with no inbreeding.

Effective population size

Contemporary effective population size was calculated using two different single-sample methods; sibship frequency and linkage disequilibrium. Both methods assume closed populations with discrete generations (Kamath et al. 2015). Effective population size is difficult to measure, particularly in iteroparous species with overlapping generations (Kamath et al. 2015), such as the grey nurse shark. Consequently, effective population size per generation (N_e) cannot be accurately inferred; rather, the following analyses calculate the effective number of breeders (N_b) of the parent generation that produced the sampled cohort (Ackerman et al. 2017). The juveniles (≤ 1.8 m in length) were therefore removed from the dataset as this would increase the number of generations included in the analyses, and upwardly bias the effective size estimate.

Effective population size using sibship frequency was conducted in Colony2 non-GUI mode, using the same parameters that were chosen for the relative/sibling reconstruction. If the effective size of a population is small, there is a higher probability that offspring within a random sample are related; if the effective size is large, there is a lower chance that the offspring are related (Ackerman et al. 2017). The sibship assignment method is based on this premise, and calculates effective size from the proportion of full and half-sibling dyads (or first and second-degree relatives) within the sample, with respect to the census population size (Ackerman et al. 2017).

NeEstimator v. 2.01 (Do et al. 2014) was used to calculate effective population size by linkage disequilibrium (LD). Linkage disequilibrium, the non-random association between alleles at different loci, can arise from migration, selection and genetic drift (Wang 2005). In a closed population with random mating and unlinked loci, LD would be the product of genetic drift, occurring at a rate inversely proportional to the N_e (Waples et al. 2016). It is therefore possible to estimate effective size by measuring LD between loci that are inherited independently, provided the assumption of an isolated population with random mating is met (Funk et al. 2016). The input file, which excluded juveniles, was converted to a GEN file in GenAlEx v. 6.5 and imported to NeEstimator v. 2.01. The random mating model under LD was chosen, and a critical value of 0.02 was selected to represent the minimum allele frequency cut off, as per previous studies (O'Leary et al. 2013, Trask et al. 2017).

Following LD estimation of effective number of breeders, the raw estimate $N_{b(LD)}$, was then adjusted for bias due to overlapping generations using the formula from Waples et al (2014). This formula incorporates two life history traits; adult life span (AL) and age at maturity (α). AL was calculated as $\omega - \alpha + 1$, where ω is maximum age and α is age at first maturity. The maximum age for this purpose was 38, as males live to >34 years and females live to >40 years (Passerotti et al. 2014). Age at first maturity was 10 as females reach sexual maturity between 9 and 12 years (Smith et al. 2015). The equation was as follows:

$$N_{b(Adj2)} = \frac{N_{b(LD)}}{1.103 - 0.245 \times \log(AL/\alpha)} \quad [1]$$

Effective population size per generation $N_{e(Adj2)}$ was then calculated using the formula from Waples et al (2014) that adjusts $N_{b(Adj2)}$ using the same two life history traits, AL and α , as follows:

$$N_{e(Adj2)} = \frac{N_{b(Adj2)}}{0.485 - 0.758 \times \log(AL/\alpha)} \quad [2]$$

Future genetic variation

The forward simulation program BottleSim v. 2.6 (Kuo & Janzen 2003) was used to model the possible effects of genetic drift on the current levels of genetic variation of the east Australian grey nurse shark population. Five simulations were run to explore the impact of genetic drift on different effective population sizes. These were chosen to represent a broad spread of N_e possibilities: the smallest N_e previously suggested to prevent the effects of inbreeding depression (50) (Frankham et al. 2014), the former N_e estimate for this population (126), and the $N_{b(sib)}$ estimate (400) and its upper and lower bounds (258 and 820), rounded up to the nearest even number. An input file, in the form of a multilocus genotype text file, was created according to the online BottleSim guidelines. All simulations were performed with the following parameters: reproduction mode = dioecy with random mating; simulation module = diploid, multilocus, constant population size; longevity of organism = 38; age at sexual maturity = 10; sex ratio = 1:1; generation overlap = maximum 100; number of years = 500; number of iterations = 500. Population size remained constant before and during the bottleneck.

RESULTS

Preliminary analyses

The filtering process removed 5557 single nucleotide polymorphisms (SNPs), resulting in a final dataset of 3087 putatively neutral SNPs across 63 individuals. Before correction for multiple tests, 377 of the 3087 loci significantly deviated from Hardy-Weinberg equilibrium (HWE) across all individuals. Following Bonferroni correction, 19 loci significantly deviated from HWE. Expected heterozygosity (H_E) was 0.267 ($SE \pm 0.003$) and observed heterozygosity (H_O) was 0.259 ($SE \pm 0.003$) across all individuals and loci, with a non-significant fixation index ($1 - H_O / H_E$) of 0.04 ($SE \pm 0.003$). The Principal Coordinate Analysis (PCoA) axes only explained 3.08% of the variation in genetic distance, and individuals EA71, EA70, EA68, EA43 and EA35 seem to be slightly differentiated from the rest of the samples (Fig. 2).

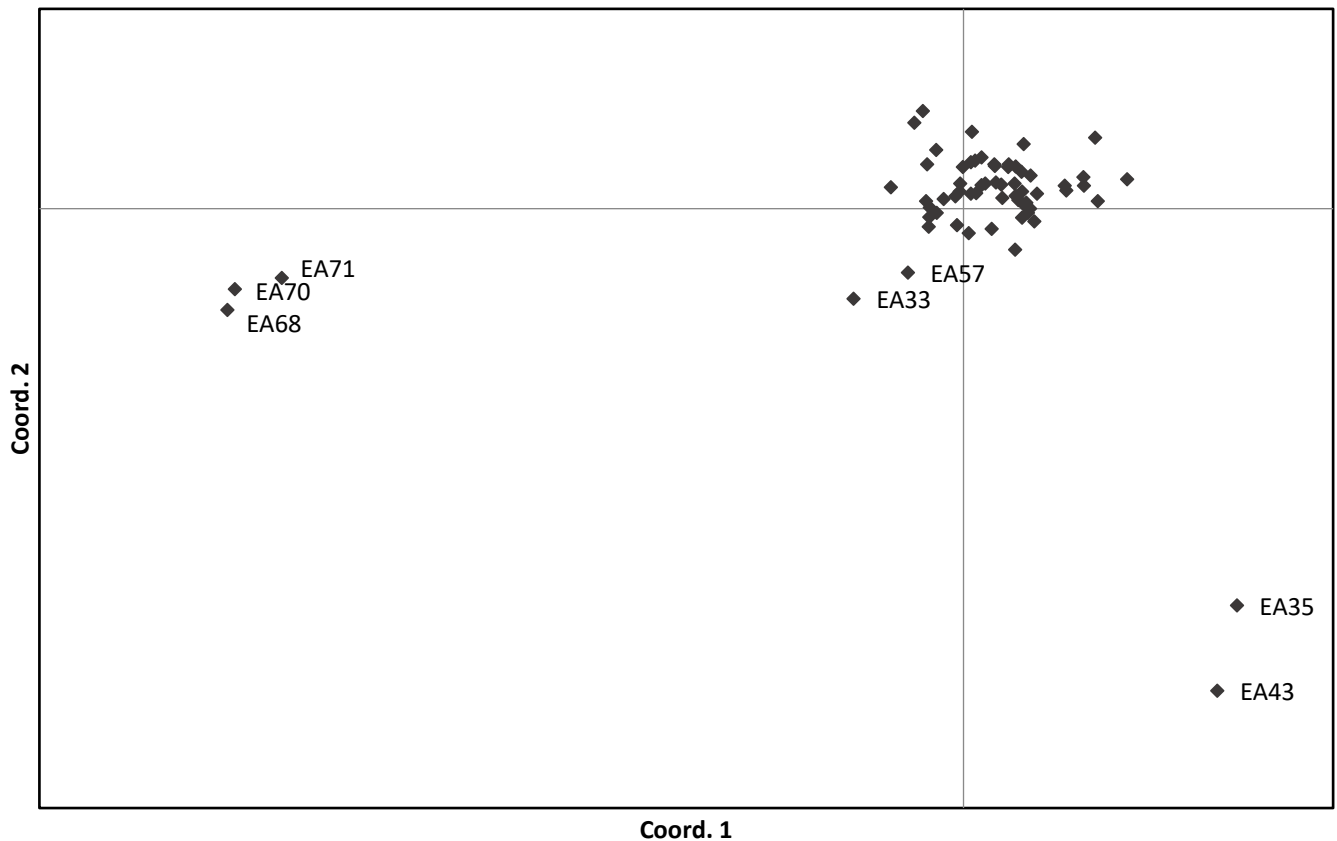


Fig. 2. Principal Coordinate Analysis of 63 east Australian grey nurse sharks (*Carcharias taurus*) showing variation in genetic distance of all individuals.

