Multiple paternity assessment and paternity assignment in wild European lobster (Homarus gammarus)

- Comparing a no-take reserve and an exploited area

Tonje Knutsen Sørdalen

NORWEGIAN UNIVERSITY OF LIFE SCIENCES
Department of ecology and natural resource management
Master Thesis 60 credits 2012

Multiple paternity assessment and paternity assignment in wild European lobster (*Homarus gammarus*)

- Comparing a no-take reserve and an exploited area

Photo: J.R.W

Master of Science Thesis by Tonje Knutsen Sørdalen

Supervisors:
Thrond Haugen, Norwegian University of Life Sciences
Halvor Knutsen, Institute for Marine Research, Flødevigen
Esben Moland Olsen, Institute for Marine Research, Flødevigen

Forord

Jeg vil rette en stor takk til veilederne mine Halvor Knutsen og Esben Moland Olsen som ga meg muligheten til å gjøre masteroppgaven hos Havforskingsinstituttet i Flødevigen. Videre vil jeg takke Hanne Sannås for opplæring og støtte, Dimitar Serbezov som har hjulpet meg på mail, Thrond Haugen og Øystein Wiig for hjelp og kommentarer mot ferdigstillingen av arbeidet. Det ble stress, men vi kom i mål! Tusen, tusen takk.

Tiden i Flødevigen har vært minnerik og utrolig fin. Foruten å være den beste plassen å være under et arbeidsår så fyltes gjestehuset opp med latter og humor fra flotte mennesker fra hele Europa. Diana Catarino, Marine, Enrique Blanco, Jan –Harand Nordahl og Henning (”the Elephant”) for det beste selskapet en kan ønske seg i en stressende periode. Jeg savner dere allerede.

Videre skylder jeg en stor takk til gode venner Anne Sørensen, Helga B. Kristensen, Tine K. Sørdalen, Kristine og Tor for å nevne noen –Tusen takk for at dere har orket å høre på pratet mitt om hummer-sex!

Sist men ikke minst Jørgen, kjæresten min, deg skylder jeg mest. Du har vært min beste venn, en å le med, en å gråte med og en å streve sammen med mot vårt felles mål; seiltur i vår egen båt langt av gårde og borte fra alt. Jeg har holdt deg oppe om natten med frustrasjoner og endeløse timer med skriving, -tusen takk for at du holdt ut. Takk for at du er min.

Abstract

Understanding the mechanisms driving mating systems is intricate for wild populations of species where behavioral observations are difficult, but nonetheless imperative for harvested species. This study investigated the occurrence and frequency of multiple paternity for the European lobster (*Homarus gammarus*) in a marine reserve (MPA) and in a heavily exploited control area on the Norwegian Skagerrak coast. Also, this study is the first to conduct a parental assignment in a wild lobster population. With three to six microsatellite loci genotyped, 81 females and ten offspring from each brood, high level of multiple paternity was discovered in both reserve (27 and 96%) and control area (3 and 90%) with no significances in regards to body size. However, significantly more cases of multiple paternity was observed in the reserve area using the most parsimonious estimate. These results demonstrate that females in all size categories may mate with more than one male after pre-molt insemination, perhaps due to altered mating behavior as result of decades of overharvest or due to high density of individuals. Of the 475 candidate males genotyped for six loci, 13 of them were assigned to offspring of 14 females but with no clear patterns for assortative mating, although 71% of the pairs consisted of a male bigger than the female. As five of the mated pairs have crossed the boundaries of the reserve in either direction there are tendencies of spill-over effects. However, eight of the pairs resided in the marine reserve which also indicating a high site fidelity. Further research to unveil the genetically significance of multiple paternity and what drives the females' choice is important for management of this high valued species.

Contents

1 Introduction and background 2
1.1 Objectives 5

2 Materials and methods 6
2.1 Fieldwork 6
2.1.1 Study area 6
2.2 Lab work 9
2.3 Statistical analysis 12
2.3.1 Properties of microsatellite loci 12
2.3.2 Multiple paternities assessment 12
2.3.3 Paternity analysis 15

3 Results 18
3.1 Properties of the loci 18
3.2 Multiple paternity assessments 19
3.3 Paternity analysis 21
3.3.1 The paired lobsters and their sizes 23

4 Discussion 26
4.1 Paternity assessment and assignment 26
4.2 Female mating pattern and preferences 27
4.2.1 Size vs. shelter and protection 28
4.2.2 Multiple matings 29
4.3 Interaction between the reserve and control area 30
4.4 Efficiency of the methods used and statistical uncertainties 31
4.5 Conclusion and future prospects 34

5 References 36

1 Introduction and background

The ecological effects of exploitation on populations of species are rarely random (Rowe & Hutchings 2003). In intensely harvested species, there is a growing awareness that targeting the largest and fastest-growing individuals has the potential to distort mating systems (Allendorf & Hard 2009). In particular, effects are strong when it comes to removing individuals from wild populations where mating system and reproductive success is depended on sexually selected traits, such as a large body size or enlarged size of prominent weaponry (Lane *et al.* 2011; Gosselin *et al.* 2005). As the vulnerability of marine species is increasingly recognized, marine protected areas (MPAs) have gained recognition for their ability to provide refuge, in which stocks of exploited species can restore and over time provide benefit to local fisheries through spillover from reserves to surrounding areas (Gõni *et al.* 2006; Moland *et al.* submitted; Planes *et al.* 2009; Christie *et al.* 2010). Marine reserves may also counteract the effects of selective fishing by protecting those old and large phenotypes which are typically targeted by fishers (Baskett *et al.* 2005). Further, protection against selective fishing will allow the demographic structure in the populations to recover and provide opportunity to study ecosystem components, such as mating systems, under natural conditions in absence of human influence (Moland *et al.* 2011; Sorin 2004).

In many marine species the level of recruitment are depended on size-specific fecundity since large and old females are generally more fecund compared to smaller females (Agnalt *et al.* 2007, 2008; Phillips 2006). Thereby, large size has often been shown to be under sexual selection (Lane *et al.* 2011; Debuse *et al.* 2003). However, when large size is also economically valuable, fishermen are opposing strong natural selection by removing those individuals from the population (Jørgensen *et al.* 2007; Fenberg & Roy 2008; Lane *et al.* 2011). In order to sustain the recruitment of economically important species like lobsters, a minimum size limit has been imposed to allow maturation before entering the fishery. Nevertheless, the most obvious biological effects of this size-selective harvesting, or "longevity overfishing", is a reduction in mean individual size in comparison to populations under less pressure, as seen in *Homarus* lobster species (e.g. Gosselin *et al.* 2003, 2005; Mercer *et al.* 2001; Beamish *et al.* 2006) and earlier onset of sexual maturity (Phillips 2006). An additional effect, especially in respect to species that can reach high age, is reduced fitness and survival prospects in targeted populations (Lane *et al.* 2011; Venturelli *et al.* 2009).

European lobster, *Homarus gammarus,* is a large and long-lived decapods crustacean distributed from the Aegean Sea, through the Mediterranean and to northern Norway (Jørstad *et al.* 2001; Anon. 2008). The lobster is considered nocturnal and solitary, preferring rocky shelters where it spends much of its time, apart from when foraging or defending the territory from intruders (Moland *et al.* 2011; Bushman & Atema 1997). Knowledge about the mating system in European lobster is still scarce, and much is derived from research on the close relative, the American lobster, *Homarus americanus* (Phillips 2006). Laboratory experiments with American lobsters have shown that females that are ready to molt (shedding of exoskeleton) approach males who are residing in shelters. Chemical cues excreted from males, in the form of urea, are playing a role in communication and if a female is accepted she will molt and subsequently mate (Bushmann & Atema 1997). A sperm package, spermatophore, is deposited in the seminal receptacle of the female along with a sperm plug that is thought to prevent additional mating until her next molt (Bushmann & Atema 1997). The female will stay in the shelter guarded by the male until her new exoskeleton is hardened and she is strong enough to leave. The post-copulatory guarding of female, and the formation of sperm plug, is thought to prevent her from additional matings until the next molt (Gosselin *et al.* 2005).

Several studies have found that female decapods can be promiscuous, that is, mate with more than one male during mating season (Thiel & Hinojosa 2003). Such behavior has recently been observed in wild populations of Norway lobster, *Nephrops norwegicus*, through detection of multiple paternal microsatellite markers in single female broods (Streiff *et al.* 2004). In this species, six out of 11 sampled females off the Portuguese coast had multiple sirings with up to three different males contributing to each brood. Interestingly, multiple sired females tended to be larger in size (Streiff *et al.* 2004). Similar results were also found in populations of the same species outside Island and in the Irish Sea (Phillips 2006). However, contradicting results have been reported from two populations of the spiny lobster species, *Panulirus argus*, where evidence for female promiscuity was detected in Brazil but not in a population in Florida Keys (MacDiarmid & Butler 1999).

Various hypotheses have been suggested to explain adaptive benefits of female promiscuity as a mating strategy. Notable explanations are concerned with (1) increased genetic variability for avoiding inbreeding (see Yue & Chang 2010; Kraus *et al.* 2004; Bretman *et al.* 2009), (2)

willingness to re-mate when identifying a superior and more attractive male, and (3) female cryptic choice in which males are chosen after copulation based on genetic compatibilities (Jennions *et al.* 2000; Thiel & Hinojosa 2003). In recent years another hypothesis, concerning sperm-limitation, has received increased attention as it also addresses the problem with size-selective harvesting. Studies on lobsters have shown that larger males are producing and passing more sperm to successive mates than smaller ones (McDiarmid & Butler 1999; Gosselin *et al.* 2003), thereby ensuring higher fertilization success (Sato *et al.* 2010; Rowe & Hutchings 2003). If those large males are removed from a population they may constrain the reproductive potential of large females who instead have to confine themselves to smaller males (Sato *et al.* 2010). Further, as a larger female require more sperm to successfully fertilize all her eggs, she might result to promiscuity and seek out additional mates, as observed in laboratory studies of American lobsters. Females were indeed more restless if they had mated with a smaller male, and more prone to mate again if the opportunity was presented (Gosselin *et al.* 2005). This has also been observed in other crustacean species (*Chionoecetes opilio*, Sainte-Marie *et al.* 2002; *C. sapidus*, Hines *et al.* 2003). Considering that many lobster fisheries have management regulations to protect berried females (Rowe 2002; Agnalt *et al.* 2007), sperm limitation is suggested to affect decapods mainly due to skewing of the sex ratio (Gosselin *et al.* 2005). For the Coconut crab, *Birgus latro*, Sato *et al.* (2010) found that overharvesting of large male individuals coincided with lower sperm concentration received by females, and consequently production of fewer fertilized eggs.

Promiscuity in wild populations of the American lobsters has been investigated in eastern Canada where the occurrence of female promiscuity was contrasted in an area with low fishing pressure compared to two heavily fished areas. Multiple paternity, the contribution of more than one male for each brood, was found in the two populations with heavy fishing pressure but not in the least exploited population. The phenomenon was explained as depletion of large males, and consequently smaller males to receptive female ratio, leading to sperm-limitation in the harvested areas (Gosselin *et al*. 2005). Occurrence of multiple paternity has not been found the European lobster at present time (Ferguson *et al.* 2002; Hughes *et al.* 2001) but an extreme population reduction due to high fishing pressure may have led to a state in which promiscuity is no longer possible due to very low mate encounter, as suggested by Phillips (2006).

