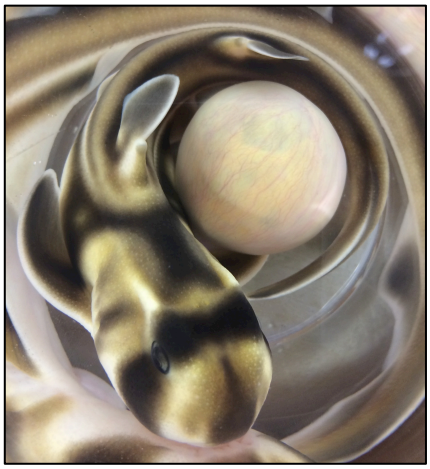
Female reproductive philopatry and male-mediated gene flow in NSW Port Jackson Sharks



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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

Understanding genetic population structure and the reproductive ecology of marine species is becoming increasingly important when developing conservation plans, due to the anthropogenic threats now facing marine ecosystems and species. Population structure in Port Jackson Sharks, *Heterodontus portusjacksoni*, was investigated using ten polymorphic microsatellites and the mitochondrial DNA (mtDNA) control region (adults: $n = 89$; embryos: $n = 75$) from two locations in New South Wales (NSW) – Sydney (SYD) and Jervis Bay (JB). MtDNA diversity was moderately low and nuclear DNA diversity was intermediate for all NSW adults. Significant structure was detected between SYD and JB using mtDNA but not microsatellites. Mean AIc values were significantly higher for females compared to males in JB but not SYD. These results reveal population genetic substructure in NSW. Females and males migrate inshore during the austral winter for breeding, however only females exhibit reproductive philopatry. Therefore, it appears the males are facilitating gene flow between populations. This study did not detect fine-scale structure within JB, however further research focusing on localized structure is needed to accurately assess this. Philopatric behaviour in *H. portusjacksoni* means the breeding sites and mating behaviours of this species should be considered when developing conservation management strategies.

Additional key words

Elasmobranch; *Heterodontus portusjacksoni*; population genetics; mating system; mtDNA; microsatellite

Introduction

Rapid human population growth over the last century has been accompanied by considerable pressures being placed on marine environments and resources (Darimont *et al.* 2015; Dulvy *et al.* 2003). Over-exploitation of marine resources has put many species at risk of population declines, depleted genetic diversity and extinction (Hutchings 2000). Sharks in particular are frequently affected through targeted commercial and by-catch fishing pressures (Worm *et al.* 2013).

Removing large predators from marine ecosystems can cause serious trophic cascades and disrupt the relative abundance of lower trophic level species (Heithaus *et al.* 2008; Myers *et al.* 2007).

Depleted populations of predators can lead to serious and permanent shifts in marine ecosystems and trigger alternate equilibrium states (Britten *et al.* 2014; Ruppert *et al.* 2013). Population declines of predatory shark species are known to have highly destructive effects on community processes and biodiversity, resulting in the loss of commercially important fish stocks (Myers *et al.* 2007).

Sharks are particularly vulnerable to over-exploitation due to their K-selected life history traits (i.e. long gestation periods, late sexual maturity, long life spans, slow growth and low fecundity). Once they are overfished, sharks can take decades to return to previous population levels (Musick *et al.* 2000). According to the IUCN Red List criteria, recent estimates indicate that extinction now threatens 25% of all known elasmobranch species due to over-exploitation and/or bycatch (Dulvy *et al.* 2014; IUCN 2015). Yet, there is currently a lack of information regarding the population status of many shark species, and this impedes the establishment and implementation of successful management strategies (Field *et al.* 2009).

The human threats that affect sharks, such as overfishing, are further complicated by the complex and varied reproductive modes in sharks, including oviparity, placental viviparity and aplacental viviparity. The extent of parental investment accompanying each of these reproductive modes generally varies, therefore influencing species-specific mating strategies (Conrath & Musick 2012). Additionally, the reproductive strategies of many shark species are still unknown, impeding the development of effective conservation strategies. Inter- and intra-specific variation in mating systems (the reproductive and/or sexual behaviours exhibited by animals) can fundamentally influence population sustainability dynamics, particularly if reproductive strategies are fitted to local conditions due to plasticity or selection. For example multiple paternity and polyandry can directly affect individual fitness, levels of genetic variability and inbreeding within a population, and subsequently affect the adaptive potential of a species (Avise *et al.* 2002; DiBattista *et al.* 2009; DiBattista *et al.* 2008).

Reproductive philopatry, where individuals show fidelity to particular nursery and/or breeding sites, has important implications for the spatial management of species. In sharks, the dispersal of females and males frequently varies (Mourier *et al.* 2013; Pardini *et al.* 2001). Nursery sites are often used to increase the survival of offspring by providing juvenile sharks with prey and decreased densities of predators (Springer 1967). Increased juvenile survival associated with nursery sites has possibly led to selection for female reproductive philopatry. This has been demonstrated for many bentho-pelagic shark species (see Chapman *et al.* 2015 for a review), including lemon sharks (*Negaprion*

brevirostris; Feldheim *et al.* 2014); blacktip reef sharks (*Carcharhinus melanopterus*; Mourier & Planes 2013); leopard sharks (*Triakis semifasciata*; Nosal *et al.* 2014); sandbar sharks (*Carcharhinus plumbeus*; Portnoy *et al.* 2010) and the bull shark (*Carcharhinus leucas*; Tillett *et al.* 2012). Selection pressure for female philopatry is probably higher in viviparous species, due to the trade-off between costs associated with parental investment and the advantage of higher reproductive success. Accordingly, reproductive philopatry is less likely in males because they generally provide little parental investment. As a result, males commonly exhibit higher levels of dispersal, and mediate gene flow between populations (Portnoy *et al.* 2015; Portnoy *et al.* 2010).

Examining the reproductive ecology of sharks is problematic due to sampling issues associated with observational studies *in situ* and the migratory patterns of many species (Speed *et al.* 2010). Further complicating this are the complex behaviours such as sexual segregation, varied adult and juvenile migratory cycles and home ranges exhibited by many shark species (Portnoy & Heist 2012). Additionally, a relatively homologous morphology across shark species leads to issues with correctly identifying species (Quattro *et al.* 2006). Moreover, tagging studies can be expensive which limits the number of individuals tagged, making it difficult to detect behavioural patterns in a population. Ultimately these factors make it difficult to unravel complex population structures using tagging and tracking, and commercial fisheries studies alone.

Molecular tools are increasingly being used to examine the extent and patterns of population subdivision occurring in sharks and many other marine organisms. High-resolution mitochondrial and nuclear markers, when used together can examine levels of genetic variation and detect patterns of population structure (Portnoy & Heist 2012). Because mitochondrial DNA (mtDNA) is maternally inherited it does not undergo recombination like nuclear DNA, which is biparentally inherited. These different modes of inheritance means genetic fixation (as a result of reproductive isolation) occurs more quickly in mtDNA, compared to most nuclear DNA regions (Portnoy & Heist 2012).

Nuclear microsatellite markers generally contain elevated levels of genetic diversity, which is useful for fine scale population discrimination. Utilizing both microsatellite and mtDNA markers is common when examining population structure, because contrasting mtDNA and nuclear DNA patterns can elucidate differences between male and female behaviour. Various mitochondrial regions have been used for elasmobranch studies, but most utilise the non-coding control region (Barker *et al.* 2015; Clarke *et al.* 2015; Taguchi *et al.* 2015; Vignaud *et al.* 2014). Microsatellite markers display varying levels of polymorphism between and within species and as such it is

necessary to employ multiple markers to assess population structure. Therefore, studies examining philopatry and sex-specific behaviour in sharks generally use both microsatellite and mitochondrial markers (Dudgeon *et al.* 2012; Hernández *et al.* 2015; Pardini *et al.* 2001).

The Port Jackson Shark, *Heterodontus portusjacksoni*, is an epibenthic, oviparous shark endemic to Australia, ranging from the Queensland (QLD) and New South Wales (NSW) border down into Victoria and around to Carnarvon in mid-Western Australia (WA) (Last & Stevens 2009). *H. portusjacksoni* migrate inshore to shallow coastal rocky reefs for breeding during the austral winter (Powter 2006). Here, females usually deposit two egg capsules at a time into rocky crevices, overall contributing approximately 16 each breeding season (Powter 2006). However, egg capsule mortality due to predation is approximately 83-89% per season. Given the low mean annual fecundity of *H. portusjacksoni,* each female therefore only contributes 1.8-2.7 surviving offspring to the subsequent generation (Powter & Gladstone 2008). Egg mortality is consequently an important factor influencing the biology and population dynamics of *H. portusjacksoni.*

To date, one study has examined the population structure of *H. portusjacksoni* using genetic techniques (O'Gower & Nash 1978). Allozyme variation indicated two subpopulations of *H. portusjacksoni* occurring in different biogeographic regions – northeastern NSW (including some of QLD) and from northern Victoria to WA. O'Gowerand Nash (1978) suggested more localized and fine-scale groups of *H. portusjacksoni* persist in these two biogeographic regions, yet the extent of these fine-scale groupings and their population structure has not yet been studied using molecular techniques. Fine scale spatially mediated genetic data are essential to understanding the mating systems that influence population structure and genetic diversity present in *H. portusjacksoni* populations. This in turn will assist the development and implementation of effective conservation strategies and fisheries management programs for this species.

H. portusjacksoni are frequently caught as bycatch in the south-eastern and eastern scalefish and shark fisheries, ranking the fifth most caught species by longlines and gillnets in 2005 (Walker *et al.* 2005). Under present fishing practices, *H. portusjacksoni* is listed as least concern on the IUCN red list (IUCN 2015), however recent demographic models suggest it is potentially vulnerable to future fishing practices due to its comparatively low fecundity and moderate resilience to the effects of fishing (Tovar-Avila *et al.* 2010).

