Genetic parentage assessment in the crayfish *Orconectes placidus*, a high-fecundity invertebrate with extended maternal brood care

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Abstract

Microsatellite data have recently been introduced in the context of genetic maternity and paternity assignments in high-fecundity fish species with single-parent-tended broods. Here we extend such analyses to an aquatic invertebrate, the crayfish *Orconectes placidus*, in which gravid females carry large numbers of offspring. Genetic parentage analyses of more than 900 progeny from 15 wild crayfish broods revealed that gravid females were invariably the exclusive dams of the offspring they tended (i.e. there was no allomaternal care), and that most of the females had mated with multiple (usually two) males who contributed sometimes highly skewed numbers of offspring to a brood. Within any multiply sired brood, the unhatched eggs (or the hatched juveniles) from different fathers were randomly distributed across the mother's brood space. All of these genetic findings are discussed in the light of observations on the mating behaviours and reproductive biology of crayfishes.

Keywords: *de novo* mutations, invertebrate, maternity, mating system, microsatellites, multiple paternity

Introduction

'Common and lowly as most may think the crayfish, it is yet so full of wonders that the greatest naturalist may be puzzled to give a clear account of it.'

Roesel von Rosenhof, 1755, as quoted in Huxley (1884)

Descriptive accounts abound on the reproductive biology and mating behaviours of crayfishes (Pippitt 1977; Berrill & Arsenault 1984; Holdich & Lowery 1988; Momot 1988; Riggert *et al.* 1999; Holdich 2002; see also the website http://zoology.byu.edu/crandall_lab/lablinks.htm). During copulation, a spermatophore (sperm packet) is transferred to the opening of a female's seminal receptacle. This leads to a sperm storage vessel (Holdich & Reeve 1988) that, after mating, may be sealed by a sperm plug (Crocker & Barr 1968; Stein 1976; Berrill & Arsenault 1984; Momot 1988). When oviposition begins, a female cups her abdomen under her cephalothorax, forming a spawning chamber into which is secreted glair, a mucous substance that stiffens

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when contacted by water. She turns on her back and expels eggs into this mucilaginous gel, where a chemical dissolves the spermatophore, and the eggs and nonmotile sperm come into contact for fertilization (Holdich & Reeve 1988). The glair then hardens and the eggs attach to oosetae (bristles) on the female's pleopods (swimming legs).

In addition to providing protection, female crayfish fan and groom the eggs and hatchlings (Mason 1970; Payne 1978; Bechler 1981). The young resemble miniature adults. They generally remain attached to the mother through several moults, but occasionally leave her to feed for short periods (and with greater frequency as they grow), returning quickly to her when danger threatens (Little 1975). The time from egg extrusion to complete juvenile independence is approximately three months for most orconectids (Momot 1988).

Premating interactions in crayfishes often include aggressive and nonaggressive interactions between the sexes and intermale agonistic encounters (Mason 1970; Pippitt 1977; Bechler 1981; Berrill & Arsenault 1984). Females evidence mate preferences by fleeing, fighting or remaining passive to approaching males (Berrill & Arsenault 1984; Pippitt 1977). In *Orconectes rusticus*, large roving males in

nature have been observed to encounter and disrupt copulating pairs (Berrill & Arsenault 1984), and for this or other reasons, at least some females might mate with two or more partners. Multiple mating by both sexes has been noticed in the laboratory in several crayfish genera (Berrill & Arsenault 1984; Reynolds 2002), but such observations do not document multiple sires for a clutch nor can they assess the frequency of multiple paternity in the wild.

With this observational backdrop, we employed polymorphic molecular markers to investigate genetic maternity and paternity in *O. placidus* (Cambaridae). This species is found under rocks and litter in small streams and rivers of the Tennessee, Cumberland and lower Ohio drainages on the western edge of the Cumberland Plateau from southern Illinois to northern Alabama (Bouchard 1976; Page 1985; Hobbs 1989; Taylor *et al*. 1996). In particular, we address whether individual females often carry progeny sired by two or more males, and if so, to quantify the number of mates, paternal skew and the microspatial distributions of offspring in each brood.

Our broader goal is to extend to an invertebrate animal the kinds of microsatellite analyses recently introduced to unveil genetic mating systems and surreptitious reproductive behaviours in several native fish species with large clutches and extended offspring care (Avise 2001; Avise *et al*. 2002). Although similar genetic parentage analyses have been conducted regarding sperm competition in several insects and other invertebrate species (e.g. Birkhead & Møller 1998), and with respect to relatedness and kin selection in social insects (e.g. Peters *et al*. 1995; Bernasconi *et al*. 1997; Oldroyd *et al*. 1997), there have been few such reports with the goal of illuminating reproductive natural histories of wild aquatic invertebrates.