If female European lobsters prefer larger males we might expect to find non-random mating pattern, at least in populations with low harvest. “Negative assortative mating” occurs if the mated pairs in a population are composed of individuals with unlike appearance, like size discrepancies, more frequent than expected by chance (random mating) (Hedrick 2011). However, constricted variation in individuals as an effect of low population density and skewed sex ratio may result in a constrained development towards “positive assortative mating” in which the size of the sexes evens out. Notably, an increase in multiple matings of larger females in populations under strong fishing pressure may be the only option to uphold the reproduction.

1.1 Objectives

Facing the challenge of rebuilding a stock of European lobster which is at its lowest record in history in Norway, four experimental no-take lobster reserves and three adjacent control areas were established along the Skagerrak coastline in 2006. The purpose was to monitor how small-scale reserves can effect local lobster populations in temperate waters (Knutsen *et al.* 2009). Additionally, the reserves are serving as baselines for biological studies of natural behavior.

The aim of this study was to explore the mating system of the European lobster under two conditions, one being a protected marine reserve and the other a heavily fished control area (no regulation). The exploitation rate in the fished site was estimated to be as high as 83% during the lobster fishing in 2011 (Wiig 2012). In this thesis I focused on three objectives. First, I investigated the occurrence of promiscuity and multiple paternity for broods in the two sites in relation to three size categories of the females. Second, a parental assignment was conducted in order to investigate the relation between mate choices by females in relation to size of both sexes, in a protected and in an exploited area. Third, I investigated the level of connectivity in females between the two sites in order to elucidate the spill-over dynamics of a small-scale lobster reserve.

2 Materials and methods

2.1 Fieldwork

2.1.1 Study area

This study was conducted between May and October 2011 on the Norwegian Skagerrak coast at two close locations; one being a lobster reserve and the other a control area. The lobster reserve (centered at 58° 25'N, 8° 45'E) is situated in sheltered water outside the Institute of Marine Research (IMR), Flødevigen, Arendal, southeastern Norway. With one km^2 in size, it extends from farthest end of inlet to include several small skerries and eventually envelope the some larger Island of Ærøya at southeast (Fig. 1). Water depths are moderately shallow on the north side of Ærøya, whereas it increases abrupt to depths below 50 m at southern and more exposed side of Ærøya Island. Upper substrate (1 – 2 m) is mostly dominated by photosynthetic macroalgaes and mud flats in the deeper basin, whereas boulders and rocks of various sizes are increasingly abundant beyond depth of 10 m. The underwater topography was an important criterion for making this area an experimental lobster reserve as it is a characteristic habitat for lobsters in Skagerrak (Moland *et al.* 2011).

The control site (centered at 58° 24'N, 8° 44'E) is in the semi-sheltered basin of Sømskilen (fig. 1), with size and habitat composition comparable to that of the reserve (Pettersen *et al.* 2009). The area includes about 500 meters of shoreline and runs 1.5 km South-East across the basin. Inside the control area the groups of islets Halvorsholmene stands out as a popular lobster fishing ground. The control area is at its deepest in the Sømskilen basin with a maximum of 30 meter in the outer part around Halvorsholmene. The river Nidelva has one of its three outlets in Sømskilen and emits freshwater into the basin, and as it mostly stays in the surface layer, it creates a halocline of less saline water only in the first two meters below sea level (Olsen & Moland 2010). As in the reserve, shallow substrate around islets is dominated by macroalgaes while rocks and mud flats are found in deeper parts (Espeland *et al.* 2010). Although reserve and control sites are adjacent they are separated by 1700 m (Moland *et al.* submitted) stretched from the reserve center to control center (Fig. 1).

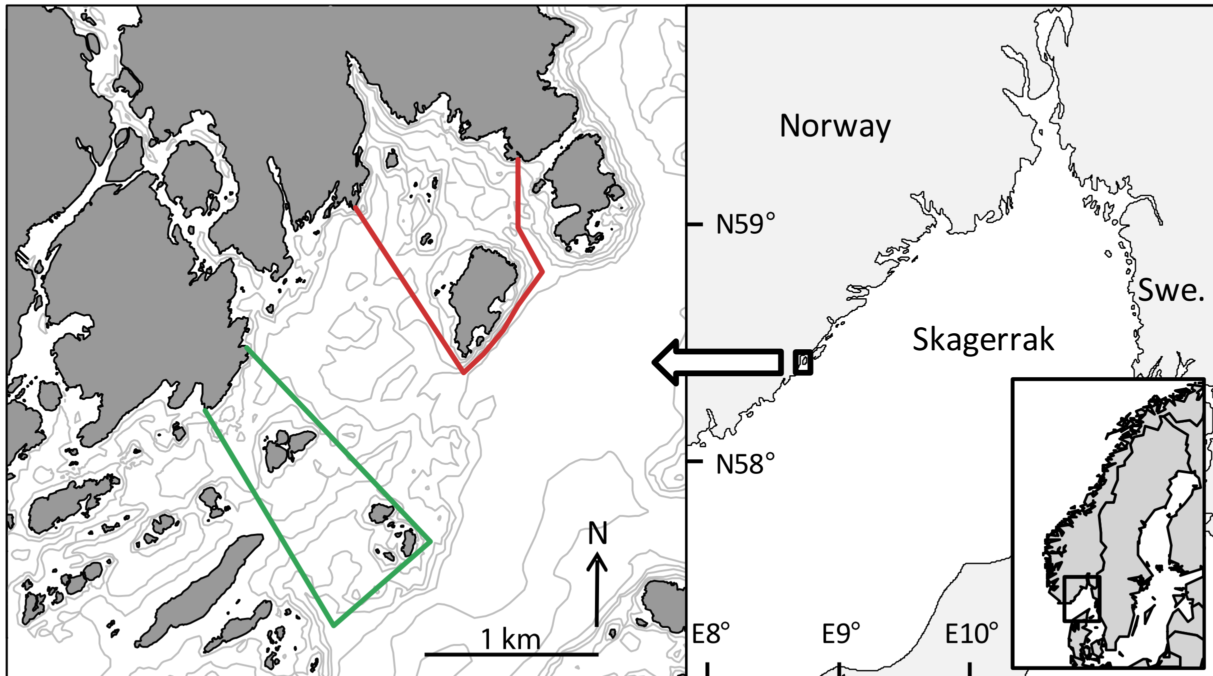

Fig. 1 Study sites. Left panel: The Flødevigen Lobster reserve enclosed in red frame and the control enclosed in green frame. Right panel: Location on the Skagerrak coast (Swe: Sweden). Lower framed picture show the Scandinavia peninsula with Norway and Sweden. Right side picture from Moland *et al.* 2011.

2.1.2 Sampling procedure

Lobsters were caught using Parlour pots with a mackerel-baited bag that were sunken at depths between 8 – 30 m with navigation from echo sounder. The pots had no escape openings, like conventional pots have, in order to maximize the size range of captured lobsters. GPS positions of the traps were noted on drafted maps to spot them easily the following morning. A few eel traps were also set out at shallower depths along skerries. The idea was to include alternative catching methods taking into consideration that some lobster may avoid entering pots. All pots and eel traps were attached to a yellow buoy with proper markings. Up to 30 pots and 10 eel traps were positioned in each of the two areas and left for either 24 h (during week days) or 72 hr (during weekends). Traps were set in presumably good lobster habitats spread across the study area.

As standard procedure, all lobsters were measured to nearest millimeter with ruler or by vernier calipers. Measurements obtained were carapace length (*CL*) from eye socket to

posterior fringe of carapace, body length (*L (tot)*), width (*AL*) and cheliped (*crusher claw*). Individuals were sexed by examining first pair of pleopods (swimmerets) and if missing any appendages, such as a leg or an antenna, this was noted. Information on traps depth point was also registered. In this particular study only *CL* measurement were used.

All lobsters were tagged with a plastic streamer tag (T-bar anchor 1, Hallprint Pty. Ltd, Holden Hill, South Australia) inserted through ventral musculature in first abdominal segment at first time capture or tag loss. Inserting tag in muscle prevents loss of tags during molting process when the exoskeleton is shed. Tags were inserted with a standard tag applicator (see Moland *et al.* 2010). The 5 cm long tag carries information on site of capture and individual number. Moreover, a small piece of tissue at 5th pair of swimmeret was cut off and stored in labeled 96% ethanol-filled Sarstedt Heparin tubes for genetic profiling.

Ovigerous females had their external roe sampled for genetic studies from June to November 2011. A small cluster off eggs were picked close to each of the female's 10 abdominal swimmerets (pereiopods) and placed in 96% ethanol-filled tubes. Each tube was numbered 1 – 10 in accordance with the positioning of the swimmerets. If multiple paternity are present in the broods then the chance of finding different parental genotype may increase by taking samples across the whole spectra of the roe. Also, since the scope of the paternity study only choose to include females fertilized in 2011, roe consisted of ready to, –or hatching eggs, was not sampled for genetics because mating had occurred in 2010. This decision was based on various studies of reproduction cycle and embryonic development in European and American lobsters (Agnalt *et al.* 2007; Pandian 1970; Perkins 1972). Keeping track of females previously sampled was done by listing tag numbers in a separate water resistant sheet that was kept with the fishing gear at all times. Thus, listing tag numbers reduced repeatedly sampling events of the same female.

The goal of sampling at least 50 ovigerous females from each of the two areas by end of September was achieved in the reserve but not in the control area. To get hold of more samples from the control area, contact and open arrangements was established with local fishermen at the beginning of the annual lobster fishing which starts 1 October. Whenever a fisherman reported catch of ovigerous females, tagged or un-tagged, within the boundaries of the control area, we met up and conducted sampling of eggs, tissue and measurements. At the end of the season, 30th of November 2011, eggs and tissue from 38 females had been sampled

in the control area and 81 females sampled in the reserve area. The total catch and release of all fished lobsters in the two areas combined in 2011 landed at 803. Since scientific research fishing had also been conducted in 2010, an overall record of 858 male genetic samples was available for this study.

2.2 Lab work

2.2.1 Selecting samples for analyses

Information on the lobsters was compiled into databases (Excel) together with data available from 2010. Having genetic samples from two seasons allow for more candidate fathers to be tested but also include potential fathers that were not sampled with the ovigerous females. If same male was sampled both in year 2010 and 2011 the latest sample was selected over the older ones because of the fresher tissue (higher DNA quality) and the more recent length measurements. Altogether 269 male tissue samples from reserve and control area were collected in 2011 ($n_{2011\ reserve}$ = 156, mean *CL* = 94.6 mm, SE ±1.3, range: 53 – 140 mm; $n_{2011\ control}$ = 113, mean *CL* = 87.2 mm, SE ±1.2, range: 55 – 125 mm). From the 2010 collection, 206 samples from reserve and control area were used ($n_{2010\ reserve}$ = 120, mean *CL* = 101.6 mm, SE ±1.6, range: 66 – 141 mm, $n_{2010\ control}$ = 86, mean *CL* = 88.8 mm, SE ±1.3, range: 60 – 121 mm). The 475 individuals from the two areas represent potential fathers of the broodstock collection.

