Investigating the reproductive behaviour of the green sea turtle (Chelonia mydas) using parentage analysis

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November 10, 2023

Abstract

The reproductive systems of natural populations can greatly impact their genetic diversity by preventing or encouraging inbreeding. It is therefore crucial to have a comprehensive understanding of the mating system to evaluate a population's ability to maintain genetic diversity over time. In this study, we examine the mating system of an endangered population of green sea turtles in Tetiaroa, French Polynesia. We determine if different mating behaviours serve as strategies to avoid inbreeding. We genotyped 107 nesting females and 1483 hatchlings from 549 nests and used 23 microsatellite markers to reconstruct the genotypes of the fathers. We assessed the level of inbreeding and relatedness of the parent pairs and explored the correlation between relatedness and fitness parameters in the offspring. We determined the mating behaviours of both males and females and investigated if specific behaviours were linked to different levels of relatedness. Our results showed that 27 fathers and 31 mothers were responsible for the genotypes of 445 hatchlings from 105 nests. Global Fis was significant, and levels of relatedness were higher than expected through random mating, indicating inbreeding and non-random partner selection. However, we did not find any mating behaviours that were associated with lower relatedness levels than the general population, suggesting that they are not part of an inbreeding avoidance strategy. Ultimately, this study illuminates the reproductive system of green turtles and shows that this population is susceptible to inbreeding. Additionally, our research demonstrates the effectiveness of parentage analysis in understanding the reproductive behaviour of elusive species.

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Running title : Green Turtle Reproductive Behaviour

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Conflict of interest disclosure

The authors declare no conflict of interest.

Ethics Approval

Chelonia mydas sample collection was authorized and coordinated by the Direction of the Environment of French Polynesia. Non-lethal skin and muscle biopsies were performed, which do not require any other specific permits. Samples were exported to France for processing with CITES permits ndeg FR1298700118-E and ndeg FR2098700187-E. All samples remain the DIREN's property.

Abstract

The reproductive systems of natural populations can greatly impact their genetic diversity by preventing or encouraging inbreeding. It is therefore crucial to have a comprehensive understanding of the mating system to evaluate a population's ability to maintain genetic diversity over time. In this study, we examine the mating system of an endangered population of green sea turtles in Tetiaroa, French Polynesia. We determine if different mating behaviours serve as strategies to avoid inbreeding. We genotyped 107 nesting females and 1483 hatchlings from 549 nests and used 23 microsatellite markers to reconstruct the genotypes of the fathers. We assessed the level of inbreeding and relatedness of the parent pairs and explored the correlation between relatedness and fitness parameters in the offspring. We determined the mating behaviours of both males and females and investigated if specific behaviours were linked to different levels of relatedness. Our results showed that 27 fathers and 31 mothers were responsible for the genotypes of 445 hatchlings from 105 nests. Global F_{is} was significant, and levels of relatedness were higher than expected through random mating, indicating inbreeding and non-random partner selection. However, we did not find any mating behaviours that were associated with lower relatedness levels than the general population, suggesting that they are not part of an inbreeding avoidance strategy. Ultimately, this study illuminates the reproductive system of green turtles and shows that this population is susceptible to inbreeding. Additionally, our research demonstrates the effectiveness of parentage analysis in understanding the reproductive behaviour of elusive species.

Keywords: genotype reconstruction, mating behaviour, fitness, relatedness, multipaternity, inbreeding

Introduction

Reproductive systems strongly influence genetic diversity in natural populations (Clegg et al., 1992). Loss of genetic diversity may lead to inbreeding depression, loss of adaptive potential, and accumulation of deleterious alleles, and ultimately to extinction (Charlesworth, 2009). The number of breeders, their reproductive success, and the mating strategies they use are key elements present in the mating system of a population (Sugg and Chesser, 1994). A good understanding of these factors is essential to assess the intrinsic capacity of natural populations to maintain themselves through generations and to maintain their genetic diversity (Anthony and Blumstein, 2000). Several components of the mating system may either mitigate or favour inbreeding. They may be precopulatory such as the operational sex-ratio (OSR, i.e. relative number of breeders from both sexes, (Emlen and Oring, 1977)), reproduction frequency, and partner choice and number (Blouin and Blouin, 1988; Taylor et al., 2014). Postcopulatory factors include sperm storage and sperm competition (Michalczyk et al., 2011).

OSR is a key feature of the mating system, especially for species with a temperature-dependent sex determination (TSD) like the marine turtles (Standora and Spotila, 1985). The primary sex-ratio of TSD species is solely dependent on environmental conditions and can be heavily biased under climate warming situations (Janzen, 1994). The primary sex-ratio of several marine turtle populations is female-biased (Casale et al., 2000; Jensen et al., 2018; Santidrian Tomillo et al., 2014; Zbinden et al., 2007), but the OSR is usually much more balanced (Hays et al., 2022), mitigating concerns about the vulnerability of a population. However, as highlighted by Wright et al. (2012a), if the OSR reflects a small number of males that breed more frequently than females, it will still lead to a loss of genetic diversity and inbreeding. Due to the high energetic cost of reproduction, which may include long migrations between foraging and nesting grounds, female marine turtles typically reproduce at intervals of several years (Hays et al., 2014). For the green turtle, *Chelonia* *mydas* , reproduction frequency in females is estimated between 2 and 5 years (Seminoff et al., 2015). In contrast, males can mate more frequently, and annual migration to breeding grounds has been observed in loggerhead (*Caretta caretta*), leatherback (*Dermochelys coriacea*) and green turtles (James et al., 2005; Limpus, 1993; Wibbels et al., 1990). Knowing the reproduction frequency of both sexes is thus important to correctly interpret the OSR concerning inbreeding risk.

Both male and female marine turtles can reproduce with several partners (i.e. polygynandry), which tends to equilibrate the OSR (Jensen et al., 2013). Polyandry is observed through multiple paternity in clutches, which has been found at various degrees in all marine turtle species. For green turtles, it ranges from 15% to 92% of the clutches depending on the populations (reviewed in Lee et al., 2018). Multiple paternity is a direct consequence of seasonal sperm storage in females, a capacity well established in Testudines (Pearse and Avise, 2001) and observed in six of the marine turtle species (Crim et al., 2002; FitzSimmons, 1998; Kichler et al., 1999; Phillips et al., 2013; Sakaoka et al., 2011; Theissinger et al., 2009). Satellite tracking and direct observations showed that breeding usually occurs before the beginning of the nesting season and all the clutches laid within a season are usually sired by the same fathers (Hays et al., 2022). However, sperm storage across multiple seasons has not been formally demonstrated in marine turtles, as opposed to terrestrial and freshwater turtles (Owens, 1980; Whitaker, 2006), but several studies have suggested that it is likely (Howe et al., 2017; Theissinger et al., 2009; Wright et al., 2013).