Allelic differentiation

After the removal of sample sites with <5 individuals, 3038 SNPs across 55 individuals from five locations remained. Analysis of allelic frequencies among these sites provided no evidence for allelic differentiation among the sampled locations. Pairwise F_{ST} for Flat Rock, South Solitary Island, Fish Rock, Forster and Sydney were low and did not significantly differ from 0. These values ranged between -0.003 and 0.0045 (Table 1). Note that due to the weighting scheme of Weir & Cockerham's F_{ST} estimate, resulting F_{ST} values can be negative and should be interpreted as 0.

Table 1. Allelic differentiation, represented by pairwise F_{ST} , between grey nurse shark (*Carcharias taurus*) sampling locations at Flat Rock (n = 13), South Solitary Island (n = 6), Fish Rock (n = 22), Forster (n = 5), and Sydney (n = 9).

	Flat Rock	Sth Sol Is	Fish Rock	Forster	Sydney
Flat Rock	0				
Sth Sol Is	0.0028	0			
Fish Rock	-0.0022	-0.0001	0		
Forster	-0.0069	0.0063	-0.0024	0	
Sydney	-0.0012	-0.003	-0.0005	-0.0045	0

Spatial genetic structure

Following the removal of individuals and sites without location data, 3076 SNPs for 58 individuals among eight locations remained. A Mantel test and spatial autocorrelation analyses provided no evidence for fine scale genotypic structure. The Mantel test showed no significant linear relationship between geographic distance and genetic distance, and therefore no evidence for isolation-by-distance ($R^2 = 0.0002$) (Fig. 3). Spatial autocorrelation analyses on all 58 individuals using unequal distance bins (0 km, 250 km, 500 km, 750 km, 1000 km, 1250 km and 1500 km) also revealed no significant correlation between genetic relatedness and distance ($\omega = 23.68$, $P = 0.063$), demonstrating that individuals within sampling locations were not more genetically similar than individuals at different sampling locations (Fig. 4). In addition, spatial autocorrelation on the 32 adult males ($\omega = 17.995$, $P = 0.096$) and 19 adult females ($\omega = 14.522$, $P = 0.188$) separately, provided no evidence for sex-biased dispersal or philopatry (Fig.5 & 6).

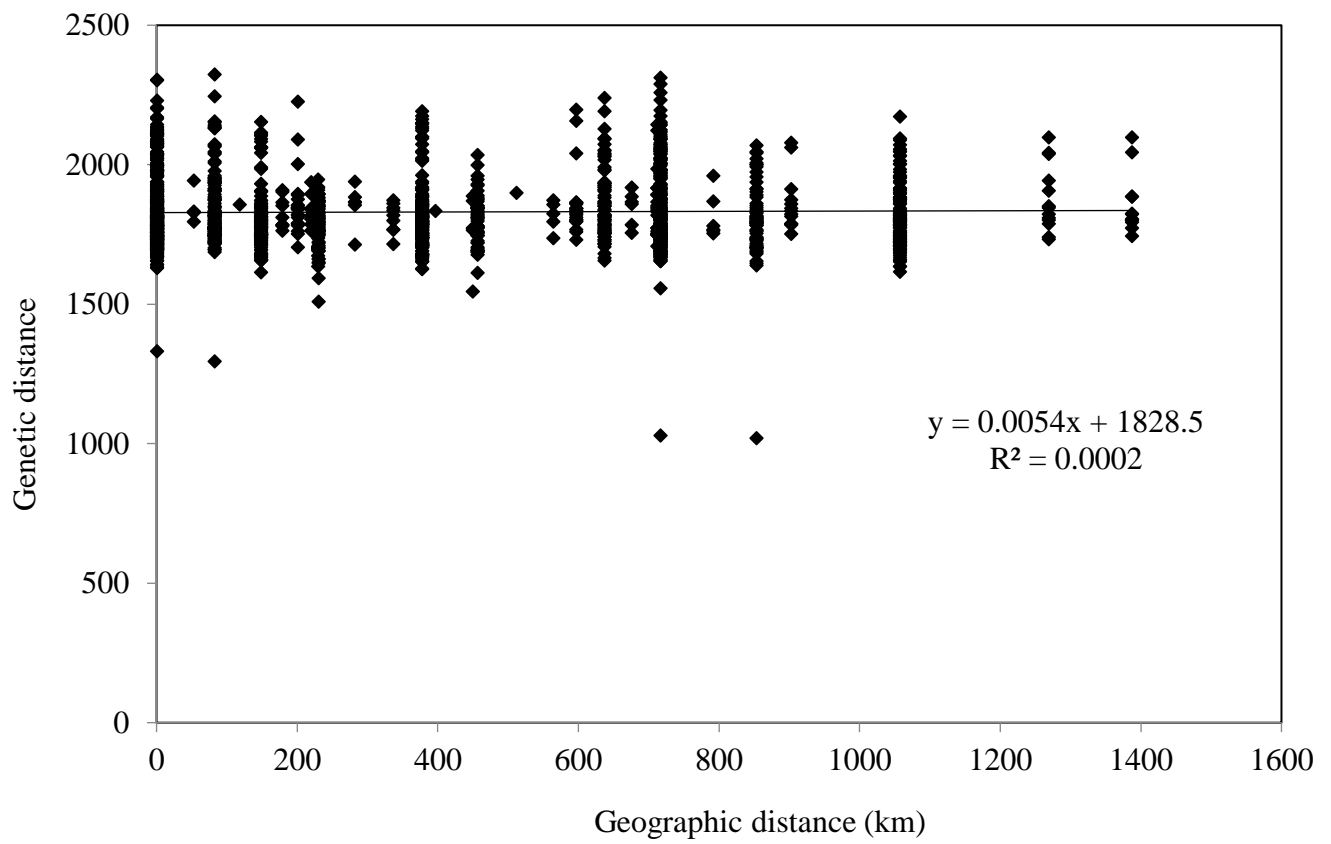


Fig. 3. Mantel test to investigate patterns of isolation-by-distance on 58 eastern Australian grey nurse sharks (*Carcharias taurus*) with location information at 999 permutations.