As some eggs were sampled in early June 2011, they could have been fertilized by males in 2010 and therefore ready to hatch the following summer and autumn 2011 (Agnalt *et al.* 2007; Pandian 1970). This is because females carry sperm up to a year prior to fertilization of the eggs which happens externally when the eggs are extruded. Also, the embryos are brooded externally while being attached to the abdomen for up to 10 months (Agnalt *et al.* 2007). Because the scope of the study limits the potential fathers to recent time (residing in the areas between 2010 and 2011), older and more developed eggs may have higher probability of being fathered by unsampled males. Separation of the eggs in development stage 1 (freshly fertilized eggs) and late stage (developed eggs) were therefore necessary.

Criteria were based upon Perkins (1972) pioneer work on embryos of the American lobster, *Homarus americanus*, where he found the relationship between time past, seasonal water temperature and embryos' eye indices measured in eye pigments. Membrane of the eggs will quickly bleach to an orange color in 96% ethanol, and reveal any black eyes of the embryos. Visual inspection thus allowed for discrimination between developed eggs with larger black eyes and freshly fertilized eggs with very tiny eyes. All eggs sampled in June, and additional three from the reserve and four from the control area were removed on suspicion of being in development stage 2. Further, additional 17 randomly selected females from reserve were set aside with the use of a lottery system. This was done to scale sample size down to onset goal, resulting in 50 eggs samples from the reserve (mothers mean *CL* = 95.5 mm, SE ±1.7, range: 72 – 127 mm) and 31 eggs samples from the control area (mothers mean *CL* = 91.5 mm, SE ±1.8, range: 78 – 123 mm).

2.2.2 DNA extraction

DNA was extracted from the sample material using Omega Bio-Tec inc. extraction kit (E.Z.N.A. tissue DNA kit). The procedure followed the manufactures user guide, only deviating by preparing HiBind DNA mini columns with equilibration buffer (100 µl) and double sterilized water (100 µl) separately. A petri dish was soaked in 96% ethanol and burned clean before using it as a cutting plate for samples. Scalpel, pincer and scissors were dipped in alcohol between handling of individual samples. Sample tubes not provided by Omega Bio-Tec were sterilized by autoclaving. One egg from each batch was chosen, put in sample tube and crushed with pincer, making sure the limited amount of cells inside membrane were utilized by the reagents. Swimmeret tissue was cut into pieces of 1 mm or 30 mg and the rest kept as backup. All samples were lysated over night in buffer (TL) and enzyme (OB Protease). Buffer (BL) and absolute ethanol was added before solutions were transferred to prepared HiBind mini columns and added buffer (HB). Solutions were spun and washed twice with DNA wash buffer and then transferred to sterile Eppendorf sample tubes. Elution of eggs solutions were done on heater holding 70℃ with 50 µl preheated elution buffer for five minutes. Tissue solutions were eluted with 100 µl elution buffer. Random testing of extracted tissue and at least three out of the batch of eggs from each female were tested for DNA yield on a spectrophotometer which gave a good indication on whether extraction had succeeded.

2.2.3 PCR amplification of microsatellite loci

Primers for six loci, *HGC 111, HGC 131*, *HGC 120, HGD 106, HGD 111* and *HGC 118* developed for European lobster by André and Knutsen (2009) were used to amplify microsatellites. The forward primer oligos were labeled in sets with three different fluorescent labels specified by Beckman Coulter. PCR amplification in 96-well plates were done in one triplex (*HGD 106, HGD 111* and *HGC 118*), one duplex (*HGC111* and *HGC 131*) and one simplex (*HGC 120*) as the latter amplified best at slightly different conditions. PCR protocol for PCR reaction mix of 9 µl master mix and 1 µl DNA extract per well were as follow: 1 µl of 10x buffer [10 mM Tris-HCl (pH 9)], 3 µl of dNTP (0.3 mM), 0.1 – 0.16 µl of Qiagen *Taq* DNA polymerase (0.5 – 0.8 U) and 0.09 – 0.3 µl of each primer (0.15 µM *HGC 111*, 0.3 µM *HGC 131*, 0.1 µM *HGC 120*, 0.18 µM *HGD 106*, 0.22 µM *HGD 111*, 0.09 µM *HGC 118*)(forward and reverse). The mixture was filled up with distillated H_2O to total volume and 1 µl of DNA extract. PCR protocol for the triplex (*HGD 106, HGD 111* and *HGC 118*) and duplex (*HGC 111* and *HGC* 131) consisted of an initial denaturation step at 95℃ for five min, followed by 35 cycles of denaturation at 95℃ for 30 s, primer annealing 56℃ for 60 s, sequence extension at 72℃ for 60 s and final extension step for 15 min. PCR protocol for the simplex (*HGC 120*) deviated by reducing the primer annealing and sequence extension for by 30 s each. This was done to give appropriate signal strength when separated by the capillary instrument.

2.2.4 Fragment analysis

Samples were analyzed using the fluorescence detection method in which fragments are separated by detecting light emitted by fluorescent labels. The fragments were separated using the automated capillary instrument CEQ8000 from Beckman Coulter that is detecting three fluorescent labels simultaneously. Sampling solutions were prepared in 96 wells sample tray in accordance specifications and protocol provided by supplier with sample loading solution (SLS), Size standard 400 (red color) and PCR products. Total volume per well were 38 µl including 0.5 µl Size standard and 3 – 4 µl PCR product mixture, were the duplex and simplex were combined in a pool plex. After checking each well for air bubbles, a drop of mineral oil was added as a seal to prevent evaporation of mixture during the fragment analysis. As the primers used amplify fragments with length range between 146 and 302 (bp), all fragments were run using instrument method “*frag3*” with a standard ranging from 60 to

400 bp. Raw data produced was analyzed using CEQ8000 Genetic Analysis System Software version 8.0. Genotype profile was generated but all individuals were visually inspected rather than relying on automated scoring. Questionable results, like alleles suspected being false or not fully present (allelic drop-outs), were gathered in repeat-runs and replicated.

Of the analyzed tissue, 1.1 % of the parental and 2.6 % of the eggs did not amplify due to either limited amount of DNA available or degraded DNA which consistently failed to amplify for a number of loci.

2.3 Statistical analysis

2.3.1 Properties of microsatellite loci

Loci characteristics were calculated using CERVUS 3.0.3 (Kalinowski *et al.* 2007; Marshall *et al.* 1998). The allele frequencies were estimated from all males and females combined in order to get the best estimate of heterozygosity as it is not confounded with the presence of relatedness of offspring. Eggs attached to mothers are not considered to be independent samples of the population. CERVUS was used to calculate polymorphic information content (PIC value; represent polymorphism within a population), the observed and expected heterozygosities and estimate potential null-allele rates. Microchecker 2.2.1 (Van Oosterhout *et al.* 2004) was also used for testing for presence of null-alleles. Allele frequency heterozygosity (genetic differentiation) between the reserve and control area, and linkage disequilibrium within the GENEPOP 4.0 (Raymond & Rousset 1995) software were also tested.

2.3.2 Multiple paternities assessment

Three approaches were used to evaluate multiple paternities for the 81 females with broods profiled for three loci (*HGD 106, HGD 111* and *HGC 118*). The first being a simple visual assessment approach of the progeny array: the contribution of male parent was taken to be half of the numbers of alleles recorded at the loci. Only if the sum of distinct alleles at one locus exceeded four at minimum two loci, to allow for the possibility for mutation at one loci,

broods were put up for screening for the next three loci (*HGC 120, HGC 111* and *HGC 131*). This resulted in three broods (H1006/ 580, H1224/ 912 and H1203/ 933) being genotyped for all of their ten eggs together with the males and females. Broods that did not deviate in allelic numbers were only genotyped for two eggs per brood; taken from 1st and 10th swimmerets. Since those swimmerets are furthest apart, they could have higher chance of being fertilized by different fathers than periopods closer together because of the design of the genitalia. The visual assessment method may however underestimate the actual number of true fathers as it assumes that males are heterozygotes and that no alleles are shared between mothers and father(s) (Gosselin *et al.* 2005).

Second, another conservative method based on a multilocus approach was applied, where the number of fathers was derived using the software GERUD 2.0 (Jones 2001; Jones 2005). The program has been extensively used for parentage analysis in wild populations (e.g. Gosselin *et al.* 2005; Mäkinen *et al.* 2007; Panova *et al.* 2007; Reisser *et al.* 2009; Jones *et al.* 2010; Yue & Chang 2010). GERUD uses an exhaustive algorithm to reconstruct the minimum number of parents that can explain the offspring array taking into account information from Mendelian expectations and expected frequencies of genotypes in the population. The only prerequisite is that all the offspring in the array have one of the parents in common. It is also recommended to only use the two to four most polymorphic loci as much computational power is needed to solve the algorithm with more than five loci (Jones 2005). For every offspring in the brood, mothers' observed allele is subtracted from each locus to obtain the paternal alleles. To determine the ability for GERUD to correctly calculate the number of sires in the brood, GERUDsim2.0, a simulation approach, is assessing confidence based on the loci used in analysis.

GERUD does not accept mismatches between mothers – offspring so if one of the subjects had missing genotype(s), that sample had to be taken out. Because two of the offspring from each brood had been genotyped for six loci, their genotype had to be cut down to three to fit with the eight remaining siblings. This resulted in a variable number of offspring being taken out from each broods in order for GERUD to run the analysis. Eight broods (six from reserve and two from control area) were also excluded from all subsequent analysis because nearly all offspring deviated from their mother. See Tab. 1 for description of the remaining 73 ovigerous females used throughout all subsequent analysis, divided into equal size classes which will be used to compare sizes for the single and multiple mated females. Size classes

denotes small (S ≥ 88 mm *CL*), medium (M = 89 – 98 mm *CL*) and large (L = 99 < mm *CL*) for comparison in the discussion of the results. GERUD has an upper limit of detecting six multiple fathers per brood. Expected exclusion probabilities for each of three loci (*HGC 118, HGD 106* and *HGD 111*) were calculated with the criteria that one parent was known with certainty and the other parent unknown.

Tab. 1 Size classes of the female lobsters. Mean carapace length (*CL*) and standard error, median with minimum and maximum length in parentheses for three size classes (S = small; M = medium; L = large) of 73 female European lobster at the two sampling sites, reserve and control (All values are in mm).

	Site	
Size class	**Reserve**	**Control**
S	78.66 (SE±4.7)	84.00 (SE±2.6)
	84.00 (73 - 87)	85.00 (78 - 87)
M	92.07 (SE±2.7)	91.80 (SE±3.1)
	91.00 (89 - 97)	91.50 (89 - 95)
L	104.36 (SE±2.7)	107.83 (SE±5.7)
	101.00 (98 - 127)	103.0 (99 - 123)

Because GERUD can only estimate the minimum number of males, the analysis was repeated in COLOY v2 as a third approach. COLONY v2 is a full-pedigree likelihood based program that estimates the most probable number of father configurations instead of a minimum and thus have no maximum limit of number of fathers. More importantly, the program also allows both female and males to be polygamous, which is a prerequisite for testing multiple paternities in regard to female promiscuity. COLONY software uses a full-pedigree likelihood method to infer sibship and parentage among individuals. All individuals are divided into subsamples of offspring, mothers and candidate fathers from which individuals are assigned to various numbers of family clusters. The algorithm in COLONY calculates the likelihood of one pedigree cluster and compares the likelihood to other possible pedigrees to find the best cluster with maximum likelihood (Jones & Wang 2010; Karaket & Poompuang 2012). COLONY assumes all sample of individuals are taken from a randomly mated and large population (Wang 2004).