Materials and methods

Collections and laboratory procedures

Crayfish were collected by seine on 29 April 2000 and 23 April 2001 from the West Fork Drakes Creek (Barren River system), near Portland in Sumner County, Tennessee. One additional female was collected on 29 April 2000 in the Red River (Cumberland River system), also near Portland, on the Sumner/Robertson County line. The samples were stored immediately in 95% ethanol. One adult male voucher specimen (TU 7095) was deposited at the Tulane University Museum of Natural History.

To construct a genomic library, muscle tissue was taken from the chelae of a single adult. A standard phenol/chloroform protocol was used to extract DNA, that then was digested with the restriction enzyme *Mbo*I. After electrophoresis through a 2% agarose gel, the desired DNA fragments (200–800 bp) were purified using the Prep-A-Gene DNA Purification System (Bio-Rad). These fragments were ligated into a phagemid vector (pBluescript II SK from Stratagene) that first had been digested with *Bam*HI and then dephosphorylated. The resulting ligations were heat-shock transformed into *Escherichia coli* (XL2-Blue MRF' Ultracompetent Cells from Stratagene). The cells were plated on Luria-Bertani–ampicillin plates for overnight growth.

Clones containing microsatellite DNA were identified following Jones & Avise (1997). After purification, positive clones were sequenced using T7 or T3 primers (gamma P32 end-labelled) and the *fmol* DNA Cycle Sequencing System (Promega). Primers then were designed for the regions flanking the microsatellites.

From adults, DNA was extracted from chelae tissue as described above. From whole embryos (after removing the yolk) and from half-bodies of juveniles, DNA was extracted using 50–150 µL of Gloor & Engels buffer (Gloor & Engels 1992) following procedures described in Jones & Avise (1997).

Each microsatellite locus was amplified in 10 µL total volume containing appropriate $MgCl_2$ concentrations (Table 1), 1× Promega *Taq* buffer, 0.25 U *Taq* DNA polymerase, 0.1 mM each dNTP and 0.15 µM of each primer. Polymerase chain reaction (PCR) cycling conditions were as follows: an initial denaturation step for 2 min at 95 °C; 30 cycles of denaturation (1 min, 95 °C), annealing (1 min, at temperatures suitable for each locus; Table 1), and extension (1 minute, 72 °C); and a final extension for 3 min at 72 °C.

For an initial assessment of genetic variation in the adult population, candidate loci were assayed by electrophoresis

Table 1 Summary of information for the crayfish microsatellite loci surveyed. GenBank Accession nos: AY112993–AY112995

Locus	Primer sequences (5′ → 3′)	repeat	Anneal temp. (°C)	$MgCl_2$ conc. (mM)	No. of alleles	Excl. prob.
2.6	GATCGAAGTAACTGCTGTTCG TGCCCATTCAGTTTTCCGTAG	$[AC]_{41}$	50	1.5	22	0.87
2.12	GACCCAATTGCTGCCCGGAAGTG CGTCCAGTACTTGGACGTGAAATC	$[GA]_{33}$	56	2.0	19	0.84
3.1	TTCAGGGGCGAGAAAGTTGTGAC GTGGGAAGGGGTAAGGGAGAG	$[GA]_{94}$	60	1.0	19	0.58

Combined exclusion probability for all three loci = 0.99.
Combined exclusion probability for loci 2.6 and 2.12 = 0.98.

Table 2 Sample sizes, progeny stage and deduced genetic paternity in 15 crayfish broods

Dam's ID	Number of progeny		Progeny stage	% progeny for primary father	Number of sires	
	Assayed*	In brood			Minimum	Adjusted‡
CF1	44	412	hatchlings	100	1	1.0
CF2	59	279	hatchlings	—†	2	2.0
CF3	70	164	hatchlings	91	2	2.1
CF4	65	257	hatchlings	94	2	2.1
CF5	41	235	hatchlings	100	1	1.0
CF6	65	138	hatchlings	88	3	3.2
CF7	41	514	hatchlings	100	1	1.0
CF8	55	368	hatchlings	100	1	2.0
CF9	236	297	hatchlings	85	4	4.9
CF10	45	245	unhatched (in eggs)	100	1	1.0
CF11	43	259	unhatched (in eggs)	59	2	3.2
CF12	41	42	hatchlings	54	2	2.0
CF13	41	370	unhatched (in eggs)	100	1	1.0
CF14	45	160	unhatched (in eggs)	96	2	2.0
CF15	20	20	hatchlings	95	2	2.0
Total or mean	911	251		90.2	1.8	2.0