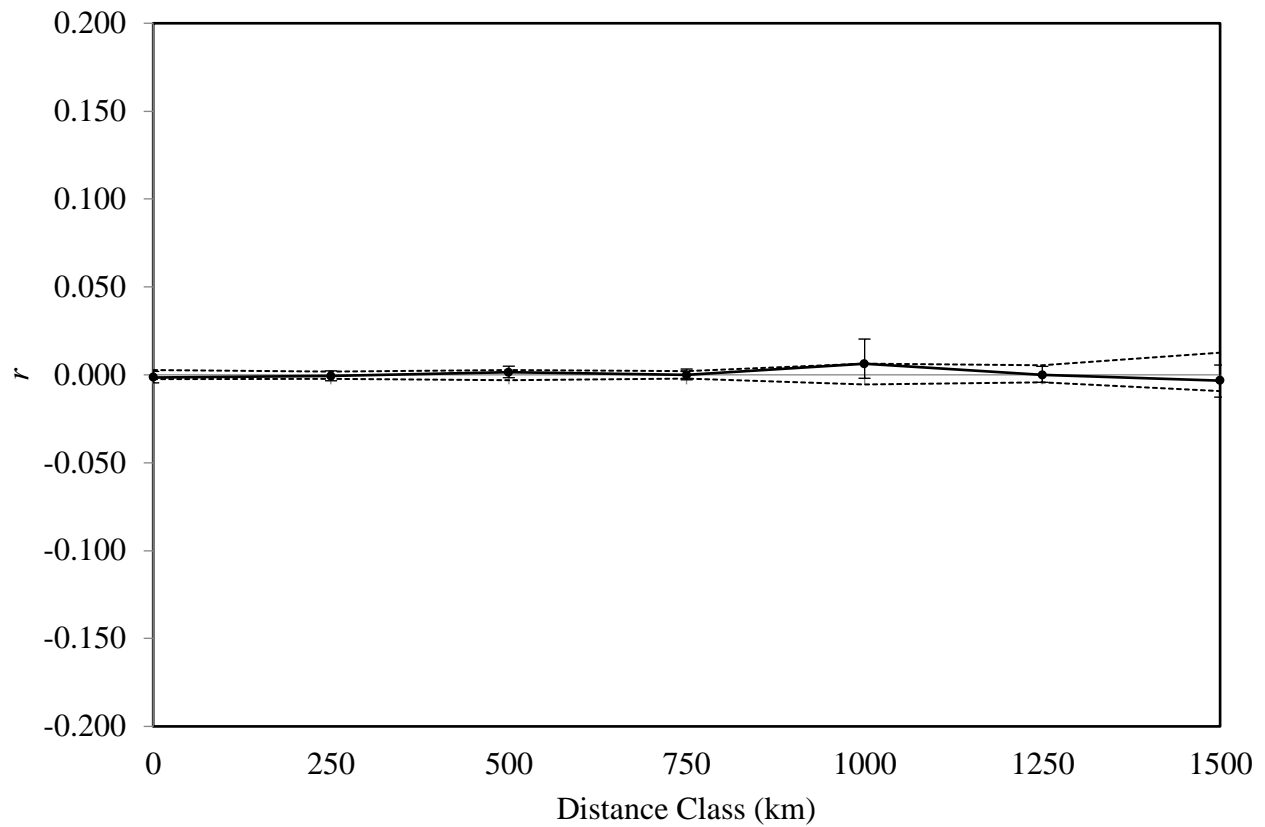


Fig 4. Spatial autocorrelation (r) estimates for distance classes 0 km (within the same location) up to 1500 km, for all eastern Australian grey nurse sharks (*Carcharias taurus*) with location information ($n = 58$). Error bars show the 95% confidence intervals estimated from 1000 bootstrap resampling. The dotted lines represent the upper and lower 95% confidence intervals from the null mode of no spatial structure, determined by 999 permutations.

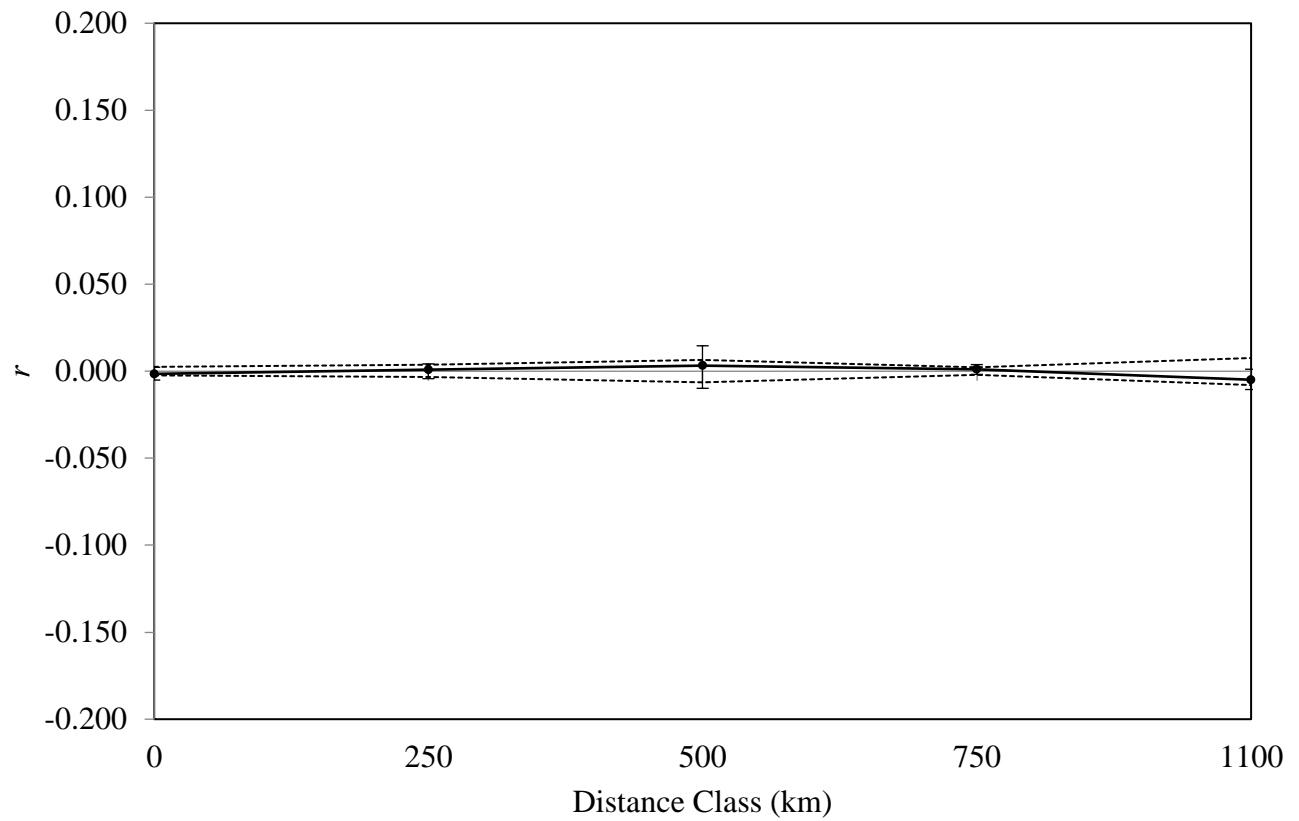


Fig 5. Spatial autocorrelation (r) estimates for distance classes 0 km (within the same location) up to 1500 km, for all adult male eastern Australian grey nurse sharks (*Carcharias taurus*) with location information ($n = 32$). Error bars show the 95% confidence intervals estimated from 1000 bootstrap resampling. The dotted lines represent the upper and lower 95% confidence intervals from the null mode of no spatial structure, determined by 999 permutations.

