(100 nmol kg^{-1}), KBr (92 μmol kg^{-1}), $SrCl_2 \cdot 6H_2O$ (13 μmol kg^{-1}), $AlCl_3$ (100 nmol kg^{-1}), LiCl (70 nmol kg^{-1}), KI (60 nmol kg^{-1}), H_3BO_3 (3.23 μmol kg^{-1}), RbCl (250 nmol kg^{-1}). To achieve defined trace metal conditions for [Zn^{2+}] or [Fe(III)$'$], 1 mmol kg^{-1} of $Na_2EDTA \cdot 6H_2O$ and varying amounts of $ZnCl_2$ or $FeCl_3$ were added to the media, which after pH adjustment with NaOH was stored for 24 h in the dark to allow chemical equilibration. In a control experiment at low, widely used culture media [EDTA] of 6 μmol kg^{-1} the same maximum growth rates were observed, indicating that the comparatively high [EDTA] applied here did not have any detrimental effect on *E. huxleyi*.

Trace metal speciation

[Zn^{2+}] and [Fe(III)$'$] were calculated from total metal concentrations using an equilibrium complexation model. Therefore, conditional stability constants for Fe(III) complexes with chlorine, fluoride, sulphate and the Fe(III) hydroxides (see refs 20, 21 and references therein) were included and, for the sake of consistency, thermodynamic stability constants[22] were used for all EDTA complexes with Fe, Cu, Co, Mn, Zn, Ca, Mg, the protonated forms of EDTA, and $ZnCO_3$ and $ZnSO_4$ after correction for ionic strength (salinity 34) with ion activity coefficients obtained either by Pitzer modelling[23] or Davies approximation. Light-induced photo-dissociation of Fe–EDTA complexes was accounted for following the model given in ref. 24. On the basis of this model two pH-dependent factors, linearly interpolated to 15 °C, were calculated and corrected for the lower photon-flux density and the light/dark cycle in our experiments (resulting in factors of 1.5 at pH 7.9 and 3 at pH 8.35). The absolute values for [Fe(III)$'$], however, crucially depend on the selected set of hydrolysis constants, particularly on β_3^*, which is so far not well constrained (see Table 2 in ref. 24 and references therein for details). The total zinc and iron concentrations in the natural sea water before nutrient addition were 7 and 1 nmol kg^{-1}, respectively and the [Zn^{2+}]/[Zn_{total}] in the zinc experiments was about 1/75,000.

Sampling and measurements

The carbonate system was calculated from pH and total dissolved inorganic carbon using the dissociation constants of ref. 25 as refitted in ref. 26. The pH was measured using the recommendations of ref. 21 and the total dissolved inorganic carbon was measured using a photochemical approach[27]. To calculate growth rates, cell counts were obtained at the beginning and end of incubations on a Coulter Epics XL-MCL flowcytometer. For measurements of cellular particulate organic carbon (POC) and nitrogen (PON) and total particulate carbon (TPC), subsamples were filtered on precombusted (500 °C) Whatman GF/F filters at the end of the experiments and stored at −25 °C. Before analysis POC filters were fumed for 2 h with concentrated HCl. POC, PON and TPC were analysed on an ANCA-SL 20-20 Europa Scientific mass spectrometer after 2 h of drying the filters at 60 °C. $CaCO_3$ was calculated by subtracting POC from TPC. $CaCO_3$ production rates were calculated from growth rates and cellular $CaCO_3$ contents. Owing to the experimental approach used in this study (see above), these parameters did not change significantly over the course of the experiments.

Received 24 January; accepted 10 May 2004; doi:10.1038/nature02631.