*Number of offspring genotyped at two or more loci.
†Could not be determined due to allelic sharing between fathers.
‡For allelic sharing by adults, using the HAPLOTYPES program. This is still an underestimate, however, because HAPLOTYPES assumes equal contributions by all sires.

of ^{32}P-labelled PCR products via 6% acrylamide gels. After sufficiently polymorphic loci were identified, all adults and progeny were genotyped by electrophoresis of fluorescently tagged PCR products via a 4.2% acrylamide gel, using an ABI377 automated sequencer. Forward primers were labelled with a 5′ 6-FAM dye (Integrated DNA Technologies, Inc.), and 0.6 μL of each PCR product was electrophoresed with 2.1 μL deionized formamide, 0.5 μL loading buffer and 0.3 μL GeneScan ROX 500 Size Standard (Applied Biosystems). Data were analysed using GENESCAN Version 3.1 and GENOTYPER Version 2.5 software.

Parentage analyses

Allele frequencies in the population were estimated from the collection of adults, and genotypic frequencies were tested for Hardy–Weinberg equilibrium (HWE; Guo & Thompson 1992) using the program GENEPOP (Raymond & Rousset 1995). Genetic exclusion probabilities for these loci were calculated under the assumption that one parent (the mother in this case) was known (Selvin 1980).

When broods are large and it is not feasible to sample all individuals, the computer simulation program BROOD (DeWoody *et al*. 2000a) can assist in determining the approximate mean number of progeny (*n*) that should be assayed from each mother to detect all contributing sires. Parameters input into BROOD include a hypothetical adult population size, observed allele frequencies in the adult sample, an estimate of the number of offspring carried per dam, and the possible number and relative contributions of different fathers to a typical brood. For the current simulations, we arbitrarily set the population size at 500, and assumed that 10 males at most contributed equally to each clutch of 300 progeny.

Offspring were categorized into two distinct life-history stages (Table 2): hatchlings (present in 11 broods) and unhatched eggs (4 broods). The progeny carried by each gravid female were usually arranged in discrete groups along her tail, corresponding to her last four pairs of pleopods (Fig. 1). Based on the outputs from the BROOD simulation, an attempt was made to genetically analyse 15 progeny sampled at random from each of these four groups (60 offspring total) from each female. For one female (CF9), 236 among her total of 297 offspring were genotyped. For all females, progeny were genotyped at two of the microsatellite loci (*2.6* and *2.12*), and when necessary, a third locus (*3.1*) was employed on a subset of offspring in an effort to resolve any remaining ambiguities about genetic parentage within a brood.

For each offspring at each locus, the paternally derived allele was deduced by subtracting from the progeny's diploid genotype the allele it had inherited from its known mother. The number of different paternal alleles observed

Fig. 1 Photograph of progeny attached to the four rows of pleopods underneath the abdomens of two female *Orconectes placidus*.

in a brood (divided by two, and rounded up to the nearest whole integer) gives a *minimum* estimate of number of sires (Kellogg *et al*. 1998). Some microsatellite alleles were shared among fathers, so we also used the computer program HAPLOTYPES to statistically upwardly adjust such estimates of sire numbers (DeWoody *et al*. 2000a). This program does not, however, take into account the possibility of skew in paternal contributions to a brood.

Results

Collections and microsatellite markers

Fifty-six adult individuals were collected, including 15 females with fertilized eggs or juveniles attached to their pleopods. Each brooding female carried on average 250 offspring (range 20–514). The developmental stage of all offspring appeared to be identical within a brood (consistent with the mode of oviposition described above), but differed noticeably among broods (Table 2).