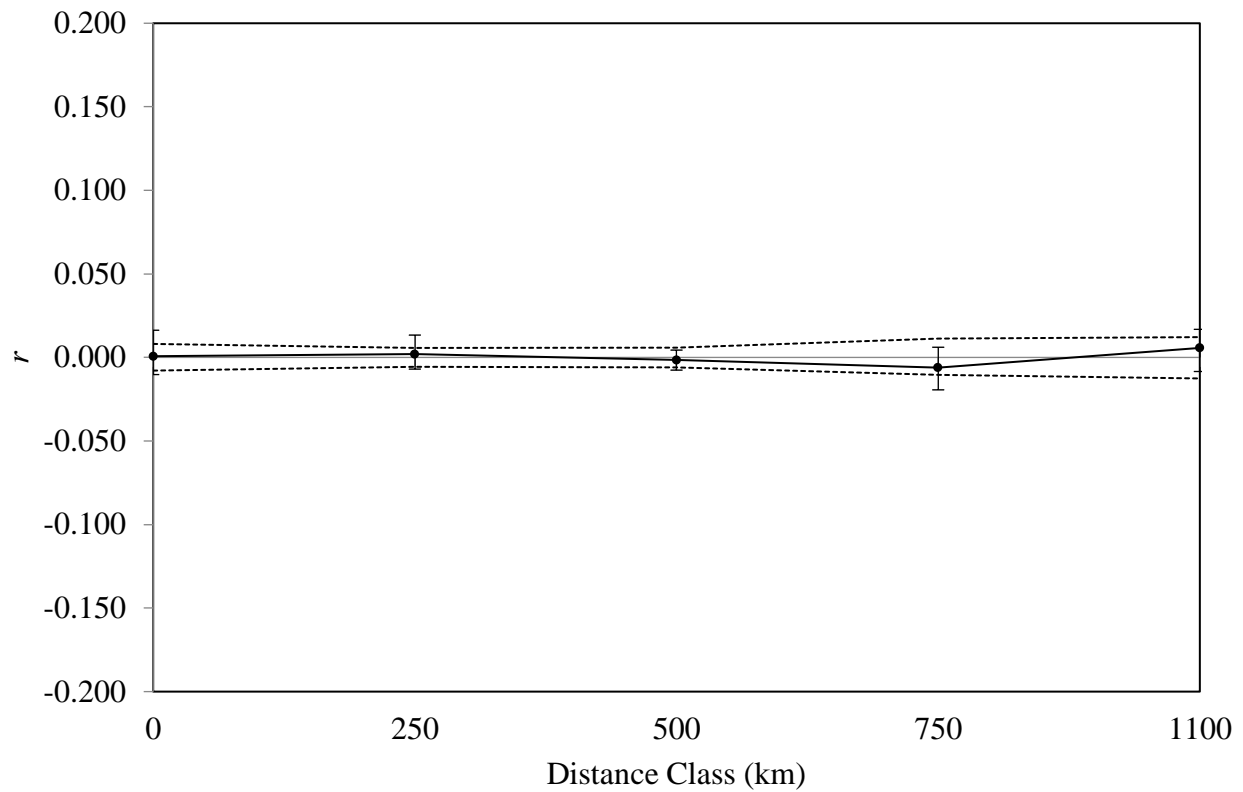


Fig. 6. Spatial autocorrelation (r) estimates for distance classes 0 km (within the same location) up to 1500 km, for all adult female eastern Australian grey nurse sharks (*Carcharias taurus*) with location information ($n = 19$). Error bars show the 95% confidence intervals estimated from 1000 bootstrap resampling. The dotted lines represent the upper and lower 95% confidence intervals from the null mode of no spatial structure, determined by 999 permutations.

Relatedness

Pairwise relatedness was calculated between the five sampling sites with ≥ 5 individuals ($n = 55$). Significantly high relatedness within a sample site was not found, providing no evidence for intergenerational site fidelity (Fig. 7). Sibship reconstruction in Colony2 for the total dataset ($n = 63$) identified five pairs of first-degree relatives and eight pairs of second-degree relatives with 100% probability. The distance between related individuals ranged from 0 km (within the same location) up to 560 km for one pair (Table 2).

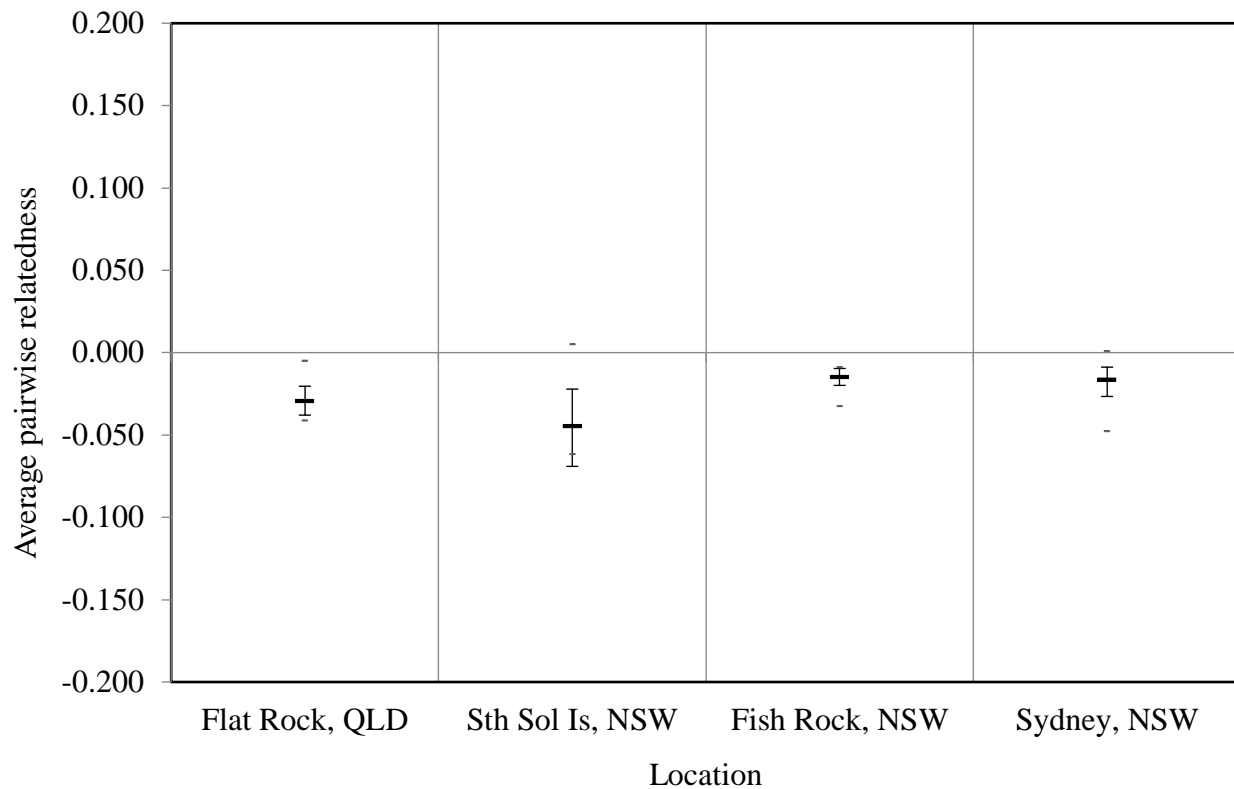


Fig. 7. Average pairwise relatedness per location ≥ 5 grey nurse sharks (*Carcharias taurus*). The upper and lower error bars represent the 95% confidence interval around the average relatedness, as determined by 1000 bootstraps. The upper and lower horizontal markers represent the 95% confidence interval of no difference in relatedness among the locations, as determined by 1000 permutations.

Table 2. The sampled location and approximate distance between first degree and second degree related pairs of eastern Australian grey nurse sharks (*Carcharias taurus*). Ind 1 and Ind 2 represent the ID for first and second individual respectively. The “-” represents missing geographic data.

Ind 1	Sampled location	Ind 2	Sampled location	Distance (km)	Relationship
EA40	Flat Rock, QLD	EA32	Forster, NSW	562	First degree
EA43	Flat Rock, QLD	EA35	Fish Rock, NSW	410	First degree
EA68	NSW	EA70	NSW	-	First degree
EA68	NSW	EA71	NSW	-	First degree
EA70	NSW	EA71	NSW	-	First degree
EA53	Coffs Harbour, NSW	EA55	Coffs Harbour, NSW	0	Second degree
EA53	Coffs Harbour, NSW	EA63	Fish Rock, NSW	80	Second degree

Ind 1	Sampled location	Ind 2	Sampled location	Distance (km)	Relationship
EA33	Fish Rock, NSW	EA57	Fish Rock, NSW	0	Second degree
EA55	Coffs Harbour, NSW	EA63	Fish Rock, NSW	80	Second degree
EA58	Fish Rock, NSW	EA63	Fish Rock, NSW	0	Second degree
EA15	Forster, NSW	EA67	Wattamolla Beach, NSW	280	Second degree
EA51	Halliday's Point, NSW	EA14	Tollgate Islands, NSW	500	Second degree
EA14	Tollgate Islands, NSW	EA69	NSW	-	Second degree

Effective population size

The effective number of breeders (N_b) was estimated from adult grey nurse sharks ($n = 57$) using two single sample methods, which resulted in similar estimates (Table 3). $N_{b(sib)}$ calculated in Colony2 using sibship frequency was 399 (95% CI: 257 - 820) assuming random mating, which was comparable to the $N_{b(LD)}$ estimate of 316.2 (95% CI: 307.2 - 325.6), calculated using linkage disequilibrium (LD) in NeEstimator2 (Table 3). Bias correction of $N_{b(LD)}$ for overlapping generations gave $N_{b(Adj2)}$ of 318.29 (95% CI: 309.23 - 327.75), which was then adjusted to find $N_{e(Adj2)}$ of 380.95 (95% CI: 370.11 - 392.28).