It has been proposed that females would benefit from polyandry and sperm storage. The 'good genes' hypothesis relies on the assumption that by mating with different partners, better quality sperm would outcompete lower quality sperm, which would lead to an increased fitness of embryos sired by the dominant father (Kokko et al., 2002). However, while many studies have attempted to prove this hypothesis, no correlation has yet been found between multipaternity and fitness parameters in the clutches (Jensen et al., 2006; Lee et al., 2018; Lee and Hays, 2004; Wright et al., 2013, but see Howe et al., 2017). On the other hand, the 'genetic compatibility' hypothesis predicts that paternity would be biased towards genetically dissimilar males to avoid inbreeding (Bretman et al., 2009; Zeh and Zeh, 1997). In an attempt to test this latter hypothesis in a hawksbill turtle population in the Republic of Seychelles, Philips et al. (2013) did not find any correlation between relatedness and paternity contribution in the clutches. Ultimately, all of these studies tend to conclude that polyandry is likely to occur as an energy cost trade-off between mating several times and avoiding mating harassment (i.e., convenience polyandry, Lee and Hays, 2004). Male sea turtles are known to actively and aggressively attempt mating, and females may try to avoid it (Booth and Peters, 1972). Thus, levels of multipaternity may simply reflect the density of breeders on reproductive grounds and the number of encounters between the two sexes (Lee et al., 2018).

Understanding the mating system of sea turtles is challenging, notably due to the lack of observations of males and breeding grounds. For species as elusive as these, molecular analyses can provide important insights. The use of microsatellite markers allowed for the genotypes of unsampled fathers to be reconstructed and to thus assess the mating system parameters in several marine turtle populations (e.g. Bernatchez and Duchesne, 2000; Figgener et al., 2016; Horne et al., 2022; Phillips et al., 2013; Wright et al., 2012). Together, these studies reveal that regional variations in these parameters are the rule rather than the exception, and many regions are still lacking this key information for their populations.

French Polynesia, in particular, is an archipelago composed of 118 islands distributed over an exclusive economic zone of 5 million km^2, a surface as wide as Europe. The country is thought to host approximately 1,000 female green turtle breeders, although no recent assessment exists (Groombridge and Luxmoore, 1989; Seminoff et al., 2015). In the centre of French Polynesia, Tetiaroa Atoll (Society archipelago) is one of the major nesting grounds. It is estimated that around 100 females nest annually on the atoll (Seminoff et al., 2015; Touron et al., 2018), and mating behaviours are observed around the island (Gaspar, pers. comm.). This location provides a unique opportunity to closely investigate the mating system of green turtles with molecular marker analyses. Here, we genotyped nesting females and hatchlings from Tetiaroa with 23 microsatellite markers, reconstructed male genotypes, and conducted parentage analysis to study the mating behaviours of both sexes.

The aim of this study is threefold. First, we characterize the level of inbreeding and relatedness in the population of green turtles nesting in Tetiaroa and explore their effect on fitness, by measuring three fitness parameters in the clutches: the number of eggs, successful hatchlings, and dead embryos. Second, using reconstructed pedigrees, we determined the features of the mating system and nesting parameters of this population. These parameters, which are key to inbreeding depression dynamics, include the OSR, the reproductive frequency, the level of multipaternity, the female's nesting intervals, and the number of partners for the males. Third, we test whether the mating strategies deployed reduce the overall relatedness and contribute to avoiding inbreeding by comparing the relatedness of couples involved in these strategies with the overall relatedness of the population. To our knowledge, this work is the first to explore the relatedness bias of mating behaviours in green turtles. It improves our general understanding of the drivers of mating strategies in sea turtles.

Material and Methods

Study site and sample collection

Sampling was conducted on Tetiaroa atoll (16deg59' S, 149deg34' E), French Polynesia, during eleven nesting seasons from 2010/11 to 2020/21. Sample collection was authorized and coordinated by the Direction of the Environment of French Polynesia. Tetiaroa atoll has a total surface of 6 sq. km, about 585 hectares of sand, and is divided into 12 islets (Figure 1). Between 2010/11 and 2017/18, 53 to 1316 nesting events per season (July - April) were recorded by the local NGO *Te mana o te moana* (Touron et al., 2018). Biopsies of approximately 0.5cm^3 of skin and muscle tissues were collected from the posterior fin of all the observed females and on all dead hatchlings and embryos found. For a total of 6 nests, more than 10 hatchlings were sampled. The laying date was either recorded when laying was directly observed, or estimated when the nest was discovered. In this case, a confidence interval of +- 3 days was applied to all of the estimations. Since 2010/11, monitoring has gradually increased and by 2016/17, almost all nests were sampled each season. However, nesting females were not always observed. (Touron et al., 2018). Nest parameters such as clutch size, number of hatchlings (estimated from empty eggshells), and number of dead embryos were recorded for each nest. Samples were stored in 90% ethanol and kept at 4degC or -20degC until processing.

Molecular analyses

Total genomic DNA was extracted using the QIAamp 96 DNA QIAcube HT Kit and the QIAcube DNA extraction robot (QIAGEN, Hilden, Germany) following the manufacturer's protocol with modification as described in Dolfo et al. (2023). Samples were genotyped at 23 microsatellite loci using five multiplex reactions as per Dolfo et al. (2023) (GenBank accession number: OQ162049-OQ162073). Allele sizes were visually assessed using GENEMAPPER software v.5 (Applied Biosystems). All ambiguous peak profiles were considered as missing data and only individuals with missing data which occurred at 3 loci or less were kept for the analyses. MICROCHECKER v.2.2.3 (Van Oosterhout et al., 2004) was used to identify null alleles, stuttering errors, and large allele dropout. In total, 1592 hatchling and female genotypes were kept for further analyses.

Conceptual workflow

To answer the three goals defined above, analyses were carried out with the following conceptual workflow (Figure 2). First, the population state was described through the number of breeders, the OSR, and the level of inbreeding and relatedness between reconstructed couples. The effect of the relatedness on the fitness parameters in the clutches was investigated. Then, the reproductive behaviours of the population were described for both sexes, i.e. the reproduction frequency, nesting and/or reproductive parameters within a season, and levels of multipaternity. Finally, we tested whether these behaviours mitigate or favour inbreeding by comparing the relatedness of couples involved with the relatedness of all reconstructed couples.

Identification of recaptured individuals

The R package *RClone* (Bailleul et al., 2016) was used to identify duplicate genotypes in the dataset and to detect recaptured individuals. Using the allelic frequencies of the dataset, *RClone* simulates a reproduction event and compares the genetic distances between genotypes in the simulated and the observed populations. This allows for a threshold to be determined, below which genotypes in the observed population may be too similar to occur as a result of reproduction. Thus, the genotypes of all individual pairs that had less than 5 differences at any locus were double-checked with GENEMAPPER, and the data were crosschecked with field information to identify recaptured individuals.

Calibration of the set of loci

To test the accuracy of the motherhood assignment provided by the software COLONY v.2.0.6.6 (Jones and Wang, 2010), we calibrated the assignment probability based on known mother-offspring relationships. The probability of assignment of a mother was plotted against its true motherhood, based on the known mother-offspring relationships from the field, which involved 58 mothers and 275 hatchlings from 105 nests. COLONY uses a full likelihood model to assign sibship and parentage relationships using multilocus genotype data. Its algorithm allows for missing data and genotyping errors. Three long runs were performed with high likelihood precision, allowing polygamy for both sexes under an inbreeding model, and assuming an error rate of 0.01 for both allelic dropout and genotyping error. It is assumed that hatchlings sampled in 2010/11 did not reach sexual maturity by 2020/21 based on the mean age at maturity of 25 years (Chaloupka et al., 2004), therefore, we did not consider the occurrence of overlapping generations. All the hatchlings were considered as potential offspring and all the females were candidate mothers.

