Marine reserves and selective fishing shape mating behaviour, secondary sexual trait and growth in European lobster

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Preface

To my supervisors over the years, thank you Halvor Knutsen and Esben M. Olsen at the Institute of Marine Research (IMR) and University of Agder (UiA), for support, for believing in me and for giving me the freedom to pursue my own ideas, even though some did not turn out as expected. To Leif Asbjørn Vøllestad at the University of Oslo (UiO) for your time and patience, for encouragements and for regularly asking me how things are going. I also thank all the national and international co-authors for all your invaluable help. I have been fortunate to build my work on the unique lobster survey, for which I am very grateful to Even Moland and Esben M. Olsen. Thank you also to the fishermen in Arendal for helping me sample egg-bearing females for my first paper when I didn't think I would get hold of any more.

A very special gratitude goes out to all the staff and researchers at Flødevigen research station (IMR) for taking so good care of me all those countless hours, days even years I spend working here. I came to you as a master student and after the first Christmas party I didn't want to leave. You people have made it memorable in so many ways. A special gratitude goes to Svein Erik Enersen and Hanne Sannæs for teaching me some of the secrets of catching lobster in all sorts of weather. I also cannot thank you enough Hanne, for devoting your time instructing me the way around the genetic lab and for sharing my frustration over single column extractions, poor DNA quality and outdated fragment machine(s). Diana, Susanna, Mats, Rebekah, Torkel, Katinka, Angela, Caren and all other fellow visiting student that have come by Flødevigen over the years; thank you for all the good times!

I am very grateful to Anne Berit Skiftesvik, Howard Browman, Caroline Durif and Reidun Bjelland for their warm-heartedly support and for inviting me into their group. Thank you for your hospitality and for entertaining discussions around the dinner table in Austevoll. I am sure there will be many more to come.

Little did I know how this thesis would turn out when I first started on this journey. After failing with experiments and almost succumbed under the weight of laboratory work, I found inspiration in the work of Jeffrey A. Hutchings and Sherrylynn Rowe, who sparked my interest in the topic of the interaction between fishery selection and mating systems. These perspectives have undoubtedly shaped the focus of my work.

My deepest gratitude goes first and foremost to Kim, first as my colleague then also as my best friend and father of our son. Thank you for bringing me so much inspiration, love and happiness, and for encouraging me to do my very best even when the days are long, and the energy is low. Countless hours have been spend discussing what exactly it is that drives us to do what we do, imagining a better world and then contemplating new ideas and projects to do. I'm grateful and proud to have you as my partner and I doubt I would have made it here without you and your help. I am also very thankful for the support from my family and friends for believing in what I do, even though I know you find my choice of profession a bit strange.

Lastly, I dedicate this thesis to my son, Julian and all other children who are the future stewards of the ocean. I sincerely believe you will do a better job than us.

Arendal, February 2019
Tonje Knutsen Sørdalen

Contents

Summary

Well-functioning mating systems are perceived as vital for population growth rate and resilience to environmental change, but we know surprisingly little about the interplay between human-induced mortality and the mating systems of exploited marine species. Fishing and hunting can reduce densities, shift sex ratios and often targets individuals with the largest body size or with the most conspicuous characters (e.g. horns and claw). These traits tend to be sexually selected traits important for the outcome of competitive interactions and mate attraction. Thus, human harvest probably has profound, but not straightforward, effects on mating patterns and the strength and dynamics of sexual selection in many species. Marine protected areas (MPAs) are implemented worldwide with the aim of restoring exploited species and ecosystem functioning and they should also have the ability to conserve species' mating patterns and secondary sexual traits. In this thesis, I have empirically examined several aspects of the mating system in wild European lobster (*Homarus gammarus*) and how it responds to intensive fisheries selection, but also whether MPAs can rescue a species' natural mating patterns and secondary sexual traits targeted by the fishery. I have used both genetic analysis methods and morphological data from three lobster reserves on the Norwegian Skagerrak coastline and adjacent area open to fishing as control, and also explored the typical female fertilization pattern in United Kingdom (UK). In **paper I**, I sampled DNA from males and egg-bearing females in one of the lobster reserves and fished area across multiple years and compared paternity data for any differences in mating behaviour. The results confirmed size-assortative mating, with females' preference for males with a body size larger than their own, however, the size difference within each pair were much larger in the reserve than in the fished area (22.5% compared to 6.4% in the fished area). Two cases of multiple paternity were also found in the fished area. Male size (body and claws) strongly influenced male mating success, but only in the reserve as selection differentials on these traits were not significant in the fished area. Lastly, estimation of sexual selection gradients on male traits found the selection to be acting strongest on relative claw size (claw size relative to body size), rather than on absolute claw and body size. In **paper II**, I joined a paternity study in a fished region of the coast of UK where we found no incidence of multiple paternity on egg-bearing females and concluded single paternity to be the common fertilization pattern. In **paper III**, I show that legal-sized male lobsters have larger relative claw sizes inside MPAs compared to same sized in fished areas (up to 8.4% larger). There were no differences between areas for females, which have

smaller claws than males. This study is possibly the first to document the usefulness of MPAs in preserving a trait under strong sexual selection and under ongoing harvest selection in the same study system. In **paper IV**, I also found support for a positive MPA effect on body growth for legal-sized lobsters and most clearly for moult increment of females. It suggests that this may be caused by the catchability of lobsters in the trap fishery to be positively correlated with high growth rate and that lobster undergo intensive and selective fishing pressure against faster growing individuals. In sum, the findings in this thesis suggest that (1) selective fishing has the ability to weaken sexual selection with potential consequences for rates of fisheries induced evolution, (2) that MPAs can be highly effective in preserving sex-specific variation and phenotypic diversity and (3) that even small-scale MPAs can help maintaining the scope for sexual selection in a sedentary species with complex mating behaviour and life-histories such as the lobster.

List of papers

This dissertation is based on the following four research papers (published or manuscripts), which will be referred to in the following text by their Roman numerals.

I. **Harvesting changes mating behavior in European lobster**
Sørdalen, T.K., Halvorsen, K.T., Harrison, H.B., Ellis, C., Vøllestad, L.A., Knutsen, H., Moland, E. and Olsen, E.M. 2018.
in *Evolutionary Applications* 11(6): 963-977 (doi:10.1111/eva.12611).

II. **Genotype reconstruction of paternity in European lobsters (*Homarus gammarus*)**
Ellis, C.D., Hodgson, D.J., André, C., Sørdalen, T.K., Knutsen, H. and Griffiths, AG.F. 2015.
in *PloS One* 10 (11): 1-14 (doi:10.1371/journal.pone.0139585).

III. **Marine reserves rescue an important secondary sexual trait in male European lobster**
Sørdalen, T.K., Halvorsen, K.T., Vøllestad, L.A., Moland, E. and Olsen, E.M.
Manuscript.

IV. **Improved body growth of lobster inside marine protected areas compared to intensively fished areas**
Sørdalen, T.K., Halvorsen, K.T. and Olsen, E.M.
Manuscript.

1. Introduction

1.1 The interplay between harvesting, marine protected areas and mating systems

Oceans and coastal ecosystems are dynamic systems, but human activities are causing unprecedented changes to the natural seascape at a pace much faster than species can keep up with (Halpern *et al.* 2008). The majority of commercially (Worm *et al.* 2009) and some recreational (Cooke and Cowx 2004) fished species are now regarded as fully- or overexploited with many examples of declining and collapsed stocks (Jackson 2001; Myers and Worm 2003; Hutchings and Reynolds 2004). Fishing can be a strong selective force and have been shown to shift phenotypic distributions and drive evolutionary change on contemporary timescales (Heino *et al.* 2015 and references therein). Fisheries selection for smaller body size and earlier maturity has been extensively studied, but fishing may also be selective on behaviour, morphological and physiological traits. For example, on elongated bodies and swimming speed (Hamon and Foote 2005; Alós *et al.* 2014), slower growth rate in trap fishery (Biro and Post 2008; Biro and Sampson 2015) and behaviour along the boldness/shyness axis (Biro and Sampson 2015; Diaz Pauli *et al.* 2015; Twardek *et al.* 2017). There is also an increasing awareness that harvesting may interfere with sexual selection and mating patterns when densities, size structures and sex ratios are altered (Parker 1992; Rowe and Hutchings 2003), which is typical for many fisheries (Zhou *et al.* 2010). When population densities are reduced, individuals may use more energy and time finding mates, which in turn can increase risk of predation and starvation or limit the opportunity to mate at the optimal time, habitat, environmental conditions, or simply with the preferred mate (Kokko and Rankin 2006). This may reduce populations productivity and their resilience to environmental stochasticity (Møller and Legendre 2001; Rowe and Hutchings 2003). Moreover, harvesting has been shown to select against sexually selected characters in males, such as large body size or the size of weaponry (e.g., horns, antlers and claws); traits which are important in mate choice and intraspecific competition (Wilber 1989; Swain *et al.* 2007; Woolmer *et al.* 2013). Sexually selected characters' positive relationship with reproductive success may be even stronger than for traits solely subjected to natural selection. This is because the slope of reproductive success on male body size is higher than that of females', since more females than males will have the opportunity to mate (Kingsolver *et al.* 2001).

In the context of fisheries-induced evolution, modelling has shown that size-selective fishing may erode the size variation needed for sexual selection to work properly, and that

fisheries-induced evolution would be accelerating faster compared to a scenario assuming random mating with respect to body size (Hutchings and Rowe 2008). Nevertheless, how the strength and direction of sexual selection is affected by fishing is not straightforward (Hutchings and Rowe 2008; Urbach and Cotton 2008; Lane *et al.* 2011).

As a response to the increasing pressures on our marine ecosystems, a growing number of marine protected areas[1] (MPAs) are implemented around the globe and have become an important tool for conservation and fisheries management (Hastings and Botsford 2003; Lester *et al.* 2009). MPAs may prohibit all fishing activity (no-take area) or they can be partially protected, banning landing of specific species and/or types of fishing gear to be used. In addition, how fishers comply by these rules and how well the areas are enforced are naturally affecting the effectiveness of MPAs. If well enforced and designed in terms of quality of habitat and size, MPAs can be a valuable aid in protecting biodiversity and fragile habitats, species and populations. It is now well documented that MPAs generally have positive effects on the number, biomass, size and age structures of harvested species (Halpern 2003; Russ *et al.* 2006; Claudet *et al.* 2008; Lester *et al.* 2009; Baskett and Barnett 2015). MPAs can also provide benefit to local fisheries yield through spill-over of juveniles, adults and export of pelagic eggs and larvae to surrounding areas (Goñi *et al.* 2006, 2010; Harrison *et al.* 2012; Lorenzo *et al.* 2016; Port *et al.* 2017). Through these mechanisms, MPAs may act as a buffer against fisheries-induced evolutionary changes and help preserve genetic diversity (Berkeley *et al.* 2004; Baskett and Barnett 2015). Thus, in addition to its utility in conservation and management, MPAs can be particularly valuable as reference systems for studying natural ecological processes and population dynamics. Contrasted against similar areas open to fishing, we may also gain insights in how harvesting interfere with ecology and evolutionary trajectories (Gell and Roberts 2003). When a no-take MPA is established, the upheaval of selective fishing is expected to restore trait distributions towards the direction of pre-fishing condition inside the area, provided that enough genotypic variation remains. Beyond the many examples of improved size and age structure (e.g. Claudet *et al.* 2008; Moland *et al.* 2013; Fidler *et al.* 2018) aquatic protected areas have been shown to house more naïve fish (Januchowski-Hartley *et al.* 2013; Goetze *et al.* 2017), fish with slower flight response (Kennedy Rhoades *et al.* 2018) and more attentive male fish with better parental care (Sutter *et al.* 2012). Lastly, since traits are a product of genotypes and the environment, the trait distribution in both fished and unfished populations will be shaped by the interaction between natural and human-induced selection

[1] Hereafter I will use the terms marine protected areas (MPAs), protected areas and reserves interchangeably.

and phenotypic plasticity. Protection from fishing may therefore also affect traits indirectly if the environment is altered, for example through changes to inter -and intraspecific competition and interactions, mortality and habitat quality.

Even though the concept and challenges associated with harvesting effects on mating systems have been recognised long ago, the interaction between sexual selection and selective fishing is poorly studied empirically in the marine environment. Reasons for this lies partly in the difficulties in designing a study that have the means and the power to obtain direct or indirect observations of cryptic mating events. Therefore, elucidating mating patterns in an open ocean system would need refined methods. For instance, pairs of MPAs and fished areas may be highly valuable as field laboratories for understanding how fishing affects mating behaviour and traits under sexual selection, or perhaps equally important, for assessing whether MPAs can play a significant role in preserving functional mating systems and diversity in sexually selected traits. Furthermore, the rapid development in DNA sequencing technology and analytical methods have alleviated some of these challenges by allowing us to study individual mating success though parentage assignments techniques. Some species may be particularly good models for such studies. For example, many decapod crustaceans show strong sexual dimorphism (e.g. body size, claw size) and support fisheries worldwide. They also come with the advantage of long periods of maternal care that allow us to simultaneous collect DNA from mother and offspring, which makes large-scale parental studies in open systems a lot more achievable.

The overarching goal of my doctoral thesis was to elucidate potential effects of harvesting on the mating system of the highly valued and overexploited European lobster (*Homarus gammarus*) by characterizing mating patterns and to identify morphological traits under sexual selection and fishery selection. Most of the work is done in a framework of MPAs particularly designed for lobster and in control areas open to fishing on the Skagerrak coast. I address how selective pressure caused by human harvest (size-selective fishing) can exert maladaptive changes to these patterns and potentially drive evolutionary trait-changes, but also how management options can mitigate such unintended consequences. In the next part of the introductory of the thesis I provide a description of the study species, including mating behaviour and the fishery in Norway, with the intend to contextualize the studies presented in each paper. In the last part of the introduction I present the objectives of my work, and briefly how I address them in each paper. In section 2, I describe the study area and main methods used. Lastly, in section 3, I summarize the main results and discuss the implications of the

findings in light of fisheries and conservation management with perspectives on the role of MPAs in preserving traits under pressure by human-induced selection.

1.2 The biology of European lobster

The European lobster (Figure 1) is a large marine decapod crustacean of the family Nephropidae (clawed lobsters), which also includes the American lobster, *H. americanus* (Milne Edwards, 1837). The distribution range of European lobster cover much of the coastal shelf seas of the eastern North Atlantic from Morocco in North Africa to arctic Norway, only bound by the Black sea and the Baltic Sea (Triantafyllidis *et al.* 2005). Unlike other species of lobsters, adult European lobster do not move great distances. Instead they are stationary once they settle into a suitable habitat and typically live within limited home ranges (~0.02 km^2) (Moland *et al.* 2011; Skerritt *et al.* 2015). The larvae, on the other hand, have a pelagic phase of 1-2 months before settlement with potential to travel 10 - 100s of kilometres with currents, suggesting considerable dispersal capacity and connectivity among populations (Huserbråten *et al.* 2013; Wahle *et al.* 2013). However, a recent population study found no spatial genetic structure throughout the species' range, but only a weak differentiation between lobsters from larger regions of the Swedish Skagerrak and the Atlantic areas to the west, with the population in Norway being a mix of these two (Ellis *et al.* 2017).

The European lobster is solitary and nocturnal and usually spend daylight hours hiding in shelters under rocks, boulders or borrows in sediments down to 60 meters of depth but may be found much deeper. They have highly developed sensory organs on their legs to detect food in the absence of light. Juvenile lobsters are mostly suspension feeders as they live completely hidden during juvenile years, whereas adults are mostly scavengers and predators on fish and a variety of ground dwelling invertebrates like molluscs, crabs, bivalves and worms (Wahle *et al.* 2013). Lobster can also cannibalise on injured or newly moulted animals at all stages in their life cycle and will eat their old shell after moulting to reabsorb much needed calcium.

Growth. Like all crustaceans, the lobster needs to shed the outer shell (exoskeleton) and replace it with a new one in order to grow; a no-linear growth process called moulting. The growth rate is influenced by two elements: how much the animal grows from one moult to the next (moult increment) and the time interval between moults. Generally, younger adults increase more in size than older lobster, and males more than females. Juveniles can moult several times a year, whereas adults usually moult once or twice every year or once every two-three years, especially when females are carrying eggs because the egg-period inhibit moulting. Water temperature and food supply are some other known determinants that

can influence the moulting schedule and growth rate (Waddy and Aiken 1995). After the old is replaced with a new soft shell, the it must remain in hiding for a couple of weeks to avoid predation until the shell is hard enough to yet again function as body armour.

Figure 1. Study species, the European lobster (*Homarus gammarus*). Photo by Tonje K. Sørdalen.

Males and females can live to become 40 - 70 years or older, respectively, although age determination is difficult to assess with certainty in many crustaceans (Sheehy *et al.* 1999). Because of the variable growth rate, lobsters recruiting to the fishery may consists of as many as seven year classes (Sheehy *et al.* 1999). The longevity and the indeterminate growth allow them to reach impressive size with only negligible senescence (Elmore *et al.* 2008). The largest specimen ever recorded (from a recovered crusher claw of 360-370 mm in Skagen, Denmark) was estimated to be a male of 650 mm total length, corresponding to a weight of 8.4-9 kilogram (Wolff 1978). Large lobsters have very few enemies other than a few big fishes, octopuses, otters and humans. A recent study has found that the lobster can produce a low-frequent buzzing sound of narrow band width, a range that corresponds with sound sensitivity in octopuses. Sound production may be used as warning signal but perhaps also be a means of communication between lobsters since they have receptors for low-frequent vibrations (Jézéquel *et al.* 2018).

Sexual dimorphism and maturation. Estimates of size at the onset of sexual maturity varies from differences in morphological, physiological and functional indices of maturation (Wahle *et al.* 2013). The timing of maturation may also depend on summer temperature and would most likely differ throughout the geographic range (Wahle *et al.* 2013). On the east coast

of Scotland, the size at maturity has been estimated to be 79- and 80-millimetres carapace length for females and male respectively, based on morphologic change in the relative growth between the body size and claw/abdomen (Lizárraga-Cubedo *et al.* 2003). In Ireland, female physiological maturity by examination of ovaries has been estimated to be 92.5-96 mm carapace length (Tully *et al.* 2001). Neither female nor male maturity schedules have been investigated in wild populations in Norway.

Relative growth in crustaceans, that is the rate of development of one part of the body relative to another, or the whole body, is useful in studies of the development of primary or secondary sexual characters. It can also inform about the emergence of physiological (i.e., ability to produce gametes), morphometric (i.e., full expression of secondary sexual characters), and functional (i.e., ability to mate and spawn) maturity when the size relationship of a body part changes relative to a reference variable (i.e. the whole body) (Émond *et al.* 2010). Adult lobster has dimorphic claws; one is a major molar-toothed (crusher) claw and the other is a minor incisor-toothed (cutter) claw. The claws have dual functionality being both tools used in foraging, and as weapons in male-male conflicts (armaments) and a signal of attractiveness towards females (ornaments) (Elner and Campbell 1981; Atema 1986). When approaching maturation, males begin to grow larger and heavier claws than females whereas the abdomen of females becomes proportionally broader to accommodate more eggs (Templeman 1935b; Mariappan, P., Balasundaram and Schmitz 2000; Émond *et al.* 2010). In females, the first pair of swimmerets (pleopods), called gonopods, are soft and feathery whereas in males they become enlarged and rigid and are used for transferring spermatophores during intercourse. Thus, the difference between males and females in allometric enlargements of claws and abdomen, and the modified swimmerets, represent sex-specific investment in secondary sexual characters that results in a clearly visible sexual dimorphism. A study on antenna morphology have also discovered sexual differences in size and distribution of the aesthetascs on antennas, a specialized organ used to determine concentration and direction of smell (Skog 2009a).

1.3 Mating system of clawed lobster

Lobster are renowned for their complicated sex life. Some 100 million years of evolution have provided ample time to refine techniques for finding mates, coerce male aggression and to court (Bracken-Grissom *et al.* 2014). Much of what we know about lobster mating system and mating behaviour derives from studies on the American lobster, but because of the strong similarity between the two species, I will use references from both species.

Female mate choice and courting. Females usually mate in a males' shelter in summer just after molting and all observations both in field and laboratory show that females, not males, make the initial mate choice (Karnofsky *et al.* 1989b; Debuse *et al.* 2003). In the weeks before moulting, a female American lobster will evaluate potential mates by making frequent visits around the neighbourhood and approach shelters of a male of interest. She is looking for a dominant male that has acquired a high-quality shelter and she is attracted by the scent of his urine-borne chemical cues which he broadcast by fanning his swimmerets from inside the shelter (Atema and Cowan 1986). The urine plays an important role as chemical communication in clawed lobster. It contains pheromones that likely convey information about level of aggressiveness or fighting abilities, sexual receptivity, sex and moult state of the signaller, although the actual molecules have not been described (Atema and Cowan 1986; Bushmann and Atema 1997; Breithaupt and Atema 2000). The urine-pheromone mix may advertise at least some important aspects of the males' quality. The urine is also critical for the establishment and maintenance of social dominance status and sexual behavior of both sexes (Karavanich and Atema 1998; Skog *et al.* 2009). The higher the dominance order of males, the more often their shelters are checked by courting females (Cowan and Atema 1985). Lobsters are solitary by nature and highly aggressive towards one another. Males are even threatening the safety of female during mating if the male aggression is not 'disarmed' prior to and during mating. The trick to reduce this dangerous aggression lies in female sex pheromones in the urine and is critical in ensuring successful courtship and normal mating behavior (Atema and Cobb 1980; Skog 2009b). If the female finds the male and his shelter to be suitable, both as a father of her offspring and as post-moult protector, she will make regular visits over the next days or weeks to deposit her urine scent until he invites her in (Atema *et al.* 1979; Bushmann and Atema 1997). She moults in his shelter, followed by one ritualized mating act (see Atema and Cobb 1980 for spicy details). Over the next week the bonded pair continue to cohabitate, a strategy believed to increase successful pre- and post-copulatory guarding of the soft-shelled female (Atema *et al.* 1979; Karnofsky *et al.* 1989b,a; Karnofsky and Price 1989b; Cowan and Atema 1990). She will stay in the shelter until her new and soft exoskeleton has hardened and she can fend for herself.