Table 3. Effective number of breeders and effective population size estimates for east Australian grey nurse sharks (*Carcharias taurus*) from the sibship frequency method ($N_{b(sib)}$), linkage disequilibrium method ($N_{b(LD)}$), correction of $N_{b(LD)}$ for overlapping generations ($N_{b(Adj2)}$), and correction of $N_{b(Adj2)}$ for N_e ($N_{e(Adj2)}$).

Method	Effective size estimate	95% Confidence intervals
$N_{b(sib)}$ random mating	399	257 - 820
$N_{b(sib)}$ non-random mating	366	242 - 698
$N_{b(LD)}$	316.2	307.2 - 325.6
$N_{b(Adj2)}$	318.29	309.23 - 327.75
$N_{e(Adj2)}$	380.95	370.11 - 392.28

Future genetic variation

Forward simulations using BottleSim v. 2.6 predicted that over the next 50 generations (500 years; assuming 10 years per generation), genetic diversity of the east coast grey nurse shark population will be lost through genetic drift. Simulations showed that a decrease in effective population size would result in an increase in the rate of genetic drift (Fig. 8). With an N_e of 820 and 400, the population is expected to retain 98% and 96% of observed heterozygosity (H_O) after 50 generations, respectively. For an N_e of 258, 126 and 50, 92%, 88% and 73% H_O is expected to be retained. Simulations predict that it would take around 17.5 generations (~175 years) at an N_e of 50, and around 39 generations (~390 years) at an N_e of 126 to lose 10% of the population genetic diversity.

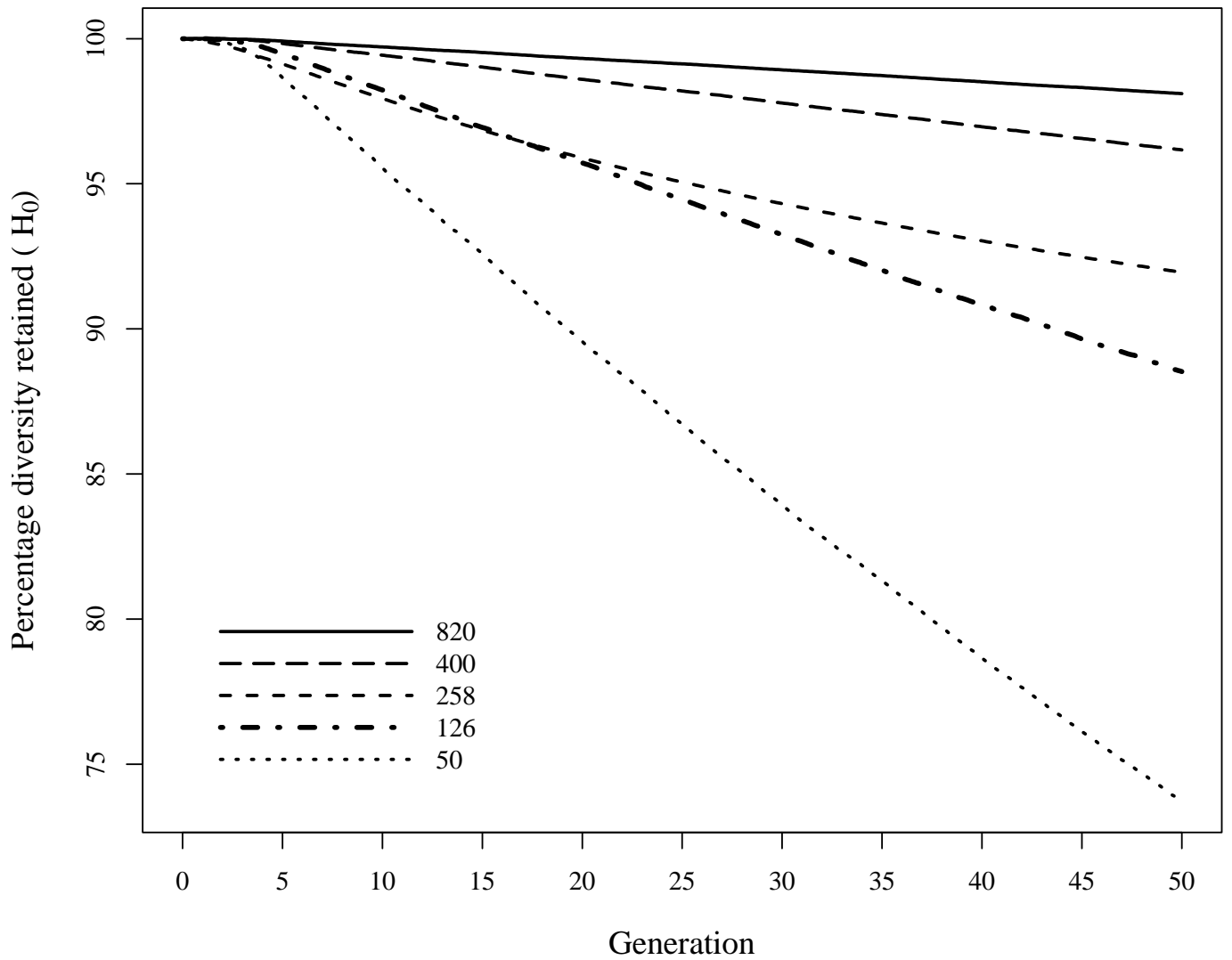


Fig. 8. Simulated loss of genetic diversity for east Australian grey nurse sharks (*Carcharias taurus*), represented as the percentage of observed heterozygosity (H_0), for different N_e values (50, 126, 258, 400 & 820) over 50 generations. Error bars indicating the 95% confidence intervals are not shown as they were not discernible.

DISCUSSION

This study demonstrates that grey nurse sharks from different aggregation sites along much of the east Australian distribution are highly connected, and that this population does not significantly deviate from genetic panmixia. Results from two single sample methods estimated the effective population size (N_e) to be around 400, and forward simulations were used to demonstrate that maintaining the current N_e of 400 will still lead to loss of genetic variation through genetic drift. These findings have significant implications for the conservation management and extinction risk of this critically endangered population of grey nurse sharks.

Analysis of thousands of putatively neutral single nucleotide polymorphisms (SNPs), continuously demonstrated high connectivity within the east coast population, through the absence of genetic structure at different temporal and spatial scales. A lack of allelic differentiation among aggregation sites was shown by F_{ST} values not significantly deviating from zero. This reveals that east coast grey nurse sharks have had sufficient gene flow among these sites over the past few generations to offset any genetic partitioning among locations via drift. Low F_{ST} values for marine populations are not uncommon, due to efficient dispersal of individuals or gametes across large distances in water (White et al. 2011). Analysis of fine scale genotypic structure, reflecting any genetic partitioning arising over shorter temporal scales, found no evidence for a relationship between geographic distance and genotypic distance. This implies that individuals are moving among aggregation sites within a single generation, or a few generations, and are therefore demographically connected. The presence of first degree relatives at different aggregation sites further supports these findings, as this means full siblings or parent/offspring pairs are dispersing within their lifetime, rather than remaining together at the same location. It should be noted that three of the grey nurse shark individuals (EA68, EA70 and EA71) identified as first-degree relatives from NSW were missing location data. These are also the same individuals that seemed to be genetically distinct from the rest of the samples (Fig. 2). As we do not know where these samples originated, a possible explanation for these findings is that these individuals were housed in aquaria in NSW. Nonetheless, these findings complement previous field observations of grey nurse sharks travelling up to 1550 km along the east coast of Australia (Otway & Ellis 2011). Spatial autocorrelation analyses of adult males and adult females separately also revealed no relationship between genetic relatedness and geographic distance for either sex, providing no evidence for sex-biased dispersal or philopatry in the east Australian grey nurse sharks. In contrast, genetic structure in many other shark species (for example, the tiger shark (*Galeocerdo cuvier*) (Holmes et al.

2017); great white shark (*Carcharodon carcharias*) (Pardini et al. 2001); and scalloped hammerhead shark (*Sphyrna lewini*) (Guttridge et al. 2017)) have been attributed to sex-biased dispersal, typically where the male is the disperser and the female is philopatric. The findings from this study support previous field observations that both adult male and female grey nurse sharks move up and down their east coast distribution in Australia (Bansemer & Bennett 2011). These movements are in synchrony with their reproductive cycle, where adults travel north to southern Queensland (QLD) and northern New South Wales (NSW) to mate, and then pregnant females aggregate at Wolf Rock in southern QLD, before travelling to the central and southern reaches of their distribution to give birth (Bansemer & Bennett 2011).