To investigate the genetic population structure and mating system of the Port Jackson Shark, a combination of genetic markers were utilised - maternally inherited mtDNA and biparentally

inherited microsatellites. The mtDNA control region and 10 novel polymorphic microsatellite markers were used to determine the level of mtDNA and nuclear differentiation within and between putative populations *H. portusjacksoni* in NSW. Specifically this study had the following aims: (1) assess the genetic diversity and population structure of *H. portusjacksoni* at two locations in NSW (Sydney and Jervis Bay), (2) describe the relatedness of adults and embryos across three breeding/aggregation sites in Jervis Bay using parentage and sib-ship analyses and (3) evaluate the likelihood of reproductive philopatry and male dispersal occurring in NSW *H. portusjacksoni* populations.

The genetic mating system of *H. portusjacksoni* has never been studied, however, given the widespread nature of female reproductive philopatry and male-mediated gene flow in elasmobranch species it is likely exhibited by this species too. Additionally, while reproductive fidelity has not yet been observed in demersal oviparous sharks, data indicate *H. portusjacksoni* exhibits site fidelity, with mating adults returning to the same coastal breeding sites over four consecutive years (Powter & Gladstone 2009), making reproductive fidelity likely in this species.

Methods

Ethics statement

A scientific collection permit for *H. portusjacksoni* eggs and tissue was obtained from the NSW Department of Primary Industries in accordance with Section 37 of the Fisheries Management Act 1994, Part II of the Marine Parks Regulation 2009 legislation (Permit # P08/0010-4.2). Sample collection and incubation of *H. portusjacksoni* embryos conformed to the criteria set by the Macquarie University Animal Ethics Committee (ARA #2014/015-3 and #2014/003-12). Adult sharks were captured by snorkelers and were released alive, at the initial capture site immediately following tissue collection. Eggs were collected by snorkelers and transported to Macquarie University Seawater Facility for incubation.

Sample collection

Adult tissue samples were collected during the 2011-2014 breeding seasons from two locations - (1) Jervis Bay NSW (JB; 35° 03’ 55” S, 150° 44’ 05” E) and (2) Oak Park (34° 06’ 95”, 151° 15’ 77”) and Bare Island (33° 99’ 13”, 151° 23’ 10”) in Sydney NSW (SYD; Fig. 1). Within JB (Fig. 2) samples were collected from three breeding/aggregation sites – Moona Moona (MM; 35° 04’ 88” S,

150° 67’ 92” E), Dent Rock (DR; 35° 06’ 62” S, 150° 68’ 13” E) and Orion Beach (OB; 35° 06’ 95” S, 150° 68’ 44” E). Sharks were caught by snorkelers and taken to shore where pre-caudal length (PCL, tip of snout to pre-caudal pit in cm) was measured and sex was recorded. Immediately following capture, each shark was scanned for an existing passive integrated transponder (PIT) tag to ensure individuals were not sampled more than once. If no tag was detected, sharks were PIT tagged intramuscularly at the base of the dorsal fin with an individually coded tag. A small portion of dorsal fin tissue (< 1 cm^2) was removed from each shark and stored in 80% ethanol for subsequent DNA extraction.

Viable *H. portusjacksoni* eggs were collected from three breeding/aggregation sites in Jervis Bay during the 2014 mating season – (1) MM (n = 19), (2) DR (n = 20) and (3) OB (n = 20). Stages of egg development were monitored and classified as per Powterand Gladstone (2008) during the 9-12 month incubation period. Tissue samples (< 1 cm^2) were collected from the caudal fins of embryos once individuals reached seven months post-oviposition. Embryo tissue was stored in 80% ethanol for subsequent DNA extraction.

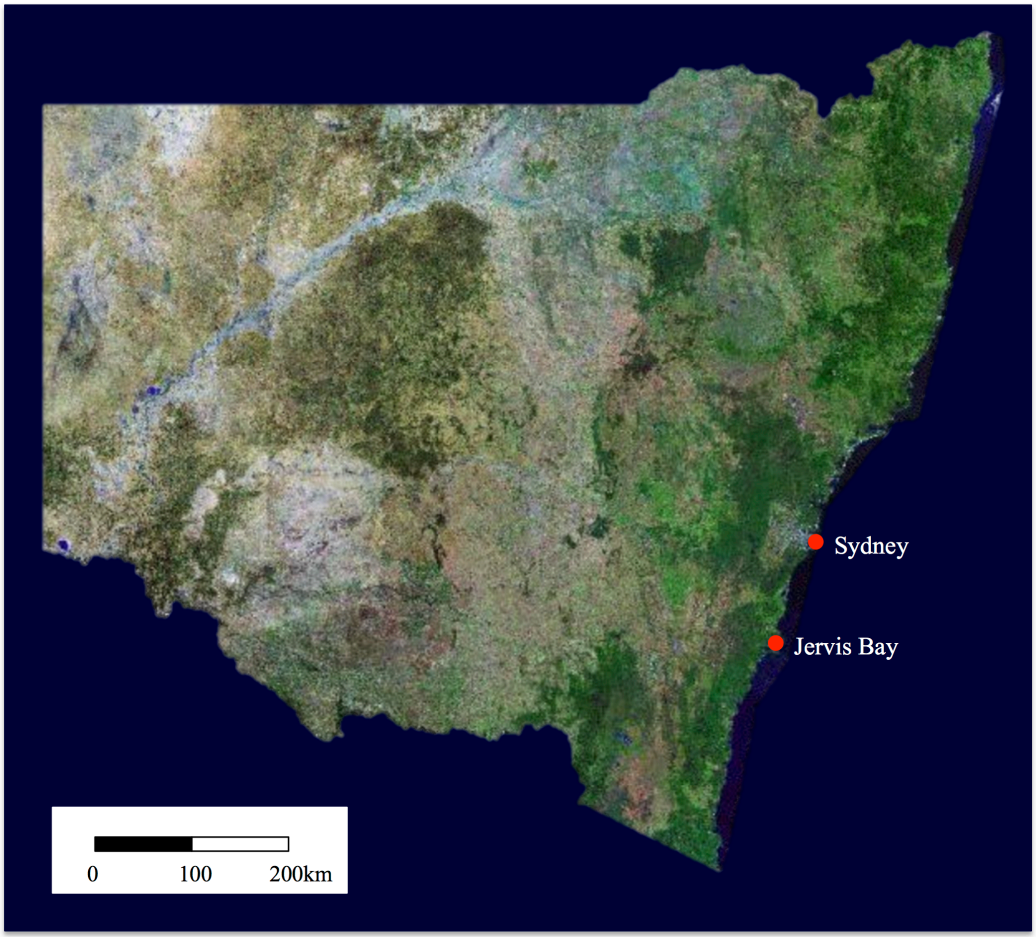

Fig. 1 Map of two sampling locations of *Heterodontus portusjacksoni* along the New South Wales coast of Australia.

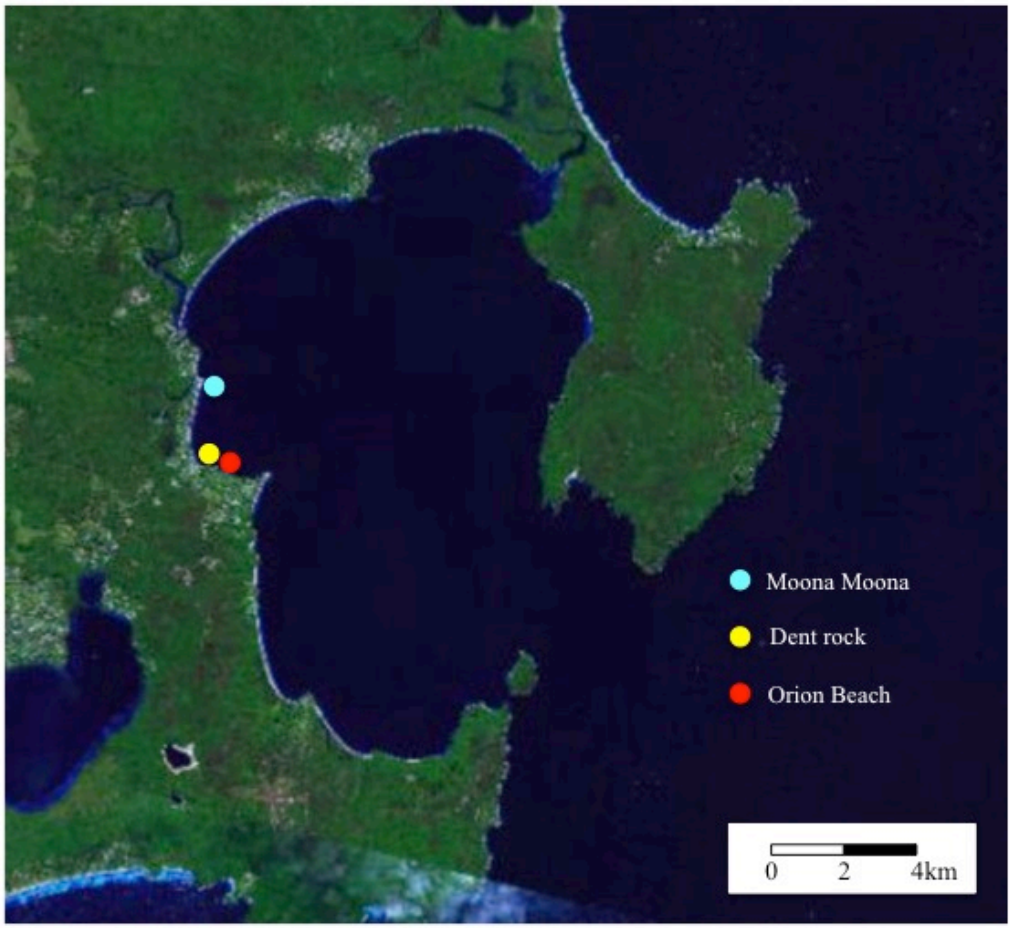

Fig. 2 Map of three sampling locations of *Heterodontus portusjacksoni* in Jervis Bay, New South Wales.