Unlike GERUD, COLONY accepts mismatches between mothers – offspring and therefore all the remaining 73 broods were analyzed without exclusions. In the analysis the error rate of genotyping was set to 0.025 as suggested by Wang (2004) and input file specifying the relationship between mothers and offspring was uploaded in the software.

2.3.3 Paternity analysis

Assigning parentage from the collection of sampled males was done by using two computer software's for parentage analysis; CERVUS 3.0.3 and COLONY v2 (Jones & Wang 2010). CERVUS was chosen because of the flexibility in regards to error rate, proportion of fathers sampled and the different proportions of loci genotyped. The CERVUS software is also reported to be robust in separating close relatives, like siblings, in which case only one could be a father (Marshall *et al.* 1998). COLONY was chosen because of higher assignment success rate and accuracy with more economic use of markers (Karaket & Poompuang 2012). Also, COLONY can infer unsampled fathers and be of use in multiple paternity assessments.

The principle behind CERVUS is to assign offspring to their respectively parents based on a pair-wise maximum likelihood approach (Jones & Wang 2010; Karaket & Poompuang 2012). The software assumes all sample of individuals are taken from a randomly mated and large population (Marshall *et al.* 1998). Candidates are first compared by how they match and differentiate in the offspring -mother -father trio by a locus by locus likelihood score for each candidate parent (fathers in this case). Secondly, the offspring are assigned to the parent with highest LOD -score (log-likelihood ratio). The score is also taking into account a simulation run based on population allele frequencies, PIC value and average non-exclusion probability of each locus in deciding paternity between two males with the same scores.

Tab. 2 Size classes of the male lobsters. Mean carapace length (CL) and standard error, median with minimum and maximum length in parentheses for three size classes (S = small; M = medium; L = large) of 474 male European lobster at the two sampling sites, reserve and control (All values are in mm).

	Site	
Size class	**Reserve**	**Control**
S	78.47 (SE±0.87)	78.18 (SE±0.80)
	80.00 (53 - 88)	81.00 (55 - 88)
M	92.95 (SE±0.35)	92.38 (SE±0.37)
	92.00 (89 - 97)	92.00 (89 - 97)
L	111.77 (SE±1.00)	104.58 (SE±1.03)
	109.00 (98 - 141)	103.00 (98 - 125)

CERVUS allows for missing data, incomplete sampling of candidate parents and scoring errors by specifying input parameters. The following four settings were implemented in the CERVUS analysis; 1) the number of candidate fathers we are confidence in having sampled was set to 0.4 which means that we believe we have 40% of all offspring fathers. The number is derived from estimations of the number of lobster in the reserve and control area based on observations of capture – re-capture studies (pers. comm. E. Moland). It is assumed that the sex ratio between adult male and females is not significantly different from 1:1.The estimate is most likely a reserved estimate as the intent is to let the program decide the paternity based on the genetic evidences provided. See Tab. 2 for description of the 474 males divided equally into small (S ≥ 88 mm *CL*), medium (M = 89 – 98 mm *CL*) and large (L = 99 < mm *CL*) size classes for simplicity. 2) The proportion of loci typed, 0.766, was calculated out of the dataset and implemented in the software's. The low rate is due to only three loci at eight of ten eggs. 3) A non-zero error rate allows for some genotypic differences between father and offspring which represents scoring errors due to misreading fragments, transcribing an allele wrongly and some error at the level of PCR. The error rate was set at 0.025 as recommended by Wang (2004). 4) Level of potential relatedness was set to zero as this information could not be obtained at this point. Finally a file with known mother -offspring relationships was uploaded into the software.

COLONY provides two different approaches. Even though the full-pedigree likelihood equation in COLONY is preferred because of the relationship inference and increased accuracy (see Wang & Santure 2009 for discussion), the program has also a pair-wise approach implemented. The pair-wise approach uses the multilocus genotype of a pair of male and offspring (a dyad) to infer their relationship (Wang & Santure 2009) and sampled males are given a likelihood probability and listed as fathers to offspring with level of confidence ranging from 50% - 95%. Results from the two approaches in COLONY will be compared with results from CERVUS and discussed briefly under chapter 3.

As in CERVUS, COLONY allows for missing data, incomplete sampling of candidate parent and scoring errors. Additionally, polygamy (see Jones & Wang 2010 for further information) was specified in accordance with information needed by the program. Thus, the same settings implemented in CERVUS were also used in COLONY to be able to compare the results, although the different software's also deviate with the respect to algorithms implemented, and is important to consider when making a direct comparison. Presence of duplicate samples of males was tested for in COLONY by checking for identical genotype entries.

Parental assignment of 730 offspring was performed with 73 known mothers and 474 male candidates with three to six genotyped loci using CERVUS and COLONY. Observed and expected assignment rates with strict (95%) confidence and relaxed (80%) confidence, given known mother, was calculated by CERVUS. COLONY was run three times with three different input formats; 1) all the genotyped data in one run, and a split into two groups; 2) offspring only genotyped for three loci together with the corresponding three loci from all males and females; 3) only the two offspring from each brood genotyped for six loci together with all males and females. The results from COLONY were compared to the observed assignment success rates given by CERVUS.

3 Results

3.1 Properties of the loci

The six loci from the adult samples exhibited medium to high polymorphism with mean number of alleles per loci at 13.3, ranging from eight for locus *HGC 118* to 19 for locus *HGC 120*. Four of the six loci showed little deviation from expected heterozygosity and corresponding high and not significant frequency of null alleles (Tab. 2). The two remaining loci (*HGC 131* and *HGC 111*) suggested deficiency of heterozygotes ($P< 0.001$) however, after correcting for multiple comparisons (FDR) (Benjamini & Hochberg 1995) only *HGC 111* remained significant ($P< 0.045$). This locus was also indicative of potential null alleles using Microchecker, though at very low frequency (less than 2%). Genetic difference between the reserve and the control area were not found to be significant for any of the loci (F_{ST} = 0.000, P = 0.117). However, linkage disequilibrium was significant for nine of the 15 combinations of loci, even after correcting for multiple comparisons (FDR).

Polymorphism information content (PIC values) ranged between 0.54 and 0.86, with an average of 0.70 (Tab. 2). This elevated level led to a very high combined exclusion probability for the whole set of loci. The average non-exclusion probabilities over six loci were 0.055 over 100 individuals for one candidate father, 0.007 over 1000 individuals for one candidate father given the genotype of known mother and 0.0002 over 10 000 individuals for a candidate parent pair. The average non-exclusion probability is the probability of not exclude an unrelated candidate parent, or a pair of parents, of an offspring at one locus (Karaket & Poompuang 2012).

Mismatching rate between mother – offspring genotype was calculated across all loci by CERVUS and were found to be a single locus mismatches in most cases, likely reflecting miss-scorings or mutations. The mismatching error rate was estimated to 2.1% of all loci comparisons (of a total of 2561 loci comparisons). The calculated error rate was variable between the six loci with *HGC 118* and *HGD 106* responsible for the highest rates,

encompassing a mean observed error rate of 0.0341 across all loci. This estimate corresponds roughly the recommended error rate of 0.025 to be used in COLONY (Wang 2004).

Tab. 2 Loci information. Microsatellite loci properties used for parentage assignment and assessing multiple paternities in 73 females and 474 male European lobsters, showing number of alleles, number of alleles genotyped (N), H_O; observed heterozygosity, H_E expected heterozygosity, F_{IS}; Hardy-Weinberg expectations, polymorphism information content (PIC), frequency of null alleles F(null). Star (*) refer to significant deviation from HWE where $P < 0.005$, calculated by CERVUS 3.0.3 and GENEPOP 4.0.

Locus	No. Of alleles	N	H_O	H_E	F_{IS}	PIC	F (Null)	NE - 1P[a]	NE - 2P[b]	NE -PP[c]
HGC 118	8	549	0.621	0.575	-0.080	0.541	-0.044	0.813	0.640	0.450
HGD 106	10	553	0.696	0.711	0.017	0.674	0.015	0.687	0.506	0.310
HGD 111	12	549	0.647	0.639	-0.012	0.597	-0.008	0.760	0.588	0.394
HGC 131	18	550	0.805	0.832	0.032	0.812	0.016	0.497	0.328	0.151
HGC 120	19	547	0.870	0.869	0.002	0.855	-0.002	0.413	0.259	0.098
HGC 111	13	550	0.722	0.754	0.043*	0.725	0.019	0.627	0.444	0.247
Average	13.33	549.66	0.727	0.730	-	0.701	-	0.055	7.1×10^{-3}	2.0×10^{-4}

[a] Average non-exclusion probability for one potential father.
[b] Average non-exclusion probability for one potential father given the genotype of a known parent.
[c] Average non-exclusion probability for a potential parent pair.

3.2 Multiple paternity assessments

GERUD do not accept missing genotypes in the data, and thus only three loci could be used in this program to assess multiple paternity. The three moderately polymorphic loci *HGC 118* (PIC = 0.54), *HGD 106* (PIC = 0.67) and *HGD 111* (PIC = 0.58) (Tab. 2) had an expected exclusion probability with one parent known, one unknown, at 0.36, 0.49 and 0.41 respectively, calculated by GERUD. Also, as some offspring had to be excluded due to missing loci, the number of offspring in each brood varied from seven to 10. Based on those premises, 13 of 73 females showed evidence of having been sired by at least two different males resulting in an average of 18% combined both areas (Tab. 3). Reserve area had 12 of the multiple sired females while control area had only one. The difference in cases of multiple sires between the reserve and control area was significant ($X^2 = 6.25$, $df = 2$, $P = 0.0179$). A total of 86 sires were inferred in the 73 females with results from GERUD, giving an average

of 1.18 sires per female. The multiple sired females from GERUD have a mean of 92.69 mm (*CL*) (SE±5.04), which is the average of the medium body size group of the ovigerous females. However, the range spanned over all body size groups (73 – 127 mm *CL*) according to Tab.1.

Three of the females, which were suspected through visual inspection having been sired by more than one male, turned out to be mismatches in mother – egg combination. Using the second set of loci genotyped for gave compatibility in GERUD and resulted in one single sire and two double sires. The benefit of screening six loci in this case saved three batches from being excluded as the eight other batches.

Tab. 3 Multiple paternity. Summary of the estimated frequency of multiple paternity in ovigerous female European lobsters expressing various paternity in two sites, a reserve (44 females) and a control (29 females) area. Total number of multiple paternity with frequencies and mean number of multiple paternity (after diagonals). Calculations were done in GERUD 2.0, which do not accept errors in data with three loci, and COLONY v2 with error rate at 0.025 using six loci. GERUD infers the minimum number of fathers whereas COLONY infers the number of most likely fathers.

	GERUD zero error rate			**COLONY** 0.025 error rate		
Nr. of Fathers	**Reserve**	**Control**	**tot. multiple paternity**	**Reserve**	**Control**	**tot. multiple paternity**
1	32	28		2	3	
2	12	1	13 (0.18)	4	3	7 (0.10)
3	-	-		11	7	18 (0.27)
4	-	-		14	7	21 (0.31)
5	-	-		10	6	16 (0.22)
> 6	-	-		3	3	6 (0.08)
Totals	44 (0.27)	29 (0.34)	13 (0.18) /1.8	44 (0.96)	29 (0.90)	68 (0.93) /3.81

Contrasting the results from GERUD and COLONY give a very broad estimate of the occurrence of multiple paternity and promiscuity of females. In the reserve, the estimated frequency of multiple paternity was 27 and 96% respectively, while the estimated frequency in the control area was 3 and 90%. Mean size of five females that both programs identified as having only one father siring their broods, and thus were truly monogamous, were on average

102.8 mm *CL* (SE±7.50) which is the average of large of body sizes groups (92 – 123 mm *CL*) according to Tab.1.