Mating and spawning events are separated in time and space as the spermatophore are stored in a seminal receptacle (sperm storage) before fertilizing the eggs (Aiken *et al.* 2004). The sperm remains viable in the storage of very large females for as long as three years and can, if enough sperm, fertilize two clutches of eggs (Waddy and Aiken 1986). Until quite recently, one of the greatest mysteries of lobster reproductive biology, puzzling scientists more

than hundred years, was understanding whether the fertilization happened internally or externally and how sperm escape from the storage of the female. The fact that the stored, immotile spermatophore is located farthest away from the opening of the storage under an impermeable sperm plug that do not change before, during and after spawning (and is only removed by moulting), have made the ejection of the stored sperm difficult to explain. At spawning, which usually occur the following spring, sperm leaves the storage via two grooves located at the side of the main opening, bypassing the sperm plug and externally fertilize the eggs as they are extruded onto the tale (Aiken *et al.* 2004). Females stores the inseminated eggs on the ventral side of her body until they hatch 9-11 months later when temperature are more favourable (Agnalt *et al.* 2007). Without a sperm supply, eggs are still extruded but are lost within a few weeks.

Although the description of the mating cycle above is the general pattern, the mating habits of clawed lobster are flexible and it is not uncommon that larger females are able to spawn in consecutive years, either by fertilizing two egg-batches with same sperm supply, or through intermoult mating (Waddy and Aiken 1986; Comeau and Savoie 2002). Intermoult mating is a beneficial strategy when a large female can skip a moulting but has used up the sperm storage and need to receive new sperm to fertilize her eggs. It is also thought to be useful for females living in areas where males are more difficult to find (Waddy *et al.* 2017).

Male mating capacity and sperm limitation. Males of clawed lobsters are known to mate with several females in a mating season (polygyny) and there is little evidence to suggest that males are particularly selective with whom they mate with. Multiple females of American lobster have been seen regularly checking in on a dominant males' shelter when he is cohabiting with a female and as soon as she leaves, a new female move in (Cowan and Atema 1985). For lobsters, bigger bodies translate into higher fecundity in both sexes. In females, egg production increases exponentially with increasing female size and large male decapods have greater sperm storages, are capable of tailoring ejaculate load to the size of the female, and replenish depleted sperm faster than smaller males (Agnalt 2008; Jivoff 1997; MacDiarmid *et al.* 1999; Kendall *et al.* 2001; Gosselin *et al.* 2003). Contrary to eggs, however, sperm are produced all-year-round. A recent study has demonstrated impressive mating capacity in mature males of American lobster, where some males were able to inseminate 30 to 54 females in one mating season and neither sex showed indication of being sperm depleted. Almost all females spawned and carried full clutches of fertilized eggs (Waddy *et al.* 2017).

Despite this recent finding there is a growing concern that the population productivity of many crustaceans, including clawed lobster, can be constrained by sperm limitation; a

scenario where a population in sum has too little sperm to fertilize all the eggs (Fogarty and Gendron 2004; MacDiarmid and Sainte-Marie 2006; Pugh *et al.* 2013; Wahle *et al.* 2013). Fisheries susceptible to sperm limitation are those managed by male-biased or male-only strategies, which is common for most crabs and for many lobster fisheries where the focus is to protect mature or egg-bearing females from harvesting (Montgomery and Liggins 2013; Smith and Jamieson 1991; Orensanz *et al.* 1998). If the fishery reduces the density and remove large males and creates a sex ratio imbalance, the mating behavior may change (Fogarty and Gendron 2004). It has been suggested that large and popular males may exhaust their sperm reservoirs after multiple copulations in a mating season with the result that females receive inadequate amount of sperm (Gosselin *et al.* 2003, 2005). Alternatively, or concurrently to the above scenario, females may turn to smaller males of lower quality if large males are not available. Multiple paternal fertilizations have been documented among individual egg-bearing females in heavily fished population of American lobster and though to be the result of individual females mating with several smaller males in attempts to avoid sperm limitation (Gosselin *et al.* 2003).

Competition and dominance. Clawed lobster of both sexes establish and maintain dominance hierarchies. Aggressive males need to pursue victories to achieve reproductive success because it is dominance and access to good shelter that are the determining factors for male attractiveness towards females (Karnofsky and Price 1989a). Male dominance is also directly correlated with mating success in aquariums (Atema *et al.* 1979; Cowan and Atema 1990). This results in strong male-male competition in lobster. Both in field and aquariums the highest ranking and preferred ("Alpha") male have been seen regularly patrol and evict other males from their shelters (Atema 1986). Observations also show that dominance in lobsters is almost entirely size dependent but that males have a higher dominant order, even if females are slightly larger (Atema and Cobb 1980; Atema 1986). The strategies in maintaining dominance order differ between the sexes and also here urine-borne chemicals are the underlying mechanisms for signalling and recognition of dominance (Karavanich and Atema 1998). Males that lose a fight will remember and try to avoid the winners in future encounters (for a couple of weeks) but aggressively fight with new unfamiliar opponents, even if they are dominant by size (Karavanich and Atema 1998; Skog 2009a). Females that lose a fight, on the other hand, will avoid encounters with a new dominant regardless whether they are familiar or not (Skog 2009a). Both sexes will know its place, but females identify, and abide by, dominance status more so than males.

Species with strong male competition invest in ornaments or weapons with the purpose

to ensure a winner in contests or display. In lobster, larger claws increase male competitive abilities and are a better predictor of victories than body size (Atema and Cobb 1980; Elner and Campbell 1981; Van Der Meeren and Uksnøy 2000). Thus, individual claw size is likely important when establishing dominance. The sexual dimorphism in claw size with the onset of maturation support this argument.

1.4 The European lobster fishery

The European lobster is one of the most valuable and sought-after species in Northern Europe's commercial and recreational fisheries. In Norway, the fishery has a long history as one of the most popular recreational fishing activities in coastal communities and used to be an important source of income for many fishermen. However, the lobster catches in Norway declined by 65 % from the 1950s to 2000s, and is today at the lowest record in history with no sign of recovery (Pettersen *et al.* 2009). Although the reason for the collapse is not known for certain, there is a consensus that it is due to increasing fishing pressure and highly ineffective regulations (Agnalt *et al.* 1999; Pettersen *et al.* 2009). The catches today have little economic value. The fishery is unreported and unregulated, and the total yearly catch is estimated to be 14 times higher than official landings (Kleiven *et al.* 2012).

In response to declining catches, a variety of management measures have been adopted, particularly in recent years. The fishery is regulated by closed season and a prohibition on fishing with exception from 1 October to 30 November on the Skagerrak coast and to 31 December in northwest. All participants must be registered prior to opening of the season (from 2017). Lobster can only be caught by one type of gear (pots) which are usually baited with mackerel (salted and rotten) and Ballan wrasse. After 2008, commercial fishers are allowed to fish with a maximum number of 100 pots, whereas recreational fishers are limited to 10 pots per person and boat. It is required that pots must be fitted with two circular escape vents (60 mm in diameter) to reduce capture of undersized individuals and a cotton tread must be installed in agreement with specifications (from 2017) to prevent ghost-fishing if lost. The minimum legal-size limit was raised to 250 mm total length (*TL*) in 2008, corresponding to 88.6- and 86.9-mm carapace length (*CL*) for males and females respectively (Paper III). In 2017, a maximum size limit at 320 mm *TL* (~116 mm *CL*) was introduced for lobster caught along the Skagerrak coastline (Kleiven *et al.* 2017). A ban on the harvest of egg-bearing females was implemented in 2008, which is a common regulation in heavily exploited crustacean populations. The scientific rationale for such a ban is to directly increase egg production and increase the population productivity by allowing females to release their larvae

before being harvested. Approximately 50% of the legal sized female lobster are protected by the ban (Figure 2).

Being one of the most popular recreational fishing activities in coastal communities, the declining stock has sparked great concern and interest (and debate) from both fisheries' management and conservationists to understand what prevents a population recovery and how small-scale MPAs can help restore lobster populations.

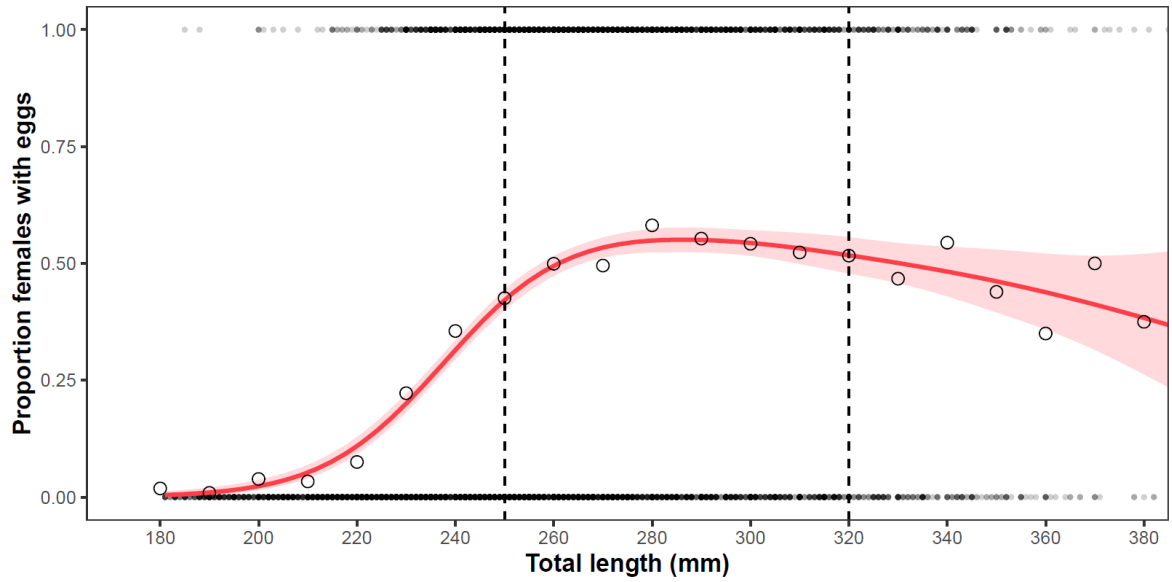

Figure 2. The proportion of females bearing eggs as a function of total length. Data is from the lobster survey in the three regions 2006 – 2018, all areas combined (n = 5577 females). A binomial gam smoother with P-splines is fitted to the data, with shaded area showing the 95 % confidence interval around the estimate. The circles show the observed proportion of egg-bearing females per 10 mm length classes, starting at 175 mm – 185 mm. The dashed vertical lines illustrate the harvest slot; minimum size limit of 250 mm (~87 mm *CL*) and the newly implemented maximum size limit of 320 mm (~116 mm *CL*), respectively.

1.5 Outline of the research

The research topics and specific objectives addressed are as follows:

1. Female mate choice and determinants of male mating success (paper I). In laboratory studies, it has been documented that female clawed lobsters preferentially choose a large male as mate and that large claw size is important when establishing dominance status. Inside MPAs on the Skagerrak coast of Norway, lobsters have rapidly become more numerous and larger (Moland *et al.* 2012), which increases the scope for mate choice. In the areas with

high fishing pressure, larger males can be few and far between. Thus, my first objective was to compare mate choice and mating patterns in and outside an MPA. I employed a parentage assignment with four years of data sampled inside both areas in order to explore to what extent there is a consistent size difference between mated pairs (size-assortative mating pattern), and if the mating pattern differ between the contrasting areas. I also compared the strength of sexual selection on body size, absolute claw size and relative claw size (adjusted for body size).

2. Female fertilization pattern in a fished population (paper II). Female fecundity increases with increasing body size and studies on other crustacean species have found the ejaculate load to be size-specific. In an intense size-selective, or male-biased fishery, populations may become sperm limited when the total production of male gametes are not able to fertilize the maximum egg capacity produced by females. Multiple female mating with several smaller males could be indicative of a sperm limited population. The objective of this study was to obtain information of paternity in lobster by estimate the frequency of multiple paternity and thus clarify the typical fertilization pattern in lobsters from an important regional fishery in the United Kingdom subjected to hatchery stocking.

3. The effect of protection on a sexually selected trait under harvesting selection (paper III). Secondary sexual characteristics such as horns, antlers and claws are the results of strong sexual selection and known to be important for the outcome of competitive interactions between males, and for female attraction. A recent study (Moland et al. *in press*) shows fisheries selection to be strongest on claw size (adjusted for body size). Since this trait was identified to be under the strongest sexual selection in males (Paper I), my objective was to investigate whether MPAs could rescue this trait from fishery selection, which should be evident as relatively larger claws in side MPAs vs. fished areas. To this end, I analysed two years of claw and body size measurements of lobsters caught in three lobster MPAs and their respective fished control areas on the Skagerrak coast of Norway.

4. Growth of lobsters in fished and protected areas (paper IV). Growth rate is a key trait for lobster reproduction since large size is correlated with better mating opportunities and fecundity. Recent experimental studies report evidence for that crustacean and fish with high growth rates and/or aggressiveness are more prone to be captured with passive gears. As such, the intensive fisheries for lobsters on the Skagerrak coast could be depleting fast growing individuals, while MPAs may serve as refuge for all phenotypes, including fast growth. My objective was to test this prediction by analysing 12 years of capture and recapture data from

the three pairs of MPA-fished areas on the Skagerrak coast (same sites as in 3), specifically by comparing annual moulting probability and moult increments adjusted for body size.

2. Methodological approaches

2.1 Study areas and sampling

In September 2006, three small-scale MPAs were established along the Norwegian Skagerrak coast to investigate if they could be used to rebuild the local lobster population and to assess the effects of lobster fishing. The three MPAs in Aust-Agder, Vestfold and Østfold county (Figure 3), have regulations that prohibit capture of lobster and use of fishing gears such as fyke nets and pots/traps. Fishing with hook and line is permitted. To monitor the effects of protection, all MPAs have one nearby area open for fishing as a control.

Lobster was caught with pots as part of the annual standardized capture–mark–recapture sampling programme conducted by the Institute of Marine Research (IMR). All individuals where tagged with T-bar tags, sexed and carapace length and total body length measured (see papers for more details on study areas and sampling protocol). In Aust-Agder county, lobster where fished more extensively throughout 2010–2013 to sample DNA (tissue) from as many males as possible for the parentage assignment. Females and offspring were sampled summer and autumn 2011 and 2012 (see paper I for details on sampling protocol). Prior to 2017, claw measurement had only been sampled in Aust-Agder county, but extended to all counties in 2017 and 2018 (see paper I and II).

The Celtic Sea and the western English Channel where chosen as study areas for genotyping of egg-bearing females in United Kingdom. Females where sampled for eggs and tissue in 2013 with similar procedure as for the parentage analysis in Norway, although the lobster were obtained with help from local fishermen (see paper II for detailed description of the study sites and sampling protocol).

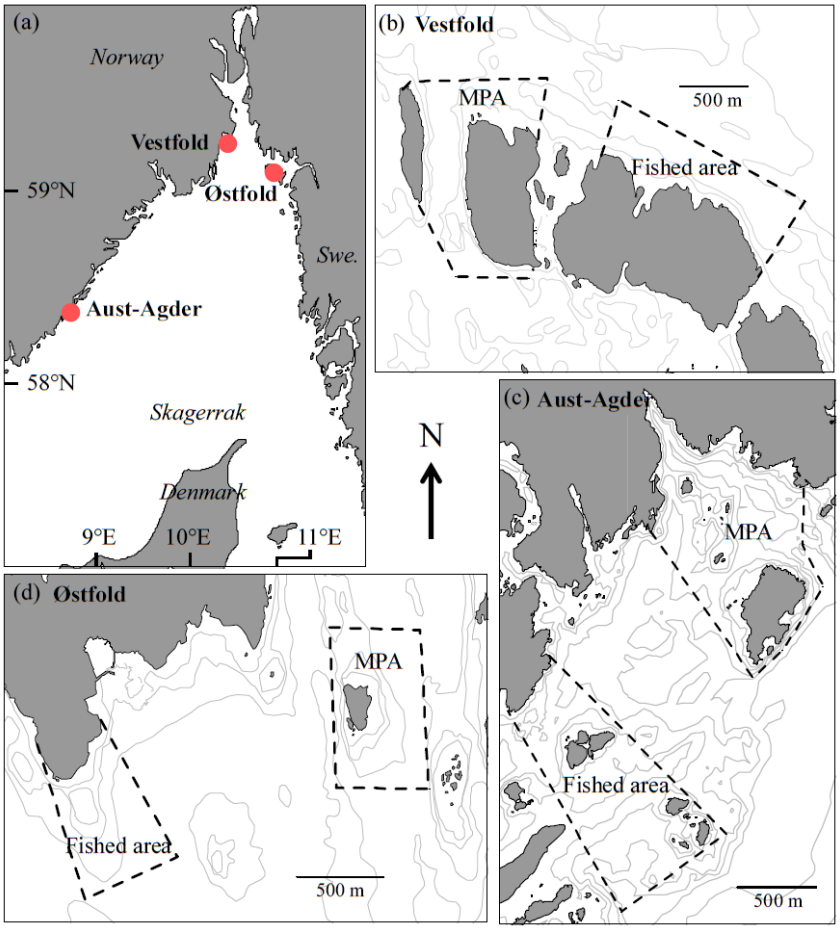

Figure 3. Study area of main focus. From top, (a) red circles shows the location of the lobster MPAs and fished control area in pairs on the Norwegian Skagerrak coast and in each of the three counties; (b) Vestfold, (c) Aust-Agder and (d) Østfold, respectively.

2.2 Genetic analysis and mark-recapture data

DNA was extracted from adult tissue and individual egg samples following protocols as described in paper I and II. Genotyping of DNA was done using 10 and 13 microsatellite loci for paper I and II, respectively. Polymerase chain reaction (PCR) was used to amplify DNA fragments before they were analysed using capillary sequencers, see paper I and II for further details of primer and PCR conditions. In paper I, I manually scored genotypes for each individual based on characteristic peaks in each locus, but because two different sequencers were used, I had to calibrate the results, so the alleles would end up being compatible (alleles with same length). Furthermore, due to allelic drop-out (i.e. null alleles), false alleles and random laboratory and genotyping errors, I calculated locus specific error rates in a series of steps which was then incorporated into the parentage analysis. This ensured the best possible

family configuration between siblings (offspring), their known mothers and the assignment of parentage among fathers. In cases where the true fathers of offspring had not been sampled, genotypes were reconstructed. Full details on descriptive population genetics, approach and estimation of error rates, and parameters and runtime in the paternity analysis can be found in main text and appendix in paper I.

When determining whether a female had been sired by more than one male in paper I, I inspected both the results that were flagged as cases of multiple paternity in the paternity analysis together with the original genotype data to not overestimate the number of multiple sired females. In paper II, all parental genotypes had to be reconstructed prior to inferring if females had mated multiple times or not (see paper II for details). In both paper I and II, we only sampled 10 offspring per female, which is a very small fraction of the total egg-mass females produces. Since males may contribute fertilization unevenly, skewed parentage (i.e. 1:9 or 2:8) among multiple males could go undetected when analysing such small number of offspring. We therefore calculated the power to detect multiple paternity under various scenarios of skewed contributions.

In paper I, I established a size relationship between mated pairs in order to analyse size-assortative mating patterns. I then calculated standardized selection differentials and standardized selection gradients on male body size and claw size (including residual claw size in the latter) to discern sexual selection on the male traits.

In paper III, a linear model was used to test whether legal sized lobster in the three MPAs had larger claws (relative to body size) than conspecifics in the contrasted fished areas. I chose to only analyse claws that was not unusual small in size, compared to body size, because they were most likely regenerated claws and thus expected to be unrelated to selective fishing mortality.

In paper IV, I estimated individual body growth of lobster from mark-recapturing data (2006-2018) over consecutive seasons by calculating the probability of moulting and the moult increment. Moulting probability was analysed with generalized linear mixed effects models and the effect of protection on moult increment was analysed with linear mixed effects models.

3. Results and discussion

3.1 Summary of main results

Up until now, there have been few studies and examples of fisheries inducing changes in sexually selected traits in marine systems. In this thesis, I have empirically examined several aspects of the mating system and sexual selection in wild European lobster (*Homarus gammarus*) and demonstrated different patterns of assortative mating in and outside a marine reserve. Furthermore, I present evidence that lobster reserves (MPAs) can rescue key sexually selected (claws) and life history (growth) traits, which are likely to be under strong harvest selection outside of MPAs. Combined, these results are important for marine conservation biology as they demonstrate that fishing can strongly alter mating behaviour, but at the same time, MPAs can be highly effective in preserving sex-specific variation and phenotypic diversity important for reproduction and productivity (Figure 4).