DNA extraction

Genomic DNA was isolated from all tissue samples using a modified salting out protocol with an initial proteinase K digestion step protocol as per Sunnucks & Hales (1996). Briefly, a 2mm^2 piece of tissue was placed in a small vial containing 10 µl of Proteinase K (10mg/mL) and 580 µl of TNES. This was vortexed and incubated for 18 hr in a 55°C water bath to facilitate tissue digestion. Following incubation, 170 µl of 5M NaCl was added to the mixture and centrifuged at 14,000 rpm for five min. Supernatant containing the DNA was placed in a new tube, precipitated with one volume of 100% ethanol and incubated at -20°C for one hour. DNA was pelleted by centrifugation at 14,000 rpm for 15 minutes. Ethanol was decanted from the mixture and the DNA pellet was

washed with 200 µl of 70% ethanol 100mM Na acetate, and then centrifuged at 14,000 rpm for 3 min. Remaining ethanol was decanted and removed with a pipette. Lastly, the DNA pellet was air dried for 3 min, resuspended in 100 µl of TE for 10-20 min (at room temperature) then stored at -20°C. Extracted DNA was visualized on a 2% agarose gel with a 1kb ladder (500µg/ml; New England BioLabs Inc.), stained with GelRed™ Nucleic Acid Gel Stain (Biotium) diluted 1:10,000 in RO water.

Microsatellite characterisation and genotyping

Genomic DNA was isolated from the dorsal fin tissue of one *H. portusjacksoni* female using a QIAGEN DNeasy blood and tissue kit (QIAGEN Inc., Valencia, California) as per the manufacturer's protocol. Extracted DNA was sent to the Australia Genomic Research Facility in Brisbane Australia for shotgun sequencing on a Titanium GS-FLX (454 Life Sciences/Roche FLX) as per (Gardner *et al.* 2011). The sample produced 46,252 individual sequences with an average fragment size of 250 base pairs. The program QDD v.2 (Meglécz *et al.* 2010) was used to screen the raw sequences for reads with more than eight di-, tetra- or penta-base repeats, remove redundant sequences, and design primers (automated in QDD using Primer3; (Rozen& Skaletsky 1999) aiming for polymerase chain reaction (PCR) product lengths of 80-480 base pairs.

Sixteen loci were chosen for further development. Loci were trailed for amplification in a 25 µl reaction containing 2X GoTaq® Colorless Master Mix 12.5 µl (2X Colorless GoTaq reaction buffer containing DNA polymerase pH 8.5, 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM $MgCl_2$), 10.75 µl Nuclease-Free water (Promega Corporation), 0.25 µl (50 µM concentration) of each forward and reverse locus-specific primers, 0.25 µl Rnase (1 mg/ml) and 1 µl of DNA. Primers were tested at annealing temperatures of 52-65°C to determine the optimal primer-specific annealing temperature. Amplification consisted of an initial denaturation of 3 min at 94°C, followed by 35 cycles of 20 s at 94°C, 20 s at the primer specific annealing temperature (Table 1) 40 s at 72°C and a final extension of 10 min at 72°C. All forward primers were 5′ labelled with a fluorescent tag: FAM (Sigma-Aldrich), NED, PET or VIC (Applied Biosystems™). For each PCR a positive and negative control were used, to ensure correct amplification and test for contamination, respectively.

PCR products were visualised on a 2% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc.) diluted 1:10,000 in RO water. Locus *PJ11* amplified to an unexpected size and locus *PJ13* amplified multiple fragments, thus both loci were excluded from further development.

Additionally, two loci (*PJ14* and *PJ16*) did not amplify at any annealing temperature between 52°C and 65°C when tested with various PCR cycles, and were subsequently removed from further optimisation. The remaining 12 loci were initially used to screen for variation in 39 individuals from a single population of *H. portusjacksoni* from Sydney, NSW (*n* = 39; Table 1). Prior to fragment analysis, PCR products were pooled based on each primer specific fluorescent tag, and purified to eliminate unincorporated primers and dNTPs using the single-step enzymatic cleanup product Exo-SAP-IT® (2 µl per 5 µl PCR product; Affymetrix Inc.). Allele fragment sizes obtained from Macrogen Inc. (Seoul Korea) were independently scored by at least two people using Peak Scanner™ Software 1.0 (Applied Biosystems™) to standardize allele sizes for each locus and ensure accuracy in genotyping.

MicroChecker 2.2.3 (Van Oosterhout *et al.* 2004) was used to check each locus for evidence of null alleles, large allele dropout and scoring error due to stuttering. Two loci (*PJ3* and *PJ4*) showed homozygote excess at the target site suggesting the presence of null alleles. There was no evidence for scoring error due to stuttering or large allele dropout at any locus. At each locus the number and range of alleles, observed and expected heterozygosity, deviation from Hardy-Weinberg Equilibrium (HWE; Table 1) and linkage disequilibrium among all pairs of loci were calculated using Genepop 4.2 (Raymond & Rousset 1995). No evidence for linkage disequilibrium was found after applying sequential Bonferroni correction for multiple comparisons (Hochberg 1988). Three loci (*PJ3, PJ4* and *PJ6*) deviated from HWE for the Sydney population following sequential Bonferroni adjustment of alpha. Consequently, further HWE tests were run on a different population of *H. portusjacksoni* (JB adults, *n* = 50). Based on initial characterisation and genotyping results, 10 microsatellite loci were chosen (*PJ1*, *PJ2*, *PJ5*, *PJ6*, *PJ7*, *PJ8*, *PJ9*, *PJ10*, *PJ12* and *PJ15*) for final genotyping and analysis of the SYD adults (*n* = 39), JB adults (*n* = 50) and JB embryos (*n* = 59).

Amplification and sequencing of mtDNA

A 1140bp fragment of the mtDNA control region (d-loop) was amplified by PCR using the primers GwF (5′-CTGCCCTTGGCTCCCAAAGC-3′) and GwR (5'-CTTAGCATCTTCAGTGCCAT-3'; (Pardini *et al.* 2001). Initial PCR conditions (using 3mM $MgCl_2$ and 55°C annealing temperature) produced the correct product size, however, also yielded additional non-specific reaction products, when visualised on a 2% agarose gel. To remove undesirable non-targeted PCR products, different PCR cycles and $MgCl_2$ concentrations were trialed. Optimum PCR conditions were carried out using an Eppendorf Mastercycler EP Gradient S thermal cycler, in 50 µl reactions as follows –

33.65 µl Nuclease-Free water, 10 µl 5X Colorless GoTaq® Flexi Buffer, 2.6 µl $MgCl_2$ (1.3mM) solution, 1 µl PCR nucleotide mix (0.2mM of each – dATP, dGTP, dCTP, dTTP), 0.25 µl GoTaq® DNA Polymerase (5u/ µl) (Promega Corporation), 0.5 µl Rnase (1mg/ml), 0.5 µl of each forward and reverse primer and 1 µl template DNA. PCR products were purified using Exo-SAP-IT® (3 µl per 15 µl PCR product Affymetrix Inc.) and then sequenced by Macrogen Inc. (Seoul Korea) using an Applied Biosystems™ 3730XL DNA Analyzer. MtDNA sequences were cleaned and aligned independently using Sequencher® 5.3 (Gene Codes Corporation) resulting in an 849bp fragment.

Data Analysis

Genetic variation

Microsatellite genotypes were screened for duplicate sampling using GenAlEx 6.5 (Peakall & Smouse 2006, 2012). Genetic variation within each population and subpopulation was estimated by calculating allelic richness, inbreeding coefficients (F_{IS}) using FSTAT 2.9.3 (Goudet 2002), number of unique alleles, number of different alleles, number of effective alleles, using GenAlEx 6.5 and observed and expected heterozygosity using Arlequin 3.5.2.2 (Excoffier& Lischer 2010).

Nucleotide (π) and haplotypic (h) diversity for mtDNA were calculated for both JB and SYD adults using Arlequin 3.5.2.2 (Excoffier & Lischer 2010). The Kimura-2-parameter (K2P) genetic distance (Kimura 1980) with a gamma distribution of 0.5 was used to take into account unequal substitution rates within sites. Using TCS 1.21 (Clement *et al.* 2000) a haplotype network was constructed using the statistical parsimony methodology (Templeton *et al.* 1992). This examined any genealogical links among mtDNA control region lineages. The maximum number of substitutions to parsimoniously link two haplotypes was estimated (with 95% confidence) by initially connecting sequences with the least number of differences. Additionally, haplotype outgroup probabilities were estimated, allowing identification of the most ancestral haplotype. Nucleotide sequences from the eight identified control region haplotypes in NSW *H. portusjacksoni* were analysed using the standard nucleotide BLAST® program (Altschul *et al.* 1990) to check for matches with any previously entered *H. portusjacksoni* individuals.

Genetic differentiation

Genetic differences between populations established *a priori* based on geographic locations were quantified by an analysis of molecular variance (AMOVA) in Arlequin 3.5.2.2. Significance between paired populations was assessed for microsatellite and mtDNA based on conventional F_{ST} and ϕ_{ST} using 10,000 random permutations following sequential Bonferroni adjustment of alpha.