In the genomic library, 20 positive clones were identified, three of which were highly variable and amplified consistently in the collection of adults (Table 1): locus *2.6*, which displayed 22 alleles, and loci *2.12* and *3.1*, which showed 19 alleles each (Fig. 2). Such highly polymorphic microsatellite loci have been reported in other orconectid crayfishes (Harris & Crandall 2000), as well as in other crayfish genera (Fetzner & Crandall 2002). All loci in the current study were characterized by variable numbers of dinucleotide repeats, but locus *3.1* was imperfect, with an occasional C replacing a G. Examinations of allelic associations in the progeny arrays revealed that all three loci assort independently. Based on this absence of genetic
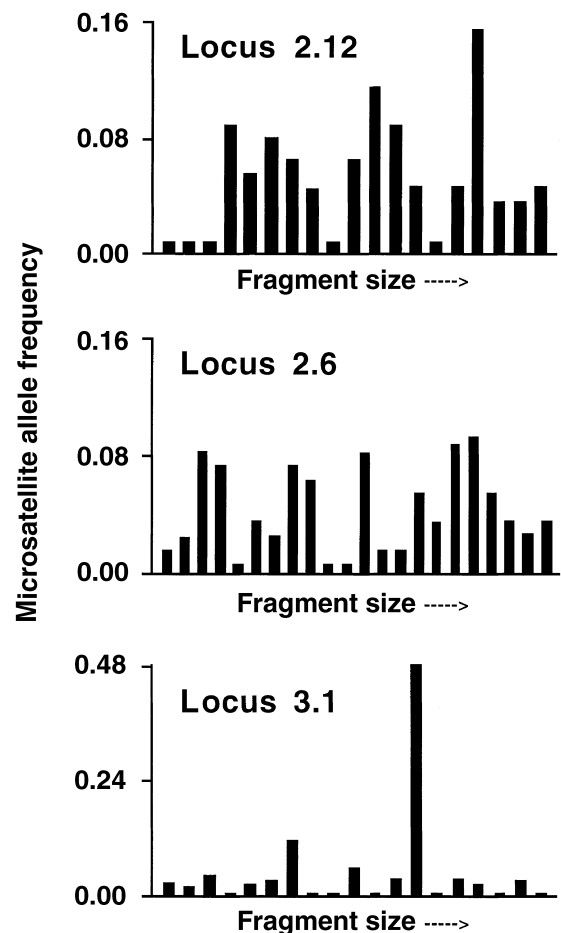

Fig. 2 Histograms of allele frequencies observed in the adult population of *Orconectes placidus* at each of three microsatellite loci. Alleles are arranged from left to right according to their rank order in size, from smallest to largest.

linkage, the overall combined genetic exclusion probability (under the one-parent-known model) was 0.99; considering only loci *2.6* and *2.12*, the combined genetic exclusion probability was 0.98.

No significant departures from HWE were detected at loci *2.12* and *3.1* in the adult population. However, locus *2.6* exhibited a marginally significant departure from HWE ($P = 0.05$) in the direction of a slight excess of homozygotes. For two reasons, we suspect that this departure is due to the presence of null (nonamplifying) alleles. First, adults displaying a single band on the gel were scored *prima facie* as homozygotes, but a few of them instead might have been heterozygotes for a null allele. Second, in the 15 broods in which progeny genotypes as well as that of the mother were available for inspection, null alleles could be documented more securely (e.g. by an absence of gel bands of maternal origin in some or all of the progeny). Using such evidence, two mothers were null heterozygotes and one was a null homozygote.

Assuming that at most 10 fathers contributed equally to a clutch of 300 young, the BROOD computer simulation estimated that a mean sample of 63 progeny (SD = 21, upper 95% CI = 103) per clutch would suffice to detect allelic contributions from all sires.

Maternity

At each locus and in every brood, alleles appeared to be inherited according to simple Mendelian rules. Furthermore, all 911 offspring genotyped in this study displayed alleles consistent with maternity by the mother who carried them. There was only one possible exception, in which one juvenile (from brood CF6) had an allele at locus *2.12* that did not match either allele in its presumptive mother. However, because this juvenile did carry alleles that were consistent with that female's genotype at the other two loci, we provisionally attribute the aberrant allele at locus *2.12* to a *de novo* mutation. If this interpretation is correct, considering all mothers we estimate the overall mutation rate at this locus in the maternal germ line to be about 1.1×10^{-3} per gamete per generation. This value is well within the range reported for microsatellite loci in other species (Weber & Wong 1993; Primmer *et al.* 1996; Schug *et al.* 1997; Jones *et al.* 1999).

Paternity

Examination of alleles and genotypes present in the offspring arrays demonstrated that 6 of the 15 broods (40%) stemmed in each case from a single sire (and, hence, consisted exclusively of full sibs). Each of the remaining nine broods (60%) apparently resulted from multiple paternity (Table 2). From single-locus counts of paternal alleles in each of the 15 progeny arrays, the minimum

number of sires per brood ranged from 1 to 4 (mean = 1.8), and the statistically adjusted estimate of the mean number of fathers was 2.0 (see footnote to Table 2). The exact sire genotypes in some instances of multiple paternity remained ambiguous (due to allelic sharing, null alleles, skewed paternal contributions and small numbers of progeny), but nonrandom associations of alleles across loci further helped to assess the approximate number of sires and, occasionally, even permitted deduction of the sire's complete multilocus genotype (see DeWoody *et al.* 2000b). For example, brood CF7 had a single sire whose genotypes at loci *2.6*, *2.12* and *3.1* could be explicitly deduced as 238/243, 183/211 and 281/318, respectively (the numbers refer to allelic designations based on gel mobility).