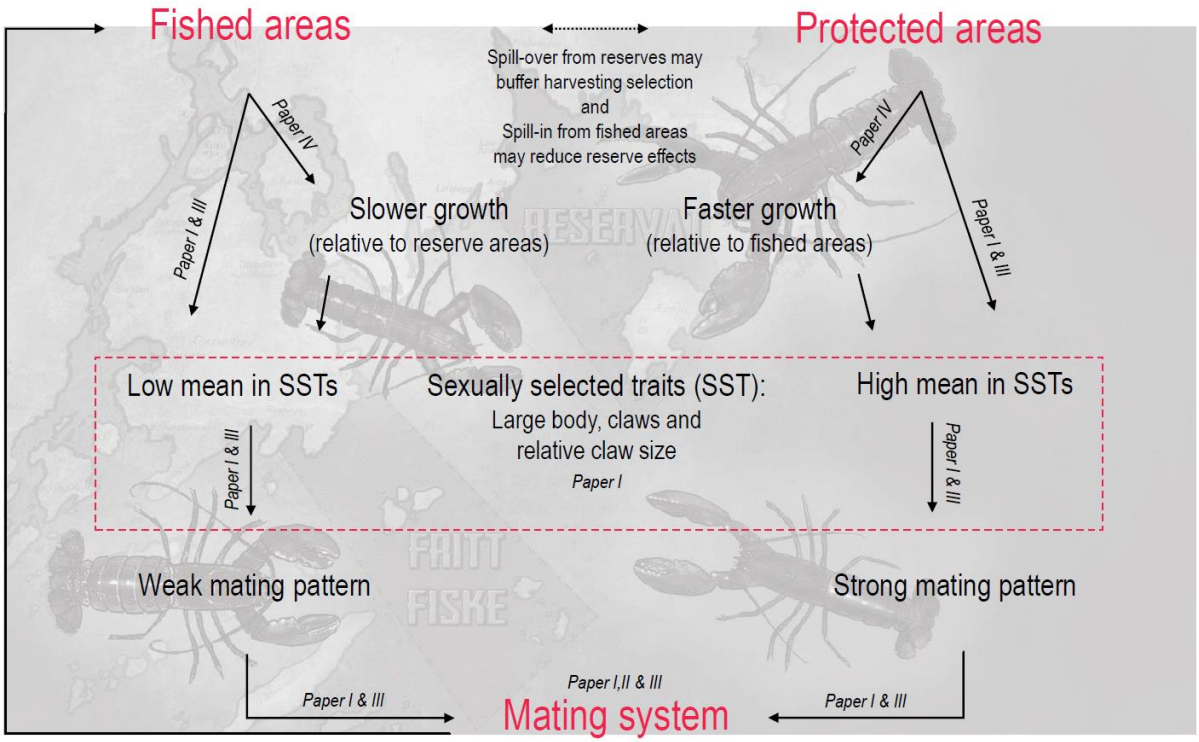

Figure 4. General overview of the main differences in growth and sexually selected traits (SST) between harvested and protected areas (MPAs) and how they interplay with the mating system of lobster. Text in italics refers to the papers from this thesis that address these topics; Female mate choice and determinants of male mating success (paper I), female fertilization pattern in a fished population (paper II), the effect of protection on a secondary selected trait under harvesting selection (paper III) and growth of lobsters in fished and protected areas (paper IV).

In **paper I**, "Harvesting changes mating behaviour in European lobster", paternity data from genotyped males and egg-bearing females confirmed females' preference for males with a body size larger than their own (the 43 known males assigned would almost exclusively be larger). However, the size difference between mated females and males were significantly larger in the reserve (22.5% compared to 6.4% in the fished area). We also found positive selection differentials on male body and claw size inside the reserve, but not in the fished area. These results suggest that when females have the opportunity to choose among a greater diversity of male traits, they mate with large males with large claws. Contrary, the mating pattern is weakened if the population is subjected to selective fishing pressure. When estimating sexual selection gradients on male traits, we found the selection to be acting strongest on relative claw size (claw size relative to body size), rather than on absolute claw and body size. Single paternity on female egg-batches was the prevalent fertilization pattern in both the reserve and fished area, however, two females from the fished area showed evidence of being sired by two different males (2 out of 97 broods analysed). Of males with known identity, eight had mated with more than one sampled female and seven of these males were from the reserve, but they did not differ in size from those with a single mating. Thus, this study present novel empirical support for how fishing affects mating behaviour in wild lobster. The study in **paper II**, "Genotype reconstruction of paternity in European lobsters" was conducted at the same time as the study in paper I, but the results were presented prior to the paternity estimates in paper I. The study was undertaken in a fished region of the coast of United Kingdom (UK) and found no incidence of multiple paternity in any of the 34 egg-bearing females genotyped and concluded single paternity to be the common fertilization pattern in European lobster (at least in this region). However, because only a limited sample of offspring were used in both paper I and II, none of the studies had statistical power to detect contribution from a secondary male with high confidence if the contribution was highly skewed in favour of one male (9:1). Thus, besides the two cases that were discovered by chance in paper I, it is possible that additional cases of multiple paternity were present among the samples in both our studies but went undetected. After large relative claw size was found to be under strong sexual section in paper I, the same trait was later linked to increased risk of being captured in the fishery (Moland et al. *in press*). In **Paper III**, "Marine reserves rescue an important secondary sexual trait in male European lobster", we show that legal-sized male lobsters have larger relative claw sizes inside MPAs compared to equal sized lobster in fished areas (up to 8.4% larger). We did not find any difference in female claw size between MPAs and fished areas, most likely because claws are male-only sexually selected trait. This study is

possibly the first to document the usefulness of MPAs in preserving a trait under strong sexual selection and under ongoing harvest selection in the same study system. In **Paper IV**, "Improved body growth of lobster inside marine protected areas compared to intensively fished areas", we also found substantial support for a positive MPA effect on body growth for legal-sized lobsters. The differences in growth, a combination of moulting probability and moult increment per year, between MPAs and fished areas, increased as lobster became larger in size. The MPA effect was most clear for moult increment of females. Even though MPAs have higher densities, which should increase competition over resources that would expectedly limit individual growth rate, lobster in the protected areas still grow substantially faster than in fished areas. It suggests that catchability of lobsters in the trap fishery may correlate with high growth rate and that lobster undergo intensive and selective fishing pressure against faster growing individuals.

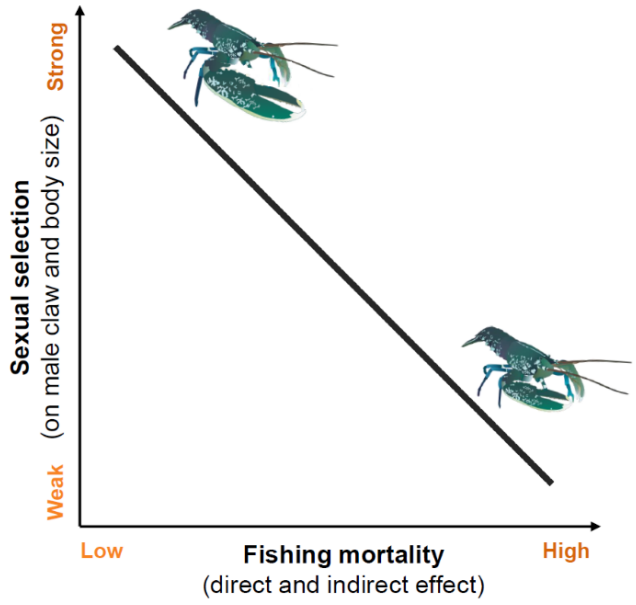

Figure 5. A simplified illustration describing the inverse relationship between fishing mortality and sexual selection and the gradual expression of male claw and body size in European lobster. Fishery driven trait changes are likely to operate under two processes; directly by selective removal of legal sized (larger and faster growing) males with larger claws and indirectly by lower the population density, thus weakening sexual selection on male traits. As fishery selection increases (towards high pressure), sexual selection for larger traits diminishes (towards weak selection). Protected from fishing mortality, MPAs can serve as hot spots for high variance in phenotypic traits and ensure stronger sexual selection (towards strong selection).

This thesis discloses a pattern of two opposing selective pressures that can be described as an inverse relationship; the lobster fishery directly selects and remove large (above legal

size) and faster growing males with larger claws, traits which are sexually selected (Figure 5). In fished areas, the reduced mean and variability in male traits would then leave females with a reduced opportunity for mate choice which results in a weak mating pattern. Additionally, the fishery is also affecting the population indirectly by lowering the density of available mates. The more individuals are dispersed, the lesser the ability of males with desired phenotype to attract and mate with females (Shuster and Wade 2003; Kokko and Rankin 2006), which would further attribute to weaker mating pattern and subsequently diminish sexual selection on male traits. If the fishery continues to target lobster with a higher chance in gaining reproductive success and relaxes sexual selection, fisheries-induced evolution could accelerate even faster than one would expect from fishing pressure alone (Hutchings and Rowe 2008; Urbach and Cotton 2008). In our case, a result from such selection could be smaller and less productive lobster. However, as I also show in this thesis, even small-scale MPAs have positive effects on claw and growth, traits that plays a key role in the lobsters mating system. Allowing females to choose among a broader assortment of males, and males to establish dominance relationships, should strengthen the overall sexual selection and reduce the likelihood for sperm-limitation.

3.2 Future prospects

This thesis highlights the need for a better understanding of how fisheries affect the mating system of exploited species and how we can consider such effects. There is still much we could investigate using this study system. For instance, what are the long-term, even lifetime, fitness benefits and costs of having much larger claws, such as energy expenditure, reproductive success and natural mortality? It would also be interesting to study behaviour around baited traps more closely to gain a better understanding of why some lobster are more prone to capture than others. Lastly, modelling studies could also be helpful to further explore what consequences the different management regulations and spatial protection has on trait distributions, productivity and evolutionary trajectories.

The vulnerability to exploitation differs between mating systems, yet we lack knowledge of mating patterns and mating behaviour, and how they respond the selectivity of fisheries, for most commercially important species (Rowe and Hutchings 2003). Part of the reasons is that studies of natural mating behaviour in marine animals can be a challenging task, especially for the most cryptic species. However, as shown in this thesis, the combination of parentage analysis and replicated MPAs with control sites could be an insightful tool and

should be applicable for many other species as well. For instance, many heavily exploited species have parental care, such as maternal brooding in crustaceans and paternal nest-guarding in fishes (e.g. salmonids, wrasses, sunfish), and thus are easy to sample for paternity analysis. It should be even possible to study the mating behaviour of broadcast spawners with these methods. For example, the coastal cod in Skagerrak have been found to have limited genetic connectivity between local cod populations and has high site fidelity (Olsen *et al.* 2008; Knutsen *et al.* 2011), but parentage studies have even succeeded on a much larger scale on coral reef fishes (Harrison *et al.* 2012).

Intra- and sex-specific variations are often ignored in stock assessments and fisheries research (Hanson *et al.* 2008), but we need to identify sexually selected traits (phenotypes) and measure them so we know if species are caught in a crossfire between sexual and fisheries selection. Many sexually selected traits manifest as morphological dimorphism and are easy to identify, such as body shape in salmon, claws in crustaceans or colours in wrasse and sunfish. Others may be more cryptic, such as mating calls in cod and differential quality in parental care behaviour in wrasses and largemouth bass. We must therefore look beyond body size and conduct in-depth studies when exploring sexually important traits.

Managing fisheries resources is complex and an evolving process. Alternative management regulations reducing or changing size selectivity are often encouraged (Froese 2004; Jørgensen *et al.* 2007; Zhou *et al.* 2010), such as restricting harvest of large individuals through gear modifications (e.g., reducing entrance diameter in traps) or maximum size limit (Zimmermann and Jørgensen 2017). A slot-size limit is now in effect for lobster on the Skagerrak coast when a maximum size limit was introduced as of the 2017 fishing season. Protecting both large and small individuals should increase variation in body size and a wide range of correlated life history traits. However, as other sexually selected traits may succumb to fisheries selection (i.e. relative claw size in lobster), slot size is no silver bullet. Another promising option that should be at the forefront in management and conservation is the establishment of interconnected networks of large and fully protected marine reserves that can function cooperatively by exchanging individuals (and genes). In particular, MPAs as "hot spots" should be able to work synergistically with slot size limits since spill-over of large individuals, likely to possess other attractive characters too, would be protected from fishing and contribute to population productivity outside of reserves. The positive MPA effect identified in this thesis are likely to show only a fraction of the many benefits of protection and I strongly encourage future studies into the depths of behavioural, ecological and fitness consequences of housing species in high densities and in all size and age classes.

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Paper I

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ORIGINAL ARTICLE

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Harvesting changes mating behaviour in European lobster

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Abstract

Removing individuals from a wild population can affect the availability of prospective mates and the outcome of competitive interactions, with subsequent effects on mating patterns and sexual selection. Consequently, the rate of harvest-induced evolution is predicted to be strongly dependent on the strength and dynamics of sexual selection, yet there is limited empirical knowledge on the interplay between selective harvesting and the mating systems of exploited species. In this study, we used genetic parentage assignment to compare mating patterns of the highly valued and overexploited European lobster (*Homarus gammarus*) in a designated lobster reserve and nearby fished area in southern Norway. In the area open to fishing, the fishery is regulated by a closed season, a minimum legal size and a ban on the harvest of egg-bearing females. Due to the differences in size and sex-specific fishing mortality between the two areas, males and females are of approximately equal average size in the fished area, whereas males tend to be larger in the reserve. Our results show that females would mate with males larger than their own body size, but the relative size difference was significantly larger in the reserve. Sexual selection acted positively on both body size and claw size in males in the reserve, while it was nonsignificant in fished areas. This strongly suggests that size truncation of males by fishing reduces the variability of traits that sexual selection acts upon. If fisheries continue to target large individuals (particularly males) with higher relative reproductive success, the weakening of sexual selection will likely accelerate fisheries-induced evolution towards smaller body size.

KEYWORDS

assortative mating, *Homarus gammarus*, marine protected areas, mating behaviour, parentage analysis, sexual selection

1 | INTRODUCTION

Humans depend on healthy ecosystems for valuable goods and services, but human activities are also considered to be one of the strongest selective forces in nature (Palumbi, 2001). For instance, harvesting is virtually always nonrandom and disproportionally removes certain phenotypes from the population (Allendorf & Hard, 2009; Hutchings & Rowe, 2008). Typically, harvesting targets large individuals due to marked preferences or management regulations imposing minimum-size limits (Beamish, McFarlane, & Benson, 2006; Berkeley, Chapman, & Sogard, 2004). There is mounting empirical evidence showing that such size-selective harvesting can drive

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contemporary evolution of life-history traits (Enberg et al., 2012; Heino, Díaz Pauli, & Diekmann, 2015; Uusi-Heikkilä et al., 2015) with consequences for population productivity and persistence (Jørgensen, Ernande, & Fiksen, 2009; Jørgensen et al., 2007; Kuparinen & Merilä, 2007). However, far less attention has been dedicated to the interaction between human-induced mortality and mating systems of exploited populations (Fenberg & Roy, 2008; Lane, Forrest, & Willis, 2011; Rowe & Hutchings, 2003). More so, the potential contribution of male phenotype to populations' reproductive success, and factors underlying variation in the intensity of sexual selection on male traits, remains largely ignored (Uusi-Heikkilä et al., 2015). This paucity of research is surprising for several reasons. First, harvesting tends to select against sexually selected characters, such as the size of weaponry (e.g., horns, antlers and claws) and body size; traits that are generally important in mate choice and intraspecific competition for access to mates (Swain et al., 2007; Wilber, 1989; Woolmer, Woo, & Bayes, 2013). Coltman et al. (2003) demonstrated this effect in bighorn sheep (*Ovis canadensis*) when the harvest of the larger and sexually dominant males for trophies led to artificial evolution towards smaller horn size and a reduction in male body size (Coltman et al., 2003; Pigeon, Festa-Bianchet, Coltman, & Pelletier, 2016). Second, if harvesting alters sex ratios (e.g., Kendall & Quinn, 2012), this will likely influence the opportunity and strength of sexual selection (Kokko, Klug, & Jennions, 2012; Kokko & Rankin, 2006). Third, the strength of sexual selection on fecundity and mating success can be stronger than that generated by natural selection (Kingsolver et al., 2001; Siepielski, DiBattista, Evans, & Carlson, 2011), illustrating the necessity of considering sexual selection when predicting evolutionary rates and trajectories of harvested populations.

To the best of our knowledge, parentage assignment techniques have never been used to directly address how harvesting may potentially disrupt natural processes of sexual selection in the marine environment. Hutchings and Rowe's (2008) modelling work on the Atlantic cod (*Gadus morhua*) showed that if reproductive success increases with body size and harvesting decreases its mean and variation, the overall strength of selection for smaller body size is stronger than expected by fishing alone. Disentangling how harvesting might affect the stability of a mating system is no trivial task, especially in many marine species which are not easily observed in their natural environment. Most studies of sexual selection and mate choice have been limited to controlled environments and model species (Rowe, Hutchings, Skjæraasen, & Bezanson, 2008; Uusi-Heikkilä, 2012), but discrepancy in results between laboratory and field studies underscore the need for more research on mating behaviour in the wild (Lane et al., 2011; Mobley, Abou Chakra, & Jones, 2014). Considering that many commercially fished species are regarded as fully- or overexploited (Worm, Hilborn, Baum, & Zeller, 2009), few locations for these species remain where natural mating dynamics are likely to be intact (Fenberg & Roy, 2008; Rowe & Hutchings, 2003). No-take marine reserves, where population demographic characteristics such as density, sex ratios and size composition are expected to be restored towards baseline conditions (Berkeley et al., 2004; Birkeland & Dayton, 2005), are therefore particularly valuable as reference systems when exploring fisheries effects on mating systems (Butler, Bertelsen, & MacDiarmid, 2015).

We investigated potential effects of harvesting on the mating system of the European lobster (*Homarus gammarus*) by comparing paternity data from a lobster reserve and an adjacent area open to fishing across multiple years. The clawed lobsters, consisting of European lobster and the American lobster (*Homarus americanus*), are long-lived iconic species with high commercial value and therefore subject to intense fishing pressure (Anonymous 1995; Kleiven, Olsen, & Vølstad, 2012). The Norwegian lobster fishery is regulated by closed season, minimum legal size (>250 mm total length, TL) and a ban on the harvest of egg-bearing females (since 2008). Laboratory studies show that when a female is ready to mate, she will seek out a male and preferentially choose a large individual as mate (Bushmann & Atema, 1997; Karnofsky, Atema, & Elgin, 1989a,b; Skog, 2009a). Given that sperm limitation may occur in many crustacean species (Hines et al., 2003; Jivoff, 2003; Kendall & Wolcott, 1999; Kendall, Wolcott, Wolcott, & Hines, 2002; MacDiarmid, Butler, & Butler, 1999; Sato, Ashidate, Jinbo, & Goshima, 2006), females would expectedly prefer to mate with males of similar or larger size to ensure passing of sufficient sperm. In addition, males should also favour large females as egg production increases exponentially with increasing female size (Wahle, Castro, Tully, & Cobb, 2013). Our first objective was to determine to what extent there is a consistent relative size difference between mated pairs in the two areas and whether size-assortative mating—the nonrandom association of body size between mated individuals—exists. Probably because of the disparate conservation regulations between the areas (and sexes due to mandatory return of egg-bearing females in the fished area), the mean size differences between males and females are smaller in the fished area relative to the reserve (Figure 1). We therefore predicted that females should mate with males of smaller sizes in the fished area compared to females in the reserve, thus creating a weaker pattern of size-assortative mating.

Body size has been shown to be under sexual selection in many crustaceans (e.g., Bertin & Cézilly, 2003; Karnofsky & Price, 1989). In clawed lobsters, male–male competition is intense and males fight over shelters and contest dominance (Atema, 1986; Skog, 2009b). Males have relatively larger claws than females (Debuse, Addison, & Reynolds, 2001; Templeman, 1935, 1944) and larger claws increase a male's competitive abilities (Atema & Cobb, 1980; Elner & Campbell, 1981), so claw size should therefore be under strong sexual selection. Thus, our second objective was to estimate and compare the strength of sexual selection, within a breeding season, on two male traits: body size (carapace length, CL) and absolute and relative claw size (width of crusher claws, CW). Aligning with our hypothesis of weaker size-assortative mating in the fished area, we predict that selection differentials, that is, the difference in these mean trait values between successful and unsuccessful males, to be larger in the reserve than the fished area because of the reduced trait variability in the fished area.

Our results contribute to a broader understanding of fisheries-induced evolution by quantifying fisheries-induced changes to mating systems and sexual selection, relevant for developing management tools aimed at mitigating long-term negative impact of selective harvesting. Specifically, we argue that fisheries targeting large males with high reproductive success can lead to a weakening of sexual selection which could further accelerate fisheries-induced evolution towards less productive (smaller) phenotypes.