Multiple mated females were not found to have body size as a common feature because promiscuity was found across all size categories. Using the COLONY estimates gave no significant correlation between female *CL* size and multiple paternity in either the reserve (Spearman correlation, n = 44, r = -0.184, P = 0.2326) or in the control area (Spearman correlation, n = 29, r = -0.019, P = 0.922). Using the parsimonious estimate from GERUD give a close to, but not a significant correlation between female *CL* size and multiple paternity in the reserve (Spearman correlation, n = 44, r = -0.264, P = 0.0838) and no significance in the control area (Spearman correlation, n = 29, r = -0.204, P = 0.289).

3.3 Paternity analysis

As one male failed to amplify any loci, the candidate males summed up to 474 along with 730 offspring and their 73 mothers. COLONY does not exclude individuals despite missing loci, but rather assign them in a lower score. Further, COLONY automatically tests for identical samples during the analysis, by comparing genotype entries in which 30 males were shown to have a genotype duplicate. Explanations and implications this may have are discussed in chapter 4.

The first run in COLONY, with input files of all the available genotypes (three and six loci array) for offspring, gave what could be described as “assignment inflation” using the full-pedigree likelihood method (data not shown). Six different males have presumably managed to fertilize all the 56 females that appeared in the result list, with one male even siring 27 of the females. The pair-wise approach returned two assignments within relaxed (80%) and strict (95%) confidence, resulting in only two (0.27%) assignments for the 730 offspring. The second run in COLONY using input files with all offspring genotyped for three loci together, with the concurrent three loci in mothers and males, returned very low assignment rates using full-pedigree likelihood. Also, no assignments exceeded relaxed confidence level using pair-wise approach.

The third run in COLONY included only offspring genotyped for six loci, two in each batch of 10 eggs. No results were inferred with full-pedigree likelihood. However, the pair-wise approach gave a successful assignment of 14, or 9.5%, of the 147 offspring within a relaxed and strict confidence level (Tab. 4). In the 14 offspring, COLONY found 12 fathers associated with 13 different females, presenting one male to have successfully fertilized and fathered two broods. Moreover, many of the male individuals appearing throughout first and second run re-appeared in the results from third run. For example, four of the six males that dominated the assignment list in the first run were still assigned to females but only to one female each.

Tab. 4 Paternity assignment. Paternity results from COLONY v2 and CERVUS 3.0.3 for 474/ 470 male European lobsters with error rate sat to 0.025. Number of males assigned to paternity and totals (with percentage) using pair-wise approach and maximum likelihood approach (ML) with confidence in paternity for the two sites, reserve and control. COLONY was run with six loci and two offspring from each brood while CERVUS was run with three and six loci data, and all offspring. COLONY identified 14 males while CERVUS identified 15 males.

	COLONY Confidence of paternity					**CERVUS** Confidence of paternity		
Site	**Assign. 95%**	**Assign. 90%**	**Assign.80%**	**Assign. 80%**	**Unassigned**	**Assign. 95%**	**Assign. 80%**	**Unassigned**
Reserve	6	5	0	0	264	5	8	259
Control	0	1	2	0	196	0	2	197
Totals	6 (0.013)	6 (0.013)	2 (0.04)	0 (0)	460 (0.97)	5 (0.011)	10 (0.022)	456(0.97)

Results from CERVUS, which used the same input file as the first run in COLONY, returned a result very similar to the third run in COLONY. Offspring typed at fewer than three loci was excluded by the program leaving 705 offspring. Four males from the reserve were also excluded for the same reason leaving 470 males for the analysis. CERVUS assigned fathers to 15, or 4.0%, of offspring within a relaxed (80%) and strict (95%) level of confidence (Tab. 4). This is 18% fewer assignments then expected based on the rate calculated in CERVUS' simulation.

Contrasting the results between COLONY and CERVUS demonstrate that the pair-wise and the maximum likelihood approach gives very similar results. CERVUS are missing two assignments in comparison to COLONY but on the other hand, CERVUS supply an assignment that COLONY has overseen. Combined results present a list of 13 males assigned to offspring belonging to 14 different females. One male was found to be father of two offspring of two different females. As regards to assigning fathers to offspring, both programs have succeeded in assigning ~3% of the candidate males to paternity.

3.3.1 The paired lobsters and their sizes

Ten females from the reserve area were assigned to males while only four females from the control area had confirmed assignments to respective males. Interestingly, eight of the 13 males were individuals caught in 2010. Based on studies on growth of the European lobster (Agnalt *et al.* 2007) we would expect that males have molted one time since they were sampled in 2010. Since the fertilization occurred in 2010, all the 2011 individuals, males and females caught that year, were slightly smaller in size at the actual time of mate encounter. This mean that by account for the growth in one year for the eight 2010 males, adding 7 mm adjustment of the *CL* based on Agnalt *et al.* (2007) reports on average molt increment, evens out the size differences due to capture date. By doing so, 10, or 71%, out of the 14 pairs now compose of either larger or at the same size males, compared to females. The male and female relationship with *CL* size is plotted with linear regression to visualize the pairs in relation to their sizes (n = 14, r^2_{adj} = 0.134, P = 0.198) in Fig. 2.

The smallest female (78 mm *CL*) and the smallest male (66 mm *CL*) are both in the small category of sizes, based on average size of studied individuals (73 females, 475 males). Further, the biggest female (112 mm *CL*) and the biggest male (141 mm *CL*) are found in the large category of sizes. Overall, the females size ranges are represented in all of the male size categories, meaning that the females have mated with small, medium and large males. Not surprisingly, the results also show that the individuals mating within the reserve area are of bigger sizes than the one and only pair from control area, and compared to pairs that interacted across the borders in either direction. On average, males were 9.7% larger than the female counterpart. However in four cases the female was actually larger than the male. The biggest size discrepancies between the pairs in favor of both sexes were two couples in which

the male was 39% larger than the female and opposite, a female was 27% larger than her male (Tab. 5).

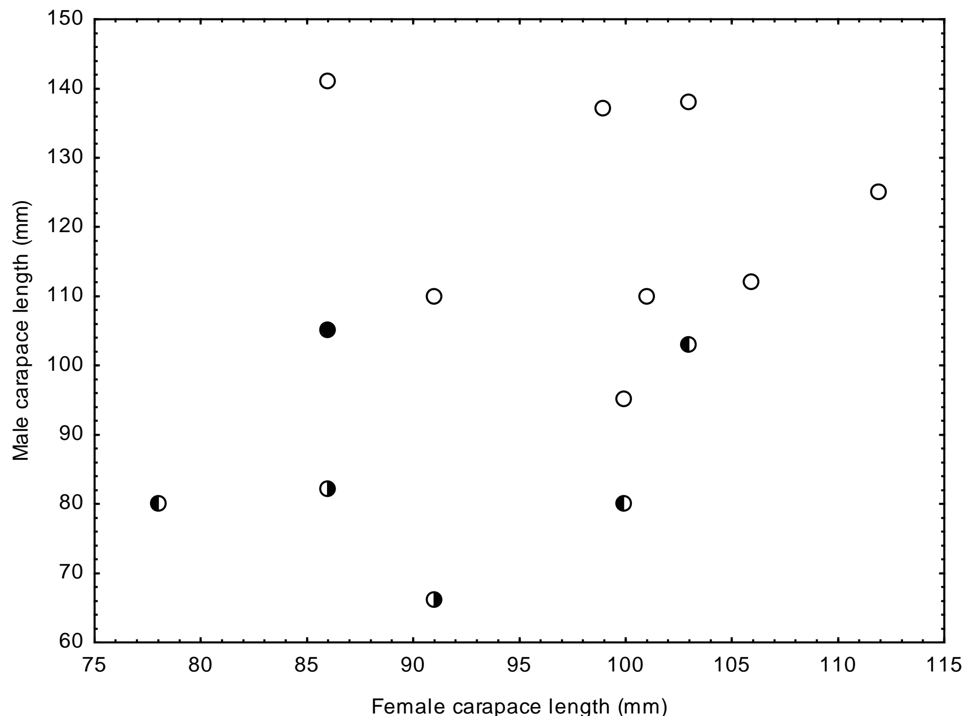

Fig. 2 Relationship between females and males *CL*. The *CL* measurements in mm of the European lobster females and the males (2010 males adjusted for growth (7 mm)) that formed pairs. Circles are divided into four different patterns seen from females' mate choice (see tab 5): white circle denotes reserve – reserve pairing, black circle denote control – control pairing, left black circle denotes control – reserve pairing and left white circle denotes reserve – control pairing (n = 14, r^2_{adj} = 0.134, P = 0.198, regression equation; y = 19.2743 + 0.9047*X).

Most of the females from the reserve were parried up with males from reserve, while only one of the four females from the control area had found a mate in the same area. The other three females had presumably gone into the reserve area to find mates. One male from the reserve area (*R-685*) was even found to be father of two offspring of two females (*C-1180* and *C-1106*) belonging to the control area. Further, one other male from the reserve area was also assigned to one female from the control area. Altogether, the results show that five of the 14 pairs have moved and inter-mixed across the two areas (Tab. 5).

Tab. 5 Paired lobsters inferred. Female (F) and male (M) European lobsters that were found to have been mated in the reserve and the control area, and the *CL* measures in mm, for both sex. Star (*) denotes 2010 males adjusted for one molting (7 mm *CL*). Size differences between the pairs are measured in percentage. "Interaction" refers to where the females have found a male, from either the reserve or the control area, in accordance with the direction of the arrow.

Female ID	Male ID	F size (*CL*)	M size (*CL*)	Size diff. pair	Interaction
R-971	R-284	101	110*	female 8% smaller	♀ ***Res*** ↔ ***Res*** ♂
R-1139	R-375	86	141*	female 39% smaller	♀ ***Res*** ↔ ***Res*** ♂
R-718	R-397	99	137*	female 28% smaller	♀ ***Res*** ↔ ***Res*** ♂
R-580	R-410	106	112*	female 5% smaller	♀ ***Res*** ↔ ***Res*** ♂
R-728	R-510	112	125*	female 10% smaller	♀ ***Res*** ↔ ***Res*** ♂
R-678	R-571	100	95*	female 5% larger	♀ ***Res*** ↔ ***Res*** ♂
R-699	R-668	91	110	female 17% smaller	♀ ***Res*** ↔ ***Res*** ♂
R-881	R-70	103	131*	female 21% smaller	♀ ***Res*** ↔ ***Res*** ♂
R-1034	C-122	86	82*	female 4% larger	♀ ***Res*** → ***Cont*** ♂
R-1171	C-602	91	66	female 27% larger	♀ ***Res*** → ***Cont*** ♂
C-1106	R-685	78	80	female 3% smaller	♀ ***Cont*** → ***Res*** ♂
C-1180	R-685	100	80	female 20% larger	♀ ***Cont*** → ***Res*** ♂
C-819	R-917	103	103	female = male	♀ ***Cont*** → ***Res*** ♂
C-1082	C-705	86	105	female 18% smaller	♀ ***Cont*** ↔ ***Cont*** ♂

4 Discussion

4.1 Paternity assessment and assignment

Male lobsters are known to be promiscuous. In females on the other hand, promiscuity has not been fully acknowledged, despite of presumptive evidences observed in the wild and in the laboratory (Gosselin *et al.* 2005; Streiff *et al.* 2004). This study demonstrates female promiscuity in European lobsters. There were no difference in magnitude between a control area and a marine reserve using the highest estimation (COLONY) but in contrast, significantly more cases of multiple paternity was found in the reserve compared to the control area using the parsimonious estimation (GERUD). Although there is a large discrepancy in the results, this study shows that female European lobster in all size categories may mate with more than one male after pre-molt insemination.