Table 1. Description of 12 microsatellite loci developed to genotype *Heterodontus portusjacksoni* where T_A: annealing temperature; N_S: number of individuals scored; N_A: number of alleles; H_O/H_E: observed heterozygosity/expected heterozygosity; loci marked with * deviate from Hardy-Weinberg expectations following sequential Bonferroni adjustment of alpha. (Note; *PJ3* and *PJ4* were excluded from further analysis due to the presence of null alleles). Results are only shown for Sydney adults (*n* = 39).

Locus	Primer sequence 5'-3'	Genbank accession no.	Repeat motif	T_A (°C)	Size range (bp)	N_S	N_A	H_O/H_E	H-W
PJ1	F: CTTAGCAGGTCAGGCAGCAT R: AAACCACTGAATGGCACCTC	to be lodged	$(AC)_{11}$	59	262-268	39	3	0.56/0.52	0.1369
PJ2	F: GTGAGCAGAAGTGGCAAACC R: TCCAGGCCATTGTTACGAAG	to be lodged	$(AT)_{12}$	61	254-264	39	6	0.62/0.67	0.5619
*PJ3**	F: GCTCGCACTGATGATGTCTG R: CTTCCCAAGTGACTGATGGG	to be lodged	$(AT)_{11}$	59	204-224	39	8	0.36/0.74	0.0000
*PJ4**	F: AAGCTGTTGCTGGTAGCGTT R: AGACAGCCAGAGACAGATGAA	to be lodged	$(AGAT)_{21}$	61	189-297	39	22	0.74/0.92	0.0000
PJ5	F: GCGCAGAGTGTAAAGAGGGA R: GCAGCTATTGAAGGACTGAATAAA	to be lodged	$(AAAG)_{16}$	59	106-174	38	13	0.84/0.87	0.9171
*PJ6**	F: AGGTGCGGTTATGAAACACG R: ATCCCGTGAAGAACTGACCA	to be lodged	$(AC)_{12}$	59	139-143	39	3	0.15/0.23	0.0010
PJ7	F: AGCTGGGAGTTTCACTTGGA R: CTGGCGATCTAGCAGCAAGT	to be lodged	$(AG)_{12}$	59	131-137	39	3	0.44/0.39	0.8463
PJ8	F: GGTCAAGTGTCAGTAGGCCG R: CGTTGGCGTAAACCAAACTC	to be lodged	$(AG)_{14}$	56	120-130	39	6	0.77/0.70	0.6289
PJ9	F: TGGCACTATTGTTTCACGGG R: TTGCTGCAGCTAAGGCTGTT	to be lodged	$(AAAC)_{10}$	60	88-124	39	10	0.74/0.81	0.4388
PJ10	F: AAATCAATCAGTCTGCCTATCAA R: ATACGGAGATTGGGAAAGGG	to be lodged	$(AGAT)_{14}$	58	103-123	39	6	0.74/0.70	0.9775
PJ12	F: ATAGGTGTCGGGAGCATGAA R: TGACTAGTTAGTTCAGTTGGCCTG	to be lodged	$(AG)_{11}$	59	91-100	39	4	0.59/0.55	0.9457
PJ15	F: AGCAGCTCATCCATCTCACG R: ACTGAGGGAGCACCACACTG	to be lodged	$(AC)_{17}$	59	84-112	39	13	0.82/0.83	0.7161

To validate microsatellite F_{ST} results, the number of genetically distinct populations along the NSW coast was estimated using the Bayesian model-based clustering method as implemented in Structure 2.3.4 (Pritchard *et al.* 2000). Structure 2.3.4 probabilistically calculates the most likely number of clusters (K) by computing the log-likelihood value of the data whilst allocating each multilocus genotype to a genetic cluster. Monte Carlo Markov chain (MCMC) runs were carried out using a burn-in period of 10,000 iterations followed by runs of 10^6, for K values ranging from one to four. To check for convergence, five independent runs were performed for each K value. Analysis was conducted twice – firstly with populations defined *a priori* and secondly without any population information. Due to the close geographical proximity of SYD and JB populations, and likelihood of moderate gene flow, the admixture model using correlated allele frequencies was chosen (Falush *et al.* 2003). To determine the number of populations (K) most consistent with the empirical dataset, the mean log-likelihood of the dataset was maximized for each K value (Pritchard *et al.* 2000).

Sex-biased dispersal

Based on F_{ST} results, sex-specific comparisons were performed for SYD and JB adult populations using mean corrected assignment indices (AIc) and mean relatedness values. Maximum likelihood estimates of pairwise relatedness coefficients were calculated between sexes (MF) and within sexes (FF; MM) in each adult population using ML-Relate and computing 5000 iterations (Kalinowski *et al.* 2006). This program calculates maximum likelihood estimates of relatedness (r) using the downhill simplex routine. R is defined as mean relatedness among sharks, $R = 2F_{ST}/(1 + F_{IT})$, where F_{IT} is the inbreeding coefficient of individuals relative to random mating (Queller & Goodnight 1989). Differences in mean relatedness between males and females at each location was assessed using a two-sample randomization test with 10,000 iterations in RT 2.1 (Manly 1997).

AIc values for all adults were calculated in Geneclass 2.0 (Piry *et al.* 2004), using the Bayesian classification method (Rannala & Mountain 1997). This method accounts for differences between populations and the sampling error associated with estimating allele frequencies. Assignment test values were corrected for population effects by using the approach of Favre *et al.* (1997), whereby for each individual the mean log-likelihood of the population was subtracted from the individual log-likelihood. Sex-based AIc differences were tested for using the non-parametric Mann-Whitney U-test. Negative mean AIc values indicate higher than estimated incidence of rare genotypes in a population, implying high dispersal frequency and positive mean AIc values indicate the more philopatric group. This method allows each population or subpopulation to be tested independently and hence is able to detect dispersal bias at different geographical scales (Prugnolle & De Meeûs 2002).

Table 2. Summary of allelic patterns and genetic variability in NSW *H. portusjacksoni* based on 10 microsatellite loci; N_S number of individuals sampled (male : female); N_{UA} mean number of unique alleles; N_E mean number of effective alleles; AR mean allelic richness; N_A mean number of different alleles; H_0 mean observed heterozygosity; H_E mean expected heterozygosity; F_{is} inbreeding coefficient

Population	**N_S (M:F)**	**N_A ± SE**	**N_{UA} ± SE**	**N_{EA} ± SE**	**AR ± SE**	**H_O**	**H_E**	**F_{IS}**
JB - DR	14 (7:7)	5.30 ± 0.78	0.10 ± 0.10	3.46 ± 0.61	5.25 ± 0.78	0.621	0.606	0.013
JB - MM	17 (8:9)	6.40 ± 1.21	0.50 ± 0.31	3.76 ± 0.70	5.87 ± 1.03	0.594	0.634	0.093
JB - OB	19 (10:9)	6.20 ± 1.20	0.10 ± 0.10	3.28 ± 0.42	5.53 ± 0.97	0.608	0.648	0.088
JB – all	50 (25:25)	7.80 ± 1.46	0.50 ± 0.31	3.79 ± 0.68	5.62 ± 0.93	0.606	0.651	0.078
Sydney	39 (19:20)	6.70 ± 1.25	0.20 ± 0.13	3.57 ± 0.65	5.64 ± 0.83	0.628	0.627	0.011
All Adults	89 (44:45)	8.10 ± 1.53	8.1 ± 1.53	3.81 ± 0.72	8.08 ± 1.53	0.616	0.648	0.051

Table 3. Percentage frequency and haplotypic (*h*) and nucleotide (π) diversity ± SE (using Kimura 2P, Gamma = 0.5) of mtDNA control region haplotypes (1-8) for NSW *H. portusjacksoni*, where N_H is the number of haplotypes and N_{PS} is the number of polymorphic sites.

Site	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**H7**	**H8**	**N_H**	**N_{PS}**	***h* ± SE**	**π ± SE**
JB-OB (*n* = 15)	67	0	20	0	13	0	0	0	3	3	0.5333 ± 0.1259	0.00153 ± 0.00114
JB-DR (*n* = 11)	64	0	9	0	27	0	0	0	3	3	0.5636 ± 0.1340	0.00142 ± 0.00110
JB-MM (*n* = 15)	53	0	13	7	27	0	0	0	4	4	0.6667 ± 0.0991	0.00173 ± 0.00124
JB - all (*n* = 41)	61	0	15	2	22	0	0	0	4	4	0.5720 ± 0.0677	0.00152 ± 0.00108
SYD (*n* = 34)	26	0	29	3	24	3	12	3	7	7	0.7950 ± 0.0326	0.00201 ± 0.00134
All NSW (*n* = 75)	46	0	21	3	23	1	5	1	7	7	0.7031 ± 0.0343	0.00195 ± 0.00129

Parentage and sibship analysis

Parentage analysis was conducted for 59 embryos and 50 adults from JB, using the maximum-likelihood approach in (1) Cervus 3.0.7 (Marshall *et al.* 1998), (2) Colony 2.0.5.9 (Jones & Wang 2010) and verified using ML-Relate (Kalinowski *et al.* 2006). Sibship reconstruction was conducted for the embryos using firstly Colony 2.0.5.9 and then verified in ML-Relate. Due to the convergence problems that are common when employing maximum-likelihood approaches, each analysis was replicated three times using the same information. All replicate analyses returned identical results.