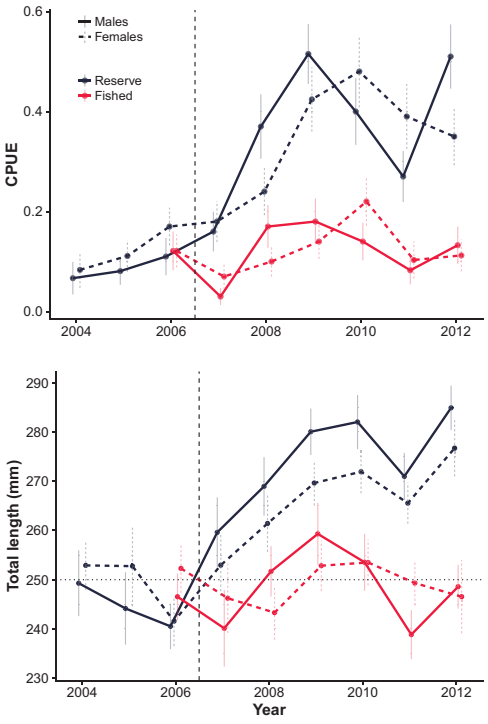

FIGURE 1 Catch and size distribution of the lobster population in reserve and fished area. Mean catch-per-unit-effort (CPUE) of legal sized European lobster (upper panel) and total body length (mm) from the annual research trap survey prior to establishment of the reserve (2004–2006) and after (2006–2013, indicated by vertical stippled line), with reserve in dark grey and fished area in red colour. The error bars depict standard error around the mean. Sex is separated with males in solid line and females in stippled line. The stippled horizontal line denotes the minimum legal size for lobsters in Norway (25 cm)

2 | MATERIALS AND METHODS

2.1 | Study system and lobster sampling

The study was conducted in an area open to fishing and in a designated lobster reserve established in September of 2006, located at the Skagerrak coast in south-eastern Norway (Figure 2). The reserve and the monitored fished area are separated by a distance of ~800 m, and mark–recapture data suggest very little exchange of individuals between the two areas (Thorbjørnsen, 2015). Temporal trends in catch-per-unit-effort and length data from a standardized research trapping survey are presented in Figure 1. Briefly, this annual survey samples lobsters using standard parlour traps set at 5–30 m depth during 4 days in late August/early September. The reserve and fished area are fished with the same effort (100 hauls per year), see Moland et al. (2013) for details. Of the egg-bearing females sampled, 108 were caught from June to September in 2011 and 2012 (60 from the reserve and 48 from the fished area). Because more lobsters are caught in the reserve, seven additional females were obtained from the fished area with help from local fishermen during the ordinary fishing season in October–December 2012 to achieve a balanced sample size in the two areas. Males were fished extensively throughout 2010–2013 from June to December in order to include as many paternal candidates as possible in the parentage analysis. Most of the males were sampled as part of the standardized research trapping survey described above. Additionally, males were sampled when fishing for females in 2011 and 2012 and in conjunction with another study in the fished area in 2011 (Wiig, Moland, Haugen, & Olsen, 2013). Captured lobsters were sexed, measured and individually tagged with externally visible T-bar tags (TBA2, 45 × 2 mm, Hallprint). Claw width and carapace length (CL—rear of the eye socket to the rear of the carapace) was measured to nearest millimetre. A small piece of tissue from the tip of the foremost pleopod was stored in

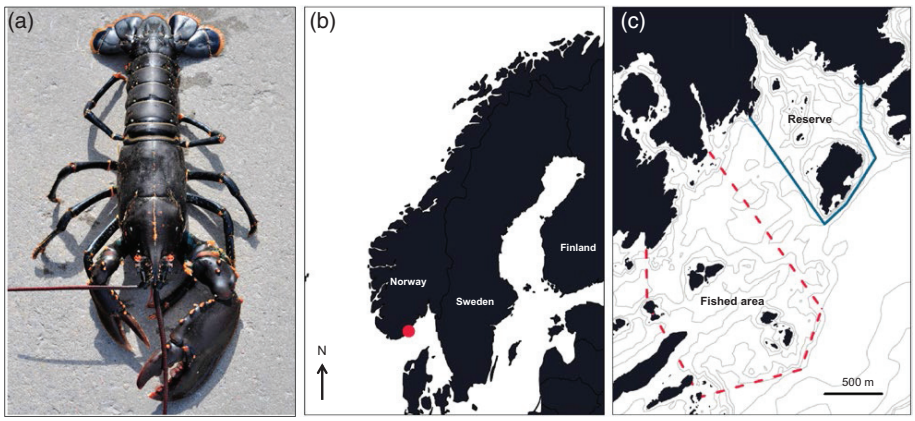

FIGURE 2 Sampling location. Study species, European lobster (*Homarus gammarus*) (a), study area on the Norwegian Skagerrak coast (circular marking) (b) and Flødevigen lobster reserve and fished area (c). Solid line: reserve boundary, stippled line: monitored fished area.

pure ethanol for later genetic profiling. All lobsters were released at the sampling site. Where males were recaptured in successive years, the freshest tissue sample was genotyped to ensure the highest DNA quality.

2.2 | Genetic sampling of female and offspring

Captured egg-bearing females were also measured and individually tagged with T-bar tags. In addition, tissue samples were collected along with samples of offspring, where one egg was randomly sampled at the top of the egg mass near each of the ten pleopods and stored in separate vials with ethanol (a total of ten vials with eggs from each female). Sampling fertilized eggs of each female allowed us to deduce the genotype of the father of each offspring based on the known mother–offspring genotype combination, which should help increase the likelihood of finding the actual fathers when running parentage assignment with the sampled males.

2.3 | DNA extraction and microsatellite genotyping

DNA was extracted from 60 females from the reserve area and 55 females from the fished area, all 650 males sampled ($n_{reserve}$ = 331, n_{fished} = 319), and a total of 1,150 offspring from the 115 females. All individuals were genotyped with ten microsatellite loci developed for European lobster (see André and Knutsen 2009 for primer sequences). The DNA was extracted with E.Z.N.A. Tissue DNA Isolation kit (Omega Bio-Tec inc.) and PCR product amplified on MyCycler ™ (Bio-Rad) using fluorescent-dyed forward primers (Life Technologies). The loci were pooled into one triplex (loci *HGD106*, *HGD111* and *HGC118*), three duplexes (loci *HGC111* and *HGC131*, *HGC129* and *HGA8*, *HGB4* and *HGB6*) and one simplex (locus *HGC120*). Fragment analysis of PCR products was carried out on capillary sequencers CEQ™8000 (Beckman Coulter) and ABI ™ 3130xl (Applied Biosystems) and manually scored using GENEMAPPER v3.7 (Applied Biosystems) and CEQ™ 8000 GENETIC ANALYSIS SYSTEM v 8.0, respectively. As the length of the alleles slightly differed between the instruments, MSATALLELE (Alberto, 2009), a script build on R, was used to bin the scored raw sizes from both fragment analysers and correctly calibrate the results from the two. To control cross-contamination of samples, a negative control was included in each 96-well plates used for PCR and electrophoreses. All candidate males with assigned parentage in the initial analysis were re-extracted and re-amplified to rule out errors. The assigned genotypes were also checked manually three times to minimize scoring errors. Genotypes that could not reliably be solved after three repeat-runs were left as missing. Individuals for which genotypes were missing at five or more loci were considered of poor DNA quality and excluded from further analysis. See Appendix S1.1 for additional details.

2.4 | Genetic analysis

The identity check function in CERVUS v. 3.0.3 (Kalinowski, Taper, & Marshall, 2007; Marshall, Slate, Kruuk, & Pemberton, 1998) enabled us to identify and remove duplicate samples by checking for identical genotype entries. Such duplicates may be due to tag-loss and thus repeated tagging. CERVUS identified 38 duplicated genotypes among the 650 sampled males. When size and expected annual growth were compared in the recapture data for the 38 males, tag-loss was confirmed as the most probable cause in all cases, and these duplicates were subsequently removed from the candidate file.

Genetic variation within samples was estimated for the adult samples only. We estimated the genetic differentiation between the lobster sampled in the reserve and the fished area using Wright's F_{ST}, with Weir and Cockerham's (1984) estimator, θ, in GENEPOP v. 4.5. Allele frequency heterogeneity between localities (years pooled) was tested using FSTAT v.2.9.3.2 (Goudet, 1995). As there was no significant genetic differentiation between the two areas (F_{ST} from 0.000 to 0.002, all $p > .99$), all samples were pooled in subsequent analysis. The fixation index (smallF, F_{IS}) was measured at each locus with FSTAT. One sample *t* test was used to assess whether F_{IS} estimates differed significantly from zero. Pairwise linkage disequilibrium for each pair of loci was tested with a likelihood ratio statistic using the Markov chain algorithm of Raymond and Rousset (1995) in GENEPOP v. 4.5 as well as deviations from Hardy–Weinberg equilibrium (HWE, exact test). All critical significance levels for multiple testing were adjusted with R-package fdrtool, after Benjamini and Hochberg (1995). Finally, genetic diversity estimates including allele number at each locus (N_A) and the theoretical exclusion probability given one parent is known for each locus and combined (EXC) were estimated with GERUD 2 (Jones, 2005). Locus-specific genotyping error rates, allelic dropout (ε_1) and false allele (ε_2), were estimated with a combination of methods (see Table 1 for error rates). For details on descriptive population genetics, and approach and estimation of error rates, see Appendix (S1.2–4).

2.5 | Paternity and multiple mating analyses

Genotypes from seven males were excluded due to missing data (≥5 missing loci), and eighteen females had eggs from which DNA yield was insufficient to allow successful genotyping of the batches. Altogether, a total of 612 males, 97 females ($n_{reserve}$ = 51, n_{fished} = 46) and 967 eggs were used (and pooled) in the final parentage analysis (Table 2). We assigned parentage using COLONY v 2.0 (Jones & Wang, 2010; Wang, 2004), a full-pedigree likelihood program (Markov chain Monte Carlo method) that provides the most probable configuration in assigning sib-ship and parentage among individuals. We allowed both females and males to be polygamous, a prerequisite for testing multiple paternities in regard to both sexes. We accepted only paternities assigned with 95% confidence or higher. This helped minimize false-positive and false-negative assignments and avoided overestimating the level of multiple paternity in the population. Although not all fathers were sampled, COLONY can infer their genotypes from the pedigree analysis to the number of mates to each female and infers the most likely number of fathers contributing to

TABLE 1 Description of loci used in the paternity analysis and error rates

Locus	N_a	H_O	H_E	EXP	Uncorrected *p*-value	F_{IS}	*F* (null)	ε_1 [a]	ε_2 [b]
C118	9	0.619	0.587	0.370	.073	−0.060	−0.031	0.013	*0.010*
D106	9	0.703	0.709	0.494	.431	0.013	0.012	0.012[m]	*0.010*
D111	12	0.645	0.631	0.405	.909	−0.024	−0.012	0.000	*0.010*
C131	13	0.806	0.830	0.669	.231	0.025	0.012	0.012[m]	0.023
C120	19	0.844	0.870	0.745	.001	0.009	0.008	0.023	0.013
C111	9	0.725	0.735	0.529	.017	0.021	0.001	0.001[m]	0.018
A8	14	0.712	0.818	0.661	.000	0.116	0.062*	0.062[m]	*0.010*
B4	9	0.606	0.606	0.399	.000	−0.004	0.001	0.006	0.010
B6	11	0.738	0.818	0.646	.000	0.001	0.044*	0.044[m]	*0.010*
C129	14	0.706	0.779	0.582	.000	0.096	0.040*	0.040[m]	*0.010*
Average	11.9	0.710	0.738	0.999		0.023	0.014	0.021	0.012

Number of alleles N_a and observed (H_O) and expected (H_E) microsatellite heterozygosity for the adult European lobster at Flødevigen area, south-east Norway in 2010–2013. Also given are the expected exclusion probabilities (EXP) of the second parent: the probability of excluding a randomly chosen nonfather when the mother is known, critical *p*-value for HWE test (a = 0.05); F_{IS}, inbreeding coefficient; *F*(null), loci denoted "*"showing null alleles at high frequency, frequency of null alleles; ε_1, allelic drop-out rate; ε_2, false allele rate. The samples are based on 727 (612 males and 115 female) lobsters. EXP and *average* EXP calculated by GERUD2 according to the equations in Dodds et al. (1996).
ε_1 [a] = Allelic drop-out rate estimated from Pedant and Micro-checker, the latter is denoted "[m]."
ε_2 [b] = False allele rate estimated from Pedant. Where Pedant estimated 0.000, 0.010 was implemented in COLONY2, shown in italic.

TABLE 2 Summary results on European lobsters used in the analysis separated in year and area

	2010		2011		2012		Years pooled	
Area	Reserve	Fished	Reserve	Fished	Reserve	Fished	Reserve	Fished
Females								
No. females (No. of offspring)	–	–	42 (420)	27 (269)	9 (90)	19 (188)	51 (510)	46 (457)
Mean carapace length (CV), mm	–	–	96 (0.11)	91 (0.10)	105 (0.15)	94 (0.13)	97 (0.12)	92 (0.11)
No. offspring assigned candidate male	–	–	296 (70%)	89 (33%)	57 (63%)	69 (36%)	353 (69%)	158 (34%)
Males								
No. males (No. of candidate assigned)	98 (20)	80 (5)	148 (11)	111 (8)	28 (5)	96 (5)	274 (36)	287 (18)
Mean carapace length (CV), mm	104 (0.18)	90 (0.13)	95 (0.17)	88 (0.15)	101 (0.19)	88 (0.16)	99 (0.18)	88 (0.15)
Mean claw width (CV), mm	58 (0.25)	46 (0.18)	51 (0.24)	44 (0.21)	55 (0.32)	44 (0.22)	54 (0.26)	45 (0.20)
St. Selection diff* carapace (*p*-value)	**0.80** (<0.01)	−0.37 (0.53)	**0.45** (0.01)	0.29 (0.33)	**1.66** (0.06)	0.43 (0.37)	**0.78** (<0.01)	0.16 (0.48)
St. Selection diff* claw width (*p*-value)	**0.94** (<0.01)	−0.25 (0.67)	**0.67** (<0.01)	0.22 (0.45)	**1.66** (0.08)	0.7 (0.19)	**0.93** (<0.01)	0.22 (0.34)

For females, number of females and number of offspring in parentheses, mean carapace length in mm with corresponding coefficient of variation (CV), the number and percentage of offspring assigned candidate males. For males, number of candidates and assigned males in parentheses, mean carapace length and crusher claw width in millimetres with corresponding coefficient of variation (CV), standardized selection differentials (diff*) for body size and claw width with confidence value (*p*-value) in parentheses. Significant selection differentials are in bold. Only paternity assigned at 95% confidence is reported and counts the number of matings by known males, including males that have mated with multiple females and hence appear more than once in the counts.

each batch. Where COLONY inferred more than one sire in a batch, visual inspection of genotypes and changes made by COLONY based on the error rates, helped minimize an overestimation of multiple paternity cases (due to for example contamination from mother's DNA, multiple reconstructions of alleles suggested with almost equal probabilities). Inferred multiple paternities were only accepted

as true cases of multiple paternity if offspring differed from the first male at five or more loci, did not show sign of scoring error (mismatching mothers genotype) and if the loci in question had not been calculated by COLONY due to missing alleles. The input files in COLONY were set up with two replicate runs and analysed with the highest precision settings with full-likelihood, and with very long runtime on a PowerEdge M820, Linux CentOS 6.7 machine. For more details on the settings used, see Appendix (S1.3).

The probabilities of detecting multiple paternal contribution (PRDM) were quantified using the software PRDM (Neff, Pitcher, & Repka, 2002). PRDM uses Monte Carlo simulations to calculate PRDM under various scenarios of skew between the fertilization contributions of multiple males based on population allele frequencies. When determining the frequency of multiple sired batches, we inspected the results that were flagged as cases of multiple paternity by COLONY along with the original genotype data. This is because COLONY can alter loci in accordance with error estimates and propose alleles in cases where genotypes are missing and that could overestimate multiple paternity cases. The offspring batches would only be resolved as cases of multiple paternity if the genotype of an offspring could not be resolved by the first male by at least five loci, did not show evidence of contamination/amplification issue or had loci altered by COLONY.

2.6 | Size-assortative mating

We first compared the overall size of females and males in the whole data set in both areas with a two-tailed *t* test. For analysing the size relationship between mated pairs, we had to account for the fact that lobsters were captured across several mating seasons. The majority of females have a biennial reproductive cycle, whereby spermatophores received during mating are stored for 9–11 months and used to externally fertilize eggs prior to incubation for a similar duration, after which they moult and remate (Agnalt, Kristiansen, & Jørstad, 2007; Aiken, Mercer, & Waddy, 2004). Thus, the egg-bearing females with newly extruded (black) eggs sampled in 2011 and 2012 most likely mated some time in 2010 and 2011. The sampling year of inferred fathers differed from the sampling year of the female in most of the mated pairs (70%). For these pairs, it was necessary to estimate male size for the time at which corresponding females were sampled prior to analysing size-assortative mating patterns. To this end, we used mark–recapture data from the reserve and fished area from 2004-2016 and extracted males that had been captured in two consecutive years at any point within this period. First, we estimated the probability of moulting as a function of carapace length at the first capture with a logistic regression. We inferred that moulting had occurred if the size difference was 5 mm or higher from the previous year; smaller differences were assumed to be measurement errors (Agnalt et al., 2007). We then estimated the yearly growth increment as a function of carapace length at the time of first capture for individuals who had moulted, using a linear regression. The predicted values from these two models were included in the calculation of adjusted carapace length for males with mating success using the following formula:

$$CL_{adjusted} = CL_{measured} + (Year_f - Year_m) \times \hat{g} \times \hat{p}_{moult} \quad (1)$$

where CL is the male carapace length (mm), Year is the year of sampling for males (m) and females (f), and $\hat{g}$ and $\hat{p}_{moult}$ are the estimated yearly growth increment (in mm) and probability of moulting, respectively, as predicted from $CL_{measured}$ using linear and logistic regression. The model predictions showed that almost all males below 90 mm CL (the minimum legal size) moulted annually and that the probability of moulting decreases to below 0.75 in larger sizes classes (>113 mm CL). The overall probability that males of all sizes would moult once every year was >0.5 (see Appendix S2, Figure S1).

With the adjusted male sizes, we used a linear model to test for assortative mating (a positive correlation between female and male body size in mated pairs) and tested whether such patterns differed between areas, comparing models with Area x female size – interaction (Equation 2) against a model with only an additive area effect with the likelihood ratio test.

$$CL_{male} = CL_{female} + Area + CL_{female} \times Area \quad (2)$$

We excluded six putative matings between mates sampled in different areas. Males were duplicated in the data file if the same male has mated with multiple females.

2.7 | Selection on male traits

Standardized selection differentials on male body size and claw size were calculated, subtracting the mean trait value of potential fathers from the mean of successful fathers in each area (Arnold and Wade 1984). The size of maturity for males is not known in this population, but to reduce the probability for including immature males among potential fathers, only males with 80 mm or larger CL were included in the selection differential calculations. Size at maturity for European lobster in Scotland has been estimated to 80 mm CL for males and 79 CL for females (Lizárraga-Cubedo, Tuck, Bailey, Pierce, & Kinnear, 2003). However, the smallest berried female in our sampling was 73 mm CL, compared to 82 mm in the Scottish study; thus, we consider a potential father threshold of 80 mm CL and above to be conservative and appropriate for our study system. Prior to calculations, trait values were mean-centred and scaled to a standard deviation of one in each area–year combination (Lande & Arnold, 1983). Significance of selection differentials was assessed with two-tailed t tests. Also, a linear model was used to compare the body size (CL) of males that were successfully assigned and, thus successfully sire offspring, to males that had not.

$$CL_{male} = Assigned + Area + Year + Assigned \times Area \quad (3)$$

Of interest was whether the difference in mean trait value between successful and unsuccessful males would be larger in the reserve (a significant interaction effect between area and assignment). Year was included as an additive effect in the model to account for variable trait distribution among sampling years. To test whether the proportion of males assigned differed between areas, we used univariate generalized linear models for each year (2010–2012), where

Assigned (0, 1) was the binomial response variable and Area the predictor.

Because males were sampled over three seasons and females in two, the aforementioned selection differentials may not reflect pure sexual selection, as mortality (both fishing and natural) would evidently determine the prospects of obtaining mating success in the different years. Although we maintain that sexual selection is likely to be the primary mechanism underlying these selection differentials, we also conducted a more specific analysis of sexual selection, where we included only males sampled in 2010 because they represent the population at the time of reproduction and also had a sufficient number of assigned paternities (in following year) to warrant further analyses (2010: 24 out of 245; 2011: 5 out of 272). We estimated standardized selection gradients, which capture the sensitivity in the fitness function when trait values change, and therefore better represent the strength and shape of selection than selection differentials alone (Kingsolver, Diamond, Siepielski, & Carlson, 2012; Matsumura, Arlinghaus, & Dieckmann, 2012). Selection gradients were estimated from logistic regressions (Janzen & Stern, 1998) on male body size (CL) and claw size (CW), with mating success (*s*) as the response variable (0 or 1). Claw width and body size were strongly correlated traits (r = .90) and could therefore not be included in the same model due to high collinearity (Lande & Arnold, 1983; Zuur, Ieno, & Elphick, 2010). Thus, to include both traits, we extracted the residuals from the linear regression between claw width and carapace length and used the residual claw size, which is then a measure of relative claw size (CW_{res}), as covariate together with CL. We also fitted a model only including relative claw size (CW_{res}). To evaluate whether trait-fitness relationships differed between areas, we also included models testing for an interaction effect with area for each of the traits (CL, CW and CW_{res}) using the following model structures:

$$\text{logit}(s) = \text{CL} + \text{Area} + \text{CL} \times \text{Area} \quad (4)$$

$$\text{logit}(s) = \text{CW} + \text{Area} + \text{CW} \times \text{Area} \quad (5)$$

$$\text{logit}(s) = \text{CL} + \text{CW}_{\text{res}} + \text{Area} + \text{CW}_{\text{res}} \times \text{Area} \quad (6)$$

We also explored whether the data supported stabilizing (i.e., nonlinear), rather than directional (i.e., linear) selection on male size, as recent studies have shown that male mating success might be highest for intermediate sized males (Uusi-Heikkilä, Kuparinen, Wolter, Meinelt, & Arlinghaus, 2012). For this, we ran models including a squared term for absolute size (body or claw), exemplified for CL below:

$$\text{logit}(s) = \text{CL} + \text{CL}^2 \quad (7)$$

All selection gradient models (full and reduced) were compared with the Akaike information criterion, corrected for small sample size, which was used to determine the most parsimonious model. We estimated approximate selection gradients ($\beta_{avggrad}$) for each trait with the Janzen-Stern logistic regression approach (Janzen & Stern, 1998). Mean standardized selection gradients on claw and body size were calculated by multiplying $\beta_{avggrad}$ by the trait value's mean and dividing by its standard deviation (Matsumura et al., 2011). The mean standardized selection gradient is recommended for comparing strength of selection across studies but is not applicable for trait such as relative claw size, which has no natural maximum and minimum value (Hereford, Hansen, & Houle, 2004 and Matsumura et al., 2011). All statistical analyses were performed in R 3.2.4 (R Core Team, 2016).