It should be noted that as grey nurse sharks do migrate along their east coast distribution in sync with their breeding seasons, it is possible that this study did not fully capture the spatial genetic patterns of the east coast population. As samples were collected opportunistically during different months over an eight-year period, related individuals that would usually aggregate together could have been sampled in separate locations. Therefore, this sampling regime may have failed to identify signals of spatial genetic structure.

Many approaches have been used to estimate the total population size of the east Australian grey nurse sharks. The most recent estimates using photographic mark-recapture were 2142 (95% CI: 1465 - 3249) using the program MARK, and 2049 (95% CI: 1216 - 2883) using Bailey's Binomial Modification (Smith & Roberts 2010). Previous estimated total population sizes include 410-461 (95% CI: 148 - 766) using mark-recapture with cattle-ear tags (Otway et al. 2004b) and 1893 (95% CI: 1556 - 2232) using four separate photoidentification surveys and an open-population model (Bansemer 2009). Previous genetic techniques based on six microsatellite loci estimated an N_e of 126 using the program ONESAMP, and a total population size of 1000-1500 individuals (Ahonen & Stow 2009). While total population size is useful for conservation management, these estimates differ from N_e in that they do not offer information on the number of reproductive individuals, or the level of genetic diversity within a population. In contrast, N_e quantifies the level of inbreeding and genetic drift within a population, providing valuable knowledge on the genetic variation and persistence of a population.

As the east coast population of grey nurse sharks approximates panmixia, it offered a sound basis for estimating effective size, as most estimators of N_e assume unlinked loci and closed populations that are in Hardy-Weinberg equilibrium, with no mutation, migration or selection (Wang 2016). Furthermore, the SNP dataset provided a powerful tool to estimate N_e as it used the information of thousands of loci.

Two single sample methods, sibship frequency and linkage disequilibrium (LD), produced similar estimates of the effective number of breeders (N_b) for the east Australian population. Given that when populations have long, overlapping generations, it is not possible to accurately infer the N_e per generation, instead the N_b of the previous cohort who gave rise to the sampled cohort is estimated. As the sibship frequency $N_{b(sib)}$ estimate of 399 (95% CI: 257 - 820) is based on individual genotypes, it reflects a very recent time frame; the effective size of the parental cohort. The temporal scale that the $N_{e(LD)}$ estimate of 380.95 (95% CI: 370.11 - 392.28), reflects is less clear. As LD is a product of genetic drift and is built up over many generations it may not reflect the previous generation alone (Wang 2005, Waples 2005, Trask et al. 2017). Instead it is possible that the N_e estimate from the LD method may reflect a short-term harmonic mean of the previous few generations. Nevertheless, both the sibship frequency and LD method provided similar contemporary effective population sizes, and this increases the confidence that these estimates are reflective of the true effective size of the east coast population of grey nurse sharks.

Estimates of N_e are often used to assess the extinction risks of a population. The previous N_e values that were recommended to avoid inbreeding depression, and to maintain evolutionary potential were 50 and 500, respectively (Jamieson & Allendorf 2012, Frankham et al. 2014). More recently, an $N_e \geq 100$ to avoid inbreeding depression in the short term, and an $N_e \geq 1000$ to retain evolutionary potential, were proposed (Frankham et al. 2014). In relation to these more recent recommendations of N_e , the east coast grey nurse shark population does not have a large enough effective size to maintain sufficient evolutionary potential and may be prone to extinction. This could have severe consequences in the future, particularly with ongoing anthropogenic-related mortalities and predictions of greater environmental stochasticity.

The contemporary N_e estimates from this study reflect the past few generations of grey nurse sharks in east Australia, and therefore are applicable to the time post-European settlement. Between the 1950's and 1970's, east Australian grey nurse sharks were fished for oil, skin, flesh and fins, and targeted by fishermen due to their aggressive appearance (Department of the Environment 2014a). While there are no robust historical records of grey nurse shark population size, a reduction in the number caught as bycatch and in shark meshing programs since the 1930's indicates that the population has decreased substantially (Department of the Environment 2014a). As such, contemporary N_e estimates can be used to gain information on the effects of recent anthropogenic-related population decline on genetic variation. However, due to the long generation time and longevity of grey nurse sharks, it is likely that

we have not yet seen the effects of a population bottleneck in their genetic makeup. Future ongoing genetic studies which monitor genetic variation and N_e would offer great insight into whether a population bottleneck has occurred, and what this means for the persistence of the east coast population of grey nurse sharks.

To account for this delay in seeing the effects of a population bottleneck in current genetic variation, forward simulations were used to model the effects of future genetic drift. All simulations, regardless of the initial N_e , showed that genetic diversity of the east coast population would erode over the next 50 generations. N_e and genetic drift are inversely related, so simulations showed that the higher the N_e , the lower the effects of genetic drift. These simulations demonstrated that an N_e of 50 and 126 was not enough to maintain 90% of current genetic variation (the recommended level of variation to avoid the effects of inbreeding depression) over the next 50 generations (Frankham et al. 2014). It was also evident that even if the east coast population continues to maintain the current N_e of around 400 individuals, loss of genetic variation will occur over the next 50 generations. These predictions from the forward simulations have implications for the management of this population, because at present, grey nurse sharks are killed as a result of human-related activities.

Grey nurse shark mortalities occur despite the many marine protected areas (MPAs) spread along the east coast that are designed to protect the east Australian population. As of 2012, 26 MPAs were managed specifically, or in part, for the conservation of grey nurse sharks; 19 of which were listed as key aggregation sites in the 2002 Recovery Plan for the grey nurse shark (Lynch et al. 2013). These reserves are managed cross-jurisdictionally as they are spread across state waters of QLD and NSW, and Commonwealth waters, and therefore vary in their classification (critical habitat, buffer zones, sanctuary or marine national park zones, and habitat protection zones) (Lynch et al. 2013). This means that there are disparities in the level of protection and activities permitted within these areas, and consequently, the level of protection a grey nurse shark receives will depend upon which MPA it is in. Furthermore, while grey nurse sharks may be protected within these sites, they do not remain in the same location. Therefore, the sharks may still be exposed to anthropogenic activities, and potentially mortality, when they are moving between MPAs and aggregation sites. As such, if fatalities are occurring in between the various reserves, MPAs may only be partially effective in protecting the east coast grey nurse shark population.

The impact of recreational scuba diving and fishing, particularly line fishing, in protected areas has been of prime concern (Bansemer & Bennett 2010, Smith et al. 2010, Robbins et al. 2013, Department

of the Environment 2014a, Smith et al. 2015). Accidental capture of grey nurse sharks is not uncommon, and aside from direct mortalities from capture and stress, many individuals are observed retaining fishing gear such as hooks, which could cause delayed fatality from punctured organs (Bansemer & Bennett 2010, Robbins et al. 2013, Department of the Environment 2014a). Hook and line fishing was listed as a key threatening process to grey nurse sharks in 2002. Approximately 12 individuals are killed a year through recreational fishing in south-east Australia, and it is likely that many more deaths go unreported (Otway et al. 2004a, Department of Primary Industries 2011). Similarly, the number of grey nurse sharks captured by commercial fisheries is probably underreported, but extremely detrimental to the east Australia population. Between 2002 and 2007, 23 grey nurse sharks were killed as a consequence of commercial fishing bycatch in NSW, while between 2007 and 2012 only 5 interactions were reported (Department of the Environment 2014a). Due to the long generation time and longevity of this species, these mortalities could have severe repercussions for this populations persistence, so the monitoring of these interactions needs to be more regulated in the future.