Colony 2.0.5.9 conducts simultaneous inference of multiple relationships among individuals and as such performs with greater statistical power compared to pairwise parentage analysis (Sieberts *et al.* 2002; Walling *et al.* 2010). Additionally, Colony 2.0.5.9 also assesses statistical confidence at an individual-level established from the proportion of iterations that a specific relationship occurs and by the probability of configurations (Wang & Santure 2009). It also accounts for genotyping errors – this study assumed a rate of 0.01 per locus for both typing errors and allelic dropouts.

In contrast, Cervus 3.0.7 takes a less conservative approach with confidence levels based on simulated population means, which can increase the overall amount of parentage assignments, however at the possible cost of increased inaccurate assignments (Walling *et al.* 2010). Similar to Cervus 3.0.7 and Colony 2.0.5.9, ML-Relate calculates the likelihood that each pair of individuals are unrelated, parent-offspring, full-siblings or half-siblings, then reports the relationship with the highest likelihood.

Cervus 3.0.7 was first used to find highly likely maternal and paternal assignments between JB embryos and adults across all three breeding sites – OB, DR, and MM. Assignment to potential parents was carried out using a strict 95% and an 80% confidence interval and simulated for the entire JB population. All adult males and females from JB (males = 25, females = 25) were included as candidate fathers and mothers respectively and the probability of a candidate mother/father being present in the sample population was set very low (0.05) due to a lack of population estimates for *H. portusjacksoni*.

Known mother- and father-offspring pairs as inferred by Cervus 3.0.7 were identified during parentage analysis in Colony as known maternity and paternity. Using this approach, adults were separated by sex and a polygamous mating system was assumed for both sexes, thus permitting the assignment of half-siblings. A long-run using high likelihood precision and genotyping error rate of 1% was implemented. Due to the absence of population estimates for *H. portusjacksoni,* the prior

probability that the true parent was present in the sample was calculated from the proportion of sampled mothers and fathers previously assigned to embryos in Cervus 3.0.7.

Maternal and paternal assignments were only accepted if Cervus 3.0.7 assigned a parent at 95% pair confidence and this assignment was the supported by either Colony 2.0.5.9 or ML-Relate. Alternatively, 80% parental assignments generated from Cervus 3.0.7 were also accepted if the assignment was verified in both Colony 2.0.5.9 and ML-Relate. Similarly for sibship reconstruction, half-sib and full-sib relationships produced by Colony 2.0.5.9 were only accepted if validated in ML-Relate.

Results

Genetic variation

Screening in GenAlEx 6.5 indicated no duplicate samples in the dataset based on identical genotypes at 12 microsatellite loci. Evidence for null alleles and deviation from HWE at locus *PJ3* and *PJ4* meant these two loci were excluded from analyses. Deviations from HWE were found at locus *PJ6* for SYD (P = 0.0010) and *PJ7* for JB adults (P = 0.0047). Since HWE deviations were not consistent across populations, both *PJ6* and *PJ7* were included in the analyses. Tests for linkage disequilibrium did not identify significant deviations from the null hypothesis of independent assortment. Measures of nuclear genetic variation including number of alleles, number of unique alleles and number of effective alleles was higher in the JB than SYD population (Table 2). Inbreeding coefficients (F_{IS}) were positive but very low for all populations.

Sequence alignment from the 849bp fragment of mtDNA control region for JB and SYD adults revealed seven polymorphic sites, defining seven unique haplotypes. All haplotypes were closely related (Fig. 3), with four haplotypes identified in the JB adults and seven in the SYD adults, with three unique to only SYD (Table 3). An additional haplotype (Haplotype 2) was identified in only in DR and MM embryos. Embryos were excluded from the haplotype network (Fig. 3). Intermediate haplotypic (h) and low nucleotide (π) diversity was observed for all populations (h: 0.5333-0.7950; π: 0.00142-0.00201; Table 3), however both were higher in SYD compared to JB. The highest frequency haplotype that was continuously distributed throughout all of NSW was haplotype 1 (H1) and haplotype 5 (H5) was identified as the ancestral maternal lineage, also distributed across all populations in NSW. All microsatellite loci and mtDNA haplotypes are novel and will be submitted to GenBank. There were no pre-existing *H. portusjacksoni* nucleotide sequence matches to the eight identified NSW haplotypes, when each sequence ran through the standard nucleotide BLAST® program.

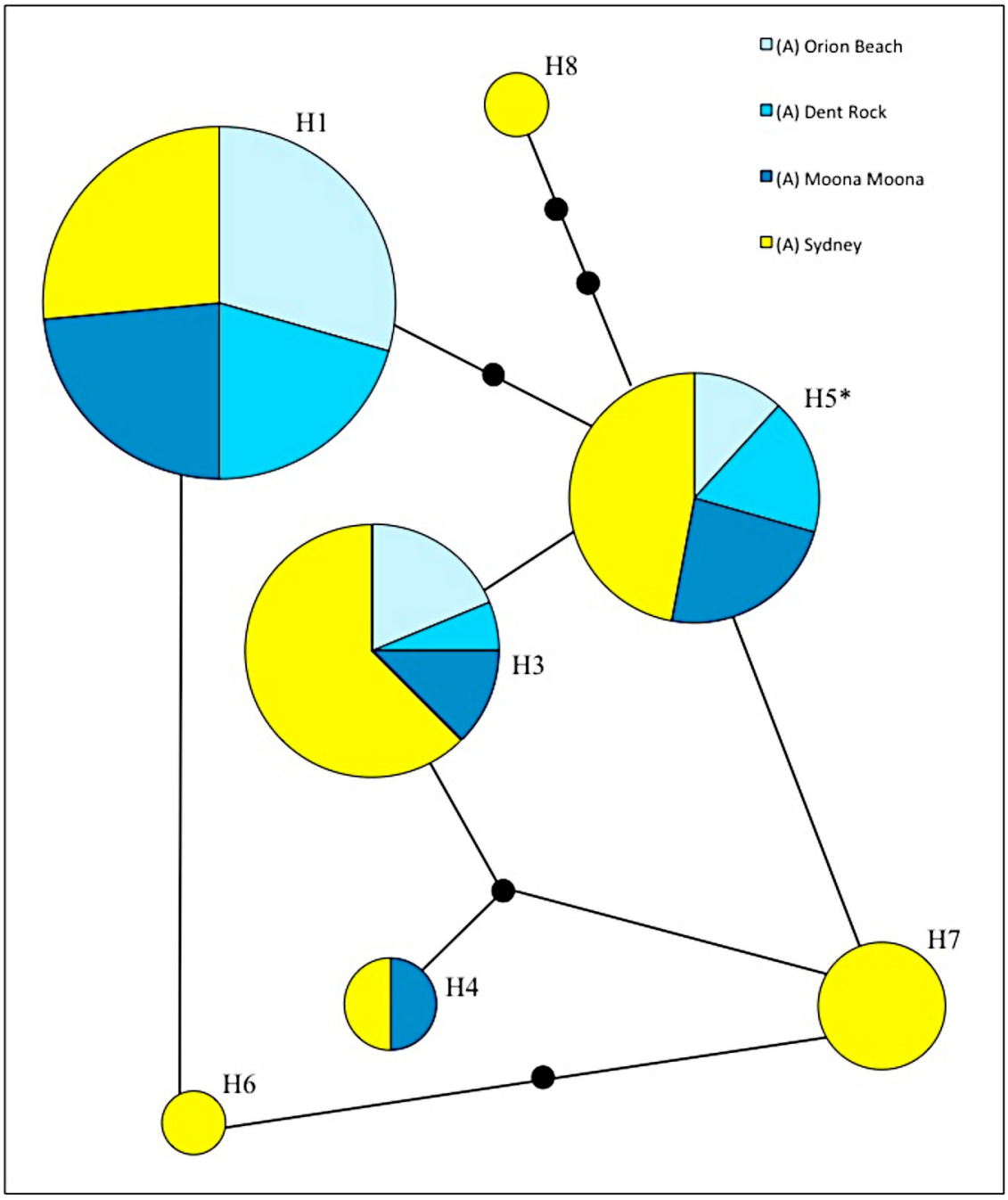

Fig. 3 Parsimony network of mtDNA control region haplotypes identified from *H. portusjacksoni* in all NSW collection sites. Connections between haplotypes represent one base-pair difference and additional intercepting lines represent additional single point mutations. H5 was identified as the ancestral lineage based on coalescence theory and is denoted with *. Circle area is scaled to reflect the frequency of individuals with a particular haplotype (1.5 cm^2 : 1 individual).

Genetic differentiation

Highly significant population structure based on F_{ST} and ϕ_{ST} for mtDNA data was detected between SYD and JB adults (F_{ST} = 0.08222, $P < 0.001$; ϕ_{ST} = 0.122287, P <0.001; Table 4). Low but significant population structure was detected between SYD and JB embryos for mtDNA. Greater genetic differentiation was detected between SYD and JB using ϕ_{ST} compared to conventional F_{ST}. Significant population structure was not detected between SYD and JB using microsatellite data. Pairwise F_{ST} comparisons between all JB locations, for adults and embryos, using microsatellite and mtDNA data did not detect any population structure following sequential Bonferroni adjustment of alpha (Table S1). The Bayesian clustering approach in Structure 2.3.4 confirmed microsatellite F_{ST} results. Using this approach, nuclear genetic structure could not be detected within NSW adults with the presence and absence of population information, for a range of K values (results not shown).

