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Single paternity within broods of the brown crab *Cancer pagurus*: a highly fecund species with long-term sperm storage

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ABSTRACT: Declining population sizes reported for a number of marine species have emphasised the need for information on mating systems to be incorporated into conservation and management strategies, particularly for exploited species. The brown crab *Cancer pagurus* L. supports a major fishery; however, many aspects of the species' mating system, such as paternity patterns, are unresolved. In this study, 3 microsatellite loci conferring a high degree of statistical power were used to determine whether broods from 18 ovigerous females collected from the English Channel had been fertilised by multiple males. Despite the capacity of this species for long-term storage of sperm and the suspected potential for females to use sperm from multiple males simultaneously, no evidence of multiple paternity was detected. Hypotheses as to the predominant processes leading to single paternity are discussed. In systems where females exhibit genetic monogamy the effective population size is constrained by the number of females. Such systems may be particularly susceptible to declines if females are removed. This has important implications for management of *C. pagurus* populations, as fishery data indicate that females are more heavily harvested than males.

KEY WORDS: Crustacea · Cancridae · Paternity · Mating system · Sperm competition · Effective population size · Management

INTRODUCTION

Many marine species have experienced substantial population declines in recent years (Dulvy et al. 2003). For a number of commercially exploited species, such as the Atlantic cod *Gadus morhua*, declines have occurred in spite of enormous efforts to ensure sustainability through the collection of data pertaining to abundance, distribution, and age-specific metrics of commercial catches (Hutchings 2003). This has highlighted the need to incorporate knowledge of the ecology of target and non-target species into conservation and management strategies (Rowe & Hutchings 2003). It is increasingly recognised that different mating systems can confer different susceptibilities of populations to certain conservation risks and affect the ability of populations to respond to changing environmental conditions and selection pressures such as fishing (Rowe & Hutchings 2003). As a result, the number of studies of mating systems among marine species is growing (Mäkinen et al. 2007, Hyde et al. 2008, Rowe et al. 2008), although the available information does not yet reflect the significant effect these systems can have on population sustainability (Rowe & Hutchings 2003).

The brown crab *Cancer pagurus* L. (Brachyura, Cancridae) is distributed along northeast Atlantic coastlines from the Lofoten Islands of Norway to Portugal (Christiansen 1969). It is commercially exploited throughout its geographical range with total landings of 46 280 t reported for 2005. As is common in the family Cancridae, mating is observed to occur between a soft bodied (recently moulted) female and a male whose exoskeleton is intact (Edwards 1966, 1979,

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Hartnoll 1969). Courtship is prolonged, with pairings of premoult females and intermoult males for periods of 3 to 21 d prior to female moulting and mating (Edwards 1966). Pairing has been observed to continue for 1 to 12 d after mating (Edwards 1966). This extended attendance by the male is presumed to represent pre- and postcopulatory mate guarding (Edwards 1979), which is common in species with soft-shelled female mating (Hartnoll 1969). Sperm plugs occluding the vulva and blocking the vagina become visible in the female soon after copulation (Williamson 1904). Sperm are stored in spermathecae pending spawning. The time gap between insemination and spawning is poorly documented and seems highly variable. For larger females, spawning may occur within a few months of insemination; however, it is also common for spawning to be delayed for up to 15 mo (Pearson 1908). Females may use one supply of stored sperm to fertilise multiple batches of eggs, with reports of 3 successive batches of eggs being fertilised without intervening moults (Pearson 1908). Despite extensive research on the fisheries and biology of *C. pagurus*, many important aspects of the mating system are unknown. In particular, the genetic mating system (i.e. the occurrence of single versus multiple paternity) has not been described.

Multiple paternity within broods has been reported in the brachyuran crabs *Chinoecetes opilio* (Roy 2003) and *Cancer magister* (Jensen & Bentzen 2002; the latter being the closest known phylogenetic relative of *Cancer pagurus* (Harrison & Crespi 1999). However, the limited information available on paternity patterns in brachyuran crabs suggests that a single male typically sires all or a very large fraction of eggs in a brood and that multiple paternity is uncommon (Diesel 1989, 1991, Koga et al. 1993, Jensen et al. 1996, Urbani et al. 1998). Due to the limited period of female receptivity, as well as the occurrence of both mate guarding and sperm plugs, it might be predicted that the incidence of multiple mating and paternity in *C. pagurus* would be low, at least within a single moult (Hartnoll 1969, Edwards 1979). However, if sperm is retained across moults, the capacity of *C. pagurus* for long-term sperm storage presents the opportunity for sperm from multiple copulations or males to be available to the female for fertilisation of subsequent broods.