3 | RESULTS

3.1 | Lobster samples and population genetics

The proportion of loci typed over all individuals was 0.946 (adults and offspring; 0.983 and 0.934 respectively, see Appendix S2, Table S1 for females and eggs analysed) and the genetic diversity was high across all loci (H_E = 0.738; Table 1). The number of alleles per locus ranged from 9 to 19, and the observed heterozygosity ranged from 0.606 to 0.844. We estimated the combined exclusion probability to be .9998 given a known maternal genotype, indicating sufficient power to distinguish between two randomly selected candidate males (though the effect of error rates is not accounted for in the estimation, equation from Dodds, Tate, McEwan, & Crawford, 1996). No parentage was assigned to males sampled in 2013, so all males from this year were removed from further analysis, reducing the number of males to 561 (Table 2). See Appendix (S1.3–4) for additional details.

3.2 | Mating patterns

A total of 511 (52.8%) offspring were assigned a known father (Table 2). We assigned eggs from 54 females to one of 43 males (7.7% of the 561 candidate males) with high confidence. Of those 54 known matings, 36 (66.7%) involved 27 males from the reserve. Assignment probability differed between the areas in 2010, assigning 19.2% of the (total number of) matings to males in the reserve and 6.4% to males in the fished area (GLM: β = 1.376, t = 2.384, df = 154, p = .017). COLONY inferred genotypes from 41 unsampled males that sired offspring with 42 females. There was little exchange of individuals across area boundaries, although five females (reserve = 3, fished = 2) had mated with males from the opposite area. Two of these interarea pairs involved a large male from the reserve, estimated to have been ~140 mm CL at the time of mating.

Colony initially flagged 24 of the broods to be cases of multiple matings, but after inspecting the assignment results, we concluded that most of the broods probably were sired by one male only because of lack in support of a second sire. However, two (2.0%) of the broods showed evidence of being sired by a second male and therefore concluded to be multiply mated females. The paternal contribution among the multiply mated females was highly skewed in favour of a primary male (9:1 ratio) in both these cases (see Appendix S2 in supplementary information, Table S1). The power to detect multiple paternity with only ten offspring genotyped at ten loci exceeded >99% confidence assuming equal contribution. We could also detect a skew down to 70:20:30

(three sires) with a confidence of more than 95% using nine offspring; however, the skew in favour of a primary male observed in the results (9:1) could only be detected with a 65% confidence. This suggests that, in addition to the two confirmed cases of multiply mated females, some of our single mated females may in fact also be multiply mated.

Of the inferred males with known identity, eight (reserve = 6, fished = 2) had mated with more than one female, of which five had mated with two females and three had mated with three females. Polygamous males were not significantly larger than males with only one recorded paternity (GLM: β = 5.956, *t* = 1.333, *df* = 53, *p* = .188). On average, the level of polygamy was higher for males, with females mating with 1.01 males and males (known and unknown) mating with 1.16 females.

3.3 | Size-assortative mating

Across all sampling years, females were larger than males in the fished area (Table 2; *t* test: *t* = 2.12, *df* = 68.6, *p*-value = .037), but not in the reserve (Table 2; *t* test: *t* = −0.57, *df* = 98.5, *p*-value = .57). Interarea pairs (*n* = 5) were removed prior to analysing the area-specific size-assortative mating pattern (see Appendix S2 in supplementary information, Figure S2). In the reserve, all but two pairs (2 out of 34) consisted of a larger male mating with a smaller female, with an average size difference of 22.5% (*t* test, *t* = 6.1799, *df* = 48.27, *p* < .0001). Females in the fished area also paired with males of larger sizes, as all but three of the 15 pairs had a male larger than the female, with the average size difference smaller (6.4%) and marginally statistically significant (*t* test: *t* = 2.034, *df* = 28.35, *p* = .051). There was a strong positive size-assortative mating pattern (GLM: β = *0.838*, *t* = 3.560, *df* = 46, *p* = .0009, multiple R^2 = .50, Figure 3). An additive area effect was supported over an interaction effect (LRT; χ^2 = 1.479, *p* = 0.224), with females mating with larger males relative to their own size in the reserve compared to the fished area (GLM: Area: β = 17.65, *t* = 3.722, *p* = .0005).

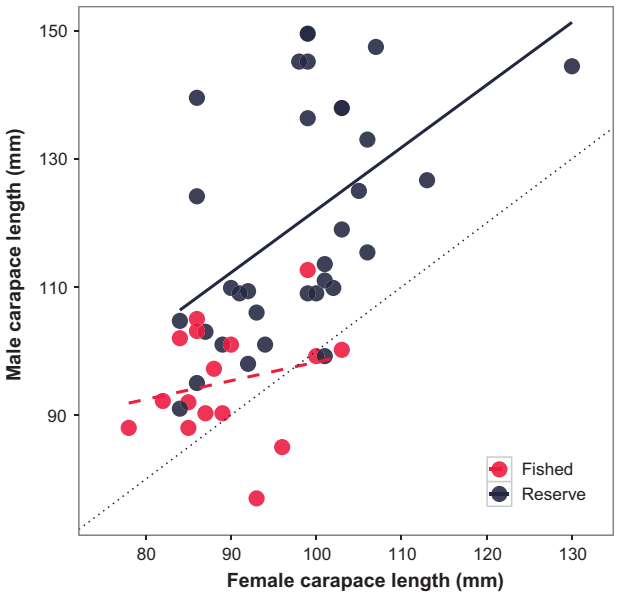

FIGURE 3 Size-assortative mating. The relationship between body size (carapace length) of male (corrected sizes, see Materials and methods) and female European lobster that formed pairs (*n* = 51) in fished (red) and reserve (dark grey) area in the four-year period. Interarea pairs are excluded. Area represents female capture point and male CL is adjusted according to the year of the mating event. Value 1.0 and black stippled line (isometry, *Y* = *X*) marks where females and males are equal in size

3.4 | Sexual selection

Across all sampling years, selection differentials on body size (CL) and claw size (CW) were significantly positive in the reserve, while they were more variable and nonsignificant in the fished area (Table 2). Correspondingly, the standardized trait difference between successful and unsuccessful males was larger in the reserve for both body size

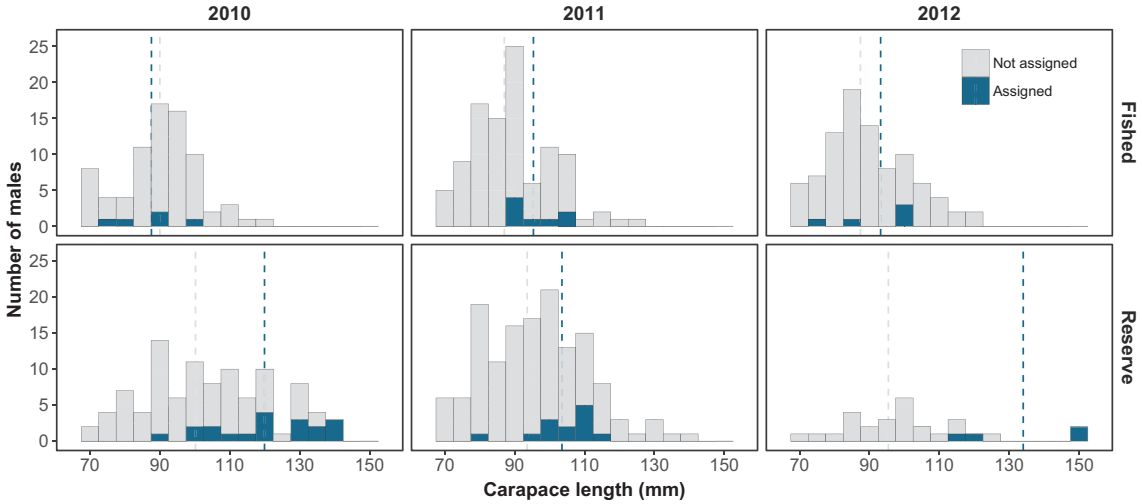

FIGURE 4 Males with parentage. Length distributions (carapace length, CL in mm) of male European lobsters with (blue) and without (light grey) confirmed assignment in the four sampling years. Vertical lines indicate mean lengths in each group

TABLE 3 Model selection

Model number	Structure	P	AICc
1	CL + CW_{res}	3	**128.60**
2	CW_{res}	2	129.98
3	CL + CW_{res} × Area	5	132.26
4	CW_{res} × Area	4	134.06
5	CW	2	135.24
6	CW × Area	4	138.18
7	CL	2	143.97
8	CL × Area	4	147.65
9	Null	1	151.04

Logistic regression modelling on selection of male European lobster from 2010 using reproductive success as the response variable. P, number of parameters; AIC, Akaike information criterion score. Explanatory variables (standardized): CL, carapace length; CW, claw width; area, reserve and fished; $CW_{res,}$ relative claw size (residuals from claw body size regression). The model with lowest AIC is indicated in bold.

and claw size (GLM: parentage × Area; CL: $\beta = 0.76$, $t = 2.44$, $p = .02$ and CW: $\beta = 0.88$, $t = 2.85$, $p = .005$, Figure 4). The subset of data (2010 mating season) used for estimating sexual selection gradients did not support an area effect on mating success (Table 3). Instead, a model containing only additive effects of body size and residual claw size on male mating success had the lowest AIC_c score and therefore the most support (Table 3). A simpler model excluding the effect of body size also received some support (Table 3). Using the most parsimonious model for inference, sexual selection was positive on body size and strongly positive on residual claw size (Table 4). For comparison, univariate selection gradients were significantly positive on all three traits and supported over more complex models including a squared term representing stabilizing or disruptive sexual selection (Table 4, Figure 5).

4 | DISCUSSION

We investigated the mating system of the exploited European lobster in its natural environment inside and outside a coastal marine reserve to establish whether harvesting can affect mating patterns and sexual selection. Our genetic parentage assignment clearly demonstrates a positive size-assortative mating pattern, where females have a strong disposition to mate with comparatively larger males. Moreover, we show that this within-pair size difference was larger in the reserve than in the fished area. We also documented that male size (body and claws) strongly influenced their mating success inside the reserve, while selection differentials on these traits were weaker and not significant in the fished area. Sexual selection was stronger on relative claw size, rather than on absolute claw and body size. Overall, our findings suggest that fishing can greatly affect mating patterns, with potential consequences for reproductive output and the rate and trajectory of fisheries-induced evolution.

4.1 | The effect of fishing on mating patterns

Having been afforded protection from fishing for almost a decade, lobsters in the reserve might display a good depiction of what can be considered more "natural" mating behaviour, given that females have access to a wider diversity of male phenotypes. Therefore, the increased scope for sexual selection on male traits is the likely explanation for the higher positive selection differentials in the marine reserve relative to the fished area. Although females in the fished area tended to mate with males larger than themselves, the average difference in body size between sexes was much smaller (♂ > ♀; 6.4%) than in the reserve (♂ > ♀; 22.5%). These results are in line with those for wild-mated female American lobsters obtained by Gosselin, Sainte-Marie, and Bernatchez (2003), who found a positive size-assortative mating pattern in larger females caught in an area of moderate fishing pressure, but a random mating pattern in a site more heavily fished. In the fished area, the lower density of lobsters and the fact that females were about the same size as males in this area imply that females would have more difficulties finding a larger mate. When individuals are more sparsely distributed, sexual selection is likely to be relaxed through lower encounter rates between mates and competitors behaviour(Arnqvist, 1992; Conner, 1989). Further, high fishing mortality of large lobsters should free up more good shelters than would typically be available to smaller males, whose

TABLE 4 Sexual selection estimates

Model no.	Trait	β	*SE*	*z*-value	*p*	$\beta_{avggrad}$	β_{μ}
1	CW_{res}	1.320	0.394	3.350	<.0001	0.965	–
	CL	0.424	0.227	1.868	.06	0.310	–
2	CL	0.609	0.201	3.033	.002	0.512	3.555
3	CW	0.835	0.203	4.111	<.0001	0.657	3.039
4	CW_{res}	1.544	0.391	3.949	<.0001	1.170	–

Sexual selection operating on body size and relative claw size in male European lobster sampled in Flødevigen during 2010. For each trait, the table gives Janzen-Stern logistic regression coefficients (β) and their corresponding standard error (*SE*), *z*- and *p*-value, the approximate selection gradients ($\beta_{avggrad}$) and the mean standardized selection gradient (β_{μ}). Traits of interest are carapace length (CL), claw width (CW) and residual claw width (CW_{res}), where residuals from the linear regression between carapace length and claw width are used as a proxy for claw size relative to body size. All traits were scaled to a standard deviation of 1 and mean-centred prior to analysis.

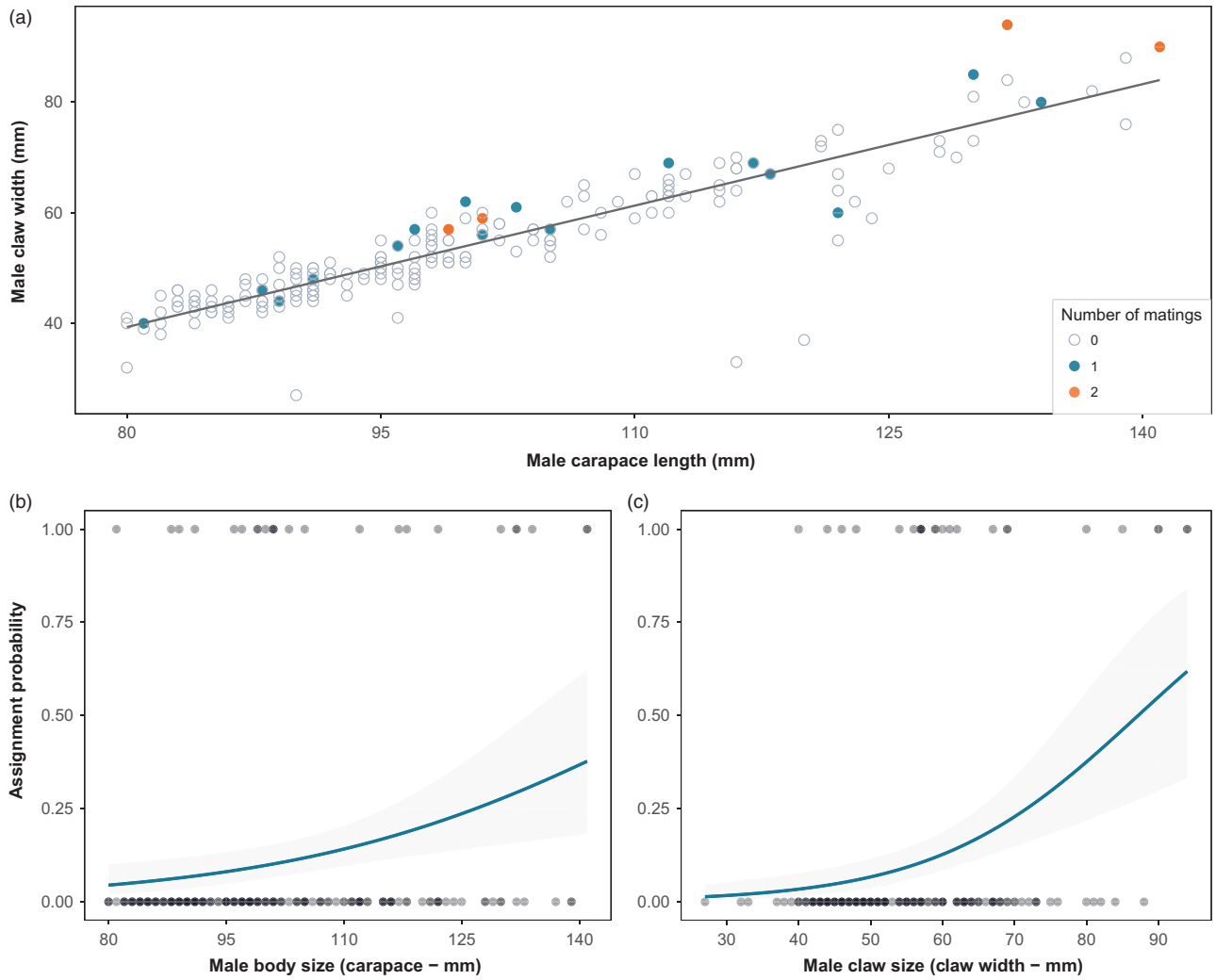

FIGURE 5 Sexual selection on traits in male European lobsters. (a) Correlation between male carapace length and claw width among males sampled in 2010. Filled coloured circles are showing number of matings (0, 1 and 2) for each male. These residuals were used to estimate sexual selection on relative claw size (Table 4). (b) Probability of mating success as a function of body size among 2010 males (Model 5, Table 3), and (c) probability of mating success as a function of claw size among 2010 males (Model 8, Table 3)

occupancy of better shelters ought to increase their chances with females (Atema, 1986; Cowan & Atema, 1990; Debuse, Addison, & Reynolds, 1999, 2003).

High fishing mortality could also explain the lower assignment probability for males in the fished area in 2010, as a male should have lower chances of surviving and successfully mate the following year, relative to a similar sized male in the reserve. This implies that the selection differentials may not be purely due to sexual selection but may also reflect an unidentified component of fisheries selection against large individuals. Arguably, results from this work show that selective harvesting is indeed affecting mating patterns, but we only compare the trait distributions of males with and without paternity on the broods (females) sampled. The consequences for true fitness and selection can therefore only be inferred. However, we find no reason to assume different trait distributions between the observed and unobserved fathers.

Contrasting no-take reserves and complementary monitored control areas where harvesting continues as usual may be one of the best options available to study the effects of harvesting regimes in situ. To test the generality of the findings, and indisputably attribute spatial variability in ecology to that in fishing pressure, the approach should be tested using multiple pairs of reserves and fished areas. Moreover, temporal replicates, tracking several selection episodes and ideally also including natural and fisheries selection, could be used to estimate life time fitness and to test individual consistency and temporal stability in sexual selection.

4.2 | Drivers of sexual selection in clawed lobsters

Female choice appears to play an important role in driving the positive assortative mating pattern; some of the largest males had mated with small females, while the largest females never mated

with small males. Females can have a direct benefit from choosing larger males. First, female clawed lobsters usually moult in a males' shelter, where she will mate soon after and cohabitate for some time, a strategy believed to increase successful pre- and post-copulatory guarding of the soft-shelled female (Atema, Jacobson, Karnofsky, Oleszko-Szuts, & Stein, 1979; Cowan & Atema, 1990; Karnofsky & Price, 1989; Karnofsky et al., 1989a,b). Secondly, large male decapods have greater sperm reserves, are capable of tailoring ejaculate load to the size of the female and replenish depleted sperm faster than smaller males (Gosselin et al., 2003; Jivoff, 1997; Kendall, Wolcott, Wolcott, & Hines, 2001; MacDiarmid et al., 1999). Thus, the narrow time window of receptiveness to mate, the need for protection during moulting and sperm quantity are plausible reasons for females to choose larger males. On the other hand, males could be reluctant to mate with smaller females if this comes at the cost of lower mating opportunities with a larger, more fecund female due to the time-out period of the mating event. Nevertheless, it is reasonable to assume that male lobsters are less choosy than females, since an intermoult male can produce sperm all-year-round and is able to inseminate multiple females within a breeding season (Waddy et al., 2017).

The univariate selection gradients (mean standardized) on both claw (3.04) and body size (3.56) were relatively high, both being well above the median (1.93) calculated from 140 published estimates (Hereford et al., 2004). Interestingly, relative claw size appears to be the trait driving sexual selection in male lobsters, along with weaker selection on body size according to the most parsimonious multivariate model. Sexually selected structures like claws, with dual function of combat and display, are likely to be honest signals of male quality to competitors and choosy females (Berglund, Bisazza, & Pilastro, 1996; Grafen, 1990). Relative claw size might therefore be a better measure of male quality than absolute claw and body size, which could simply be due to chance survival to old age. Fitness benefits accruing to large males with relatively large claws are well documented in Fiddler crabs, where large-clawed males win more competitions and attract more females than small-clawed males (Christy, 1983; Oliveira & Custodio, 1998; Pratt & McLain, 2002). In both European and American lobster, larger claws are found to increase male competitive abilities and to be a better predictor of victors than body size (Atema & Cobb, 1980; Elner & Campbell, 1981; Van Der Meeren & Uksnøy, 2000). Note that we did not find support for stabilizing selection on body and claw size, implying that also very large individuals maintain high male–male competitiveness and/or female attraction.

4.3 | Multiple matings and sperm limitation

Single paternity on female broods was the prevalent fertilization pattern, but two females caught in the fished area had evidence of being sired by two different males (2 out of 97 broods analysed). In contrast, a recent study in a region of the United Kingdom found no incidence of multiple paternity in the European lobster (Ellis et al., 2015). Both cases found in our study had contributions highly skewed in favour of a primary male and only a single offspring from each brood deviated from the other nine siblings. Multiply-sired crustacean broods have often shown to have high level of paternal skew (e.g., Bailie, Hynes, & Prodöhl, 2011; Streiff, Mira, Castro, & Cancela, 2004; Yue et al., 2010). However, because of our method with only a limited sample of offspring, we did not have statistical power to detect a secondary parental sire of 9:1 skew with high probability. Thus, besides the two cases that were discovered by chance, it is possible that additional multiple sired broods were present among our single sired broods but went undetected.