Within each multiply sired clutch, the contributions from different fathers usually were highly skewed, with one sire sometimes responsible for > 85% of the assayed progeny (Table 2). Only in broods CF11 and CF12 did each of the two fathers achieve nearly equal paternity. In these (or other) broods, there was no evident microspatial localization of full-sibs (Fig. 3). In other words, progeny from the different sires were scattered randomly across the different pleopod groups.

Discussion

Little was known previously about the reproductive consequences of crayfish mating behaviours in nature. Individuals (brooding females especially) are secretive, mostly active at night, and seldom observed mating in the wild; and in any event the apparent social mating system of any species can differ appreciably from the actual or realized genetic mating system. Here we present microsatellite data that shed new light on the genetic mating system of *Orconectes placidus*. The primary empirical conclusions from this genetic parentage analysis are as follows: (i) > 50% of the broods were multiply sired, typically by just two or a few males; (ii) each gravid female was the exclusive mother of all the progeny she carried, meaning that no juvenile transfers among dams were detected and each multiply sired clutch consisted of a mixture of full-sib and half-sib offspring; (iii) the genetic contributions of different sires to a brood were often but not invariably highly skewed; and (iv) the offspring from different sires were randomly distributed among the pleopod groups on the dam's abdomen.

Multiple mating by females

No deduced paternal genotype (or that of any adult male collected) was encountered in more than one brood. Nonetheless, because of the relatively small number of gravid females sampled and the unknown true size of the breeding population, we cannot conclude that individual
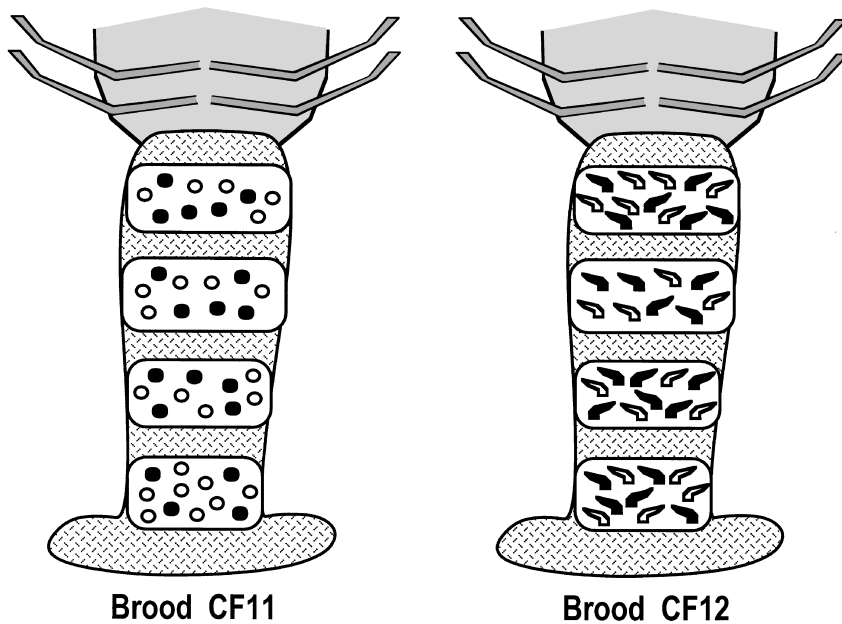
Fig. 3 Diagrams of two female crayfish abdomens showing the microspatial arrangements of sampled offspring from the two males (open vs. closed symbols) who in each case had sired nearly equal numbers of young in the brood (Table 2). Left, unhatched eggs in brood CF11; right, hatchlings in brood CF12. Pictured for each female are offspring sampled from the four distinct rows of pleopods (the spatial positions of offspring *within* a row were not monitored and have no meaning here).

males typically mate with a single female. Unequivocally, however, our data do demonstrate the converse — that females often mate successfully with multiple males.