Only a few published studies have covered the frequency of multiple matings in crustaceans, but the few available ones show variation both between and within species. The estimated rate of multiple paternity found in this study depended on programs used and were 27 and 96% for the reserve, and 3 and 90% for the control area. These estimates appear to be much higher compared to what was found in the American lobster (13%; Gosselin *et al.* 2005), as well as in other decapods such as that of Norway lobster (55% of 11 broods; Streiff *et al.* 2004), of the snow crab, *Chionoecetes opilio,* (3.8% of 79 broods; Roy 2003) and in the crayfish species, *Orconectes placidus,* (40%; Walker *et al.* 2002). On the other hand, the results from this study are closer to that of the Porcelain crab, *Petrolisthes cinctipes,* (80% of 10 broods; Toonen 2004) and to the newly discovered freshwater shrimp, *Caridina ensifera blue* (100% of 20 broods) (Yue & Chang 2010). Nevertheless, the studies referred to above suggest that multiple paternity is a rather common mating behavior in crustaceans.

Paternity assignments have been extensively used in field studies to document paternity in populations where observations of matings and certainties of true parentage can be highly unreliable (Sorin 2004). This study is the first to assign paternity to offspring in a wild population of decapods. Two seasons of intensive sampling led to a collection of 474

individual males from the reserve and control area that were successfully genotyped and labeled as potential fathers. CERVUS and COLONY managed to assign 13 males to offspring of 14 females (out of 73) with >80% confidence, of which 10 (71%) of the pairs consisted of a male either at the same size or bigger than the female. Contrasting the size differences within the pairing show that one female was 39% smaller, and another female 27% larger than their mates of choice. The variation in mate choice by size was previously thought to not be compatible due to physical limitations. The female American lobsters could apparently not mate with males >7% smaller or >21% larger than herself (Phillips 2006). However, a more recent investigation on female receptacle load in laboratory demonstrated that successful matings displayed a vast variation in mate sizes. In that particular study, one female was 29% larger than the male, and a male was 67% larger than the female (Gosselin *et al.* 2003). The results herein on the European lobster are well within this range and show that females not necessary choose larger males. That said, because larger males molt less frequent than smaller males (Phillips 2006) there is a chance that not all of the 2010 males corrected for molting have in fact molted. Therefore, the difference in size between sexes may be slightly overestimated. Not finding a clear pattern in females' choice of mates may be a result of small sample size.

4.2 Female mating pattern and preferences

The relative importance of female mate choice and competition in lobster mating system is probably influenced by several factors. How selective a female can allow her to be depends much on the ratio of mature males to receptive females, the variance in quality of the mates available and the cost of breeding. The latter is uttered as the chance of getting injured or killed in the process of the mating (see Phillips 2006; Shuster & Wade 2003; Gosselin *et al.* 2003). Covert strategies like female cryptic choice are not thought to influence *Homarus* species due to the physiology of the seminal receptacle with mixing of the sperm (for discussion see Gosselin *et al.* 2003). Also, because of the partially reversed sex roles were it is mostly the female sex that is exerting mate choice, there should be no need for concealed strategies (Bushman & Atema *et al.* 1997; Gosselin *et al.* 2005). As the American lobsters' sperm is not motile, like other decapods crustacean, sperm competition hypothesis is neither not perceived as likely for the European lobster (Gosselin *et al.* 2005). Therefore, the results

from this study are discussed based on the importance of male size and promiscuity in females mating strategy.

4.2.1 Size vs. shelter and protection

Of the 14 females assigned a male, ten had found a mate of a larger size which coincide with theories about mate choice in lobster (i.e. McDiarmid & Butler 1999). In *Homarus* species, the level of sexual competition is mainly expressed between the males while they are defending shelters used for hosting receptive females (Debuse *et al.* 2003). A large male can attract many females by dominating good shelter grounds (Bushman & Atema 2000). From females' perspective, larger males possess larger shelters (more space for the female to fit in) and can provide more protection (fight off disturbances) of the female when she is in a vulnerable state during molting (Bushman & Atema 2000; Phillips 2006; Gosselin *et al.* 2003). Interestingly, prolonged male guarding has been seen correlating with increased male size, possibly reflecting males' self interest in protecting high fecund females, especially if it's a chance the female will re-mate (see Gosselin *et al.* 2003; Rondeau & Sainte-Marie 2001 for discussion) but is also benefitting the female as it decreases her risk of getting injured (Debuse *et al.* 2003; Bushman & Atema 2000). Although the importance of large males has been thoroughly discussed and emphasized in literature, there are evidences from studies on the European lobster that females may not judge the males entirely by his size of appearance. Debuse *et al.* (2003) found that females were not so selective of male size when shelters were abundant. At least four of the males assigned to females in our study were smaller than the female counterpart but as both study areas are considered good lobster habitats, shelter may have compensated for smaller size and ultimately favored the smaller males. Females' selection for size may perhaps be more of an encouragement for male to dominate shelter ground and thus protection of the female (Debuse *et al.* 2003). In fact, Gosselin *et al.* (2003) examinations of receptacle loads in American lobster concluded that small males are probably responsible for most matings in the wild, especially in the most exploited stocks. However, they also found some weak tendencies towards positive size assortative matings in the least exploited area. In the present study, no assortative mating was detected but that has also been proved to be difficult to observe in wild populations as a pattern of size assortative mating can be confounded by changes in environmental conditions, or masked by genetic drift (Serbezov *et al.* 2010). In addition, promiscuity behavior would also lead to decreased intensity of sexual selection because a wider representation of the adult population is contributing to the next

generation (Serbezov *et al.* 2010). The result, as it seems in this data, is a picture of a species with a flexible mating system that perhaps is a product of environmental uncertainties.

4.2.2 Multiple matings

Female mating behavior is acting out stronger in pre-molt females than inter-molt females (Bushman & Atema 2000) but inter-molt courtship and insemination has long been recognized as an alternative mating behavior in the American lobster (McDiarmid & Butler 1999; Gosselin *et al.* 2003, 2005). The same behavior was also recently documented in European lobster (Skog 2009). What factors influence the frequency of such mating, with consequence of multiple paternity, are largely unknown (Yue & Chang 2010). One hypothesis is that when the female receive little investment from the male, from either cause of sperm-limitation or because of covert male ejaculation strategy (see discussion Gosselin *et al.* 2003; McDiarmid & Butler 1999), females tend to mate again. When Gosselin *et al.* (2005) found multiple matings in exploited sites in the American lobster, promiscuity as compensation for deficient sperm reserves was concluded as the most probable cause. This study on European lobster however, got the opposite result with even the most parsimonious estimate, more multiple mated females were found in the reserve than in the exploited area. There is probably little sperm-limitation due to male deficiency in this reserve because it has been closed for fishing since 2006. The catch and release data two years back points to a high density of lobsters in the reserve and, and even sex ratio in both the reserve and the control area. Further, eight out of the ten males from the reserve assigned to females were over 100 mm *CL,* which is in the large size class. Nevertheless, one hypothesis is that female promiscuity has been implemented at a time when many of the larger males had been harvested because one of the benefits of promiscuity is that the genetic variation increases and can improve the populations' ability to withstand stochastic events and selection pressure (Rowe & Hutchings 2003). The act of promiscuity may still be practiced as *Homarus* lobsters are long-lived species and it may take time to adjust to a new environment after decades of overharvesting. If this hypothesis is true, we might see less occurrence of multiple paternity in the future (McDiarmid & Butler 1999).

From another point of view, when Ferguson *et al.* (2002) didn't find multiple paternity in the European lobster ten years ago, very low mate-encounter was considered as plausible cause (Phillips 2006). If this is the case, the results from the present study could be explained as an

increase of lobsters in the reserve and possibly demonstration of spill-over beyond reserve boundaries. As the reserve fills up, more individuals move out and by time we could expect to find even more cases of multiple paternity in the control area. This hypothesis coincides with the results of significantly lower frequency of multiple paternity in the control area, using the parsimonious estimate. Density dependent multiple paternity has barely been addressed but examples are found in free-spawning invertebrates (i.e. Johnson & Yund 2007) and in the Rock shrimp species (*Rhynchocinetes typus*, Thiel & Hinojosa 2003) but not yet in lobsters. In the Rock shrimp, Thiel & Hinojosa (2003) observed that higher density of individuals led to more harassment of the females and thereby an increase in matings. Although this hypothesis may explain the higher number of multiple paternity in reserves, this reserve is only six years old and it is highly unlikely that one generation of lobsters could change a mating system. Still, as lobster stocks within reserves rebuild demographical structures closer to historical and natural populations, investigations on what drives lobster mating systems must continue and reveal the underlying causes so that they can be made account for in management plans.

4.3 Interaction between the reserve and control area

Studies of movement of lobster species have in most cases been in the interest of commercial exploitation to determine the migration patterns, mixing of stocks and its effects of environmental variables (see Phillips 2006 for references therein). Today the agenda of marine reserves are concerned in quantifying the small and large-scale patterns of movement in relation to spill-over mechanisms through catch and release methods (see i.e. Goñi *et al.* 2006; Moland *et al.* submitted). The lobsters in this study was initially fished and tagged in two different areas where they through parental assignment method were traced to have paired across those boundaries. Of the 14 couples identified, five of them have interacted across the boundaries and in three of the cases the female has been tagged in the control area but found a male from the reserve area. The last two pairs involved a female tagged in the reserve but sired by a male from the control area. Although most of the successful assignments involved lobsters from the reserve area, both sexes of eight of the 14 couples possibly reside in the reserve permanently. Acoustic tagging studies on the European lobsters in the same reserve are suggesting high site fidelity for adult lobsters with 95% of the individuals residing within

the boundaries of the reserve for a period of 242 days (Moland *et al.* 2011). Further, reported limited migration in and out of the reserve by Moland *et al.* (submitted) also supports site fidelity in lobsters. On the other hand, no genetic differentiation between the two areas was found in this study which denotes that genes are exchanged regularly between the areas. Combining these results with that of the parental assignments imply that the lobsters' search for mate can be quite limited to a small area, and the females may often choose to pair up with a close neighbor. Over time, more mixing of individuals between the areas are suggested as the reserve will restore the demographic structure and individuals relocate to lesser crowded areas (Goñi *et al.* 2006). Also, we might see a "demographic diffusion" into the control area which is a state where the largest males are outnumbered by smaller males and choose to relocate to avoid stress (Moland *et al.* 2011). Anecdotal, the male *C -705* that had mating success with a female from the same area, had in another study been observed residing in a rather small patch in the control area from September 2011 until it was caught by recreational fishermen during the lobster fishing in November 2011 (Wiig 2012).