Multiple paternal fertilizations have been documented in American lobster populations and linked to sperm limitation due to fisheries-induced sex ratio imbalance (Gosselin, Sainte-Marie, & Bernatchez, 2005). Whether it is cause for concern for our European lobster remain unknown, but the finding that females mate with relatively smaller males (presumably with lower sperm storages) in the fished area indicates that the likelihood of sperm limitation is present. As for males, we found eight individuals with known identities that had mated with more than one sampled female, but they did not differ in size from those with a single mating. Seven of these males came from the reserve, where the higher population density suggests increased opportunities for males to monopolize and mate with multiple females (Kokko & Rankin, 2006; Shuster & Wade, 2003).

4.4 | Implications for fisheries-induced evolution and management perspectives

When mating is nonrandom for traits under opposing harvest selection (e.g., when larger males are both preferred by females and targeted in fisheries), a reduction in mean and variability in these traits due to fishing is expected to lead to faster harvest-induced evolution than under the assumption of random mating (Hutchings & Rowe, 2008). To our best knowledge, our study on European lobster provides the first empirical support for weakened sexual selection due to fishing. If fisheries continue to target individuals (particularly males) with higher relative reproductive success, the weakening of sexual selection will likely accelerate fisheries-induced evolution towards smaller and less productive body size.

Despite the potential ramifications for rates of fisheries-induced evolution, sexual selection tends to be left out of the equations in studies assessing this subject, with potential consequences for their conclusions (Hutchings & Rowe, 2008; Urbach & Cotton, 2008). The reason could be that obtaining data for estimating sexual selection is often more challenging than for natural and fisheries-induced selection. In spite of this, we encourage inclusion of a sexual selection component in future studies of fisheries-induced evolution because the genetic variation underlying sexually selected characters may be much higher than for nonsexually selected traits (Pomiankowski & Moller, 1995). Therefore, we may anticipate stronger evolutionary effects than on other phenotypic traits (Urbach & Cotton, 2008).

A general objective in an evolutionarily enlightened management framework should be to minimize harvest-induced evolution

and loss of adaptive potential in populations (Jørgensen et al., 2007). Accounting for evolutionary processes in management can potentially increase long-term yield, the resilience to population collapse and ecosystem stability (Zimmermann & Jørgensen, 2017; Mollet, Poos, Dieckmann, & Rijnsdorp, 2016). Fishing that can maintain or increase the variability of sexually selected traits (that correlate genetically with body size) are predicted to slow evolution towards smaller body size relative to the scenario of random mating (Hutchings & Rowe, 2008; Uusi-Heikkilä, Lindström, Parre, Arlinghaus, & Kuparinen, 2016). This may be achieved by changing the selectivity of fishing, such as restricting harvest of large individuals through gear modifications (e.g., reducing entrance diameter in traps) or maximum size limit/harvest slots (Hutchings and Fraser 2008, Zimmermann and Jørgensen 2017). A shift in management towards protection of large individuals can also restore age and size structure and balance sex ratios (Birkeland & Dayton, 2005; Tiainen, Olin, Lehtonen, Nyberg, & Ruuhijärvi, 2017; Halvorsen, Sørdalen, Durif, & Vøllestad, 2016), which should have positive effects on populations productivity and environmental resilience (Arlinghaus, Matsumura, & Dieckmann, 2010; Gwinn et al., 2015; Matsumura et al., 2011).

Long-term overfishing has left the European lobster in Norway at a historically low level and profoundly diminished the prospects of individuals reaching a high age or large size. Thus, fisheries-induced evolution may have already left considerable footprints. For the 2017 fishing season (starting 1 October), a maximum size limit of 320 mm total length (~116 mm CL) was implemented for lobster caught along the Skagerrak coast. As for the benefit of spatial management, a handful of small reserves established along the coastline are unlikely to have any strong effects on the evolutionary trajectory. If, however, the number and size of reserves are increased, with sexual selection recovering within, the potential for increased reproductive output from large females (mated with large males) and spill-over of larger, more "attractive" males from the reserves could possibly strengthen sexual selection and buffer fisheries-induced evolution in fished areas (see also: Baskett and Barnett 2015).

In conclusion, our paper presents novel empirical support for how fishing affects mating behaviour in wild European lobster. Selective fishing reduces the phenotypic variability for sexual selection to act upon, but at the same time, the strength of sexual selection may be relaxed through lowered density and biased sex ratio. Sexual selection is an integral part of evolution and should therefore be mandatory to consider in evolutionary enlightened management.

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CONFLICT OF INTEREST

None Declared.

DATA ARCHIVING STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.1b1f023.

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SUPPORTING INFORMATION

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Supplementary information S1: methods and results

S1.1 DNA extraction and genotyping

The DNA was extracted with E.Z.N.A. Tissue DNA Isolation kit (Omega Bio-Tec inc.) using columns. The procedure followed the manufacturers guidelines, only deviating by preparing HiBind DNA mini columns with equilibration buffer (100 µl) and ddH_2O (100 µl) separately. One offspring sampled from each pleopod was chosen (a total of 10 per female), crushed, and placed in a separately labelled tube. Pleopod tissue sampled from the female and male was cut into pieces of 30 mg before being digested overnight. To mitigate the low DNA concentration in some of the offspring samples, we eluted each sample in 50 µl buffer.

We carried out PCR amplifications on MyCycler ™ (Bio-Rad) using fluorescent- dyed forward primers (Life Technologies). One microliter of DNA was used for the PCR amplification, for a 10-µL total reaction volume, in one triplex (loci *HGD106*, *HGD111* and *HGC118*), three duplexes (loci *HGC111* and *HGC131*, *HGC129* and *HGA8*, *HGB4* and *HGB6*) and one simplex (locus *HGC120*). Cycling condition for the triplex and duplex (*HGC111* and *HGC131*) included an initial 5-min phase of denaturation at 95℃, followed by 35 cycles of 95, 56, and 72℃ for 30, 60, and 60 s, respectively, ending with a final elongation step at 72℃ for 15 min. Cycling condition for the simplex deviated by reducing the primer annealing and sequence extension to 30 s, while for the two duplexes (*HGC129* and *HGA8*, *HGB4* and *HGB6*) cycling number was increased to 40.

Fragment analysis of PCR products was carried out on capillary sequencers CEQ™8000 (Beckman Coulter) and ABI ™ 3130xl (Applied Biosystems). Loci *HGD106*, *HGD111*, *HGC118* and half of samples with loci *HGC111*, *HGC131*, and *HGC120* were separated on CEQ8000, the other half along with all 2013 samples for loci *HGD106*, *HGD111*, *HGC118* and all of *HGC129*, *HGA8*, *HGB4* and *HGB6* were separated on ABI3130xl. For samples run on CEQ™8000, total volume per well was 38 µl including 0.5 µl size standard and 3 – 4 µl PCR product mixture, where the duplex and simplex were combined in a pool-plex. Genotypes were scored manually using CEQ™ 8000 GENETIC ANALYSIS SYSTEM v 8.0. For samples run on ABI ™ 3130xl, PCR products were diluted 1:10 with ddH_2O and pooled with 0.8 µl (*HGC129, HGA8)* and 0.6 µl (*HGB4, HGB6)* respectively. Samples were then prepared by adding 0.15 µl of -250 LIZ (Applied Biosystems) internal size standard and 8.33 µl of formamide Hi-Di sample loading solution (Applied Biosystems) to 2 µl of the diluted PCR mixtures.

S1.2 Genetic analysis and genotyping errors

Genotypes were manually scored for each individual based on characteristic peaks using GENEMAPPER v3.7 (Applied Biosystems). As the length of the alleles slightly differed between the instruments, MSATALLELE (Alberto, 2009), a script build on R, was used to bin the scored raw sizes from both fragment analysers and correctly calibrate the results from the two. All females and ~30% of males and eggs were screened on both sequencers for calibration. The script was modified to 2, 3 and 4 base pair repeats to suit the loci specifications.

Locus-specific genotyping error rates were estimated with a combination of methods. CERVUS was used to compare observed heterozygosity (H_O) and expected heterozygosity (H_E), and to identify any mother-offspring mismatches. We then used maximum likelihood (ML) programs PEDANT v. 1.0 (Johnson & Haydon, 2007) and MICRO-CHECKER v. 2.2.1 (Van Oosterhout *et al*., 2004) to model approximations of allelic drop-out (ε_1) and false allele (ε_2) error rates, with two different sample sets. First, PEDANT was used to estimate locus-specific ε_1 and ε_2 separately by using repeat genotypes. Depending on the locus, ~7-8% of all adult and offspring samples were genotyped twice and used as replicates. Second, to better account for the presence of null alleles in the drop-out model, which also causes a homozygote excess similar to allelic drop-out, the frequency of null alleles was assessed with MICRO-CHECKER based on all adult samples. When implementing the locus specific error rates (ε_1 and ε_2) for any given loci in the paternity analysis, we used MICRO-CHECKER's estimates as the ε_1 error rate when MICRO-CHECKER returned higher estimates than PEDANT's drop-out model. This is because PEDANT is based on a subset of samples while MICRO-CHECKER is based on all adult genotypes. Further, if the model of ε_2 error rate in PEDANT returned zero for any loci, a conservative non-zero value of 0.01 was specified to allow some genotypic mismatches in the parentage analysis (Wang, 2004) (see **Table 1** for error rates).

S1.3 Paternity analysis

We assigned parentage using COLONY v 2.0 (Jones & Wang, 2010a; Wang, 2004), a full-pedigree likelihood program (Markov-chain Monte Carlo method) that provides the most probable configuration in assigning sib-ship and parentage among individuals. All individuals are divided into subsamples of offspring, mothers and fathers from which individuals are assigned to various numbers of family clusters. The algorithm calculates the likelihood of one pedigree cluster and compares the likelihood to other possible pedigrees to identify the most parsimonious cluster. Besides assigning parentage based on candidate parents, COLONY also reconstructs genotypes for the missing parent. Sampling a wild population, we expect to have

incomplete representation of true fathers in the analysis. The assumed proportion of true fathers among the paternal candidates sampled was set to 50% based on results from preliminary runs in COLONY and CERVUS where we tested how various proportions affected the assignment rate. With very little variation in assignment results, COLONY showed robustness in handling uncertainty in the sampling rate, which is also supported elsewhere (Jones & Wang, 2010b; J Wang & Santure, 2009). COLONY allows both females and males to be polygamous, which is a prerequisite for testing multiple paternities in regard to both sexes. We chose to let COLONY update allele frequencies during assignment runs, since the total number of male contributions was inflated when allele frequencies were fixed. We accepted paternities assigned with 95% confidence or higher. This helped minimize false positive and false negative assignments and avoided overestimating the level of multiple paternity in the population. The best configuration given by COLONY was used to determine the number of males fathering the offspring. Although not all fathers were sampled, COLONY can infer their genotypes from the pedigree analysis to the number of mates to each female. The input files were set up with two replicate runs and analysed with the highest precision settings with full-likelihood, and with very long runtime on a PowerEdge M820, Linux CentOS 6.7 machine. No parentage was assigned to males sampled in 2013, so all males from this year were removed from further analysis, reducing the number of males to 563.

S1.4 Population descriptive

Significant linkage disequilibrium was detected in three out of 45 pair comparisons (6.6%) after accounting for multiple testing. Though COLONY does not implicitly account for linkage between loci, low levels of linkage disequilibrium are unlikely to have a large effect on the outcome of parentage analysis give the size of the sampled population (Amos *et al*.,1992; J Wang & Santure, 2009). Disequilibrium has not been found in a more wide-ranging population of European lobster in Norway (André & Knutsen, 2009), south-western UK (Ellis *et al*., 2015) or in the Irish sea (latter lost significance after Bonferroni correction) (Watson *et al*., 2016). Higher level of affinity between lobsters within our smaller study system would be more expected than within samples gathered across larger areas. Five loci (*C120, A8, B4, B6* and *C129*) did not conform to HWE after accounting for multiple testing ($P < 0.05$). Three of the loci (*A8, B6* and *C129*) also showed potential evidence of null alleles at frequencies 0.040 – 0.062, which could partially explain their deviation from HWE and cases of mismatches between maternal and offspring genotypes. Null-alleles at loci *A8* and *C129* were also found in Great Britain (Ellis *et al*., 2015). Systematic genotyping errors and hidden genetic structure

from sampling in a local area could also explain why five loci were out of Hardy-Weinberg equilibrium (Dakin & Avise, 2004), where none were found in the more wide-ranging population study of European lobster in Norway.

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Supplementary information S2: figures and table

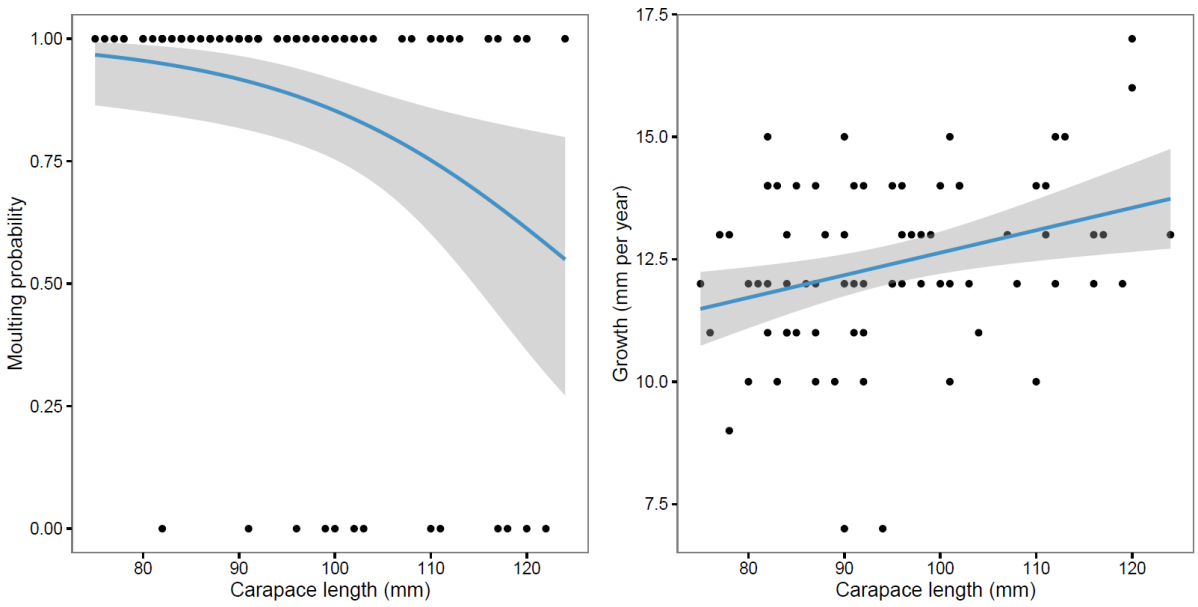

Figure S1. Predictions of yearly size change for males. Model predictions for molting probability and growth increment per year for those who had molted (increased the length with more than 5 mm). The data are from male lobster captured in consecutive years in a mark-recapture survey conducted in the study area of Flødevigen, Norway, 2006-2016. The predictions are used in the calculation of adjusted male body size when males with mating success were sampled in a different year than the females.

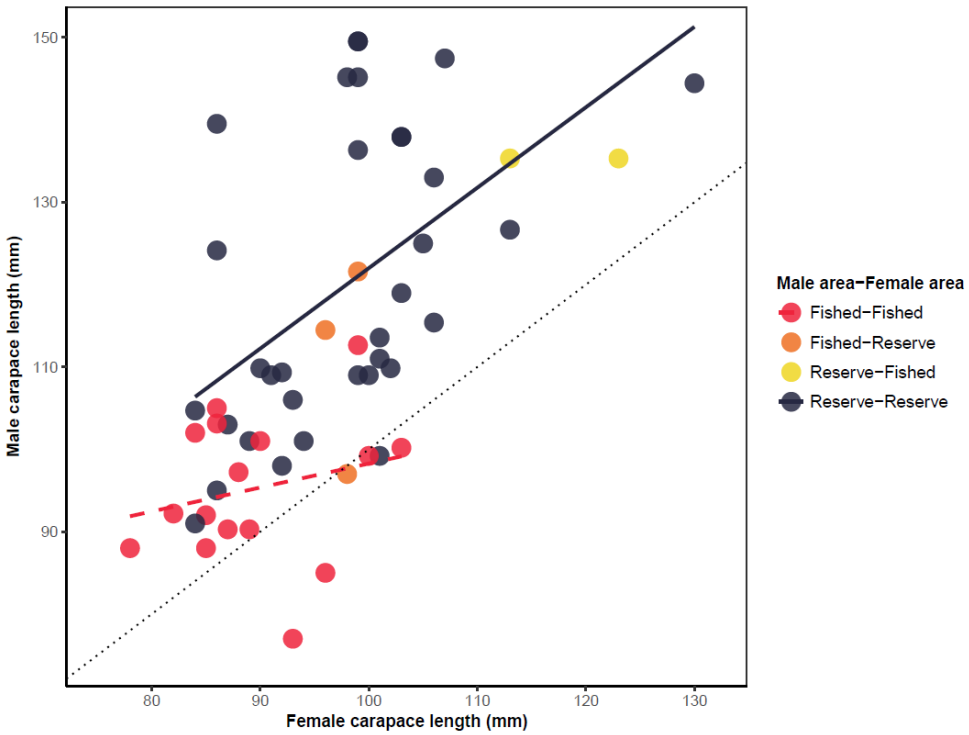

Figure S2. Size-assortative mating (all pairs). The relationship between body size (carapace length) of male (corrected sizes, see Materials and methods) and female European lobster that formed pairs (n = 51) in fished (red) and reserve (dark grey) area, and inter-area pairs where the male was from fished area and female was from reserve area (orange), and visa-versa (yellow) in the four-year period. Male *CL* is adjusted according to the year of the mating event. Value 1.0 and black stippled line (isometry, Y = X) marks where females and males are equal in size.

Table S1. List of 97 female European lobster analysed for paternity separated in area and year of sampling. ID of female, number of offspring analysed per female, average number of loci typed for each females' offspring, the ID of primary (#1) and, in two cases, the secondary (#2) male, fertilization skew of paternity (in proportion) in each egg batch as determined by COLONY, and as determined by manual inspection (given as SP = single paternity/ MP = multiple paternity). Letter (R = reserve/ F = fished) denotes which area the known males (n = 44) were sampled and absence of a letter denotes the genotype ID (n = 57) of the most likely father of the egg batches. (^{m}) denotes males that have mated with more than one female and are duplicated in table.

Area/ year	Female ID	Offspring analysed	Average no, of loci per offspring	male #1	male #2	Fertilization skew, determined by Colony	SP by male #1, or MP, determined by manual inspection
Reserve 2011	507	10	9,6	R-0153		100	SP
	639	10	9,9	R-0626[m]		100	SP
	678	10	9,6	R-0571[m]		100	SP
	681	10	9,2	36[m]		100	SP
	699	10	9,8	R-0668[m]		100	SP
	715	10	9,9	R-0079[m]		100	SP
	718	10	9	R-0397		100	SP
	725	10	8,8	R-0066[m]		100	SP
	728	10	9,8	R-0510[m]		100	SP
	736	10	10	R-0537		100	SP
	886	10	9,9	R-0771		100	SP
	921	10	10	R-0668[m]		100	SP
	922	10	10	R-0760		100	SP
	944	10	10	R-1361[m]		100	SP
	946	10	9,6	41		100	SP
	949	10	9,7	13		100	SP
	953	10	9,8	42		100	SP
	966	10	9,1	R-0586		100	SP
	971	10	10	R-0284		100	SP
	984	10	9,8	R-1618		100	SP
	1017	10	9,6	R-0066[m]		100	SP
	1029	10	9,6	3[m]		100	SP
	1046	10	9,4	R-0668[m]		100	SP
	1053	10	9,7	6		100	SP
	1055	10	9,6	R-0070[m]		100	SP
	1056	10	9,7	R-1680		100	SP
	1128	10	9	11		100	SP
	1130	10	10	R-0211		100	SP
	1131	10	9,8	12		100	SP
	1133	10	9,8	R-0626[m]		100	SP
	1147	10	9,7	R-1361[m]		100	SP
	1171	10	9,9	14		100	SP
	1172	10	9,7	R-0408		100	SP
	625	10	9,8	R-0079[m]		90:10	SP
	754	10	9,7	F-0992[m]		90:10	SP
	933	10	10	F-1111		90:10	SP
	1048	10	9,3	5		90:10	SP
	580	10	9,5	R-0410		70:30:00	SP
	881	10	9,8	R-0070[m]		70:30:00	SP

	1034	10	8,3	R-1154		70:30:00	SP
	912	10	10	36[m]		50:50:00	SP
	1139	10	9,5	R-0375		80:10:10	SP
Fished 2011	806	10	9,9	F-0455[m]		100	SP
	807	10	9,6	37		100	SP
	819	10	9,7	38		100	SP
	820	10	10	F-0108		100	SP
	833	10	10	39		100	SP
	839	10	9,9	33		100	SP
	864	10	9,9	40		100	SP
	1006	10	7,8	1		100	SP
	1011	10	8,6	2		100	SP
	1041	10	8,5	F-0992[m]		100	SP
	1065	10	9,5	7		100	SP
	1071	9	8,3	F-1371		100	SP
	1082	10	7,6	F-0705		100	SP
	1095	10	7,6	F-1093		100	SP
	1103	10	9,3	8		100	SP
	1106	10	9,8	9		100	SP
	1118	10	9,9	10		100	SP
	1122	10	9,8	F-0538		100	SP
	1174	10	9	F-0795		100	SP
	1175	10	8,8	15		100	SP
	1176	10	9,6	16		100	SP
	1178	10	10	18		100	SP
	1180	10	9,7	20		100	SP
	1038	10	5,7	4		90:10	SP
	1177	10	9,4	17	F-1296	90:10	**MP**
	1179	10	9,8	19		90:10	SP
	842	10	9,5	F-0131		80:10:10	SP
Reserve 2012	1617	10	10	R-0373		100	SP
	1640	10	10	34		100	SP
	1642	10	9,7	F-0704		100	SP
	1648	10	8,1	31		100	SP
	1663	10	9,5	R-0759		100	SP
	1683	10	9	R-0611		100	SP
	1647	10	9,3	35		90:10	SP
	1665	10	8,4	R-0635		90:10	SP
	1632	10	7,9	R-0011		80:20:00	SP
Fished 2012	1208	10	8,4	R-0510[m]		100	SP
	1211	10	9,6	F-1210		100	SP
	1221	10	7,5	F-1248		100	SP
	1223	10	8	3[m]		100	SP

1236	10	9,6	23		100	SP
1294	10	10	F-1350		100	SP
1311	10	9,9	29		100	SP
1331	10	10	30		100	SP
1348	10	9,9	R-0510[m]		100	SP
1369	10	9,8	32		100	SP
1218	10	8,3	F-0992[m]		100	SP
1222	10	8,5	21		90:10	SP
1230	10	9,8	22		90:10	SP
1291	10	10	24[m]	26	90:10	**MP**
1300	10	9,5	27		90:10	SP
1363	10	9,2	F-0357		90:10	SP
1383	10	9,4	28		90:10	SP
1252	8	7,8	24[m]		88:12:00	SP
1255	10	8,8	25		60:30:10	SP

Paper II

PLOS ONE

RESEARCH ARTICLE

Genotype Reconstruction of Paternity in European Lobsters (*Homarus gammarus*)

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Data Availability Statement: Microsatellite genotypes of 312 individuals (13 spatial samples of 24 individuals), used in the calculation of regional allele frequencies, microsatellite characterisation, and tests of HWE, linkage and null alleles, are available on the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.v176m.