To determine the effect of genotyping errors on the accuracy of motherhood assignment, the above calibration was run with three different subsets of markers to determine which one would best perform in assigning true mother-offspring relationships: subset 1 comprised all 23 loci described in Dolfo et al. (2023); in subset 2 the loci which were deviant from Hardy-Weinberg equilibrium, as emphasized by the authors, were removed, leaving 14 loci (list in Supplementary Data 1); in subset 3 the loci which were given the highest error rate by COLONY were removed (CMY19, CMY22, and CMY35), leaving 20 loci. Based on this calibration (Supplementary Data 1) all 23 loci were retained for the analyses.

Parentage analyses

COLONY was run with 23 loci in the same configuration as for marker set calibration, to assign candidate mother-offspring relationships and to infer the genotype of unknown parents. For genotyped mothers (n=107), an offspring assignment probability threshold of 0.9 was used based on the results of the above calibration. For genotypes that were reconstructed, COLONY only provides a probability per locus. A probability threshold of 0.9 is usually a good choice for conservative results, however, in our case, this threshold would discard 93% of the genotypes. Thus, probability thresholds from 0.4 to 0.9 were examined to determine the best trade-off between conservative and robust results (i.e. number of samples retained) (Supplementary Data 2). Finally, only reconstructed loci with a probability superior to 0.8 were retained for the analyses, while the others were considered as missing data. Like genotyped individuals, individuals with missing data at more than 3 loci were discarded.

Finally, the dataset was checked for inconsistencies (for example, when more than one mother was found for a single nest) and the least likely relationship (i.e. with the lowest probability) was removed. Ultimately, a parent-offspring relationship was only retained when both parents (assigned or reconstructed) passed the different thresholds: 0.9 for assigned mothers, 0.8 for reconstructed loci, and less than 3 missing loci for a given genotype.

From parentage assignments, OSR, female and male reproduction frequencies were determined. Additionally, within-season parameters such as multipaternity, female nesting interval, and the number of partners for males, were reported. The effect of multipaternity on fitness parameters (clutch size, number of hatchlings, number of dead embryos) was investigated by performing a two-tailed Student's t-test on the average.

Relatedness and inbreeding

The inbreeding coefficient (F_{is}) was calculated with GENETIX v4.05.2 (Belkhir et al., 2004) on all genotyped females and one offspring per nest (n=665). Pairwise relatedness coefficients (r) were calculated with the

R package *related* v1.0. (Pew et al., 2015) for all parent pairs found using COLONY, using sample allele frequency. Simulations were performed based on the observed allele frequency to determine which of the four relatedness estimators implemented in the package performs best on our data (i.e. is the closest to expected relatedness values). A hundred simulated genotypes for each of the four relationships were generated and their $r$coefficient was calculated (parent-offspring, expected $r = 0.5$; full-sibling, expected $r = 0.5$, half-sibling, expected $r = 0.25$, unrelated, expected $r = 0$). The Wang estimator, which yielded the best fit to expected relatedness values, was then used for relatedness calculation of reconstructed parent pairs (Supplementary Data 3). Relatedness of reconstructed vs potential parent pairs was calculated, and a two-tailed Student's t-test on the mean was performed to test for significant differences between the two relatedness distributions. To explore the effect of relatedness on fitness, clutch size, total number of hatchlings, and number of dead embryos were plotted against the relatedness of parent pairs. Additionally, the relatedness of pairs involving i) a male that mated with multiple partners (within a season or across seasons), ii) a female that mated with multiple partners (within a season or across seasons), iii) the female and dominant male from clutches with multipaternity, and iv) a female that that nested over several years, were compared with the relatedness of all the reconstructed parent pairs to determine whether any of these behaviours led to a deviation of the observed relatedness, and significant differences on the mean relatedness were tested with a two-tailed Student's t-test.

Results

Recapture identification

Genotypes were obtained from 1483 hatchlings representing 549 nests and 107 females. Sample size per nest ranged from 1 to 83, with 15 nests having more than 10 samples. The 23 loci had a meanH_e of 0.66 +- 0.04 and a meanH_o of 0.62 +- 0.04. Using the R package*RClone* , two genotype clones were identified (Table 1). They correspond to two females, both of which were sampled for the first time in 2016-2017 and resampled after 4 years in 2020-2021. One of these females was correctly identified in the field by its flipper tag, while the other was thought to be an unknown female and was given a different name at the second encounter.

Parentage analysis

After COLONY's parentage reassignment, genotype reconstruction, and filtering with the different thresholds, 105 nests were assigned to 27 genotyped mothers, and 13 were assigned to 4 reconstructed females between seasons 2014/15 and 2020/21. The total of 118 nests accounted for 448 offspring. One hatchling that was not assigned to any nest was linked with a genotyped mother. Out of these 118 nests, 70 counted more than one genotyped hatchling. Once the father's genotypes were reconstructed, a total of 27 fathers explained the genotypes of the 448 hatchlings. The operational sex ratio (OSR) ranged from 0.8 to 1.3 males per female depending on the season, averaging at 0.87, but the sample size was small with fewer than 10 males and females per season. (Table 2). Thus, temporal variations between the nesting seasons were not investigated.

Inbreeding and relatedness in the population

Global F_{is} was significant ($F_{\text{is}} = 0.06$, p_value < 0.001) showing inbreeding in the population. Pairwise relatedness was calculated for all possible male-female dyads and reconstructed couples. Mean relatedness among reconstructed couples (mean +- sd = 0.097 +- 0.21) was higher than the mean relatedness of all possible dyads (mean +- sd = -0.003 +- 0.15) (Figure 3). This difference was found to be significant when a t-test was performed on the mean (p = 0.008). The percentage of pairs with a relatedness greater than 0.25 and 0.5 was also higher for the reconstructed couples than for the potential pairs ($r_{0.25}$: 17% vs 4%, $r_{0.5}$: 3% vs 0.17%, Table 3). When looking at the effect of the relatedness of the parents on the fitness parameters in the clutches, a slightly positive correlation was found with the number of successful hatchlings, and no correlation with the clutch size and number of dead embryos was observed. (Supplementary Data 4).

Frequency of reproduction and within-season nesting parameters

Out of the 31 females, 26 of them nested for only one season. The remaining five females were observed nesting across consecutive seasons. Among these, four had offspring in two seasons and one had offspring for three seasons, indicating an annual reproduction cycle for these females (Table 1). Each of them laid between 3 and 9 clutches sired by a unique male over the period. The interval between the first and last clutch was 359 to 673 days. For these females, we did not find any other clutch sired by other males within this period.