Whether such matings result from female choice or forced copulations is unknown. Female crayfish invest heavily in offspring care, so it is plausible that they actively seek multiple mates for potential fitness advantages that sperm from additional sires might confer (e.g. fertilization insurance, better paternal genes for the dam's progeny or higher genetic diversity within her brood). Sperm plugs have been noted in the female reproductive tracts of several orconectid species (Crocker & Barr 1968; Stein 1976; Berrill & Arsenault 1984; Momot 1988), but our genetic data indicate that even if sperm plugs are inserted routinely by O. placidus males, they are less than fully effective in blocking paternity by other males. Other suggestions consistent with this finding are that a sperm plug may function primarily to hold sperm in place (Berrill & Arsenault 1984) or shield the sperm from water (Crocker & Barr 1968), rather than prevent later inseminations.

The available genetic data do not reveal the temporal order of sperm use by females, nor do they indicate the length of time that sperm may have been stored inside the female (long-term sperm retention has been reported in some crayfish species; Albaugh 1973). In some insects with mechanisms of external fertilization roughly similar to that in crayfishes, fertilization success is often (but not invariably) biased in favour of the last-mating male (Parker 1970, 1984).

Lack of interbrood mixing

Depending on the species, female crayfish care for offspring from two to eight months (Bechler 1981; Momot 1988).

As hatchlings undergo later moults, they become increasingly independent, venturing from their mother to forage nearby but quickly returning to the safety of her abdomen (Little 1976; Jezerinac *et al*. 1995). However, our genetic data give no indication of hatchling transfers between brooding females. In other words, we observed no instances of allomaternal care.

One theoretical possibility is that the chemical pheromones known to mediate interactions between a mother crayfish and her offspring (Holdich & Reeve 1988; Little 1976) are sufficiently dam-specific to ensure that such relocations of juveniles rarely if ever occur. However, from behavioural experiments on several species of crayfish, including one (*sanborni*) in the genus *Orconectes*, Little (1976) concluded that the pheromonal cues are species specific but not brood specific: 'larvae are attracted as strongly to the other conspecific brooding females as to their own mother'. Another possibility is that hatchlings in the sampled broods were not yet old enough for movement off the female. However, this seems unlikely for the developmentally advanced offspring in several of the assayed broods (see Fig. 1). Thus, we provisionally favour ecological explanations for the apparent lack of interbrood transfer — brooding females at our study locale may seldom have been in close enough association to allow an easy relocation of juveniles among dams.

Mate numbers

In highly fecund taxa, it is often logistically prohibitive to genotype all of the brooded progeny carried by females. Thus, in this case we used the computer simulation BROOD to estimate, a priori, the approximate number of progeny

sampled per brood needed to detect alleles from all contributing sires (given the observed allele frequencies in the adult population sample, and assuming equal contributions from up to 10 different males to a clutch). That number turned out to be ≈ 60. It later became apparent, however, that > 50% of the broods had multiple sires and that paternal contributions often were highly skewed. Thus, some low-contributing sires might have been missed. Accordingly, we ran the BROOD simulations again, assuming a paternal skew (85:5:5:5) more consistent with the initial genetic findings. These simulations indicated, a posteriori, that ≈ 100 progeny per brood (SD = 40, upper 95% confidence limit = 173) might be needed to detect all contributing sires.

To pursue this issue empirically, we then sampled one clutch (CF9) nearly exhaustively. At loci *2.6* and *2.12*, we scored genotypes for 236 among the total of 297 hatchlings present. Many of these offspring also were assayed at locus *3.1*. Among the 60 progeny originally assayed from this dam, three (5%) proved genetically to have been sired by two males other than the primary father. In the survey of 236 young from CF9, the final tally of progeny attributed to the additional fathers increased to 36 (15.2%), and the minimum number of genetically detected sires increased from three to four.

This still may not be the exact sire number for this brood, however, because opposing biases of unknown relative magnitudes could apply. On the one hand, a higher number of deduced parents increases the likelihood of allelic sharing among fathers and can lead to an underestimate of the true number of sires (Fiumera *et al.* 2001). On the other hand, the number of sires could be overestimated if *de novo* mutations in paternal germ lines, rather than additional sires, account for some of the 'orphan' alleles (those that at face value appear inconsistent with a paternity assignment at one assayed locus only).

Microspatial arrangements of offspring

Because each offspring in each brood was catalogued with respect to the pleopod group to which it was attached, we can also examine the microspatial distribution of full- and half-sib progeny within each female whose offspring were multiply sired (examples in Fig. 3). In each such case, the offspring from different fathers appeared randomly distributed across the brood space.