4.4 Efficiency of the methods used and statistical uncertainties

Quantifying individual reproductive success is a crucial key to understand mating system, evolution of reproductive strategies and sexual selection in species (Sardell *et al.* 2010). More, finding variations in the strategy of promiscuity, and the reproductive success of individual males, require accurate assessments and assignments. Problems with the sampling of an undefined population size in relation to a parental assignment is obvious, however, too few loci and too low PIC-values may also have constrained this study. Despite high effort of sampling, this study of European lobster resulted in only rough estimates of the occurrence of multiple paternity in sired broods and only a small number of assigned fathers.

The probability of detecting multiple paternity increases with sample size (Wang 2004) which in this study only ranged from seven to ten offspring, due to GERUDs requirements of no missing genotypes. In the American lobster, Gosselin *et al.* (2005) pooled 100 eggs from each female and by doing so increased the possibility of scoring the reproductive contribution among the different fathers and the reproductive skew. Here, the individual eggs were also used for the parental assignment and could therefore not be pooled in the same way. Future

studies on multiple paternity should however, be separated from parental assignment to allow for increased sample size.

The creator of GERUD recommend to use the three loci with highest level of polymorphism in the initial run of the analysis of multiple paternity and then compare these results with the final run (Jones 2005). Two of the offspring in each brood were genotyped for six loci but the rest of them (75%) were genotyped for only three loci which unfortunately happened to be the least polymorphic of all six. The probability of allele-sharing between mother and father(s) increase with lower polymorphic content and could have underestimated the actual number of fathers in the progeny array. Further, the expected exclusion probabilities were also corresponding low at 0.36, 0.49 and 0.41, which means that the capability for GERUD to exclude a random individual from parentage was low (Wang 2004). It would be more efficient to test the markers at an early stage in the experimental design and also determine the number of offspring needed by the analysis (Wang 2004). It is also recommended to combine offspring and mothers' tissue on the same plate running the fragment analysis so the genotypes can be compared much easier and errors detected early. Doing this could have prevented the exclusion of eight of the females with offspring due to incompatibilities.

Offspring having a set of common alleles will often result in no assignments unless an increase in the number of markers. Also, analyzing parentage in wild populations where true parents are mixed with close relatives demand higher number of loci in order to differentiate between a non-parent relative and a true parent. Six loci in this study is not likely to be enough because there is a high probability that at least one non-parent relative will have a set of alleles compatible to all of the offspring loci and are falsely assigned (Kalinowski *et al.* 2007; Marshall *et al.* 1998). Tag-loss could lead to individuals being sampled several times as different individuals and bias the collection of candidate parents. COLONY gave up 30 males which were shown to have a genotype duplicate (with other 30 males) in the sample data. Whether or not these males were used in the assignment algorithm was not tested at this point. Since CERVUS is giving a score to all the candidate males, two males with the same score, perhaps duplicates, are rejected as potential fathers to an offspring. That said, the rejection could have falsely excluded a true parent and should be investigated further. The shortage of loci used in this study may also have assigned a non-true father to offspring. Male *C-602* is only 66 mm *CL* and were most likely not mature at the time of fertilization. There is however spatial differences between the sexual onset of maturity in the European lobster (see

Lizárraga-Cubedo 2003). According to Agnalt *et al.* (2007) the lobsters in south-west of Norway reach sexual maturity earlier than other stocks in Europe and have reported females at 75 mm *CL*. The smallest female caught in this study was even smaller, with 73 mm *CL*. This may indicate an earlier maturity for both sexes at this location and may be an adaptation for intense harvest (Law 2000). Nevertheless, the male *C-602* is most likely a close family member of the female *R-1171* or a non-relative by chance.

Using the pair-wise method COLONY assigned 9.5% of 147 offspring to paternity while CERVUS assigned 4.5% of 730 offspring. Based on multiple runs in COLONY is seems that the programs have problems with running (progeny) genotype files with high proportion of missing data. The accuracy of the few (if any) of the assignments was much lower compared to running the program with only the individuals typed for six loci. The multiple paternity assessment (during the same analysis of parentage) had to be run using all of the available loci because it t not possible to do a multiple paternity assessment based on offspring arrays with only two individuals. Large discrepancies in results between GERUD and COLONY have also been observed in other studies (see i.e. Yue & Chang 2010; Panova *et al.* 2007). For note however, running only three loci in COLONY gave approximately the same mean number of multiple paternity as with all of the loci but could not discriminate between candidate fathers and gave only assignments of low confidence. CERVUS didn't seem to have the same trouble with high proportion of missing data and the small difference in the results between the two paternity programs probably reflect the difficulty in assigning paternity in a mating system with high relatedness (Sardell *et al.* 2010). The "assignment inflation" given by COLONYs full-pedigree likelihood approach may also be attributed to high relatedness in the individuals. However, it could be the case that using loci with little information, like *HGC 118* and *HGD 111*, may have introduced more noise and as a consequence given lower accuracy in the assignment of paternity, as proposed before by Karaket and Poompuang (2012). The error rate set to 0.025 may also be an estimate too inaccurate for this dataset and it is recommended to calculate the error rates of each locus separately during the process of repeating genotypes in the fragment analysis.

As expected, no genetic differentiation was found between the reserve and the control area ($F_{ST} = 0$, $P = 0.117$) most likely due to the proximity of the two locations. Very low genetic differentiation is also recently found along Skagerrak coastline, with only a few barely significant pair-wise comparisons (Huserbråten 2012). However, that study spanning along

the entire Skagerrak coastline do not show any systematic linked loci, while as many as nine out of 15 combinations of loci herein showed linkage. There might be more than one explanation for this result. This high level of linkage in the lobsters in this area may reflect that only a small number of breeding individuals (N_e) have contributed to an expansion of the local stock as it may have experienced population bottleneck (Hedrick 2011). An alternative explanation is that this observation of linkage is just a coincidence, as the study from Skagerrak coastline does not show any systematic pattern in linkage among loc. It is generally recommended to use markers that are approximately in equilibrium when running paternity and pedigree analysis (see Huang *et al.* 2004; Abecasis & Wigginton 2005), and a prospective expansion of this study would have to investigate this deeper. Also, more markers will with higher certainty be able to differentiate between members of family and the true parents of the offspring.

4.5 Conclusion and future prospects

This thesis is, as far as I am aware of, the first to present parentage analysis of a wild lobster species after attempting to sample as many candidate males as possible and assign them to eggs attached to females. Additionally, it is also the first time multiple paternity has been found in this species. The results from the parental assignments showed that it is possible to assign paternity to males by combining the genotype markers and information on the individuals collect from catch and release, even when the population size is unknown. By using the results from the parental assignment I was able to briefly investigate the level of connectivity between two adjacent areas, one of them being a marine reserve. Two assignment programs (COLONY and CERVUS) assigned 13 males in parentage with offspring from 14 females but the mated couples showed no clear pattern of assortative mating, but it is hard to find patterns in data with only 14 data points. More assignments could have given a clearer pattern of female European lobsters' preference for males with bigger sizes than themselves. Judging from the locations the individual in the pairing was originally sampled, most of the female lobsters had found a mate in proximity but a few (of the females) had also wandered between the areas. More cases of multiple paternity was found in the reserve compared to the control area using a conservative estimates (GERUD) which is interesting since it is the opposite from what has been found in the American lobster.

However, COLONY gave no significant difference in frequency between the reserve and the control area. area but with no correlation to size of the females in none of the possible scenarios.

For now, it is too early to explain the underlying behavioural mechanisms in the mating system of the European lobster and how it is affected by exploitation from the fisheries. Future research will be necessary to assess multiple paternity in European lobsters and this work will include genotyping more broods (more eggs in each sample) with high value markers. Second, sampling, genotyping seminal receptacles of pre- and post oviposition females and comparing the sperm contribution of first male in relation to female size could confirm the hypothesis of sperm-limitation. If this proves to be the case, then large males should be given more recognition as an important part of the fecundity of populations because protecting ovigerous females may not be enough to manage this high value species. Third, it may also be important to unveil the genetically significance of multiple paternity. If density is triggering promiscuity and multiple paternity, it may be a functional response of a population under pressure to increase the genetic diversity. Last, expanding an analysis of paternity in a local, wild lobster stock will need a more samples of male candidates in order to match cases and subsequently determine females’ choice of mates.

5 References

Abecasis, G. R. & Wigginton, J. E. (2005). Handling marker-marker linkage disequilibrium: Pedigree analysis with clustered markers. The American Journal of Human Genetics, 77: 754-767.

Agnalt, A. L., Kristiansen, T. S. & Jørstad, K. E. (2007). Growth, reproductive cycle, and movement of berried European lobsters (*Homarus gammarus*) in a local stock off southwestern Norway. ICES Journal of Marine Science, 64: 288-297.

Agnalt, A. L. (2008). Fecundity of the European lobster (*Homarus gammarus*) off southwestern Norway after stock enhancement: do cultured females produce as many eggs as wild females? ICES Journal of Marine Science, 65: 164-170.

Allendorf, F. W. & Hard, J. J. (2009). Human-induced evolution caused by unnatural selection through harvest of wild animals. Proceedings of the National Academy of Sciences, 106: 9987-9994.

André, C. & Knutsen, H. (2010). Development of twelve novel microsatellite loci in the European lobster (*Homarus gammarus*). Conservation Genetics Resources, 2: 233-236.

Baskett, M. L., Levin, S. A., Gaines, S. D. and Dushoff, J. (2005). Marine reserve design and the evolution of size at maturation in harvested fish. Ecological Applications, 15: 882-901.

Beamish, R. J., McFarlane, G. A. & Benson, A. (2006). Longevity overfishing. Progress In Oceanography, 68: 289-302.

Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society, 57: 289-300.

Bretman, A., Newcombe, D. & Tregenza, T. O. M. (2009). Promiscuous females avoid inbreeding by controlling sperm storage. Molecular Ecology, 18: 3340-3345.

Bushmann, P. J. & Atema, J. (1997). Shelter sharing and chemical courtship signals in the lobster, *Homarus americanus*. Canadian Journal of Fisheries and Aquatic Sciences, 54: 647-654.

Bushmann, P. J. & Atema, J. (2000). Chemically mediated mate location and evaluation in the lobster, *Homarus americanus*. Journal of Chemical Ecology, 26: 883-899.

Christie, M. R., Tissot, B. N., Albins, M. A., Beets, J. P., Jia, Y., Ortiz, D. M., Thompson, S. E. & Hixon, M. A. (2010). Larval Connectivity in an Effective Network of Marine Protected Areas. PLoS ONE, 5: e15715.

Debuse, V. J., Addison, J. T. & Reynolds, J. D. (2003). Effects of breeding site density on competition and sexual selection in the European lobster. Behavioral Ecology, 14: 396-402.

Espeland, S. (2010). Diel vertical migration patterns in juvenile cod from the Skagerrak coast. Marine ecology Progress Series, 405: 29-37.

Fenberg, P. B. & Roy, K. (2008). Ecological and evolutionary consequences of size-selective harvesting: how much do we know? Molecular Ecology, 17: 209-220.

Ferguson, A. (2002). Genetic diversity in the European lobster (*Homarus gammarus*): population structure and impacts of stock enhancement. Available at: http://www.qub.ac.uk/bb-old/prodohl/gel/gel.html.

Goñi, R., Quetglas, A. & Reñones, O. (2006). Spillover of spiny lobsters *Palinurus elephas* from a marine reserve to an adjoining fishery. Marine Ecology Progress Series, 308: 207-219.