In a single season, females laid between 1 and 10 clutches (Supplementary Data 5), with an average of 3.2 nests per female. The most common nesting interval was 11 to 14 days, with variations ranging from 0 to 94 days (Figure 4). Six clutches followed a first egg-laying event by only 0 to 3 days (+- 3 days), indicating that a single female can lay several clutches in a very short time interval, possibly on the same day. The size of these clutches varied between 39 and 103 eggs (mean 79 eggs), which was not different from the mean clutch size of the total sample (84 eggs, t-test p-value= 0.43) (Supplementary Data 6).

Of the 27 reconstructed males, 18 were assigned offspring in only one season. Eight males were assigned offspring in 2 seasons, with 7 of them doing so consecutively and one with a gap period of 3 years. One male was assigned offspring in three consecutive seasons. Among these 9 males, 6 sired different females in the consecutive seasons. Within one season, 24 of the 27 males sired a single female. Two males sired 2 different females, and 1 male sired 3 females in the same season.

Multiple paternity

Multipaternity was confirmed from genotype analysis in 5 of the 70 clutches in which we identified more than one genotyped hatchling (7%). Two fathers per female were responsible for all of the offspring sampled in each clutch. The occurrence of multipaternity was not correlated with the number of genotyped hatchlings per clutch, as multipaternity was found in clutches with counts of 2 to 20 genotyped hatchlings (Supplementary Data 7). One male was dominant in each clutch and sired between 60% and 66% of the genotyped hatchlings (Figure 5) (except in the clutch with only 2 genotyped hatchlings).

Out of these five clutches, two were laid by the same female, CMY0188 (1415_ONE8 and 1415_ONE9) (Figure 5). She laid these two clutches within 13 days in season 2014/15. They were sired by the same fathers *129 and *133, with the contribution of father *133 slightly decreasing in the second clutch (66% to 60%). In this season, eight other clutches laid by the same female were sired only by father *133, showing no multipaternity (Table 4). Three of them were laid before the clutches with multipaternity and counted 3, 17, and 6 genotyped hatchlings, respectively. The remaining 5 were laid after 1415_ONE9 and counted 8, 9, 7, 1, and 6 hatchlings (Table 4). Hatchlings from father *129 thus occurred only after several egg-laying events, possibly indicating mating in the inter-nesting interval.

Two other clutches laid one year apart were also sired by the same fathers (*62, *53) but these were from two different females, CMY1384 and CMY2419 respectively (Figure 5). For the female CMY1384, we found three nests in 2017/18 counting 1, 3, and 2 genotyped hatchlings, and multipaternity was found only in the second clutch, with father *53 absent from the other clutches. For CMY2419, 4 nests were identified in season 2018/19 (2, 2, 1, and 2 genotyped hatchlings) and father *62 was found only in the second clutch, the only one to show multipaternity (Table 4). Finally, the last clutch with multipaternity, 2021_ONE17, was laid by female CMY3468 in season 2020/21 and sired by fathers *97 and *337. Another clutch was laid by the same female after 36 days, from which two hatchlings were sampled. Only father *97 was found responsible for the genotype of these hatchlings (Table 4). Thus, multipaternity was never found in all the clutches laid by a female within a season.

We then investigated the effect of multipaternity on fitness. When comparing the fitness parameters in clutches with and without multipaternity, no correlation was found between the number of fathers and any of the three parameters (t.test p_values: 0.91, 0.95, and 0.83 for the clutch size, the total number of hatchlings, and the number of dead embryos respectively. Figures in supplementary data 4).

Mating behaviour and relatedness

Finally, none of the deduced mating behaviour, i.e mating with multiple partners, breeding more frequently, or having a higher share of paternity in multipaternity clutches was correlated with a change in the relatedness compared to the relatedness of all reconstructed couples (Figure 6). T-test p-values on the mean relatedness ranged from 0.16 to 0.5 (Table 3). However, fathers that were dominant in multipaternity clutches were consistently more related to the female than to the other father, although that comparison involved only 8 pairs and the difference was not significant with the t-test (p-value: 0.180, Table 3). Relatedness of couples with the dominant father ranged between 0.044 and 0.391 (mean r= 0.252), while relatedness with the non-dominant father ranged between -0.180 and 0.212 (mean r= 0.130).

Discussion

The present parentage analysis on the green turtle nesting population of Tetiaroa allowed for the genotype of 31 females and 27 males to be rebuilt from the genotype of 443 hatchlings from 105 nests, leading to an OSR of 0.87. The highly conservative thresholds we adopted in the analysis process accounted for the small number of males and females retained, which should be interpreted as a minimal number of breeders rather than the real number. As a principle, we favoured type II errors, assuming that reconstructed males and females are real and provide certainty in the following analysis. Nonetheless, patterns related to the mating system of this green turtle population emerged. First, the relatedness of couples was higher than expected by random mating, coupled with significant F_{is} , regardless of the mating behaviour considered. Neither the multipaternity nor the global relatedness was correlated with the fitness parameters in the clutches. Second, a low level of multipaternity was observed, and individuals showed some plasticity in their mating behaviours. Noticeably, both males and females appeared able to reproduce annually. Within a single season, we discover that females can lay several clutches over short time intervals, and males can sire several females, both before and during the nesting season. All of these outcomes are quite new in the depiction of green turtle reproductive behaviour and features.

Level of inbreeding and strategies to avoid relatedness in the population

Inbreeding was significant in this population (F_{is} =0.06, p_value < 0.001) and coupled with a higher relatedness than expected by chance. This indicates that these turtles breed non-randomly and overall do not avoid inbreeding. A similar situation was found by Horne et al. (2022) in a Hawaiian hawksbill turtle population, and was correlated with strong philopatry to nesting complexes less than 100 km apart. Here, the atoll of Tetiaroa has a total area of only 6 sq. km, but each female was found nesting on only one or two of the 11 islets surveyed (Supplementary Data 6). Such a level of male philopatry (< 1-2km) would be surprising given the scale, the absence of a swimming barrier around the island, and the capacity of males to connect rookeries (Bradshaw et al., 2018; Roberts et al., 2004).

The levels of relatedness observed could not be correlated with a loss of fitness when measuring clutch parameters. Philips et al. (2017) suggested that relatedness levels and parental multilocus heterozygosity can interact in both negative and positive ways in turtle populations and that relatedness alone does not explain levels of fitness, but investigating these interactions is beyond the scope of this study. None of the mating behaviour deduced from the parentage analysis (mating with multiple partners, breeding more frequently, or having a higher share of paternity in multipaternity clutches) seemed to efficiently mitigate inbreeding, as the relatedness of pairs involved in these behaviours was similar to the overall relatedness. This is in discordance with the inbreeding avoidance theory that predicts that populations would tend to avoid inbreeding with pre and postcopulatory mechanisms (Blouin and Blouin, 1988; Cornell and Tregenza, 2007; but see Szulkin et al., 2013). Inbreeding avoidance is suspected in a small population of leatherback turtles in the South West Atlantic (Vargas et al., 2022). On the contrary, mating choices in this population seem to be driven by other factors. At the scale of French Polynesia, three populations were identified regardless of their nesting islands and mitochondrial lineage (Dolfo et al., submitted). When including reconstructed male genotypes from Tetiaroa in this analysis, most of them belonged to the same “genetic population” as their female mates (Supplementary Data 8). However, in Tetiaroa, three distinct genetic populations coexist, indicating a deliberate selection of partners rather than random pairing. As proposed by the authors, these populations may be linked with phenotypical or ecotypical preferences in partner choice (e.g. carapace colour and shape) or may be conditioned to breeding grounds that occur irrespective of nesting islands. However, in Tetiaroa, breeding behaviours are observed in the vicinity of the island (Gaspar, pers. comm.), suggesting that breeders then nest on this island. Courtship is usually observed as a result of repetitive attempts by males, as females can also refuse mating (Booth and Peters, 1972). Green turtle courting behaviour should thus be investigated to further determine what may drive such a non-random mating choice.