In six of these cases, hatchlings were involved. Thus, one possibility is that progeny from different sires move around and mix extensively on their mother's abdomen during development. However, two of the multiply sired broods consisted of unhatched (and immobile) fertilized eggs (Table 2), yet likewise there was no evidence for nonrandom microspatial distributions of offspring from different sires. Thus, regardless of whether the hatchlings

later shift positions, it appears that the zygotes tracing to sperm from different spermatophores are already spatially well-mixed by the time they attach to the female's pleopods.

Conclusion

Prior molecular assessments of genetic parentage in crustaceans (Brockman *et al.* 2000) and other invertebrates (e.g. Parker 1984) have focused primarily on the topic of sperm competition and utilization (Birkhead & Møller 1998), or (for social insects) on genetic relatedness in the context of eusociality. We hope that our findings on a high-fecundity crustacean with extended parental care will motivate further genetic research on the reproductive biologies and natural histories of aquatic invertebrate species in the wild.

Acknowledgements

References

Albaugh DW (1973) A case of long-term sperm retention by a female crayfish (Decapoda, Astacidae). *Southwestern Naturalist*, **18**, 97–98.

Avise JC, ed. (2001) DNA-based profiling of genetic mating systems and reproductive behaviors in poikilothermic vertebrates. *Journal of Heredity* (special issue), 92, 99–211.

Avise JC, Jones AG, Walker D *et al.* (2002) Genetic mating systems and reproductive natural histories of fishes: lessons for ecology and evolution. *Annual Review of Genetics*, in press.

Bechler DL (1981) Copulatory and maternal–offspring behavior in the hypogean crayfish, *Orconectes inermis inermis* Cope and *Orconectes pellucidus* (Tellkampf) (Decapoda, Astacidea). *Crustaceana*, **40**, 136–143.

Bernasconi G, Krieger MJB, Keller L (1997) Unequal partitioning of reproduction and investment between cooperating queens in the fire ant, *Solenopsis invicta*, as revealed by microsatellites. *Proceedings of the Royal Society of London B*, **264**, 1331–1336.

Berrill M, Arsenault M (1984) The breeding behaviour of a northern temperate orconectid crayfish, *Orconectes rusticus*. *Animal Behaviour*, **32**, 333–339.

Birkhead TR, Møller AP, eds. (1998) *Sperm Competition and Sexual Selection*. Academic Press, San Diego.

Bouchard RM (1976) Geography and ecology of crayfishes of the Cumberland Plateau and Cumberland Mountains, Kentucky, Virginia, Tennessee, Georgia, and Alabama, part I: the genera *Procambarus* and *Orconectes*. *Freshwater Crayfish*, **2**, 563–583.

Brockman HJ, Nguyen C, Potts W (2000) Paternity in horseshoe crabs when spawning in multiple-male groups. *Animal Behaviour*, **60**, 837–849.

Crocker DW, Barr DW (1968) *Handbook of the Crayfishes of Ontario*. University of Toronto Press, Toronto.

DeWoody JA, DeWoody YD, Fiumera AC, Avise JC (2000a) On the number of reproductives contributing to a half-sib progeny array. *Genetical Research Cambridge*, **75**, 95–105.

DeWoody JA, Walker D, Avise JC (2000b) Genetic parentage in large half-sib clutches: theoretical estimates and empirical appraisals. *Genetics*, **154**, 1907–1912.

Fetzner JW Jr, Crandall KA (2002) Genetic variation. In: *Biology of Freshwater Crayfish* (ed. Holdich DM). Blackwell Science, Oxford.

Fiumera AC, DeWoody YD, DeWoody JA, Asmussen MA, Avise JC (2001) Accuracy and precision of methods to estimate the number of parents contributing to a half-sib progeny array. *Journal of Heredity*, **92**, 120–126.

Gloor G, Engels W (1992) Single-fly preps for PCR. *Drosophila Information Service*, **71**, 148–149.

Guo SW, Thompson EA (1992) Performing the exact test of Hardy–Weinberg proportions for multiple alleles. *Biometrics*, **48**, 361–372.

Harris DJ, Crandall KA (2000) Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies. *Molecular Biology and Evolution*, **17**, 284–291.

Hobbs HH (1989) *An Illustrated Checklist of the American Crayfishes (Decapoda: Astacidae, Cambaridae, and Parastacidae)*. Smithsonian Institution Press, Washington, DC.

Holdich DM, ed. (2002) *Biology of Freshwater Crayfish*. Blackwell Science, Oxford.

Holdich DM, Lowery RS, eds. (1988) *Freshwater Crayfish: Biology, Management, and Exploitation*. Croom-Helm, London.