Gosselin, T., Sainte-Marie, B. & Bernatchez, L. (2003). Patterns of sexual cohabitation and female ejaculate storage in the American lobster (*Homarus americanus*). Behavioral Ecology and Sociobiology, 55: 151-160.

Gosselin, T., Sainte-Marie, B. & Bernatchez, L. (2005). Geographic variation of multiple paternity in the American lobster, *Homarus americanus*. Molecular Ecology, 14: 1517-25.

Hedrick, P. W. (2011). Genetics of populations. Jones and Bartlett Publishers. Sudburry, Massachusetts.

Hines, A. H., Jivoff, P. R., Bushmann, P. J., van Montfrans, J., Reed, S. A., Wolcott, D. L. & Wolcott, T. G. (2003). Evidence for sperm limitation in the blue crab, *Callinectes sapidus*. Bulletin of Marine Science, 72: 287-310.

Huang, Q., Shete, S. & Amos, C. I. (2004). Ignoring linkage disequilibrium among tightly linked markers induces false-positive evidence of linkage for affected sib pair analysis. The American Journal of Human Genetics, 75: 1106-1112.

Hughes, M., Prodohl, P., Apostolidis, A., Farestveit, E., Ferguson, A., Heath, P., Jørstad, K., Katsares, V., Kelly, E., Mercer, J., Triantafyllidis, A. & Triantaphyllidis, C. (2001). The GEL project: paternity assessment and breeding structure in the European lobster *Homarus gammarus*. Conference Life Histories -Assessment and Management of Crustacean Fisheries, A Coruña, Spain. October 8–11, 2001.

Huserbråten, M. (2012). Unknown title. Master thesis, University of Oslo.

Jennions, M. D. & Petrie, M. (2000). Why do females mate multiply? A review of the genetic benefits. Biological Reviews, 75: 21-64.

Jones, A. G. (2001). GERUD 1.0: A computer program for the reconstruction of parental genotypes from progeny arrays using multilocus DNA data. Molecular Ecology Notes, 1: 215-218.

Jones, A. G. (2005). GERUD 2.0: A computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. Molecular Ecology Notes, 5: 708-711.

Jones, A. G., Small, C. M., Paczolt, K. A. & Ratterman, N. L. (2010). A practical guide to methods of parentage analysis. Molecular Ecology Resources, 10: 6-30.

Jones, O. R. & Wang, J. (2010). COLONY: a program for parentage and sibship inference from multilocus genotype data. Molecular Ecology Resources, 10: 551-555.

Jørgensen, C., Enberg, K., Dunlop, E. S., Arlinghaus, R., Boukal, D. S., Brander, K., Ernande, B., Gårdmark, A. G., Johnston, F. & Matsumura, S. (2007). Ecology: Managing Evolving Fish Stocks. Science, 318: 1247-1248.

Jørstad, K. E., Agnalt, A.-L., Kristiansen, T. S. & Nøstvold, E. (2001). High survival and growth of European lobster juveniles (*Homarus gammarus*) reared communally on a natural-bottom substrate. Marine and Freshwater Research, 52: 1431-1438.

Kalinowski, S. T., Taper, M. L. & Marshall, T. C. (2007). Revising how the computer program cervus accommodates genotyping error increases success in paternity assignment. Molecular Ecology, 16: 1099-1106.

Karaket, T. & Poompuang, S. (2012). CERVUS vs. COLONY for successful parentage and sibship determinations in freshwater prawn *Macrobrachium rosenbergii*. Aquaculture, 324–325: 307-311.

Knutsen J. A., M. E., Olsen E. M., Knutsen H. & Pettersen A. R. (2009). Rapid rebound of lobster in Norway's first reserves; IMCC 2009 Marine Protected Areas: Institute of Marine Research.

Kraus, B., Neumann, P., Praagh, J. v. & Moritz, R. F. A. (2004). Sperm limitation and the evolution of extreme polyandry in honeybees (*Apis mellifera*). Behavioral Ecology and Sociobiology, 55: 494-501.

Lane, J. E., Forrest, M. N. K. & Willis, C. K. R. (2011). Anthropogenic influences on natural animal mating systems. Animal Behaviour, 81: 909-917.

Law R. (2000) Fishing, selection, and phenotypic evolution. Ices Journal of Marine Science, 57, 659–668.

Lizárraga-Cubedo, H. A., Tuck, I., Bailey, N., Pierce, G. J. & Kinnear, J. A. M. (2003). Comparisons of size at maturity and fecundity of two Scottish populations of the European lobster, *Homarus gammarus*. Fisheries Research, 65: 137-152.

MacDiarmid, A. B. & Butler Iv, M. J. (1999). Sperm economy and limitation in spiny lobsters. Behavioral Ecology and Sociobiology, 46: 14-24.

Mäkinen, T., Panova, M. & André, C. (2007). High Levels of Multiple Paternity in Littorina saxatilis: Hedging the Bets? Journal of Heredity, 98: 705-711.

Marshall, T. C., Slate, J., Kruuk, L. E. B. & Pemberton, J. M. (1998). Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology, 7: 639-655.

Mercer, J. P., Bannister, R. C. A., van der Meeren, G. I., Debuse, V., Mazzoni, D., Lovewell, S., Browne, R., Linnane, A. & Ball, B. (2001). An overview of the LEAR (Lobster Ecology and Recruitment) project: results of field and experimental studies on the juvenile ecology of *Homarus gammarus* in cobble. Marine and Freshwater Research, 52: 1291-1301.

Moland, E., Olsen, E. M., Andvord, K., Stenseth, N. C. & Knutsen, J. A. (2011). Home range of European lobster (*Homarus gammarus*) in a marine reserve: implications for future reserve design. Canadian Journal of Fisheries and Aquatic Sciences, 68: 1197-1210.

Moland, E., Olsen, E. M., Knutsen, H., Garrigou, P., Espeland, S. H., Kleiven, A. R., André, C. & Knutsen, J. A. (In review). Lobster and cod benefit from small scale northern MPAs.

Olsen, E. M. & Moland, E. (2010). Fitness landscape of Atlantic cod shaped by harvest selection and natural selection. Evolutionary Ecology, 25: 695-710.

Pandian, T. J. (1970). Ecophysiological studies on the developing eggs and embryos of the European lobster *Homarus gammarus*. Marine Biology, 5: 154-167.

Panova, M., Boström, J., Hofving, T., Areskoug, T. & Eriksson, A. (2010) Extreme female promiscuity in a non-social invertebrate species. PLoS ONE 5: e9640.

Perkins, H. C. (1972). Developmental rates at various temperatures of embryos of the northern lobster (*Homarus americanus*). Fisheries Bulletin, 70: 95-99.

Pettersen, A. R., Moland, E., Olsen, E. M. & Knutsen, J. A. (2009). Lobster reserves in coastal Skagerrak - an integrated analysis of the implementation process. Pages 178-188 in E. Dahl, E. Moksness, & J. Støttrup. Coastal zone management. Wiley-Blackwell Scientific Publications, London.

Phillips, B. F. (ed.) (2006). Lobsters: Biology, Management, Aquaculture and Fisheries. Blackwell Publishing Ltd, Oxford, UK.

Planes, S., Jones, G. P. & Thorrold, S. R. (2009). Larval dispersal connects fish populations in a network of marine protected areas. Proceedings of the National Academy of Sciences of the United States of America, 106: 5693-5697.

Raymond, M., & Rousset, F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity, 86: 248-249.

Reisser, C. M. O., Beldade, R., Bernardi, G. (2009) Multiple paternity in sympatric congeneric reef fishes, *Embiotoca jacksoni and E. lateralis*. Molecular Ecology, 7: 1504-1510.

Rondeau, A., Sainte-Marie, B. (2001). Variable mate-guarding time and sperm allocation by male snow crabs (*Chionoecetes opilio*) in response to sexual competition, and their impact on the mating success of females. Biological Bulletin, 201: 204-217.

Roy, N. (2003) Incidence de la polyandrie chez le crabe des neiges, *Chionoecetes opilio* (Brachyura, Majidae), MSc Thesis, Université du Québec à Rimouski, Rimouski.

Rowe, S. (2002). Population parameters of American lobster inside and outside no-take reserves in Bonavista Bay, Newfoundland. Fisheries Research, 56: 167-175.

Rowe, S. & Hutchings, J. A. (2003). Mating systems and the conservation of commercially exploited marine fish. Trends in Ecology Evolution, 18: 567-572.

Sainte-Marie, B., Sevigny, J. M. & Carpentier, M. (2002). Interannual variability of sperm reserves and fecundity of primiparous females of the snow crab (*Chionoecetes opilio*) in relation to sex ratio. Canadian Journal of Fisheries and Aquatic Sciences, 59: 1932-1940.

Sardell, R. J., Keller, L. F., Arcese, P., Bucher, T. & Reid, J. M. (2010). Comprehensive paternity assignment: genotype, spatial location and social status in song sparrows, Melospiza Melodia. Molecular Ecology, 19: 4352-4364.

Sato, T., Yoseda, K., Okuzawa, K. & Suzuki, N. (2010). Sperm limitation: possible impacts of large male-selective harvesting on reproduction of the coconut crab *Birgus latro*. Aquatic Biology, 10: 23-32.

Serbezov, D., Bernatchez, L., Olsen, E. M. & Vøllestad, L. A. (2010). Mating patterns and determinants of individual reproductive success in brown trout (*Salmo trutta*) revealed by parentage analysis of an entire stream living population. Molecular Ecology, 19: 3193-3205.

Shuster, S. M., & Wade, M. J. (2003). *Mating systems and strategies*. Princeton, NJ [u.a] Princeton Univ. Press.

Skog, M. (2008). Sex and violence in lobsters -a smelly business, olfactory communication in the European lobster: Lund University, Department of Cell and Organism Biology.

Sorin, A. B. (2004). Paternity assignment for white-tailed deer (*Odocoileus virginianus*): Mating across age classes and multiple paternity. Journal of Mammalogy, 85: 356-362.

Streiff, R., Mira, S., Castro, M. & Cancela, M. L. (2004). Multiple paternity in Norway lobster (*Nephrops norvegicus*) assessed with microsatellite markers. Marine Biotechnology, 6: 60-6.

Thiel, M. & Hinojosa, I. (2003). Mating behavior of female rock shrimp; *Rhynchocinetes typus* (Decapoda: Caridea) - indication for convenience polyandry and cryptic female choice. Behavioral Ecology and Sociobiology, 55: 113-121.

Toonen, R. J. (2004). Genetic evidence of multiple paternity of broods in the intertidal crab *Petrolisthes cinctipes*. Marine Ecology Progress Series, 270: 259-263.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. & Shipley, P. (2004). Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes, 4: 535-538.

Venturelli, P. A., Shuter, B. J., Murphy, C. A. (2009). Evidence for harvest-induced maternal influences on the reproductive rates of fish populations. Proceedings of the Royal Society, 276: 919-924

Wang, J. (2004). Sibship reconstruction from genetic data with typing errors. Genetics, 166: 1963-79.

Wang, J. and Santure, A. W. (2009) Parentage and sibship inference from multilocus genotype data under polygamy. Genetics 181: 1579–1594.

Wiig, J. R. (2012) Acoustic monitoring of lobster (*Homarus gammarus*) behavior and survival during fishery. Master thesis, University of Life Sciences, Ås.

Yue, G. H. & Chang, A. (2010). Molecular evidence for high frequency of multiple paternity in a freshwater shrimp species *Caridina ensifera*. PLoS ONE, 5: e12721.