Additionally, our results on multipaternity clutches contradict the ‘gene compatibility’ theory, as the dominant father in multipaternity clutches was more related to the female than the other male. Dominance in shares of multipaternity is thought to be linked with first male sperm precedence (Fitzsimmons, 1998). This, for example, is confirmed in nests 1415_ONE8 and 1415_ONE9. In these nests, the dominant male was found in all of the other nests earlier in the season (Table 4), while the non-dominant male was not found in any of the nests. Although the latter might have been missed in the early nests, this pattern may also reflect a second mating occurring later in the nesting season. In this case, multipaternity might mitigate inbreeding by mating with other partners when the first one has a high level of relatedness. This mechanism is observed in the Whites’ skink (*Egernia whitii*), where females sometimes seek a second and less related partner outside their social group, which increases the offspring heterozygosity (While et al., 2014). In Chinese alligators (*Alligator sinensis*), polyandry females mate with males that are less related to them than monogamy females, mitigating inbreeding in the population (Wang et al., 2017). To date, very few studies have explored relatedness in multiple paternity clutches of marine turtles (Phillips et al., 2013). This hypothesis therefore needs to be confirmed with a higher number of multipaternity clutches and deserves further investigations in other populations.

Number of breeders and population density

The operational sex ratio (OSR) was slightly biased toward females (0.87:1 male: female), indicating that more females than males breed in this population. OSR in sea turtles has typically been reported as skewed towards males or even between both sexes (Hays et al., 2022). For example, Prakash et al. (2022) found an OSR of 1:1 in a population of hawksbill turtles in Fiji, and Wright et al. (2012b) found an OSR of 1.4:1 in a green turtle population in Cyprus. The primary sex-ratio of this population in Tetiaroa does not seem to suffer heavy female bias as observed elsewhere (Laloe et al., 2020), and so the discrepancy between the number of male and female breeders is likely small. However, a low level of multipaternity was observed (7% of the clutches, 16% of the females) and green turtles demonstrated multipaternity in as much as 92% of their clutches (Alfaro-Nunez et al., 2015). Our finding constitutes the lowest estimation of multipaternity level in this population, considering that 70 clutches were analysed over a total of 549 observed. Plus, the number of hatchlings genetically sampled was uneven in the clutches (most often < 10), thus the contribution of some fathers may have been missed. With no apparent fitness benefits, a low level of multipaternity may indicate low chances of encountering multiple males, directly linked to the density and number of male breeders (Lee et al., 2018). However, a high density of breeders is observed near the island (Gaspar, pers. comm.), and 11% of the males mated with multiple females within a season, indicating that males may encounter several available females. Coupled with the low level of multipaternity, it suggests that not all males are equally capable of mating with multiple females. In contrast, Phillips et al. (2013) found no male hawksbills that mated multiple times within a season and concluded that a low density and high turnover of males prevented this from occurring.

Another deduced behaviour of interest arose from the two reconstructed males *53 and *62. They both sired female CMY1384 in 2018/19 and a different female CMY2419 in the next season. This finding is surprising given the low number of breeders retained and the low level of multipaternity observed, though the probability of such an occurrence increases when the number of male breeders is low and the density in the mating area is high. This may indicate that some males are dominant on the breeding ground. Dominance hierarchies in males have been described in other species of freshwater turtles like the common snapping turtle (*Chelydra serpentina*) and the wood turtle (*Clemmys insculpta*), and in the gopher tortoises (*Gopherus agassizii*) (Pearse and Avise, 2001). Such dominance could occur regularly on the breeding ground, or be year-specific in the case where CMY2419 was sired in 2018/19 and able to store sperm until the next season. Alternatively, non-random mating choices driven by a restrictive criterium may limit the number of suitable partners for a given female, increasing the probability of mating with the same pair of males. Nonetheless, these contrasting results are the first to look into the number of male breeders in French Polynesia and show that differences between the two sexes are likely to be small and that this number seems sufficient to maintain fitness in the population despite high levels of relatedness.

Plasticity in mating behaviours

Reproduction frequency and long-term sperm storage

Parentage assignment revealed that at least 5 females (16%) returned to lay once or twice during the 11-year study period. These returning events were not observed in field surveys, as recapture identification found that only 2 females were resampled during a returning event. One of these females was among the 5 females identified from their genotype, matching the genetic approach (CMY1113, Table 1). Interestingly, these females were found nesting in consecutive seasons. According to data available so far, annual reproduction frequency has never been observed in female marine turtles. For example, Hays et al. (2014) revealed with satellite tracking that while no female loggerhead turtles migrated annually to breeding grounds in the Mediterranean Sea, 76% of the males did. Studies focusing on female reproduction frequency are based on field observations, and thus it is possible that in some cases, annual reproduction has been overlooked due to the absence of direct observation in the field.

Satellite tracking on adult females in French Polynesia revealed that several individuals stay within the territory, while others migrate to Fijian neritic feeding grounds between the nesting seasons (Craig et al., 2004; Piovano et al., 2019; DIREN personal comm.). Whether those breeding annually are those that remain in French Polynesia throughout the year needs to be determined, but it is difficult to imagine long annual migrations to Fiji ($>$ 2000km), given the time and energy that it requires, adding to the energy needed for the reproduction itself. Interestingly, the same fathers consistently sired the clutches of annually breeding females over the given period (from 359 to 673 days). This may indicate that females are capable of storing sperm across seasons or exhibiting narrow male choice for reproduction, implying fidelity to what are currently unknown mating criteria. Alternatively, cross-seasonal sperm storage has neither been demonstrated nor quantified in marine turtles, although it is suspected to occur. For example, Wright (2013) found that multipaternity was more common in returning females than in primary nesters, and proposed cross-seasonal sperm storage as one of the possible causes. Cross-seasonal sperm storage over several years has been observed in other taxa, including birds (Feldschuh et al., 2005), insects (Baer et al., 2003), and reptiles (Ewing, 1943; Levine et al., 2021). Although the possibility of multiple mating among the same individuals cannot be ruled out (Sakaoka et al., 2013), cross-seasonal sperm storage coupled with relatively sedentary life traits may help females to minimize energy costs linked with migration and mating. To date, however, adult green turtles are rarely observed outside the nesting season in French Polynesia, and no feeding ground has been identified throughout French Polynesia or nearby. Hatase et al. (2006) showed, using satellite tracking and stable isotope analysis, that 31% (n=89) of the females nesting on Ogasawara Islands, Japan, were oceanic planktivores rather than neritic herbivores. Further investigation using these complementary techniques should reveal whether females in French Polynesia also feed in oceanic habitats close to nesting grounds.