Holdich DM, Reeve ID (1988) Functional morphology and anatomy. In: *Freshwater Crayfish: Biology, Management, and Exploitation* (eds Holdich DM, Lowery RS), pp. 11–51. Croom-Helm, London.

Huxley TH (1884) *An Introduction to the Study of Zoology, Illustrated by the Crayfish*. Appleton, New York.

Jezerinac RF, Stocker GW, Tarter DC (1995) The crayfishes (Decapoda: Cambaridae) of West Virginia. *Bulletin of the Ohio Biological Survey*, New Series 10.

Jones AG, Avise JC (1997) Microsatellite analysis of maternity and the mating system in the Gulf pipefish *Syngnathus scovelli*, a species with male pregnancy and sex-role reversal. *Molecular Ecology*, **6**, 203–213.

Jones AG, Rosenqvist G, Berglund A, Avise JC (1999) Clustered microsatellite mutations in the pipefish *Syngnathus typhle*. *Genetics*, **152**, 1057–1063.

Kellogg KA, Markert JA, Stauffer JR, Kocher TD (1998) Intraspecific brood mixing and reduced polyandry in a maternal mouth-brooding cichlid. *Behavioral Ecology*, **9**, 309–312.

Little EE (1975) Chemical communication in maternal behaviour of crayfish. *Nature*, **255**, 400–401.

Little EE (1976) Ontogeny of maternal behavior and brood pheromone in crayfish. *Journal of Comparative Physiology*, **112**, 133–142.

Mason JC (1970) Maternal–offspring behavior of the crayfish, *Pacifastacus trowbridgi* (Stimpson). *American Midland Naturalist*, **84**, 463–473.

Momot WT (1988) *Orconectes* in North America and elsewhere. In: *Freshwater Crayfish: Biology, Management, and Exploitation* (eds Holdich DM, Lowery RS). Croom-Helm, London.

Oldroyd BP, Clifton MJ, Wongsiri S, Rinderer TE, Sylvester HA, Crozier RH (1997) Polyandry in the genus *Apis*, particularly *Apis andreniformis*. *Behavioral Ecology and Sociobiology*, **40**, 17–26.

Page LM (1985) The crayfishes and shrimps (Decapoda) of Illinois. *Illinois Natural History Survey Bulletin*, **33**, 404–406.

Parker GA (1970) Sperm competition and its evolutionary significance in the insects. *Biology Review*, **45**, 526–567.

Parker GA (1984) Sperm competition and the evolution of male mating strategies. In: *Sperm Competition and the Evolution of Male Mating Strategies* (ed. Smith RL). Academic Press, New York.

Payne JF (1978) Aspects of the life histories of selected species of North American crayfishes. *Fisheries*, **3**, 5–8.

Peters JM, Queller DC, Strassmann JE, Solis CR (1995) Maternity assignment and queen replacement in a social wasp. *Proceedings of the Royal Society of London B*, **260**, 7–12.

Pippitt MR (1977) Mating behavior of the crayfish *Orconectes nais* (Faxon, 1885) (Decapoda, Astacoidea). *Crustaceana*, **32**, 265–271.

Primmer CR, Ellegren H, Saino N, Møller AP (1996) Directional evolution in germline microsatellite mutations. *Nature Genetics*, **13**, 391–393.

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

Reynolds JD (2002) Growth and reproduction. In: *Biology of Freshwater Crayfish* (ed. Holdich DM). Blackwell Science, Oxford.

Riggert CM, Distefano RJ, Noltie DB (1999) Distributions and selected aspects of the life histories and habitat associations of the crayfishes *Orconectes peruncus* (Creaser, 1931) and *O. quadruncus* (Creaser, 1933) in Missouri. *American Midland Naturalist*, **142**, 348–362.

Schug MD, Mackay TFC, Aquadro CF (1997) Low mutation rates of microsatellite loci in *Drosophila melanogaster*. *Nature Genetics*, **15**, 99–102.

Selvin S (1980) Probability of nonpaternity determined by multiple allele codominant systems. *American Journal of Human Genetics*, **32**, 276–278.

Stein RA (1976) Sexual dimorphism in crayfish chelae: functional significance linked to reproductive activities. *Canadian Journal of Zoology*, **54**, 220–227.

Taylor CA, Warren ML Jr, Fitzpatrick JF Jr. *et al.* (1996) Conservation status of crayfishes of the United States and Canada. *Fisheries*, **21**, 25–38.

Weber JL, Wong C (1993) Mutation of human short tandem repeats. *Human Molecular Genetics*, **2**, 1123–1128.