Behavioural observations, physiology and phylogeny can be inaccurate predictors of realised animal mating systems (Birkhead 2000). Molecular genetic parentage analysis can permit qualitative and quantitative descriptions of paternity patterns and provide new insights into the breeding biology of species where direct observation is difficult. Burfitt (1980) investigated paternity patterns among broods from 6 ovigerous female *Cancer pagurus* sampled in the North Sea using variation at an allozyme locus. The study was hampered by the low levels of genetic polymorphism (maximum 3 genotypes) and, although unexpected allele frequencies were detected for one brood, multiple paternity could not be confirmed. In the present study, more powerful microsatellite markers were employed to investigate whether broods of eggs spawned by individual *C. pagurus* females had been fertilised by multiple males.

MATERIALS AND METHODS

Sample collection. Ovigerous crabs overwinter without feeding and are rarely caught in traps (Edwards 1979, Howard 1982). However, 18 berried females were captured as part of a beam trawl survey operated by the Centre for Environment, Fisheries and Aquaculture (CEFAS) in the western English Channel. Carapace width was measured to the nearest millimetre. Tissue samples were obtained from each female by stimulating autotomy of a cheliped. Each cheliped was then stored in 95% ethanol. Fertilised eggs (hereafter referred to as eggs) were brushed off each female and placed in a vial containing 95% ethanol labelled according to the female from which they were derived. The females were returned to the sea after sampling.

DNA extraction and amplification of microsatellites. Total DNA was isolated from female cheliped muscle tissue using a cetyltrimethyl ammonium bromide (CTAB)-chloroform/isoamyl alcohol method (after Winnepenninckx et al. 1993). DNA was also isolated from 40 individual eggs from each brood. Due to the small amount of tissue available a Chelex® extraction method (modified from Estoup et al. 1996) was used for individual eggs. Individual eggs were transferred by pipette to 0.2 µl PCR tubes and incubated at 37°C until the ethanol had evaporated. Then 50 µl of 5% chelating resin (Chelex®, Bio-Rad) and 3 µl of Proteinase K (10 mg μl^{-1}) were added to each sample. The mixture was incubated at 55°C for 3 h and boiled (100°C) for 10 min. Samples were then centrifuged at 16 060 × *g* for 5 min, and the supernatant removed and stored at –20°C. As *Cancer pagurus* is a highly fecund species with 0.5 to 2.9 million eggs produced per female (Edwards 1979, Ungfors 2007), DNA was also extracted from pooled batches of eggs. This pooling strategy permitted the subsequent screening of a greater number of offspring for non-maternal alleles. Briefly, eggs that were floating freely in ethanol were randomly sampled by pipetting ~750 µl of the ethanol-egg 'slurry' into an Eppendorf microcentrifuge tube. This was then incubated at 37°C until all the ethanol had evaporated. DNA extraction from the 'dried' egg pool then followed the same protocol as for the maternal tissue. Each pool

contained between 300 to 500 eggs and 4 pools were extracted per brood. For the maternal, individual egg and pooled egg samples, PCR was used to amplify 3 previously developed microsatellite loci (*Cpag*-4, *Cpag*-5D8 and *Cpag*-6C4B; McKeown & Shaw 2008). The loci are unlinked and free from technical artefacts (null alleles) that could confound results (McKeown & Shaw 2008). PCR amplification conditions and genotyping protocols are described in McKeown & Shaw (2008). In the case of the pooled eggs, allelograms representing the alleles present at each locus among the pooled eggs were produced.

Paternity analysis. The probability (power) of the loci to detect multiple paternity in a sample of offspring (PrDM) was estimated using the program PrDM v.1 (Neff & Pitcher 2002), which employs a Monte Carlo simulation incorporating the effects of (1) number of loci, (2) number of alleles per locus, (3) allele frequencies, (4) number of contributing sires and (5) reproductive skew among the sires. Allele numbers and frequencies estimated from genotyping of adults sampled randomly from multiple locations in the English Channel as part of a wide scale study of population genetic structure were used. Results were consistent regardless of the population sample used (or when all populations data were pooled) and so only the results based on data from the nearest geographical sampling site (Start Point: 50° 15′ N, 03° 33′ W, n = 133) are described. A conservative model of 2 contributing sires was run for scenarios of 50:50 and 90:10 paternal contributions. As the probability of detecting multiple paternity is also affected by the number of offspring analysed, the simulation was run assuming a range of numbers (10 to 50) of surveyed offspring.