For males, 6 (22%) sired different females in consecutive seasons, indicating a possible annual reproduction frequency. However, caution should be taken as this might also reflect long-term sperm storage in the case when the females could not be identified in one of the consecutive seasons. Male reproduction interval is thought to be shorter than for females due to a smaller energy cost of reproduction that allows them to reproduce more often (Hays et al., 2022). For example, the male green turtle remigration interval was 2.1 years in the southern Great Barrier Reef (Limpus, 1993), and 1 year in Hawaii (Balazs, 1983), based on tagged recapture. Wright et al. (2012a) determined the reproduction frequency of male green turtles in northern Cyprus with parentage analysis and found that 3% bred more than once within the 3 years. Overall, annual male reproduction frequency linked to a residential strategy must be considered as a strong possibility in French Polynesia, and in this population, a discrepancy between male and female reproduction frequency is not observed.

Within-season nesting parameters

Within a single season, females laid, on average, 3.2 clutches. Field estimations based on the length of the nesting season and the observed inter-nesting interval proposed that each female laid on average 6 nests in 2017/18 (Touron et al., 2018). The true number is likely to lie between these two estimations and is coherent with clutch frequencies for other green turtle populations in the Pacific Ocean (reviewed in Pilcher 2021). They vary from 1.8 in French Frigate Shoal to 7 +- 1.3 clutches per female per season in the Northern Mariana Islands (Balazs, 1980; Summers et al., 2018). Additionally, through parentage analysis, 74 nests without observed females in the field were associated with a female, demonstrating the efficiency of the analysis to supplement field observations.

Inter-nesting interval was highly variable (between 0 and 94 days) and an interval of 11 to 14 days was the most common interval in this population. This is coherent with field observations (Touron et al., 2018), and aligns with findings in other populations (reviewed in Robinson et al., 2022). As not all nests could be analysed, the longest inter-nesting interval could not be determined. However, and more surprisingly, our study reveals the ability of females to lay regular-sized clutches within a very short interval (0 to 3 days). This represents a new finding in the reproductive strategy of green turtles. This shows a capacity for plasticity in nesting behaviour, a potential advantage in allowing a population to adapt to changing environmental and weather conditions on nesting beaches if one supposes that females might search for optimal nesting conditions. Plasticity in nesting behaviour has been observed in Tetiaroa, with an increase in the nesting season length over the years (Touron et al., 2018), possibly indicating that females may look to nest during cooler months to produce more male offspring in the ongoing warmer conditions (Laloe et al., 2020).

For almost all females, clutches were sired by the same father within a season, coherent with the fact that breeding occurs before nesting (Hays et al., 2022). However, one male (*129) sired 2 clutches in the middle of the nesting season, which induced multipaternity in these clutches. The genes of this male were not found in the first three clutches nor the last five clutches laid by the same female (CMY0188) during the season, although the number of sampled hatchlings (1 to 17) was enough to detect multipaternity in some of these clutches. The first clutch sired by this male was laid 40 days after the first recorded clutch for this female. Female marine turtles are thought to be available for reproduction only for a short interval of approximately one week, before nesting (Comuzzie and Owens, 1990). Here we suggest for the first time in a natural sea turtle population that mating occurs during the inter-nesting interval (but see Sakaoka et al., 2011, on a captive population of loggerhead turtles), and provide another indication that green turtles are capable of unexpected plasticity in their mating behaviour.

Implication for the conservation

The green turtle population of Tetiaroa shows a significant level of relatedness and inbreeding, with no clear inbreeding avoidance strategy and an apparent preference for related partners that rather favour it. This indicates that the reproductive system of green turtles makes them intrinsically vulnerable to inbreeding and its potential negative consequences on the capacity of a population to maintain itself through generations. Although it does not seem to affect the fitness of Tetiaroa's population at this stage, it could have negative effects if the number of breeders is not maintained, which highlights the importance of turtle conservation programs. On Tetiaroa, a conservation and monitoring program has been in place since 2007, and an increasing number of breeders has been recorded on the island, indicating that it is likely efficient and can preserve the genetic diversity of this population (Touron et al., 2018). Finally, unexpected plasticity in green turtle mating behaviours and nesting parameters shows some adaptive capacity which may help maintain the population's resilience under changing environmental conditions.

Acknowledgements

This research was funded by the Direction of the Environment of French Polynesia. We want to thank the volunteer network coordinated by the Direction of the Environment in French Polynesia for providing all of the samples used in this work, including the NGO Te mana o te moana for the samples from Tetiaroa and Moorea and the Bora Bora Turtle Center for the samples from the Leeward Islands. Thank you to Jeanine

Almany for the English corrections.

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Data Accessibility and Benefit-Sharing

Data availability

Microsatellite sequences are available on GenBank under accession number OQ162049-OQ162073. All other data generated or analyzed during this study are included in this published article and its supplementary information files.

Benefit-Sharing

Benefits Generated: A research collaboration was developed with associations from French Polynesia providing genetic samples, all collaborators are included as co-authors, the results of the research have been shared with the provider communities and the broader scientific community, and the research addresses a priority concern, in this case, the conservation of organisms being studied. More broadly, our group is committed to international scientific partnerships, as well as institutional capacity-building.

Author Contributions

Chelonia mydas sample collection was coordinated by Miri Tatarata and Cécile Gaspar. Miri Tatarata and Serge Planes designed the research. Sample processing and analysis were performed by Violaine Dolfo, reviewed by Emilie Boissin, and supervised by Emilie Boissin and Serge Planes. The first draft of the manuscript was written by Violaine Dolfo, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Table 1: Females found nesting in multiple seasons using *Rclone*analysis (marked with °) and parentage analysis (marked with^{+}).

Ind #	Ind. ID	1st sampling season	2nd encounter	1st genotype reassignment	2nd genotype reassignm
1	CMY1113$^{\circ,+}$	2016/17	2020/21	2017/18	-
2	CMY1139°	2016/17	2020/21 +	-	-
3	CMY1104^{+}	2016/17	-	2017/18	-
4	CMY1105^{+}	2016/17	-	2017/18	-
5	CMY1101^{+}	2016/17	-	2015/16	2017/18
6	#75^{+} (reconstructed)	NA	-	2016/17	2017/18

+ Different ID given on the second encounter

Table 2: Number of females and males deduced from parentage analysis for each season, and operational sex ratio (OSR, number of males for one female)

Season	**Nb. females**	**Nb. males**	**OSR**
2014/15	3	4	1.33
2015/16	2	2	1.00
2016/17	9	8	0.90
2017/18	8	8	1.00
2018/19	3	3	1.00
2019/20	6	5	0.83
2020/21	6	7	1.17
total	31	27	0.87