Table 4. Genetic Differentiation between Sydney adults and Jervis Bay individuals (adults and embryos). Pairwise F_{ST} values calculated with microsatellite and mtDNA data, and Φst values for mtDNA. * Denotes significance for $P < 0.05$

Pairwise Comparison	**Microsatellite F_{ST} (*P* value)**	**mtDNA F_{ST} (*P* value)**	**mtDNA Φst (*P* value)**
SYD and JB adults	0.00258 (0.181)	0.08222 (0.008)*	0.12229 (0.004)*
SYD and JB embryos	0.00294 (0.143)	0.03653 (0.046)*	0.08487 (0.011)*

Sex-biased dispersal

Females from JB were found to be more philopatric than males, estimated by significantly higher assignment values among females than males (females: 0.386, males; -0.370, $P < 0.05$). In SYD, females had higher assignment values than males, however this difference was not significant (Table 5). Mean relatedness among females and males in JB was the same (and marginally higher in SYD males than females; Table 5). In JB and SYD there were no significant differences between the relatedness among females versus males ($P > 0.05$). A lack of significant F_{ST} differences between sites in JB meant tests for sex-biased dispersal were not conducted between DR, OB and MM.

Table 5. Mean relatedness between same-sex and opposite pairs ± SE obtained by jackknifing over all loci. Corrected assignment indices (AIc) for males and females from the Jervis Bay (males *n* = 25 and females *n* = 25) and Sydney (males *n* = 19 and females *n* = 20) adults. * Denotes significance for $P < 0.05$

	Relatedness			AIc		
	MM	MF	FF	Males	Females	*P*
JB Adults	0.094 ± 0.008	0.060 ± 0.003	0.105 ± 0.008	-0.370 ± 0.305	0.386 ± 0.329	0.035*
SYD	0.107 ± 0.012	0.061± 0.004	0.118 ± 0.012	-0.111 ± 0.302	0.111 ± 0.359	0.187

Parentage and sibship analysis

Parentage analysis in Cervus 3.0.7 without prior knowledge of parentage resulted in a total of five maternal and two paternal assignments when comparing adults and embryos from the same JB site (i.e. DR/DR; Fig. 4). For Cervus 3.0.7 simulations and reconstructions the probability of a candidate parent being present in the sample was set very low (0.05) due to a lack of any population estimates for *H. portusjacksoni*. Between site comparisons in JB (ie. DR/MM) produced a total of 12 maternal and six paternal assignments (Fig. 4). Three maternal and one paternal assignment generated in Cervus 3.0.7 were not accepted (Table S2 and S3) because the relationships were (i) at 80% confidence and (ii) could not be verified in Colony 2.0.5.9 and ML-Relate.

Sibship reconstruction identified more half-sibs between (*n* = 72) than within (*n* = 40) JB sites (Fig. 4). Zero full-sibs were assigned within JB and one was assigned between JB sites. The highest number of assigned half-sibs was 19 for OB within sites, and 33 for DR/OB between sites (Fig. 5). Overall, there were more mother, father, half-sib and full-sib assignments between sites than within sites in JB. For this study, parentage and sibship assignments were not regarded as highly accurate due to (i) a lack of population estimates for *H. portusjacksoni*, (ii) relatively small sample sizes for JB collection sites, (iii) lack of knowledge of any candidate parents being present in the sampled population and (iv) a clear discrepancy between full- and half-sibling assignments, with only one full-sib assigned for all embryos.

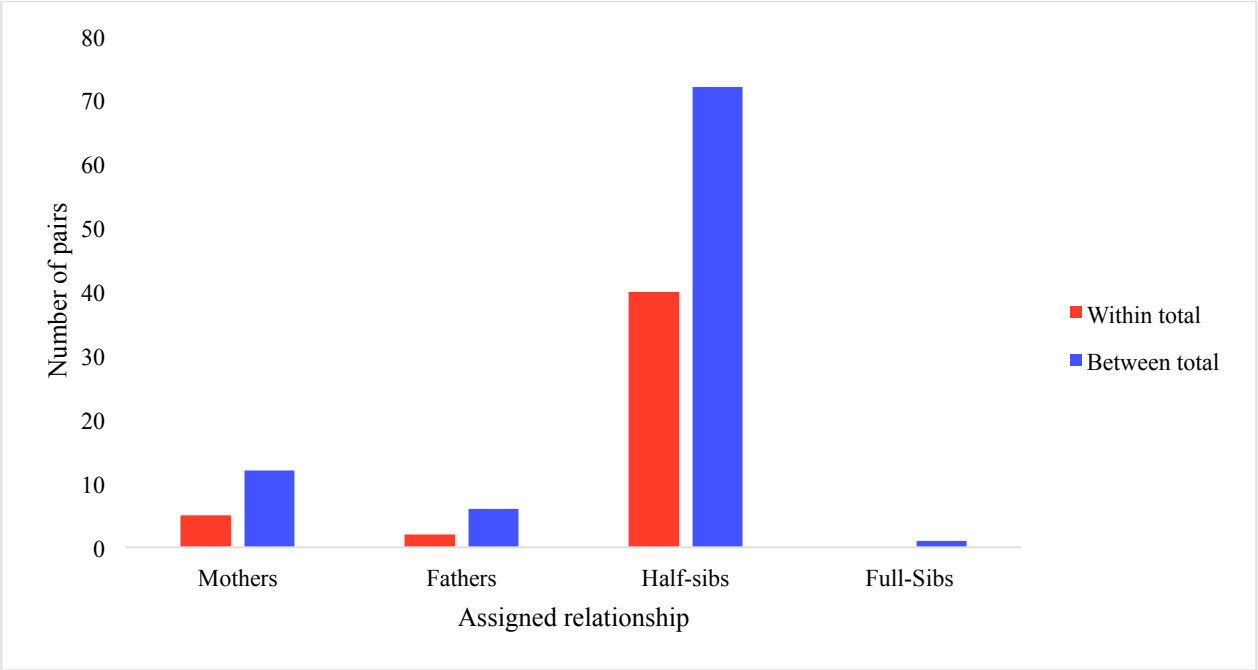

Fig. 4 Total number of parentage and sibship assignments for adult and embryo *H. portusjacksoni* within and between collection sites in Jervis Bay, NSW.

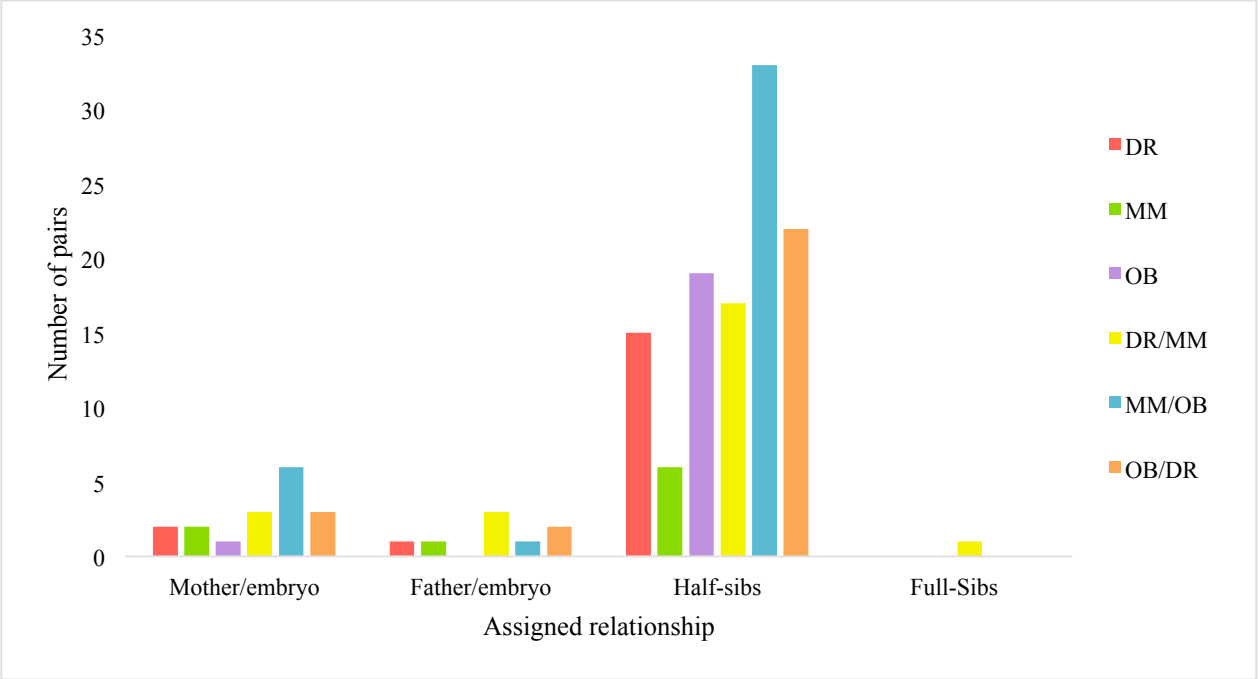

Fig. 5 Number of parentage and sibship assignments for each site or combination of sites for adult and embryos Port Jackson Sharks within Jervis Bay, NSW.

Discussion

Genetic diversity

Overall haplotypic and nucleotide diversity observed for the mtDNA control region for all sampled NSW *H. portusjacksoni* adult sharks were moderate and low respectively. Average haplotype and nucleotide diversity was higher in SYD than JB. These values are consistent with the diversity observed for near-shore and reef associated shark species, such as lemon (*N. brevirostris*) and blacktip sharks (*C. limbatus*), compared to larger pelagic species such as whale (*Rhincodon typus*) and blue sharks (*Prionace glauca*;) that travel over large geographical distances (Castro *et al.* 2007; Keeney *et al.* 2005; Ovenden *et al.* 2009; Schultz *et al.* 2008). Verifying this, Karl *et al.* (2011) compared the haplotypic and nucleotide diversity across a wide-range of reef-associated and pelagic sharks and found both measures to be statistically significantly different between habitat types, with near-shore species exhibiting lower diversity.