For each locus the maternal genotype was determined directly from DNA extracted from the female's tissue. The minimum number of fathers for each brood was then inferred using 2 methods. The first was a single locus manual assignment approach whereby the minimum number of sires was estimated to be half the number of non-maternal alleles recorded at a locus (based on both individual and pooled egg allele counts), rounded to the next upper integer if the quotient was uneven (Toonen 2004). Multiple paternity, therefore, was concluded if 3 or more non-maternal alleles were detected at any locus among offspring. The second method was a multilocus approach performed using the program GERUD v. 2.0 (Jones 2005). While this is more sophisticated than allele counting as it uses multiple loci simultaneously, it was only applicable to data derived from individuals. Briefly, following the removal of maternal alleles from each offspring genotype, the minimum number of fathers (and their genotypes) siring each brood was inferred using an exhaustive search algorithm.

RESULTS

Summary statistics for the population sample screened for the 3 microsatellite loci are reported in Table 1. Overall, the set of microsatellites provided considerable power for detecting multiple paternity within broods (Table 2). As expected, the PrDM increased with sample size. A sample of only 40 eggs (equal to the number of individual eggs assayed per brood) was sufficient to obtain a PrDM of 99.9% under the assumption of equal male contribution and 92.7% in the case of a 90:10 paternal skew. With a sample size of 50 eggs (less than that assayed via the pooling method) PrDM was 99.8 to 99.9% for the simulated models of paternal contribution.

For each brood the genotypes of the 40 individual eggs revealed the same allelic combinations (maternal and presumed paternal alleles) as detected in the respective samples of pooled eggs. Allele counts provided no evidence (i.e. >2 paternal alleles at any locus) that more than a single male had contributed to any of the 18 broods tested (Table 3). The multilocus analysis was compatible with the single-locus analysis for all broods, as it indicated that mating involving a single male and the genotyped mother was sufficient to produce the respective brood genotypes in each case.

Table 1. *Cancer pagurus.* Allelic diversity (k), observed and expected heterozygosity (H_{obs} and H_{exp}, respectively), and probabilities (p-values) of conformance of genotype proportions to Hardy-Weinberg equilibrium (HWE) for 3 microsatellite loci, based on a sample of 133 adults from Start Point (50° 15′ N, 03° 33′ W) analysed as part of a larger population genetic study. H_{obs} and H_{exp} were calculated using FSTAT 2.9.3 (Goudet 1995). HWE test was performed in GENEPOP 3.3 (Raymond & Rousset 1995)

Locus	k	H_{obs}	H_{exp}	HWE (p)
Cpag-4	27	0.908	0.941	0.591
Cpag-5D8	27	0.880	0.895	0.626
Cpag-6C4B	6	0.683	0.681	0.495

Table 2. *Cancer pagurus.* Probability of detecting multiple paternity (PrDM) for the 3 microsatellite loci for a range of egg numbers assayed (n) and assuming broods to be the product of 2 fathers with equal (50:50) or skewed (90:10) contributions. See 'Materials and methods—Paternity analysis' for simulation details

Eggs (n)	50:50	90:10
10	0.991	0.627
20	0.998	0.861
30	0.999	0.946
40	0.999	0.977
50	0.999	0.998

Table 3. *Cancer pagurus*. Designated identification (ID), collection date and carapace width of ovigerous females, and different alleles detected within each brood at each locus by genotyping of mothers and offspring. Alleles are designated by length in base pairs. Maternal alleles are in **bold**