Table 3: Mean relatedness between potential and reconstructed couples, couples involved in different mating behaviours, and Student's t-test p-values. Significant p-values are indicated in bold. $r_{0.25}$ ($r_{0.5}$): percentage of pairs with a relatedness greater than 0.25 (0.5).

	mean r	t-test p	# pairs	$r_{0.25}$	$r_{0.5}$
All potential couples	-0.003	-	2880	4%	0.17%
All reconstructed couples	0.097	**0.008 (vs potential)**	36	17%	3%
Couples with female nesting in consecutive seasons	0.294	0.16 (vs reconstructed)	5	-	-
Couples with multipaternity	0.162	0.39 (vs reconstructed)	8	-	-
Dominant male in clutches Non dominant male in clutches	0.252 0.130	0.13 (vs reconstructed) 0.18 (vs dominant)	4 4	-	-

	mean r	t-test p	# pairs	$r_{0.25}$	$r_{0.5}$
Males mating multiple times	0.143	0.50 (vs reconstructed)	16	-	-

Table 4: Father ID and number of offspring in all clutches of the 4 females involved in polyandry. Clutches with multipaternity are indicated in bold.

Female ID	**CMY0188**	**CMY0188**	**CMY1384**	**CMY1384**	**CMY2419**	**CMY2419**	**CMY346**
Season	2014/15	2014/15	2017/18	2017/18	2018/19	2018/19	2020/21
Nest number	Father ID	Offspring nb	Father ID	Offspring nb	Father ID	Offspring nb	Father ID
#1	*133	3	*62	1	*53	2	***97/*337**
#2	*133	17	***62/*53**	**3**	***53/*62**	**2**	*97
#3	*133	6	*62	2	*53	1	
#4	***133/*129**	**12**			*53	2	
#5	***133/*129**	**7**					
#6	*133	8					
#7	*133	9					
#8	*133	7					
#9	*133	1					
#10	*133	6					

Figure Captions

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Figure 1: Sampling locations on Tetiaroa Atoll, French Polynesia. Sampled nests are represented with black dots around the islets.

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Figure 2: Conceptual workflow for all the analyses performed for this study

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Figure 3: Density and boxplot of the relatedness of all the potential (full line) and reconstructed (dash line) parent pairs.

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Figure 4: Inter-nesting interval between two successive nesting events for every female between 2014/15 and 2020/21 in Tetiaroa atoll.

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Figure 5: Relative fathers' contribution in the five clutches where multipaternity was found. Each vertical bar represents one clutch and each shade of grey represents one male. The number of hatchlings in the clutches is displayed above each bar.

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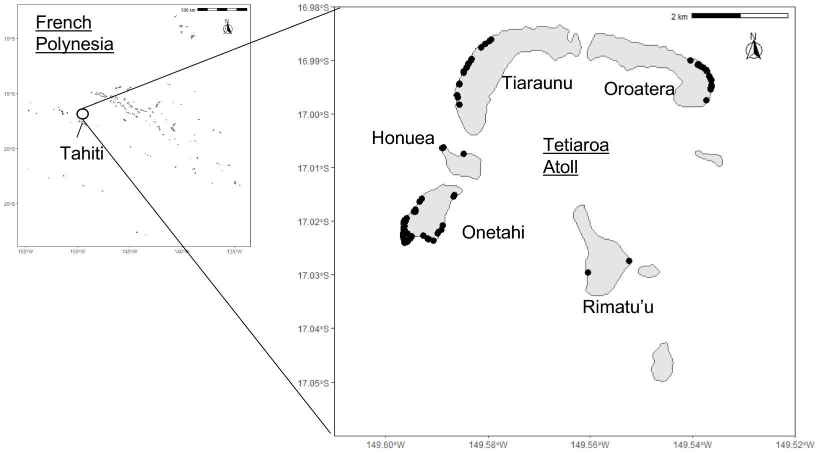
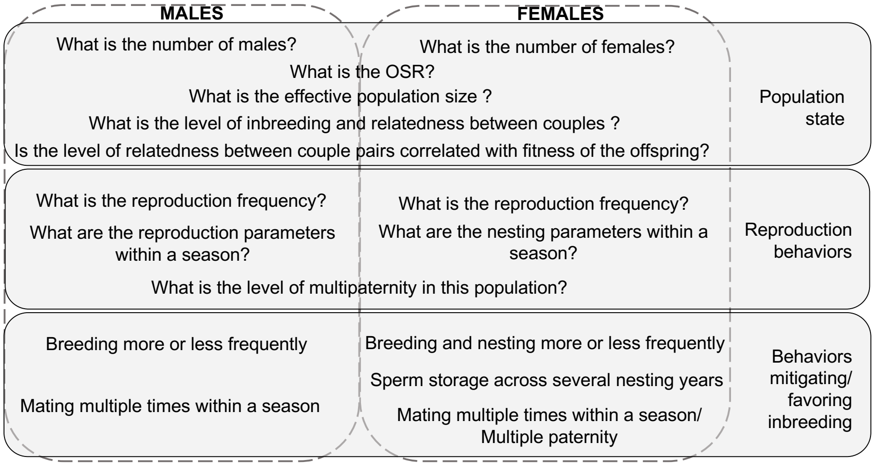
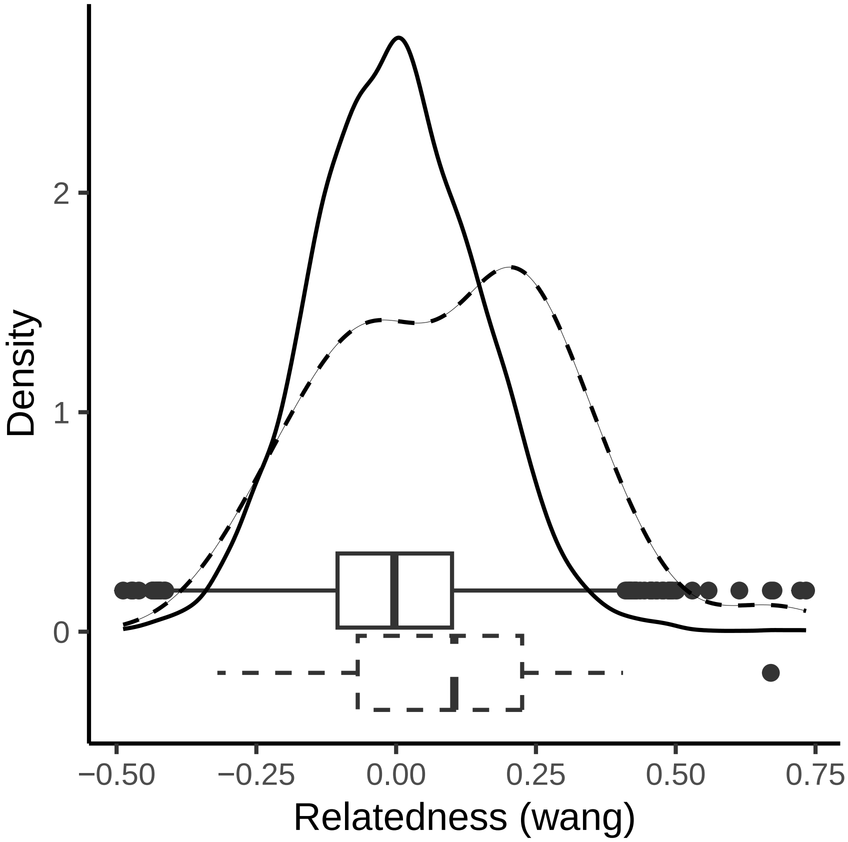
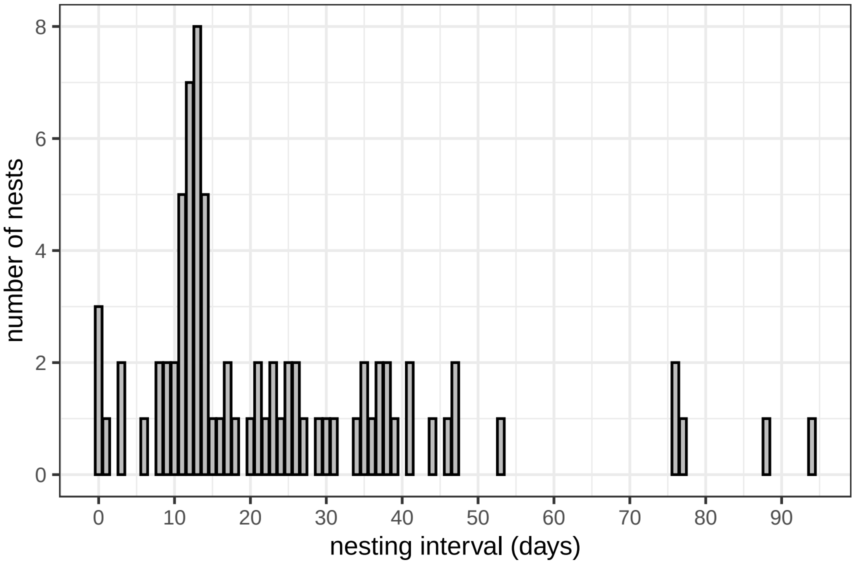
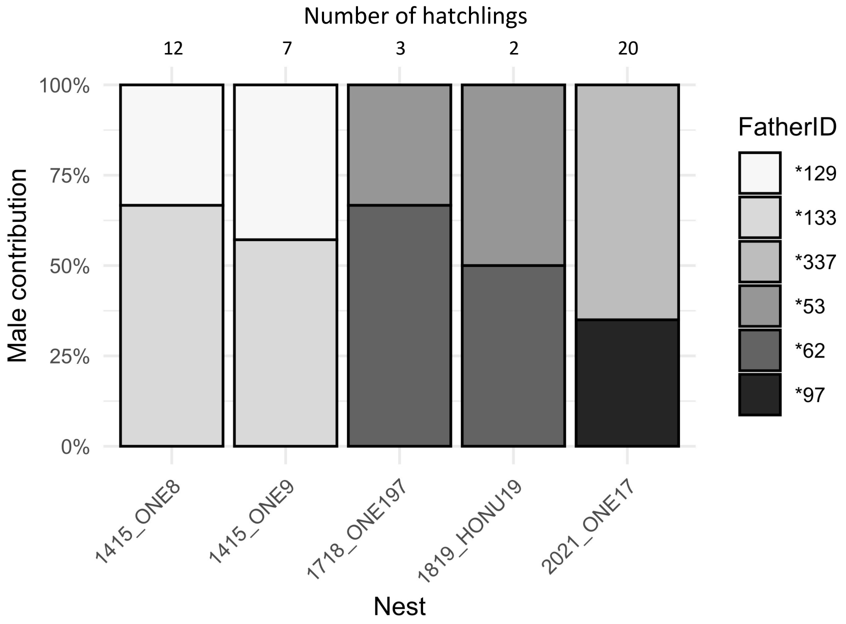
Figure 6: Relatedness of couples involved in different reproduction behaviours compared with relatedness of all reconstructed couples. Grey line: all reconstructed parent pairs, black line: multiple mating of males, two-dash line: annual nesting of females, dot line: dominant father in multipaternity clutches.