Analysis of 10 nuclear microsatellite loci showed intermediate levels of mean expected heterozygosity and alleles per locus in NSW *H. portusjacksoni*, with SYD exhibiting marginally higher expected heterozygosity than JB. Overall JB had a higher mean number of alleles per locus compared to Sydney. Interestingly, nuclear diversity values for NSW *H. portusjacksoni* were similar to that of the pelagic species such as the whale (*R. typus*; Schmidt *et al.* 2009) and white shark (*Carcharodon* carcharias; Gubili *et al.* 2009). Evidently the lower variability exhibited in the mitochondrial genome of *H. portusjacksoni* is not reflected in the nuclear genome for NSW *usjacksoni*. This is consistent with other reef-associated shark species, where levels of genetic variation in the mtDNA control region are lower than nuclear microsatellite markers (Karl *et al.* 2011).

Given the different levels of diversity between the mtDNA and nuclear genome for NSW *H. portusjacksoni* it is possible that the evolutionary processes shaping the nuclear and mitochondrial loci have contrasting dynamics. In this study, because samples were collected from sharks during the breeding season at near-shore oviposition sites, it appears that the observed diversity patterns are a result of sex-specific reproductive behaviours, explicitly female fidelity to breeding sites. Female mediated structure in both SYD and JB would mean that the mtDNA haplotypes hold evolutionary independence, but the nuclear variation would represent a random subset of the species-wide gene pool for those microsatellite markers tested, due to male dispersal. This is also consistent with population-level mtDNA, but not nuclear DNA structure that is observed here (see discussion below).

Fixation indices based on mtDNA showed highly significant genetic structure between SYD and JB adults, and SYD and JB embryos. However, no genetic differentiation was detected using microsatellites. This suggests that *H. portusjacksoni* females exhibit reproductive philopatry during the breeding season, with males facilitating gene flow among populations. Both microsatellite fixation indices and the Bayesian clustering approach in Structure 2.3.4 support the presence of a single panmictic population for *H. portusjacksoni*. Further testing for sex-biased dispersal using mean corrected assignment indices (AIc) indicated females had significantly higher AIc values than males within JB. Interestingly, this result was not reflected in the SYD population, however, it is likely that a lower sample size (n = 39) could have resulted in an inability to detect sex-biased dispersal at this location. Regardless, AIc values for females were higher than males at the SYD population, and thus further tests employing larger male and female sample sizes would be beneficial.

Collectively, these results are consistent with the growing evidence for male-mediated gene flow and female reproductive philopatry in sharks and many other marine species (Pardini *et al.* 2001; Portnoy *et al.* 2015; Tillett *et al.* 2012). Male-mediated gene flow and female fidelity to natal sites has been documented in a range of marine species including green sea turtles (*Chelonia mydas*; Lee *et al.* 2007), humpback whales (*Megaptera novaengliae*; Baker *et al.* 2013), sperm whales (*Physeter macrocephalus*; Engelhaupt *et al.* 2009), white sharks (*Carcharodon carcharias*) (Blower *et al.* 2012), lemon sharks (*Negaprion brevirostris*; Feldheim *et al.* 2014), blacktip reef sharks (*C. limbatus*; Mourier & Planes 2013) and scalloped hammerhead sharks (*Sphyrna lewini*; Daly-Engel *et al.* 2012).

In marine teleosts, even relatively sedentary species may be genetically homogenous over broad geographic distances as a result of passive larval dispersal (Shulman & Bermingham 1995). Elasmobranchs however are born or hatch fully developed and therefore gene flow occurs through the movement of juveniles or adults (Heist 2008). As such, the amount of gene flow between different populations required to reduce genetic heterogeneity to undetectable levels is considered to be only a few individuals per year (Waples 1998). Given this, it is not unlikely for fine-scale genetic population structure to be absent in many shark species. While the present study did not detect fine-scale population structure between breeding sites within JB, the structure detected between JB and SYD is consistent with the hypothesis of O'Gower & Nash (1978) that localized structure may exist within the *H. portusjacksoni* species range.

Most shark species documented exhibiting sex-biased dispersal, such as the white shark (*Carcharodon carcharias*), are pelagic species undertaking large-scale migrations on a seasonal basis (Karl *et al.* 2011). Differentiation between nuclear and mitochondrial loci for white sharks between Australia and South-Africa indicate male-biased dispersal (Pardini *et al.* 2001), however females have been shown to migrate between natal sites during the breeding season (Bonfil *et al.* 2005). Similarly, blacktip reef sharks have been shown to exhibit female reproductive philopatry, migrating to specific nursery areas that are outside their home range for parturition (Mourier & Planes 2013). Since females of these bentho-pelagic species are philopatric, it appears males are dispersing genes between populations. Due to the large focus of shark research on pelagic and bentho-pelagic species, which are generally ovoviviparous or viviparous, reproductive philopatry in an oviparous epibenthic species, such as *H. portusjacksoni,* has not been demonstrated until now.

Acoustic tagging and tracking data (Brown *et al.* unpublished data) support the genetic evidence for reproductive philopatry in *H. portusjacksoni*. Males and females both exhibit site fidelity to sampled sites within JB during the breeding season. Females visit on average 1.5 breeding sites per season and males two sites per season. However, during each mating season males spend approximately 97% and females 83% of their time in JB at any one breeding site. Additionally, acoustically tagged adult males and females from JB have never been detected by acoustic tag receivers in SYD. Given the high level of adult male site fidelity, both between breeding seasons and within seasons, it would appear that the male dispersal occurs in the juvenile phase.

The spatial ecology of *H. portusjacksoni* has been studied for adults and juvenile sharks in NSW using underwater visual surveys and tagging (Powter& Gladstone 2009). Here, results reveal the spatial ecology of *H. portusjacksoni* is strongly habitat mediated in JB. Juveniles and adults of both sexes displayed site fidelity to breeding reefs, with the majority of individuals being re-sighted at the initial tagging site up to three years later. Additionally the strength of philopatry did not differ between males and females, implying the presence of discrete breeding populations with little dispersal between reefs (Powter & Gladstone 2009). While juveniles were shown to exhibit site fidelity, the acoustic tracks of two juvenile males from this study showed that juvenile males vary significantly in their use of space across sea grass beds, with one male moving more frequently and larger distances around its initial capture site in JB. Interestingly, one male was recorded moving towards the mouth of JB, suggesting a larger use of space by juveniles and the possibility that males leave JB during this life-history stage (Powter & Gladstone 2009). The genetic structure detected in this study combined with the acoustic tracking data (Brown *et al*. unpublished data) and the strong site fidelity of adult sharks to breeding reefs as shown by Powterand Gladstone (2009) suggest male mediated gene flow is likely to occur immediately post-hatching and/or during the early juvenile

stages of this species.

Parentage and sibship reconstruction in Jervis Bay

This study also used 10 polymorphic microsatellite loci to assign parents to offspring. A total of 17 mothers and eight fathers were assigned to offspring in JB. Both paternal and maternal assignments were higher between sites, than within sites. For example in MM only two mothers were assigned to embryos, whereas at DR/OB six mothers were assigned to embryos. Additionally, sibship reconstruction resulted in 40 within site and 72 between sites half-sibs. The incidence of full-sib assignment was very low, with only one full-sib assignment for the entire dataset. Females lay up to two eggs at any one time so while there is a chance of detecting full-sibling relationships through sibship reconstruction, the probability of this is relatively low. These reconstructions were carried out using microsatellites and due to the higher number of assignments observed between sites but not within sites, these results support the lack of nuclear DNA structure among the three JB sampling locations. Although parental reconstruction was verified in three programs and sibship reconstruction was carried out in two programs results suggest a lack of fine scale population structure within JB. However, due to the small sample size of candidate fathers, mothers and embryos from JB sites, the validity of these results must be taken into consideration. Additionally, a lack of population estimates for JB meant the probability of sampling a candidate father or mother in this study was likely inaccurate, meaning any assignments provided by Cervus 3.0.7 cannot be considered robust. Further parentage analysis with larger sample sizes, reliable population estimates of *H. portusjacksoni,* and a larger number of microsatellite markers are required to increase the power of parentage and sibship analyses for this species.

Conclusions

This study demonstrates female reproductive philopatry and male-mediated gene flow in an oviparous, epibenthic shark - *H. portusjacksoni*. Analysis of mtDNA and microsatellite markers indicates genetic differentiation between two populations of *H. portusjacksoni* in NSW – SYD and JB. At a finer geographical scale within JB, no population structure was detected, however, this may have been due to the relatively limited sample sizes for each of the three JB collection sites. Additionally, two of the sampled sites in JB (DR and OB) are geographically very close to one another (within 500 m). Given that females on average visit two breeding sites per year, it is extremely likely that sharks from these sites would be genetically indistinguishable. Prior elasmobranch studies have detected population structure using only mtDNA (Clarke *et al.* 2015; Taguchi *et al.* 2015) or nuclear DNA (Schrey& Heist 2003), however, studies utilising both nuclear

and mitochondrial markers produce more reliable results because contrasting patterns of sex-specific behaviour can be examined (Portnoy *et al.* 2015).

Much remains to be learned about the reproductive ecology and life history of *H. portusjacksoni*. While genetic studies can identify behavioural patterns unable to be studied *in situ*, a holistic approach that combines tagging and tracking data with genetic analyses is more robust for detecting intricate reproductive behaviours. Understanding the environmental processes contributing to the patterns of female reproductive philopatry observed here would require both molecular and field studies. Estimating divergence times would provide further information that may help to understand the environmental factors contributing to the population structure observed in NSW. Moreover, further studies that examine other populations of *H. portusjacksoni* in NSW may help to identify possible biogeographic barriers along the NSW east coast that are influencing dispersal potential and population connectivity..