Female ID	Date collected	Carapace width (mm)	Alleles		
			Cpag-4	*Cpag*-5D8	*Cpag*-6C4B
1	29 March 2006	193	214, **217**, **244**, 271	186, 192, **201**, **234**	**172**, **180**
2	4 July 2006	158	208, **211**, **214**, 247	186, 189, **195**, **207**	**172**, 176, **180**
3	30 March 2006	162	220, **226**, **238**, 283	**186**, **189**, 207	**172**, 176
4	23 March 2006	188	250, **256**, 277, **286**	159, **180**, **192**, 231	**172**, 176, 180
5	22 March 2006	188	205, **208**, **253**, 259	**186**, 189, 192, **195**	**172**, **176**
6	24 March 2006	181	208, 214, **222**, **235**	159, **186**, **204**	172, **180**
7	12 July 2006	159	205, 250, **271**, **277**	**192**, **225**, 234, 243	**176**, **180**
8	26 March 2006	187	**214**, 256, **268**, 277	**186**, **192**, 195	168, **172**, **184**
9	10 July 2006	182	**217**, 259, 271	**168**, **192**, 195, 204	**164**, **172**, 180
10	14 August 2006	185	**244**, 268, **274**	186, **189**, **195**	172, **176**
11	19 June 2006	178	**250**, 262, **271**	**180**, 204, **207**	**172**, **176**
12	20 June 2006	147	**247**, **265**, 268, 277	183, **192**, **195**, 207	**172**, **176**, 188
13	25 March 2006	187	**211**, 214, **217**, 274	183, 189, **207**	**172**, **176**
14	25 March 2006	186	214, **220**, **268**	**183**, **186**, 204	**172**, 176
15	24 March 2006	100	**247**, 259, 265, **274**	165, **186**, **210**, 225	168, **172**, **176**
16	7 July 2006	177	229, **232**, **238**, 277	**174**, **219**, 222, 238	**172**, 176, **180**
17	24 March 2006	179	**223**, **232**, 238, 259	165, **177**, **180**, 186	**172**, **176**, 180
18	19 July 2006	145	229, **244**, 253, **283**	**168**, 201, **213**, 219	172, **176**

DISCUSSION

In this study, paternity patterns within *Cancer pagurus* broods were assessed by microsatellite analysis of (1) individual eggs and (2) pools of eggs, sampled from each of 18 ovigerous females collected in the English Channel. This approach, combining the resolution of individual egg genotypes and the screening of a large number of eggs for non-maternal alleles, along with properties of the markers employed, conferred a high degree of investigative power. As a result, the failure to detect multiple paternity in any of the 18 broods permits the robust inference of single paternity in this case.

While the detection of a single case of multiple paternity is sufficient to reject genetic monogamy, it is much more difficult to conclusively demonstrate the absence of multiple paternity. Firstly, for any species, the frequency and degree of multiple paternity can vary temporally, spatially, or phenologically (Sainte-Marie et al. 2002, Roy 2003, Gosselin et al. 2005). Substantial variation in the frequency of multiple paternity has been reported among crustaceans: 80% (of 10 broods) in the porcelain crab *Petrolisthes cinctipes* (Toonen 2004); 60% (of 15 broods) in the crayfish *Orconectes placidus* (Walker et al. 2002); 54.6% (of 11 broods) in the Norway lobster *Nephrops norvegicus* (Streiff et al. 2004); and 13% (of 108 broods) in the American lobster *Homarus americanus* (Gosselin et al. 2005). In the snow crab *Chinoecetes opilio*, multiple paternity was not detected in 2 studies (0% of 7 broods, Urbani et al. 1998; 0% of 5 broods, Sainte-Marie et al. 1999), but low rates of multiple paternity were revealed in a larger study (3.8% of 79 broods, Roy 2003). It cannot be ruled out that a low level of multiple paternity may not have been detected in the present study due to the number of broods screened. However, it can be concluded that even if multiple paternity occurs in *Cancer pagurus* it is at very low frequencies and single paternity is the predominant system.

The present study extends the prior observations of mating behaviour in *Cancer pagurus* (Edwards 1966, 1979) by demonstrating realised fertilisation outcomes and can suggest hypotheses for future study. While it is believed that male cancrids are generally polygynous and exhibit female-centred competition (Orensanz & Gallucci 1988, Orensanz et al. 1995) the range of female mating behaviors have not been fully resolved. Single paternity could indicate that *C. pagurus* females are monandrous. Monandry, at least within a single moult–mate cycle, might be expected given the limited period of female receptivity as well as the reported mate guarding behaviour and occurrence of sperm plugs. Furthermore, the fitness benefits of multiple matings are often less obvious for females than for males (Hosken 1999, Birkhead 2000, Jennions & Petrie 2000), particularly when females can store enough viable sperm from a single mating to fertilise their eggs for much of their reproductive lifetime (Hosken 1999). When in a soft-shelled receptive state, female *C. pagurus* are likely to be highly vulnerable to injuries from competing male mates (Diesel 1991), predators, and

other females. The ability of *C. pagurus* to use stored sperm to fertilise multiple successive egg clutches might benefit the female by reducing the need for additional matings and the required moults.