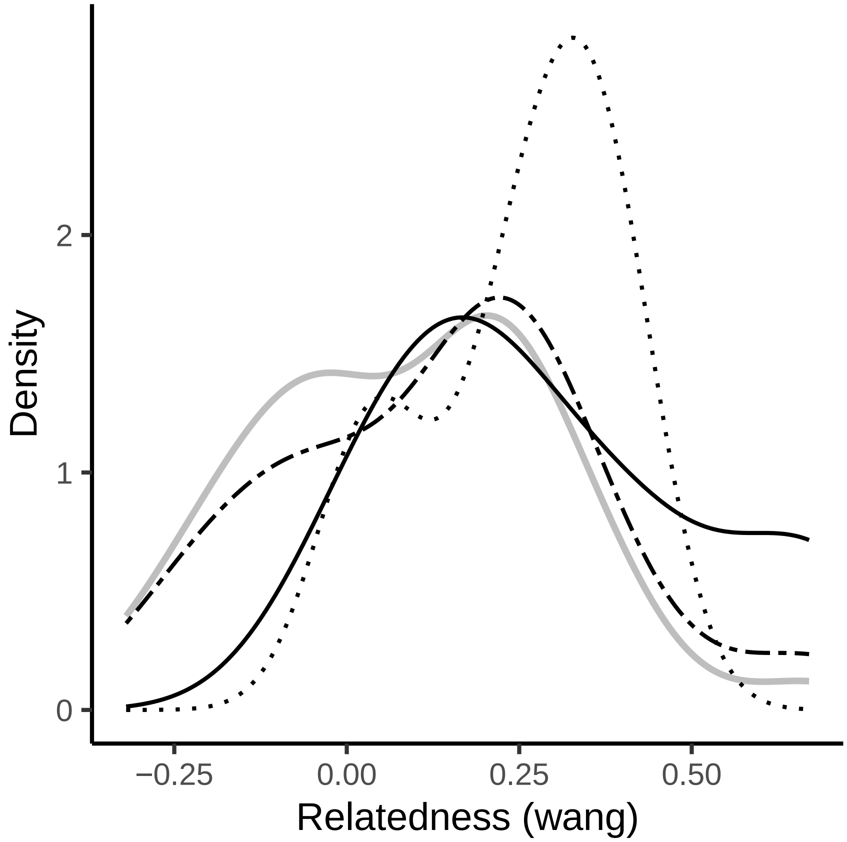





Density
2
1
0
−0.50
−0.25
0.00
0.25
0.50
0.75
Relatedness (wang)


number of nests
8
6
4
2
0
0
10
20
30
40
50
60
70
80
90
nesting interval (days)


Number of hatchlings
12
7
3
2
20
Male contribution
100%
75%
50%
25%
0%
1415_ONE8
1415_ONE9
1718_ONE197
1819_HONU19
2021_ONE17
Nest
FatherID
*129
*133
*337
*53
*62
*97


Density
2
1
0
−0.25
0.00
0.25
0.50
Relatedness (wang)