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Abstract

Decapod crustaceans exhibit considerable variation in fertilisation strategies, ranging from pervasive single paternity to the near-ubiquitous presence of multiple paternity, and such knowledge of mating systems and behaviour are required for the informed management of commercially-exploited marine fisheries. We used genetic markers to assess the paternity of individual broods in the European lobster, *Homarus gammarus*, a species for which paternity structure is unknown. Using 13 multiplexed microsatellite loci, three of which are newly described in this study, we genotyped 10 eggs from each of 34 females collected from an Atlantic peninsula in the south-western United Kingdom. Single reconstructed paternal genotypes explained all observed progeny genotypes in each of the 34 egg clutches, and each clutch was fertilised by a different male. Simulations indicated that the probability of detecting multiple paternity was in excess of 95% if secondary sires account for at least a quarter of the brood, and in excess of 99% where additional sire success was approximately equal. Our results show that multiple paternal fertilisations are either absent, unusual, or highly skewed in favour of a single male among *H. gammarus* in this area. Potential mechanisms upholding single paternal fertilisation are discussed, along with the prospective utility of parentage assignments in evaluations of hatchery stocking and other fishery conservation approaches in light of this finding.

Introduction

The reproductive behaviour and ecology of fished species can affect their vulnerability to population collapses, and their subsequent ability to recover [1]. Polyandry may arise in breeding females as a life history strategy in order to increase the genetic diversity or fitness of offspring

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[2,3], or where males are sperm limited [4]. Selective fishing may also influence the occurrence of polyandry, especially where mating strategies are dependent on age, size, or sex ratio [1,5,6]. As a result, information on the dynamics of female mating strategies is a vital component to the informed conservation management of exploited fisheries [7].

Clutch fertilisation in marine decapods varies between species and populations, from pervasive single paternity (e.g. snow crab [8]) to ubiquitous multiple paternity (e.g. squat lobsters [9]). Multiple sires have been detected within individual clutches in a variety of aquatic crustaceans (e.g. ghost shrimp [10]; Norway lobster [11]; porcelain crab [12]; Dungeness crab [13]; rock shrimp [14]; freshwater crayfishes [15]; Pacific gooseneck barnacle [16]). However, the frequency of polyandrous fertilisation remains unknown in the European lobster (*Homarus gammarus*), a high-value species exploited extensively throughout its range by trap fishing. The presence of multiple paternal fertilisations has been detected among individual egg clutches of the closely-related American lobster, *Homarus americanus* [17,18], with some evidence from the wild that increased fishing pressure disrupts the natural monandrous behaviour of some females via reductions in the abundance, size or post-copulatory mate-guarding ability of breeding males [18].

Despite supporting a highly lucrative fishery, information on the reproductive ecology of *H. gammarus* in the wild is scarce [19], and is often implied from that of the better-studied *H. americanus*. Female *H. americanus* are thought to seek out and compete for males and usually moult during a period of shelter cohabitation, whereupon a spermatophore is deposited by the male into the seminal receptacle of the female [20,21]. The male attempts to prevent further insemination from competitors by guarding the female until both her shell and a sperm plug blocking the entrance to the seminal receptacle have hardened [20,22]. Females vacate the male's shelter and usually store the spermatophore for approximately a year before spawning, whereupon it is released to externally fertilise the eggs during extrusion and oviposition [23,24]. Homarid eggs hatch following 9–11 months of development while stored ventrally along the female abdomen, at which point most mature females mate and moult again, forming a biennial reproductive cycle [21,24]. Occasionally females moult, mate and spawn annually [24], while large (>120 mm carapace length [CL]) females can go several years without moulting and may mate during intermoult if spermatophore reserves are insufficient to sire a brood [25].

It has long been established that female fecundity increases with increasing body size [24,26,27], and studies on the effects of male size in other lobster species show that ejaculate load is also size-specific and may be reduced by previous copulations [20,28]. Where the abundance and mean size of males is reduced by fishing, it has been proposed that the population may become sperm limited, with the production of larvae restricted by a lack of available spermatophore with which to fertilise the maximum egg capability of breeding females [28]. Such sperm limitation may cause females to seek additional copulations, with more than one spermatophore used to fertilise an egg clutch [18,20]. Alongside sperm limitation, other hypotheses proposed to explain observed multiple paternity in marine invertebrates have included convenience polyandry [29–31] and enforced mating [14]. Where multiple paternity has been identified among marine crustaceans, considerable skews in fertilisation success towards a single male have often been detected [9,14,16,18]. This has been proposed to result from various post-copulatory processes including spermatophore stratification [32], cryptic female choice [30] and sperm competition, although the latter was ruled out for *H. americanus* because their sperm lack motility [18,22].

We investigated *H. gammarus* paternity around Cornwall, an Atlantic peninsula in south-western UK, where lobsters are intensively fished and are also the focus of stock enhancement by a local hatchery [33]. Because physical tags having proven largely ineffective in marking

early-stage post-larval lobsters [34–36], the hatchery is interested in pursuing genetic methods of parentage assignment that have allowed the successful identification of stocked finfish among admixed wild populations [37–39]. The tissue archiving requirements and general suitability of such an application are in part dependent on the number of sires contributing to individual clutches, adding to the need for information of lobster paternity in the region. By reconstructing male genotypes from clutches of fertilised eggs, we aimed to estimate the frequency of multiple paternity and thus elucidate the typical fertilisation scenario in lobsters from this important regional fishery.

Materials and Methods

Ethics statement

Permission to obtain tissue samples from adult lobsters (for both paternity assays and population screening) were obtained from the Cornwall Inshore Fisheries Conservation Authority (IFCA), who regulate and manage the lobster fishery within coastal waters. Tissue samples were collected on board commercial vessels as part of regular fishing routines. The collection of tissue samples from adult lobsters from the Isles of Scilly did not require the permission of the Isles of Scilly IFCA since samples were obtained from animals already landed to a merchant on the mainland. Eggs for paternity assays were collected from ovigerous females captured within the six nautical mile inshore jurisdiction of Cornwall IFCA, who provided written permission for both the sampling of eggs and the temporary landing of ovigerous lobsters, which is normally prohibited by a regional bylaw [40]. The European lobster is categorised as being of Least Concern in the Red List of Threatened Species of the International Union for Conservation of Nature [41].

Sites and sampling

During March and April 2013, trap-caught ovigerous female lobsters were collected directly from selected inshore fishers temporarily permitted to land these animals by the regional fisheries management authority. Typically, the rocky Celtic Sea habitats to the north and far west support a greater abundance of lobster than the mixed substrates of the western English Channel along the southern coast [42]. As such, lobsters were sourced from two sites in each area (four sites in total, separated by a minimum Euclidean distance of 55 km) to account for any spatial variation in paternity structure (Fig 1). Where possible, samples were taken immediately upon receipt of the lobsters, although occasionally they were stored in holding tanks for a maximum of 48 hours before sampling. Sampling consisted of the removal of a small piece of maternal tissue from the tip of a hindmost pleopod, and of ten eggs from the clutch (total clutch size is specific of female size and even region, though is typically 9–13,000 for mean-sized individuals of 103 mm CL [27]). An egg was removed from both the base and the tip of the egg-mass from each of the five pairs of pleopods. Egg sampling was structured in this way to maximise the likelihood of detecting multiple paternity and because some marine decapods (though not *H. americanus* [18]) have demonstrated spatial segregation of multiple paternal fertilisations [9,10]. Twelve females were sampled from each of two Celtic Sea and English Channel locations, although insufficient DNA yields from undeveloped eggs later reduced these sample sizes. As such, 340 eggs from 34 females were genotyped successfully (Fig 1). Female carapace length (CL) was measured using a Vernier caliper and rounded down to the nearest whole millimetre, as per [43]. The assessment of a wide range of female sizes is important given the expectation that the frequency of multiple paternity may vary with female size, particularly if caused by sperm-limitation [20,28].

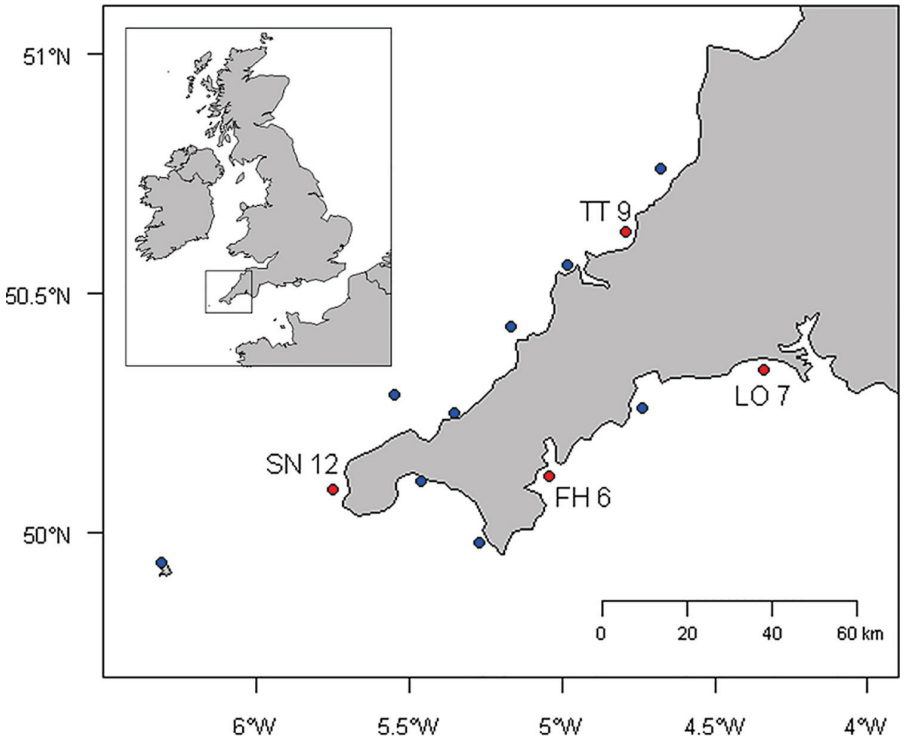

Fig 1. Map of sample sites. Map of the Cornwall peninsula showing the location of sampling sites. Red points denote the paternity sample sites Tintagel (TT), Sennen (SN), Falmouth (FH) and Looe (LO), with sample sizes denoting the number of clutches successfully tested. These four sites, and nine additional sites denoted by blue points, were each used to sample 24 individuals to provide accurate estimates of regional allele frequencies. Position relative to the UK, Ireland and continental Europe is inset.

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Microsatellite genotyping

Genotyping of tissue samples was carried out using 15 microsatellite loci; 12 previously published [19], and the three newly characterised loci (see S1 Text for development process). Maternal DNA was extracted from individual pleopod tissues and progeny DNA from whole eggs using the Wizard® SV 96 Genomic DNA Purification System (Promega). Primer oligonucleotides were synthesized by Eurofins Genomics (Eurofins Genomics), with forward primers 5'-tagged with one of four fluorescent sequencing dyes; FAM, ATTO 550, ATTO 565 and Yakima Yellow. The Mulitplex PCR Kit (Qiagen) was used to allow the amplification of all loci across four multiplexes (See Table 1 for multiplex organisation). PCR volumes of 8 μl were prepared in the following reaction mix: 4 μl Multiplex PCR Mix; forward and reverse primers at 0.48–1.33 μM (Multiplex 1, 0.88 μM, apart from HGD106, 0.48 μM; Multiplex 2, 1.00 μM; Multiplexes 3 and 4, 1.33 μM); and 2 μl template DNA (20–50 ng). PCR was conducted in a Techne Prime Elite 96 thermocycler (Bibby Scientific Ltd.), with an initial denaturation (94°C, 3 min), then 35 cycles of denaturation (94°C, 40 s), annealing (55°C, 40 s) and extension (72°C, 30 s), before a final extension (72°C, 4 min). Fragment analysis was carried out for the 312 samples using an ABI 3130 Genetic Analyzer (Applied Biosystems Inc.). Alleles were automatically sized against Genescan™ 500 LIZ™ size standard (Applied Biosystems Inc.) using Geneious 6.1 software (Biomatters Ltd.), before also being checked manually and rescored where necessary.

Table 1. Loci exclusion probabilities.

Rank	Locus	Multiplex	Exclusion Probability	
			Maternal genotype known	Neither parental genotype known
1	HGC120	4	0.732	0.575
2	HGC131b	4	0.662	0.491
3	HGD110	4	0.611	0.435
4	HGC111	3	0.494	0.314
5	HGB6	2	0.483	0.308
6	HGD106	1	0.481	0.301
7	HGC103	2	0.476	0.304
8	HGB4	1	0.430	0.251
9	HGC118	1	0.378	0.201
10	HGD111	3	0.350	0.186
11	HGD129	2	0.347	0.179
12	HGD117	1	0.320	0.178
13	HGC6	2	0.212	0.071
14	HGA8 [a]	1	*0.647*	*0.473*
15	HGC129 [a]	3	*0.543*	*0.363*

Loci are ranked via individual exclusion probabilities, assuming an assay of 10 progeny genotypes and deriving allele frequencies from a regional population survey (see S1 Text for sampling details).

[a]Loci which were removed from paternity analyses due to the presence of null alleles; as such these are ranked last and their exclusion probabilities (italicised) will be inaccurate.

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While some studies have previously pooled eggs from each pleopod region or the whole clutch into single extractions, we elected to genotype eggs individually. Pooling progeny genotypes can allow the detection of multiple paternity while boosting the number of progeny screened and the sample size of females per unit effort, but such an approach can significantly underestimate the true number of sires [9] and provides no way of estimating fertilisation skew. To prevent genotyping errors overestimating the occurrence of multiple paternity, any progeny genotype that did not support a single paternal contribution (i.e. where three or more alleles were recorded at a locus) was retested in single-locus PCR (using Qiagen Taq PCR Master Mix in place of Multiplex PCR Mix) and controlled fragment analysis procedures. The software FreeNA [44] was used to estimate the frequency of null alleles from regional population genotype data of 312 individuals (see S1 Text for sampling details).

Statistical analysis

Probabilities of detecting multiple paternal contributions (PrDM) were quantified by the software *PrDM* [45]. Using regional population allele frequencies (from 312 individuals–see S1 Text for sampling details), *PrDM* used Monte Carlo simulations to calculate PrDM under various scenarios of skew between the fertilisation contributions of multiple males; two males in ratios of 50:50, 60:40, 70:30, 80:20 and 90:10, and three males in ratios of 34:33:33, 50:25:25, 60:20:20, 70:15:15, 80:10:10 and 90:5:5. The software GERUD 2.0 [46] was used to estimate the exclusion probabilities (the probability that they exclude an unrelated individual from a putative pedigree [47]) of individual loci to enable loci to be ranked by power to assign parentage. GERUD 2.0 was used to reconstruct the minimum number of possible paternal genotypes, which were also independently assembled manually from progeny genotypes. Because GERUD 2.0 only reconstructs the minimum number of unknown parental contributions that can

explain the progeny genotypes, two-allele genotypes are presumed to be heterozygotes. Although unlikely given the number of markers used, it is therefore possible that two males displaying only homozygote or shared alleles would be reconstructed as a single male. As such, total heterozygosity calculations and heterozygote excess tests were carried out on pooled parental genotypes using GENEPOP 4.2 software [48]. The presence of heterozygote excess or significantly increased heterozygosity compared to known maternal genotypes could suggest an underestimation of the number of males contributing to reconstructed paternal genotypes.

Results

Egg DNA yields and female sizes

All eggs in intermediate and later stages of development (as evidenced by brown and red colouration) yielded suitable quantities of DNA for downstream analysis. However, 3 of 24 Celtic Sea females and 11 of 24 English Channel females possessed eggs that were either unfertilised [49] or in early stages of development (as evidenced by black and/or dark green colouration) from which DNA yields were insufficient to allow successful genotyping, reducing the actual sample sizes to 21 and 13 respectively. Of those females providing successful progeny arrays, size (CL) ranged from 94–155 mm ($n_{\text{Total}} = 34$, mean CL = 113.5 mm, SE ±2.31), with English Channel individuals (mean CL = 117.9 mm, SE ±4.26) tending to be slightly larger than those from Celtic Sea sites (mean = 110.7 mm, SE ±2.56).

Genotyping and marker power

Maternal and progeny samples that amplified effectively were screened at all 15 loci, however two loci were dropped from the analysis upon the detection of null alleles, which are known to introduce substantial errors in empirical assessments of parentage [50–52]. In this case, null alleles appear to have caused mismatches between maternal and progeny genotypes, or progeny genotypes to suggest three paternal alleles at the loci HGA8 and HGC129 (in 11 and four occasions among 68 parents, respectively). FreeNA confirmed null alleles at frequencies of 0.11 for HGA8 and 0.04 for HGC129. Null allele frequencies were zero for all other loci except HGC103 and HGD111, for which negligible frequencies of 0.02 were estimated. Because of this, only the remaining 13 markers were used in the determination of potential paternal genotypes and PrDM. The exclusion probabilities of these individual loci ranged from 0.21 to 0.73 when using ten progeny arrays and a known maternal genotype (Table 1). Note that this probability is not a measurement of the likelihood of individual loci successfully detecting multiple paternity or determining the number of sires, but of their likelihood to correctly exclude unrelated males from potential parentage via genotypic mismatch (e.g. when surveying paternal candidates). As such it is indicative of the relative power provided by each locus. The three most powerful loci were HGC120, HGC131b and HGD110.

Probability of detecting multiple paternity

With 10 progeny genotyped at 13 loci, the probability of detecting a secondary paternal contribution where one was present exceeded 0.99 assuming equal fertilisation contributions (Fig 2). The confidence threshold for the detection of additional males dropped below 95% only when the paternal contribution of secondary sires accounted for 25% or fewer of the progeny. If the paternal contribution had been highly skewed in favour of a primary male in this way, then more than 10 progeny genotypes would have been required to retain a 95% confidence level in PrDM (Fig 2). In scenarios where secondary contributions were split between two males (three sires in total), PrDM effectively remained unchanged, although for some scenarios, one or two

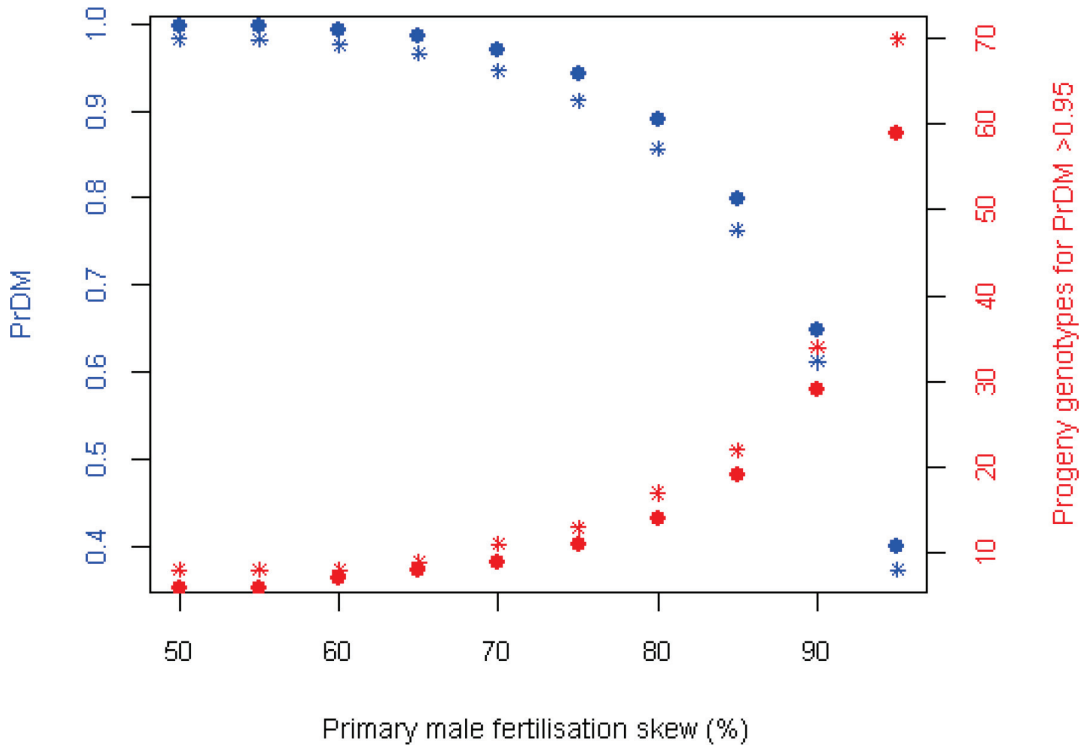

Fig 2. PrDM with skewed male fertilisation success. Variation in PrDM from 10 progeny genotypes (blue axis and data points) and the number of progeny genotypes required to achieve a 95% confidence level in PrDM (red axis and data points) under various scenarios of male fertilisation skew. Round points infer progeny genotyping at all 13 loci, while starred points infer progeny genotyping at only the three most informative loci (all amplified within Multiplex 4).