Further pressure is placed on the east coast population from shark nets and baited drum lines which have been put in place to protect beach goers from sharks. Since 1950, more than 439 grey nurse sharks have been entangled in shark nets in NSW (Green et al. 2009, Department of Primary Industries 2017) and since 1985, at least 49 have been caught in QLD nets (Shark control program: Sharks caught by type, Queensland, available at <https://data.qld.gov.au/dataset/shark-control-program-caught-type>, accessed 10 Aug 2017). While many sharks are released alive, it is unlikely that they survive, post-release (Department of the Environment 2014a). Despite recommendations by the Fisheries Scientific Committee for shark nets to be listed as a key threatening process to marine species, the listing was unsuccessful and nets continue to be used in shark mitigation programs (Department of Primary Industries 2005, Department of the Environment and Energy 2005). Sharks are transient and are obviously not bound by state borders and management, and consequently protection from human activities, such as fishing, in one location does not ensure protection of the sharks in another. As the simulations in this study have demonstrated that even maintaining the current N_e will lead to loss of genetic diversity, it is imperative that further declines in the population are prevented. Therefore, rules and regulations of protected areas need to be consistent across their east coast distribution to ensure maximum protection of the east Australian population of grey nurse sharks.

Future studies that employ systematic sampling will offer more insight into the connectivity of the east coast population. As this current study was based on data that was collected opportunistically, samples

were collected over an eight-year period at different times in the year. In addition, some aggregation sites had low sample numbers, yet this is not an uncommon set back when researching endangered species that may be low in number or difficult to find. Overall, while these analyses found no evidence for spatial genetic structure, it is possible that more methodical sampling that investigates the same aggregation sites multiple times a year, may uncover more information on spatial genetic structure. This is because the level of relatedness within aggregation sites may change throughout different times of the year or breeding season. Furthermore, an ongoing study that samples young-of-the-year would allow the calculation of the harmonic mean N_e and variance N_e of the east coast population through the temporal method. This would offer valuable information on the persistence of the population over time, as well as how long it takes for the effects of a population bottleneck to be evident in the genetic makeup of a population that is characterised by long, overlapping generations.

CONCLUSIONS

This study has demonstrated that there is high genetic and demographic connectivity among the east Australian grey nurse shark aggregation sites and that this population approximates panmixia. Two single sample methods estimated the effective population size (N_e) to be around 400, and forward simulations revealed that maintaining this N_e will lead to loss of genetic variation over the next 50 generations. As human-related mortality is ongoing, particularly from recreational and commercial fishing, legislation for the marine reserves should be uniform and overseen by a unified management board to ensure better protection of the critically endangered population of grey nurse sharks in eastern Australia.

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SUPPLEMENTARY MATERIAL

Table S1. Information on identification, sex, length, life stage, location and date of sample for all grey nurse sharks used in this study.

ID	Sex	Length (m)	Life stage	Location	Latitude	Longitude	Date collected
EA1	Male	2.3	Adult	South Solitary Island, NSW	-30.206	153.246	08-08-02
EA2	Male	2.3	Adult	South Solitary Island, NSW	-30.206	153.246	09-08-02
EA3	Male	2.4	Adult	South Solitary Island, NSW	-30.206	153.246	09-08-02
EA4	Male	2.44	Adult	South Solitary Island, NSW	-30.206	153.246	09-08-02
EA5	Female	2.56	Adult	South Solitary Island, NSW	-30.206	153.246	08-08-02
EA6	Male	2.38	Adult	Fish Rock, NSW	-30.939	153.117	26-06-02
EA7	Male	2.37	Adult	Fish Rock, NSW	-30.939	153.117	26-06-02
EA8	Male	2.61	Adult	Flat Rock, QLD	-24.513	152.561	23-07-02
EA9	Male	2.17	Adult	Fish Rock, NSW	-30.939	153.117	25-06-02
EA10	Male	2.41	Adult	Fish Rock, NSW	-30.939	153.117	26-06-02
EA11	Male	2.44	Adult	Fish Rock, NSW	-30.939	153.117	26-06-02
EA12	Female	1.96	Adult	Fish Rock, NSW	-30.939	153.117	24-09-02
EA13	Female	2.13	Adult	Fish Rock, NSW	-30.939	153.117	25-09-02
EA14	Female	1.53	Juvenile	Tollgate Is, NSW	-35.754	150.285	16-05-02
EA15	Female	1.93	Adult	Forster, NSW	-32.183	152.561	19-01-02
EA16	Female	2.51	Adult	Bronte, NSW	-33.957	151.286	13-09-02
EA19	Male	2.56	Adult	Flat Rock, QLD	-24.513	152.561	23-07-02
EA20	Male	2.15	Adult	Fish Rock, NSW	-30.939	153.117	25-06-02
EA21	Male	2.49	Adult	Flat Rock, QLD	-24.513	152.561	23-07-02
EA22	Male	2.53	Adult	Flat Rock, QLD	-24.513	152.561	23-07-02
EA23	Male	2.45	Adult	Fish Rock, NSW	-30.939	153.117	25-07-02
EA24	Male	2.53	Adult	Flat Rock, QLD	-24.513	152.561	25-07-02
EA26	Female	1.5	Juvenile	Off Hawkesbury River, NSW	-33.957	151.286	05-12-03
EA27	Female	1.66	Juvenile	Bondi, NSW	-33.957	151.286	12-12-02
EA29	Female	2.72	Adult	Wallagoot Lake, NSW	-36.789	149.983	20-06-02

ID	Sex	Length (m)	Life stage	Location	Latitude	Longitude	Date collected
EA31	Male	-	-	Flat rock, QLD	-24.513	152.561	05-06-02
EA32	Female	1.97	Adult	Forster, NSW	-32.183	152.561	09-06-04
EA33	Male	2.4	Adult	Fish Rock, NSW	-30.939	153.117	23-10-03
EA34	Male	2.26	Adult	Fish Rock, NSW	-30.939	153.117	11-11-03
EA35	Female	2.4	Adult	Fish Rock, NSW	-30.939	153.117	11-11-03
EA36	Male	1	Juvenile	Little Broughton Island, NSW	-32.623	152.351	19-03-02
EA37	Female	2.47	Adult	Flat Rock, QLD	-24.513	152.561	26-03-02
EA38	Female	2.002	Adult	Magic Point, NSW	-33.957	151.286	18-03-03
EA40	Male	-	-	Flat Rock, QLD	-24.513	152.561	20-08-99
EA41	Male	-	-	Flat Rock, QLD	-24.513	152.561	20-08-99
EA42	Male	-	-	Flat Rock, QLD	-24.513	152.561	20-08-99
EA43	Male	-	-	Flat Rock, QLD	-24.513	152.561	10-03-01
EA44	Male	-	-	NSW	-	-	-
EA45	Male	2.56	Adult	Flat Rock, QLD	-24.513	152.561	08-10-01
EA46	Female	-	-	Flat Rock, QLD	-24.513	152.561	-
EA47	Female	2.65	Adult	South Cronulla, NSW	-33.957	151.286	13-09-04
EA48	Female	2.88	Adult	Killcare, NSW	-33.957	151.286	13-09-04
EA49	Male	2.51	Adult	Cooge, NSW	-33.957	151.286	08-12-05
EA50	Male	2.49	Adult	Tuncurry, NSW	-32.183	152.561	12-05-06
EA51	Female	1.745	Juvenile	Hallidays Point, NSW	-32.183	152.561	26-06-06
EA53	Female	2.56	Adult	Coffs Harbour, NSW	-30.206	153.246	22-09-06
EA54	Male	2.15	Adult	Fish Rock, NSW	-30.939	153.117	01-12-06
EA55	Male	1.8	Adult	Fish Rock, NSW	-30.939	153.117	01-12-06
EA57	Female	2.38	Adult	Fish Rock, NSW	-30.939	153.117	01-12-06
EA58	Female	2-2.50	Adult	Fish Rock, NSW	-30.939	153.117	01-12-06
EA59	Male	1.53	Juvenile	Tuncurry, NSW	-32.183	152.561	05-12-06
EA60	Male	2.25	Adult	Fish Rock, NSW	-30.939	153.117	29-04-05
EA61	Male	2.4	Adult	Fish Rock, NSW	-30.939	153.117	22-05-07
EA62	Female	2.4	Adult	Fish Rock, NSW	-30.939	153.117	22-05-07

ID	Sex	Length (m)	Life stage	Location	Latitude	Longitude	Date collected
EA63	Female	2.1	Adult	Fish Rock, NSW	-30.939	153.117	22-05-07
EA64	Male	2.2	Adult	Fish Rock, NSW	-30.939	153.117	22-05-07
EA65	Male	2.45	Adult	Fish Rock, NSW	-30.939	153.117	22-05-07
EA66	Female	-	-	Bondi, NSW	-33.957	151.286	19-12-07
EA67	Female	2.79	Adult	Wattamolla, NSW	-33.957	151.286	18-12-07
EA68	-	-	-	NSW	-	-	-
EA69	-	-	-	NSW	-	-	-
EA70	-	-	-	NSW	-	-	-
EA71	-	-	-	NSW	-	-	-

Table S2. The number of sites and samples that were used in each analysis.