Significant questions still remain regarding the intricate mating behaviour of this species. For example, it is unknown if females mate and deposit eggs at one breeding site, or if breeding and oviposition are carried out at separate locations. Furthermore it is unknown if this species exhibits multiple paternity, polyandry and intraspecific variability in reproductive behaviours. Multiple paternity has been detected in the oviparous small-spotted catshark (*Scyliorhinus canicula*; Griffiths *et al.* 2011). Therefore it is possible that *H. portusjacksoni* could exhibit this behaviour, however, additional research is required to determine this. Further analyses across the *H. portusjacksoni* range would help to ascertain whether the mating behaviours identified in this study vary based selective pressures associated with reproductive strategy, mate encounter rates and body size, or ecological factors such as parturition habitat type that may influence juvenile survival. Filling these knowledge gaps is critical to developing location-appropriate management strategies. While *H. portusjacksoni* is not currently considered at risk of extinction (Tovar−Ávila *et al.* 2010), successful management plans that consider the reproductive behaviour and protection of breeding areas may be increasingly important in the future, because the sex-specific behaviours exhibited in this species ultimately influence its genetic diversity and population structure.

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Supplementary material

Table S1. Genetic differentiation among the Jervis Bay sites in NSW. Pairwise F_{ST} calculated with microsatellite data (matrix below) and with mtDNA (Φst) control region (matrix above). Sites are abbreviated as Dent Rock (DR), Moona Moona (M) and Orion Beach (OB), with "(A)" referring to adults and "(E)" referring to embryos from that site. Alpha adjusted to $P = 0.003$ following sequential Bonferroni correction.
* Denotes significance at $P < 0.003$

	DR (A)	MM (A)	OB (A)	DR (E)	MM (E)	OB (E)
DR (A)	-	-0.05943	-0.07285	-0.03130	-0.03870	-0.01342
MM (A)	0.01942	-	-0.04625	-0.04908	-0.03582	-0.03729
OB (A)	0.01786	0.01032	-	-0.04081	-0.05342	0.02190
DR (E)	0.02245	0.00107	0.00882	-	-0.04617	0.00281
MM (E)	0.00619	0.00510	0.00728	0.00778	-	0.03627
OB (E)	0.01471	0.01120	0.02222	0.00650	0.00970	-

Table S2.. Maternal assignments using Cervus, Colony and ML-Relate for JB adults and embryos. "Y" = mother confirmed by colony and "-"= no mother assigned by colony. ML-Relate assigned mothers as either parent offspring (PO), full sibling (FS), half-sibling (HS) or unrelated (U).

Embryo ID	Candidate mother ID (CERVUS)	Site - Embryo/Mother	Pair confidence (CERVUS)	No. paired loci mismatching (CERVUS)	Colony	ML-Relate relationship	Accepted mother?
31-DR	146-DR	DR/DR	80%	0/9	-	PO	Yes
102-DR	2-OB	DR/OB	80%	1/10	Y	PO	Yes
103-DR	5564-MM	DR/MM	80%	1/10	-	U	No
28-DR	146-DR	DR/DR	80%	0/9	-	PO	Yes
105-DR	2-OB	DR/OB	80%	0/10	Y	PO	Yes
37-DR	197-OB	DR/OB	80%	1/10	-	U	No
49-MM	5640-MM	MM/MM	95%	0/10	Y	PO	Yes
43-MM	11-DR	MM/DR	80%	0/10	Y	PO	Yes
45-MM	5213-MM	MM/MM	95%	1/10	-	FS	Yes
54-MM	4-DR	MM/DR	80%	0/10	-	PO	Yes
55-MM	197-OB	MM/OB	80%	0/10	-	PO	Yes
57-MM	85-DR	MM/DR	95%	0/10	Y	PO	Yes
128-MM	413-OB	MM/OB	80%	1/10	-	U	No
11-OB	10-DR	OB/DR	80%	0/10	-	PO	Yes
113-OB	5562-MM	OB/MM	80%	0/10	-	PO	Yes
110-OB	146-DR	OB/DR	95%	0/10	Y	PO	Yes
119-OB	37-OB	OB/OB	95%	0/10	-	PO	Yes

Table S3 . Paternal assignments using Cervus, Colony and ML-Relate for JB adults and embryos. "Y" = father confirmed by colony and "-"= no father assigned by colony. ML-Relate assigned fathers as either parent offspring (PO), full sibling (FS), half-sibling (HS) or unrelated (U).

Embryo ID	Candidate father ID (CERVUS)	Site - Embryo/Father	Pair confidence (CERVUS)	No. loci mismatching (CERVUS)	Colony	ML-Relate relationship	Accepted father?
23-DR	5-DR	DR/DR	80%	0/10	-	PO	Yes
108-DR	5638-MM	DR/MM	95%	0/10	Y	PO	Yes
96-DR	5638-MM	DR/MM	80%	0/10	Y	PO	Yes
101-DR	5642-MM	DR/MM	80%	0/10	-	PO	Yes
127-MM	371-OB	MM/OB	80%	1/10	-	HS	No
46-MM	5638-MM	MM/MM	80%	0/10	Y	PO	Yes
113-OB	5638-MM	OB/MM	80%	0/10	-	PO	Yes
116-OB	5-DR	OB/DR	80%	0/10	-	PO	Yes

Appendix

ANIMAL RESEARCH AUTHORITY (ARA)

MACQUARIE University

AEC Reference No.: 2014/003-12 **Date of Expiry:** 20 February 2017

Full Approval Duration: *21 February 2014 to 20 February 2017 (36 Months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
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Louise Tosetto	0406 189 992
Evan Byrnes	0432 761 233
Catarina Vila Pouca	0403 161 454
Sherrie Chambers	0407 796 410

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above, or
Animal Welfare Officer - 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: Spatial Learning and Memory Retention in Fish

Purpose: 4 - Research: Human or Animal Biology

Aims: To examine how long fish from contrasting environments retain spatial information

Surgical Procedures category: 3 - Minor Conscious Intervention

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex	Total	Supplier/Source
23 – Fish	Intertidal gobies	Mature adult / Any	240 +100	Wild
23 – Fish	Port Jackson Sharks	Any		
		TOTAL	340	

Location of research:

Location	*Full street address*
SIMS	Building 19, Chowder Bay Road, Mosman NSW 2088
Biology Sea Water Facility	Building E8C, Macquarie University, North Ryde, 2109
Fauna Park	209 Culloden Rd, North Ryde, 2109

Amendments approved by the AEC since initial approval:

1. *Amendment #1 - Change transport and capture methods (Exec approved 8 May 2014, ratified by AEC 15 May 2014).*
2. *Amendment #2 – Add a new species - Port Jackson Sharks (Approved at AEC meeting14 August 2014).*
3. *Amendment #3 – Addition of Evan Brynes as a Masters Student(Executive approved, ratified by AEC 11 December 2014).*
4. *Amendment # 4 – Addition of JennaLee Clark as an Associate Investigator(Executive approved, ratified by AEC 11 December 2014).*
5. *Amendment #5 – Addition of 100 Port Jackson Eggs (Executive approved, ratified by AEC 11 December 2014).*
6. *Amendment #6 – Addition of Louise Torsetto as Researcher (Executive approved, ratified by AEC 11 December 2014).*
7. *Amendment #7 – Amend the way to feeding the gobies (AEC approved 19 February 2015).*
8. *Amendment #8 – Administration of substances and amend experimental design (AEC approved 16 April 2015).*
9. *Amendment #9 – Addition of Catarina Vila Pouca as PhD Student (Executive approved, ratified by AEC 16 July 2015).*
10. *Amendment #10 - Amendment to Experimental Design – Item withdrawn by A/Prof. Brown.*
11. *Amendment #11 – Addition of Sherrie Chambers as PhD Student (Executive approve. Ratified by AEC 10 December 2015).*
12. *Amendment #12 - Amendment to length of time sharks can be held in captivity. (AEC approved 10 December 2015).*

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Professor Mark Connor (Chair, Animal Ethics Committee) **Approval Date: 18 February 2016**

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2014/015 -3 **Date of Expiry:** 17 April 2016

Full Approval Duration: *18 April 2014 to 17 April 2017 (36 Months)*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
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Student:
Nathan Bass 0432 840 314
Jennalee Clark 0404 004 882

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or **Animal Welfare Officer** - 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Movements, Migrations and Social Interactions of Wild Shark Populations**

Purpose: **7 - Research: Environmental Study**

Aims: **To examine social interactions in Port Jackson sharks (PJs) and their movements within Jervis Bay and along the NSW coast using new tag technology**

Surgical Procedures category: 4 - Minor Surgery With Recovery

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex/Weight	Total	Supplier/Source
23 - Fish	Heterodontus portusjacksoni	Any	3000	Wild
		TOTAL	3000	

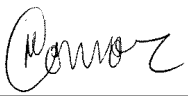
Location of research:

Location
Entire New South Wales distribution of the Port Jackson sharks (Port Stevens to Narooma), specifically: - Jervis Bay = Orion Beach, Plantation Point, Hyams Beach, Moona Moona Creek and North Bowen Island - Sydney = Oak Park (Cronulla), Bare Island (La Perouse) and Shelley Beach (Manly) - Central Coast = Terrigal Haven (Terrigal) and Cabbage Tree Harbour (Norah Head)

Amendments approved by the AEC since initial approval:
Amendment #1 – *Addition of Jennalee Clark as Associate Investigator (Executive approved 13 May 2015, ratified by AEC 14 May 2015).*

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.



Professor Mark Connor (Chair, Animal Ethics Committee) **Approval Date: 14 May 2015**

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)