Despite the vulnerability of soft-shelled females, moulting is necessary for growth, which in turn increases fecundity (Edwards 1979, Ungfors 2007). Although *Cancer pagurus* individuals are difficult to age, it is likely, based on their sizes (Table 3), that many of the females sampled in this study have undergone multiple moults, with each moult presenting an opportunity for copulation, presumably involving a different male. It is possible that sperm stores are lost during the moulting process, meaning that females may only contain sperm from a single male at any time. The absence of trans-moult sperm retention has been reported in the brachyuran crabs, *Chasmagnathus granulata* (López Greco et al. 1999) and *Aratus pisonii* (Hartnoll 1965). However, trans-moult sperm retention has been demonstrated in cancrids (Orensanz et al. 1995), including *Cancer magister* (Shirley & McNutt 1989), which, as mentioned, is the closest known relative of *C. pagurus* (Harrison & Crespi 1999). Therefore, sperm loss during moulting would seem unlikely to be the main process leading to single paternity here.

Assuming sperm is retained across moults and that additional males add to the spermathecal sperm supply during moults, single paternity could result from a number of potential mechanisms. Fresh sperm from a new mating might fertilise the majority of eggs due to depletion and/or degradation of older stored sperm. Single paternity might also reflect effective female postcopulatory sperm choice or sperm precedence mechanisms. Postcopulatory mechanisms of female sperm choice include sperm dumping, digestion or other forms of sperm sorting (Thiel & Hinojosa 2003). Sperm precedence mechanisms, resulting from male–male sperm competition, may result in a single male gaining all fertilisations due to either sperm removal or sperm stratification processes (Birkhead & Hunter 1990). Removal of previously deposited sperm could involve extraction (Waage 1979) or 'flushing out' (e.g. Rubenstein 1989). However, Orensanz et al. (1995) suggest that sperm extraction is unlikely among the Cancridae. Based on published data, sperm precedence appears to be the most likely mechanism of paternity assurance (if any) involved here. As observed for *Cancer magister* (Jensen et al. 1996), the spermathecae of *Cancer pagurus* appear to be the 'ventral' type where the vagina and oviduct open into the spermatheca in close proximity to one another. With such a structure, the last male to inseminate the female is expected to have the advantage (last male sperm precedence). Last male sperm precedence due to sperm stratification has been reported in the snow crab where out of a total of 91 females studied, 88% had mated with multiple males, but only 3.2% exhibited multiple paternity within their resulting broods (Urbani et al. 1998, Sainte-Marie et al. 1999, Roy 2003).

The predominance of single paternity in *Cancer pagurus* provides an interesting addition to the ongoing debate as to the evolutionary significance of polyandry and multiple paternity (Jennions & Petrie 2000). Proposed benefits of polyandry and multiple paternity include: (1) reducing the chances of reproductive failure due to mating with an incompatible male, such as a relative (Zeh & Zeh 2003); (2) lowering relatedness among offspring and reducing sibling competition (Yasui 1998); and (3) genetic 'bet-hedging' (Jennions & Petrie 2000), which may be beneficial in unstable environments (Yasui 1998). It is likely that local populations of *C. pagurus* are large and, therefore, naturally buffered against close-kin mating. Furthermore, the long-lived pelagic larvae have the potential for extensive dispersal, which may serve to reduce sibling competition. The large and dispersive populations of *C. pagurus* may therefore result in little selective disadvantage for single paternity.

At the population level, multiple paternity is suggested to increase effective population size (Martinez et al. 2000). Therefore, single paternity species/populations may be more prone to loss of genetic diversity if population sizes are reduced (e.g. by overfishing) than are genetically polyandrous systems. Theoretical results, however, suggest that by increasing variance in male reproductive success, multiple paternity may reduce the effective population size and genetic diversity of the next generation (Hedrick 2005). In situations where females are each fertilised by a single male in a given reproductive cycle, the number of females imposes a strict constraint on the number of males that can breed. Under this type of mating system, a decline in female numbers would result in proportional reductions in effective population size regardless of the number of males. Species like *Cancer pagurus* may, therefore, be highly susceptible to decline in effective population size (and related loss of genetic diversity) if overfishing of females occurs. In the *C. pagurus* fishery, female landings per unit effort are considerably higher than those for males for most of the year (Bennett 1995), which emphasises the importance of collecting and monitoring sexspecific landings statistics.

This study suggests that single paternity is the norm for *Cancer pagurus*. Further research should test this by investigating paternity patterns throughout the species range. Analysis of spermathecal content might also elucidate how mating behaviour (polyandry versus monandry) and/or postcopulatory sexual selection shapes paternity patterns in this economically important species.

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