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fewer progeny genotypes could still yield PrDM >0.95 (S5 Table). Estimates of PrDM based on genotyping at only the three most polymorphic loci (all amplified within Multiplex 4) were almost as powerful as those attained by all 13 loci. PrDM was <0.95 at a lower paternal skew (70:30 as opposed to 75:25), but was only decreased by 0.002–0.037 under the fertilisation scenarios investigated.

Paternal reconstruction

Reconstructions of paternal genotypes by GERUD 2.0 showed that single male genotypes explained all of the 34 progeny arrays. Of the candidate paternal genotypes, 28 were able to be reconstructed in full at all 13 loci (S4 Table). For six reconstructed paternal genotypes, it was not possible for GERUD 2.0 to resolve the paternal genotype at all 13 loci; four reconstructions were unable to determine paternal genotype at one locus and two more were unresolved at two loci. In these instances, both maternal and paternal genotypes were heterozygous and the paternal genotype possessed one allele that was shared with a maternal allele, but the progeny array contained no homozygotes to determine which allele was shared. On such occasions, GERUD 2.0 simply returned multiple single-sire genotypes that could explain the progeny array which were ranked in order of likelihood according to Mendelian segregation probability. All reconstructed male genotypes differed at multiple loci; no paternal genotype matched those provided by any other progeny array, so the clutches of all 34 females appeared to have been fertilised by 34 separate males. Total heterozygosity of reconstructed paternal genotypes was 0.68, while known maternal genotypes had a total heterozygosity of 0.69. A test for heterozygous excess

among reconstructed paternal genotypes was non-significant (p = 0.50) and comparable to that obtained for known maternal genotypes (p = 0.49). Twelve allele scores (1.6%) were altered after genotyping was repeated. Had the original scores been analysed, it would have led to four incidences of multiple paternity (all with 1/10 progeny supporting a second sire).

Discussion

Unlike many other genetic studies on aquatic crustaceans [9–16,18], our investigation found no evidence for multiple paternal fertilisations of individual *H. gammarus* broods. The loci employed ensured the statistical power to detect additional paternal fertilisations was consistently high, exceeding 99% when assuming approximately equal male representation among the progeny, and exceeding 95% wherever secondary males accounted for at least a quarter of the brood. This power to detect secondary sires is greater than that reported by Bailie *et al* [9], which failed to reach 95% at any fertilisation skew when genotyping up to 86 galatheid squat lobster progeny at only two or three microsatellites, and is commensurate with that of Gosselin *et al* [18] for *H. americanus* at equal (50:50) skews, but not at extreme (90:10) skews due to our genotyping fewer eggs. The power to detect secondary paternal genotypes with low progeny representation is important since multiply-sired crustacean broods often show high levels of paternal skew, with Bailie *et al* [9] estimating that secondary paternal fertilisations composed 14% or fewer of the majority of galatheid broods. Due to the statistical power of our method falling outside of 95% confidence limits at high paternal fertilisation skews, it is possible that multiple paternity was present but undetected in *H. gammarus* broods we assessed. It is unlikely, however; most (64%) multiply-sired broods identified by Gosselin *et al* [18] exhibited secondary fertilisation contributions at ratios where detection probability would have exceeded 95% in our study. Even applying the least frequent rate of detection in a sub-population (11%) and the maximum skew (90:10) found among multiply-sired *H. americanus* clutches [18], we would still anticipate at least three cases of multiple paternity among our *H. gammarus* samples (two from Celtic Sea sites and one from English Channel sites), of which our power of detection (65%) would have been expected to overlook only one. Overall, our results suggest that multiple paternity is likely to be absent, or rare and highly skewed in favour of a dominant male, among *H. gammarus* in this geographical region.

While the reconstruction of paternal genotypes was conservative in that it provides the minimum number of males required to explain the observed progeny genotypes, it appears to be have been accurate in confirming single paternity. Overall heterozygosity of reconstructed paternal genotypes was equal to that of all maternal individuals, and showed no evidence of heterozygous excess, suggesting no underestimation of the number of sires represented among paternal reconstructions. Alongside reconstructing sire contributions from individual egg genotypes, some studies have inferred multiple paternity via significant departures of progeny genotypes from Mendelian expectations of allele frequencies [9]. However, this method was not considered for our analysis because it was deemed potentially ambiguous and unlikely to prove informative given the size of the progeny array per brood, and because the possibility of missing additional paternal alleles across 13 loci was remote.

The prevalence of single paternity among individual *H. gammarus* broods suggests that either (i) all females copulated only with a single male; or (ii) females copulated with more than one male, but fertilisation was attained by only a single male.

In *H. americanus*, regular monandrous mating appears to be maintained by both female choice (female preference for the protection and/or spermatophore of dominant males [53]) and male competition (male efforts to prevent rival inseminations prior to the formation of a sperm plug [18]). Clear evidence of female choice has also been observed in *H. gammarus* [54],

so the same processes may well occur in both species. Where polyandry was found in *H. americanus*, Gosselin *et al* [18] proposed that female choice and/or male competition could have been altered by effects of fisheries-induced sex ratio imbalance, which may have included sperm limitation. However, male and female abundance and size distributions are approximately equal in *H. gammarus* around Cornwall [42,55], which may serve to maintain the ubiquity of monandrous mating. Male density affects the frequency of multiple paternity in many species (e.g. house mice [56]; European earwig [57]), and if the proportion of breeding males were driving variation in the occurrence of multiple paternity in lobsters, the frequency of multiply-sired clutches could follow a Gaussian distribution; both even sex ratios and extreme male depletion would be expected to lead to single paternity, with multiple paternity most frequent in an intermediate state of partial male depletion. For example, male density explains a normally-distributed dynamic in the fertilisation success of female Red sea urchins [58]. Even if female lobsters were inseminated by multiple males, spermatophore stratification may ensure last-male precedence upon fertilisation, as is the case in Snow crabs [8].

Potential mechanisms preserving single paternity in Cornwall may be weakened or absent in other *H. gammarus* stocks, however. Further assessments of paternity would be particularly valuable in stocks recovering from collapse (e.g. Norway [24,43,59]), of limited size distribution (e.g. NE England [55]), of high abundance (e.g. Lundy, UK [60–62]) and in the absence of fishing (e.g. Lundy, UK; Flødevigen, Bolærne and Kvernskjær in Scandinavia [63]). If destabilised population demography were found to affect the frequency of multiple paternity, such data could be a useful reference point as to the health of lobster fisheries. Although *Homarus* species are presumed to be polygynous [21], we found no evidence of any male fertilising multiple clutches, despite some females within individual sample sites being captured in close proximity (i.e. traps approximately 100 m apart). Sex-biased conservation measures may result in sperm limitation [28], so knowledge on paternity and the fertilisation success of individual males would benefit fishery managers in ensuring conservation legislation safeguards recruitment.

The results of PrDM simulations suggest that a different sampling regime to that which we employed would enhance power to detect multiple paternity at highly uneven skews. Genotyping 10 eggs per clutch at 13 loci amplified in four multiplexes (40 PCR reactions) gave us an estimated 65% power to detect additional males contributing just 10% of fertilisations. However, PrDM was only slightly reduced by using only the three most informative loci, which can be multiplexed together. As such, the attainment of >95% power to detect secondary males in a 90:10 fertilisation skew would have been possible with a progeny array of 34 eggs per clutch, each genotyped in a single PCR reaction (34 PCR reactions). Although this would require more DNA extractions, it may be a preferable option in future studies of parentage using these microsatellites, assuming those loci are similarly diverse elsewhere. Especially where population allele frequencies are readily available, *a priori* analysis of PrDM would be advisable to determine the most efficient sampling regime and marker panel. Further attempts to genotype *H. gammarus* eggs would also be advised to avoid clutches in early phases of development to ensure only fertilised eggs are sampled and that DNA yields are sufficient for downstream analysis.

Our findings of high allelic diversity and single paternal fertilisations in this population of *H. gammarus* bodes well for the potential utility of genetic markers in parentage assignments [64] to enable evaluations of fisheries conservation measures, and particularly hatchery stocking. As a result of the recent collapses seen in some stocks and the increased fishing pressures on others, attempts have been made in a variety of European locations, including Cornwall [33], to enhance the productivity and sustainability of *H. gammarus* fisheries via the release of cultured juveniles [36,43,59,65,66]. Genetic tagging, the establishment of hatchery origin via multi-locus assignment of parentage, has important advantages over existing tagging options

for juvenile lobsters, such as sub-lethal sampling and no restrictions on the body size of released individuals, as well as providing data for the assessment of genetic impacts on the wild target stock [36]. Hatcheries sourcing ovigerous lobsters from the wild may genotype maternal tissues directly, but paternal genotype(s) must be deduced from a sample of eggs or larvae in order to establish all possible progeny genotypes [36]. Since single paternity appears to be the regular mode of fertilisation in this region, the resolution of parentage may be achieved by genotyping many fewer progeny than would be required were multiple paternity frequent. As a result, the compilation of the anticipated genotypes of released lobsters, a necessary step before surveying the wild population, would be more affordable. The development of a genetic tagging approach may become a crucial tool with which to assess and compare different *H. gammarus* conservation strategies, particularly in light of the scarcity of methods with which to monitor recruitment and the performance of wild larvae and juveniles [21,36,67].

Conclusions

Multi-locus genotyping proved a powerful tool in the assessment of paternity in *H. gammarus*, and provided evidence only of singly-sired clutches in an important regional population. Multiple paternity was not detected, indicating it is likely to be either absent, or irregular and highly skewed in favour of a single male. The detection of only single paternity among *H. gammarus* may reflect demographic stability in sex-ratios across a wide size distribution in this region. The development of additional microsatellite markers provides greater power for further studies of parentage and population genetics in *H. gammarus*. The prospects of their potential utility in evaluations of hatchery stocking and other fishery conservation measures in Cornwall are increased by the establishment of single paternity as the dominant method of fertilisation.

Supporting Information

S1 Table. Primer sequences of tested loci. Table featuring primer sequences of novel loci tested and cause of discard where development was not achieved.
(DOCX)

S2 Table. Dataset of population genetic survey of novel microsatellite loci. Spreadsheet featuring scored genotypes of tissues collected from around the coast of Cornwall, UK. For each of 24 individuals sampled from 13 locations (n = 312), allele scores (in base pairs) are shown for the three microsatellite loci (HGD110, HGD117 and HGD129) developed during this investigation.
(XLSX)

S3 Table. Characteristics of novel microsatellite loci. Three novel microsatellite loci with associated diversity information: N_A = number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity; H-W = p-values for deviation from Hardy-Weinberg equilibrium as evidenced by exact test (p) and U-test of heterozygote excess (H_{ex}).
(DOCX)

S4 Table. Dataset of maternal and progeny genotypes forming the paternity assays. Spreadsheet featuring scored genotypes of maternal and progeny tissues, and resolved paternal genotypes. For each of 34 assays of *H. gammarus* paternity, allele scores (in base pairs) are shown at 15 microsatellite loci (including HGA8 and HGC129, both0020later dropped for evidence of null alleles). Each assay features twelve samples; maternal genotype at top, followed by the genotypes of 10 progeny, with resolved paternal genotype at bottom (italicised). Sample names compose a letter denoting capture location and size (mm) of female lobster, followed by sample type, where M = maternal, P = paternal, and for progeny, 1–5 denotes pleopod region (with 5

nearest the tail), and t = tip or b = base of pleopod.
(XLSX)

S5 Table. Estimates of PrDM at various paternity scenarios. Table shows calculations of the probability of detecting multiple paternal contributions (PrDM) and the number of egg genotypes required to achieve a 95% confidence level in PrDM. Values reflect various scenarios of numbers of sires and their fertilisation skew, and are calculated for all 13 loci (as used in this study) and the three most polymorphic loci (all from Multiplex 4). Predictions used allele frequencies obtained from a survey of 312 individuals in the south-western United Kingdom.
(DOCX)

S1 Text. Microsatellite development. Methodology and results of the characterisation of novel microsatellite loci.
(DOCX)

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Author Contributions

Conceived and designed the experiments: CE DH AG. Performed the experiments: CE AG. Analyzed the data: CE AG. Contributed reagents/materials/analysis tools: CE DH CA HK AG. Wrote the paper: CE DH CA TS HK AG.

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Supplementary information

S1 Table. Primer sequences of tested loci. Table featuring primer sequences of novel loci tested and cause of discard where development was not achieved.

Locus	Primer	Primer sequence (5'-3')	Developed / Reason Undeveloped
HGD110	HGD110F	ACGGATGGATGGATAGGTAG	Developed
	HGD110R	ATTCTCTGGCAGGTCAAGAC	
HGD117	HGD117F	GCCTACTCTCTCCTTCCTTC	Developed
	HGD117R	ACCTGTCTATCGTTCTGTTTG	
HGD129	HGD129F	CCGTGCTGAAAGGGTTAT	Developed
	HGD129R	CAAACTATTCGTCCACAAAGTC	
HGA5	HGA5F	GGTGTCCAGCAAACAATATAGG	Difficulty in consistent scoring
	HGA5R	ACCTGCACTTGTACCCACAC	
HGD121	HGD121F	AGCAGATGTAACCGAGGTAGT	Difficulty in consistent scoring
	HGD121R	GAATGAAGCACCATAACACAG	
HGC107	HGC107F	CTCTGCTCTTTCTGGTGTTG	Difficulty in consistent scoring
	HGC107R	GTCGGCACTAAACTCATCAC	
HGC121	HGC121F	TCAACCTTTCCAGACAAGTGA	Appeared monomorphic
	HGC121R	AGGAACGTAGACCCGTACAGAG	
HGC106	HGC106F	GATCGAACTCAGGTCCAC	Failed to amplify
	HGC106R	TTTGTGTGTGTATGTGTG	

S2 Table. Dataset of population genetic survey of novel microsatellite loci. Spreadsheet featuring scored genotypes of tissues collected from around the coast of Cornwall, UK. For each of 24 individuals sampled from 13 locations (n = 312), allele scores (in base pairs) are shown for the three microsatellite loci (HGD110, HGD117 and HGD129) developed during this investigation. XLSX file available on the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.v176m.

S3 Table. Characteristics of novel microsatellite loci. Three novel microsatellite loci with associated diversity information: N_A = number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity; H-W = p-values for deviation from Hardy-Weinberg equilibrium as evidenced by exact test (p) and U-test of heterozygote excess (H_{ex}).

GenBank accession number	Locus	Primer sequence (5'-3')	Repeat motif	Size range (bp)	N_A	H_E	H_O	H-W p	H-W H_{ex}
KT240103	HGD110	F: ACGGATGGATGGATAGGTAG R: ATTCTCTGGCAGGTCAAGAC	(AGAT)8	176-220	11	0.799	0.824	0.5637	0.201
KT240104	HGD117	F: GCCTACTCTCTCCTTCCTTC R: CCTGTCTATCGTTCTGTTTG	(ATAG)7	254-302	10	0.574	0.574	0.116	0.195
KT240105	HGD129	F: CCGTGCTGAAAGGGTTAT R: CAAACTATTCGTCCACAAAGTC	(AGAT)11	234-290	10	0.563	0.564	0.837	0.640

S4 Table. Dataset of maternal and progeny genotypes forming the paternity assays. Spreadsheet featuring scored genotypes of maternal and progeny tissues, and resolved paternal genotypes. For each of 34 assays of H. gammarus paternity, allele scores (in base pairs) are shown at 15 microsatellite loci (including HGA8 and HGC129, both0020later dropped for evidence of null alleles). Each assay features twelve samples; maternal genotype at top, followed by the genotypes of 10 progeny, with resolved paternal genotype at bottom (italicised). Sample names compose a letter denoting capture location and size (mm) of female lobster, followed by sample type, where M= maternal, P = paternal, and for progeny, 1–5 denotes pleopod region (with 5 nearest the tail), and t = tip or b = base of pleopod. XLSX file available on the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.v176m.

S5 Table. Estimates of PrDM at various paternity scenarios. Table shows calculations of the probability of detecting multiple paternal contributions (PrDM) and the number of egg genotypes required to achieve a 95% confidence level in PrDM. Values reflect various scenarios of numbers of sires and their fertilisation skew, and are calculated for all 13 loci (as used in this study) and the three most polymorphic loci (all from Multiplex 4). Predictions used

allele frequencies obtained from a survey of 312 individuals in the south-western United Kingdom.

Paternal skew – two sires (Primary male : Secondary male)		**50:50**	**60:40**	**70:30**	**80:20**	**90:10**
13 loci (4 multiplexes)	**PrDM with 10 eggs**	0.998	0.993	0.970	0.891	0.649
	***n* eggs for PrDM >0.95**	6	7	9	14	29
3 loci (1 multiplex)	**PrDM with 10 eggs**	0.983	0.976	0.946	0.856	0.612
	***n* eggs for PrDM >0.95**	8	8	11	17	34

Paternal skew – three sires (Primary male : Secondary males)		**34:33:33**	**50:25:25**	**60:20:20**	**70:15:15**	**80:10:10**	**90:5:5**
13 loci (4 mplxs)	**PrDM with 10 eggs**	1.000	0.999	0.994	0.971	0.890	0.648
	***n* eggs for PrDM >0.95**	5	5	6	9	14	29
3 loci (1 mplx)	**PrDM with 10 eggs**	0.998	0.996	0.986	0.955	0.862	0.616
	***n* eggs for PrDM >0.95**	6	6	8	10	16	32

S1 Text. Microsatellite development. Methodology and results of the characterisation of novel microsatellite loci.

To improve analytical power, novel loci were developed to complement the species-specific microsatellite panel already publicly available. To characterise new loci, eight tetra-repeat microsatellites, isolated from partial genomic libraries, were used to design primer pairs as described by André & Knutsen [1]. Preliminary marker tests were conducted by analysing 12 individuals (none included in paternity assays), four from each of three of the study sites; Tintagel, Sennen and Looe. Of these eight loci, five either failed to amplify (HGC106), appeared to be monomorphic (HGC121), or presented significant difficulties in scoring alleles consistently (HGA5, HGC107, and HGD121) (S1 Table). Further, comprehensive screening was conducted for the three loci that amplified reliably and were polymorphic (HGD110, HGD117 and HGD129). Comprehensive screening involved the analysis of 312 individuals;

24 from each of 13 geographic samples (including the four paternity sample sites; see Figure 1 in the main paper for locations) spanning 230 km of coastal waters from Looe (the south-eastern-most paternity sample site) to Boscastle (beyond the north-eastern-most paternity sample site) and west to the Isles of Scilly (offshore from the western-most paternity sample site). These samples were genotyped at the novel loci, as well as the existing 12 loci of André & Knutsen [1] to enable checks for linkage disequilibrium.

DNA extraction, PCR amplification and fragment analysis of loci followed the protocols listed in the Microsatellite Genotyping section in the main paper. Taq PCR Master Mix (Qiagen) used to amplify loci instead of Multiplex PCR Mix. Population differentiation among geographic samples was checked by G-tests in the web-based GENEPOP 4.2 software [2], to justify pooling samples as a single unit for the characterisation of novel loci, testing for null alleles, and the estimation of allele frequencies. Across all 15 loci, significant genic differentiation was detected among the 13 spatial samples, but not after the removal of HGA8 and HGC129, loci later found to be affected by null alleles. A G-test for overall population differentiation was then non-significant ($p = 0.07$), and only four of 91 sample pairs showed significant differentiation ($p < 0.05$), as expected by chance alone.

These genotypes were also tested in GENEPOP 4.2 for heterozygosity, linkage disequilibrium and deviation from Hardy-Weinberg expectations. All tests of linkage disequilibrium were non-significant after this threshold was adjusted to account for multiple tests [3]. No deviation from Hardy-Weinberg expectations were detected via the exact probability test ($p = 0.30$; [4]) or U-test of global heterozygote excess ($p = 0.50$; [5]). For the newly-developed loci HGD110, HGD117 and HGD129, genotyping of the 312 individuals from Cornwall (S2 Table) revealed that the number of alleles ranged from 10 to 11 and the observed heterozygosity was 0.56 to 0.82 (S3 Table). The likelihood of null alleles being present was estimated in the software FreeNA [6], which did not detect any failed amplification among alleles (estimated frequencies of null alleles were <0.0001 for all loci).

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Paper III

Paper IV