Analysis	Site number	Sample number
Hardy-Weinberg Equilibrium	9	63
PCA	9	63
Pairwise F_{ST}	5	55
Mantel test	8	58
Spatial autocorrelation	8	58
Adult male spatial autocorrelation	5	32
Adult female spatial autocorrelation	5	19
Relatedness	5	55
Colony analysis	7	57
BottleSim	9	63

JOURNAL INSTRUCTIONS

Manuscript formatting requirements and preparation tips

1. Manuscript length

The target length of Research Articles is approximately 10 printed pages (generally about 6000 words of body text). There are additional types of manuscripts that can be submitted with different page/word targets (see Author guidelines).

2. Title page

Title: The title should be concise and informative, i.e. summarizing either the subject or the most important findings of the study rather than merely the hypothesis addressed. It should have around 100 characters (ca. 15 words), and 150 characters at most (including spaces). Avoid 'A', 'An', 'The', 'On', etc. at the beginning.

Provide a **running page head** with 3 to 6 words; e.g. 'Detection of shrimp WSSV'.

Authors and addresses: If a manuscript has several authors from different institutions:

- use superscript numerals for identification;
- provide the address of each author's institution, identifying any present address(es) if applicable. Include zip or postal code but not street address or box number;
- use an asterisk (*) to refer to a footnote that identifies the single corresponding author and provide her/his e-mail.

Abstract: Limit length to 250 words. Provide concise information on your work, its significance and its principal results. Avoid literature cites, series of data, or meaningless clauses such as 'the results are discussed'.

Key words: Supply 3 to 8 key words, listed in order of importance.

3. Text

Please use continuously numbered pages and lines, 12 point font, and double spacing. Manuscripts that do not use correct English grammar, spelling and punctuation will be returned to authors without review; if you are not a native English speaker, you should have the text edited by someone who is, before submitting your manuscript. You may also wish to consult a 'How to' book such as Day & Gastel (2011; How to write and publish a scientific paper, 7th edn. Greenwood Press, Santa Barbara, CA).

Verbosity: Please eliminate verbiage; example:

Verbose – 'The speed was chosen because past studies by Miller (1995) and Smith (1998) have shown this to be slightly greater than the maximum sustained swimming speed.'

Not verbose – 'The speed is slightly greater than the maximum sustained swimming speed (Miller 1995, Smith 1998).'

Verbose – 'It has been shown that boat noise affects whale behaviour (Smith 1994).' (and similar phrases such as 'it has been reported/found that', 'it is possible/suspected that', 'results show that')

Not verbose – 'Boat noise influences whale behaviour (Smith 1994).'

Genus and species names must be in italics; write the genus name in full at first mention in each section (Abstract, Introduction, Materials and Methods, Results, Discussion) and abbreviate whenever mentioned again in the same section. When referring to a species, do not use the genus name alone, unless you have previously defined it that way; be precise when using 'sp.' (singular) and 'spp.' (plural).

At first mention in a section – 'The filter feeding of blue mussels *Mytilus edulis* was examined'.

After first mention in a section – 'Filter feeding rates of *M. edulis* increased with increasing temperature.'

Sequence data: Full sequence information is required when molecular methods are used. The sequences of novel primers must be given. Novel nucleotide or protein sequences must be deposited in the GenBank, EMBL or DDBJ databases and an accession number obtained.

Ocean acidification data reporting: When presenting methods and results reporting ocean acidification the 'Guide to best practices for ocean acidification research and data reporting' must be followed. Specifics for reporting ocean acidification data in scientific journals are outlined in Annex 1 of the 2015 addendum.

Abbreviations: Define abbreviations and acronyms in the Abstract and at first mention in the main text, and thereafter use only the abbreviation / acronym.

Equations and units: Use standard SI units. Relations or concentrations (e.g. mg per l) must be given as 'mg l⁻¹' (not mg/l). Variables are usually italicised (except for Greek letters). Italicisation should be consistent in normal, superscript and subscripted text. Example of proper spacing: ' $p < 0.05$, $r^2 = 0.879$ ' (not ' $p < 0.05$, $r^2 = 0.879$ '); but: 'we studied organisms of size $< 0.5 \mu\text{m}$ '

Figures and tables

Please consult Guidelines to Authors on Figure Preparation.

Figures, tables, and their captions should be self-explanatory; e.g. abbreviations and acronyms must be defined here. For table footnotes, use superscripted lower case letters; asterisks can be used to indicate statistical significance (must be defined in the legend).

4. Acknowledgement section

Declare all sources of funding of the study. In addition, you may wish to acknowledge any assistance you received from anyone not listed as author. Include this section before your Literature Cited.

5. Literature cited

All literature cited in the text must be listed, and all listed literature must appear in the text, using Harvard (Name-Year) referencing style. Citing references as 'in press' implies that the article has been accepted for publication; if pagination information is not available yet, the DOI should be included in the citation. Unpublished results and submitted articles should be cited as: author's name unpub. data (e.g. N. Smith unpubl. data) in the text only.

Format required for citing literature (examples):

Periodicals: Use standard abbreviations according to 'BIOSIS Serial Sources' or use the style for your selected journal in a reference managing software. A list of over 30000 journal names and BIOSIS abbreviations can be found [here](#). In addition, Endnote users may download styles for IR journals in this [zip file](#) for import into reference managing software.

- Dempster T, Holmer M (2009) Introductory editorial. *Aquacult Environ Interact* 1:1–5

Books: Write the title of the book in lower case, and give the publisher and place of publication. In the case of book series, give the series editor as well. Example:

- Hanski I (2005) The shrinking world: ecological consequences of habitat loss. In: Kinne O (ed) *Excellence in ecology, Book 14*. International Ecology Institute, Oldendorf/Luhe

Papers from books, conference reports, symposium proceedings, etc.: Give the title of the chapter, the editor(s) and title of the volume, the publisher and place of the publisher (not the location where the conference was held), and the pages of the chapter. The date cited must be the year of publication (not the year in which the conference was held). Example:

- West TL, Amrose WG (1992) Abiotic and biotic effects on population dynamics of oligohaline benthic invertebrates. In: Colombo G, Ferrari I, Ceccherelli VU, Rossi R (eds) *Marine eutrophication and population dynamics. Proc 25th Eur Mar Biol Symp*. Olsen & Olsen, Fredensburg, p 189–194

Dissertations: Write the title in lower case, the type of thesis / dissertation (e.g. MS / MSc / PhD), and give the university and its location. Example:

- Eve TM (2001) Chemistry and chemical ecology of Indo-Pacific gorgonians. PhD dissertation, University of California, San Diego, CA

Websites: Permanent databases such as FishBase, GenBank, or climatological sources may be included in the Literature Cited list; the access date must be given. URLs for printed publications also available online may be included with their citations. Example:

- Froese F, Pauly D (2009) FishBase. www.fishbase.org (accessed 13 Jan 2013)

Other website references should only be cited in the body text.

6. Final checklist

Please cross-check your manuscript using this list. Consult recent IR publications as a general guide for formatting:

- Include page numbers and continuous line numbers
- Ensure that abbreviations are defined at first mention in the abstract, main manuscript Included text and figure/table legends, and that the legends are informative
- Use periods instead of commas as decimal signs
- Correctly present your statistical results (e.g. include two sets of degrees of freedom for ANOVA results and significance/p-values of regressions)
- Cite all of the references in the text and vice versa
- Correctly label your figure axes with a title and a unit where applicable
- Create legible figures: i.e. large enough font size (at least 10 pt) with sufficient resolution for pdf viewing
- Prepare the manuscript (text, figures and tables) as a single file