1. Petit, J. R. *et al.* Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature* **399,** 429–436 (1999).
2. Mahowald, N. *et al.* Dust sources and deposition during the last glacial maximum and current climate: A comparison of model results with paleodata from ice cores and marine sediments. *J. Geophys. Res.* **104,** 15895–15916 (1999).
3. Martin, J. H. Glacial-interglacial CO_2 change: the iron hypothesis. *Paleoceanogr.* **5,** 1–13 (1990).
4. Morel, F. M. M. *et al.* Zinc and carbon co-limitation of marine phytoplankton. *Nature* **369,** 740–742 (1994).
5. Broecker, W. S. & Peng, T.-H. The role of $CaCO_3$ compensation in the glacial to interglacial atmospheric CO_2 change. *Glob. Biogeochem. Cycles* **1,** 15–29 (1987).
6. Milliman, J. D. Production and accumulation of calcium carbonate in the ocean: budget of a nonsteady state. *Glob. Biogeochem. Cycles* **7,** 927–957 (1993).
7. Baar de, H. J. W. & Boyd, P. W. in *The Changing Ocean Carbon Cycle* (eds Hanson, R. B., Ducklow, H. W. & Fields, J. G.) 61–140 (Cambridge Univ. Press, Cambridge, 2000).
8. Conkright, M. E., Levitus, S. & Boyer, T. P. *World Ocean Atlas 1994,* Vol. 1 *Nutrients* (NOAA Atlas NESDIS 1, US Department of Commerce, Washington DC, 1994).
9. Coale, K. H. Effects of iron, manganese, copper, and zinc enrichments on productivity and biomass in the subarctic Pacific. *Limnol. Oceanogr.* **36,** 1851–1864 (1991).
10. Crawford, D. W. *et al.* Influence of zinc and iron enrichments on phytoplankton growth in the northeastern subarctic Pacific. *Limnol. Oceanogr.* **48,** 1583–1600 (2003).
11. Sunda, W. G. & Huntsman, S. A. Cobalt and zinc interreplacement in marine phytoplankton: Biological and geochemical implications. *Limnol. Oceanogr.* **40,** 1404–1417 (1995).
12. Ellwood, M. J. & Van den Berg, C. M. G. Determination of organic complexation of cobalt in seawater by cathodic stripping voltammetry. *Mar. Chem.* **75,** 33–47 (2001).
13. Riebesell, U. *et al.* Reduced calcification of marine plankton in response to increased atmospheric CO_2. *Nature* **407,** 364–367 (2000).
14. Zondervan, I., Rost, B. & Riebesell, U. Effect of CO_2 concentration on the PIC/POC ratio in the coccolithophore *Emiliania huxleyi* grown under light-limiting conditions and different daylengths. *J. Exp. Mar. Biol. Ecol.* **272,** 55–70 (2002).
15. Broecker, W. S. & Peng, T.-H. *Tracers in the Sea* (Eldigio, New York, 1982).
16. Bruland, K. W. Complexation of zinc by natural organic ligands in the central North Pacific. *Limnol. Oceanogr.* **34,** 269–285 (1989).
17. Kremling, K. & Streu, P. The behaviour of dissolved Cd, Co, Zn and Pb in North Atlantic near-surface waters (30°N/60°W–60°N/2°W). *Deep-Sea Res. I* **48,** 2541–2567 (2001).
18. Lohan, M. C., Statham, P. J. & Crawford, D. W. Total dissolved zinc in the upper water column of the subarctic North East pacific. *Deep-Sea Res. II* **49,** 5793–5808 (2002).
19. Duce, R. *et al.* The atmospheric input of trace species to the world ocean. *Glob. Biogeochem. Cycles* **5,** 193–259 (1991).
20. Liu, X. & Millero, F. J. The solubility of iron in seawater. *Mar. Chem.* **77,** 43–54 (2002).
21. DOE. *Handbook of Methods for the Analysis of the Various Parameters of the Carbon Dioxide System in Seawater* (eds Dickson, A. G. & Goyet, C.) Version 2.1 ORNL/CDIAC-74 ⟨http://andrew.ucsd.edu/co2qc/handbook.html⟩ (1994).
22. Pettit, L. D. & Powell, K. J. *IUPAC Stability Constants Database* (IUPAC and Academic Software, Otley, 2001).
23. Millero, F. J. & Pierrot, D. A chemical equilibrium model for natural waters. *Aquat. Geochem.* **4,** 153–199 (1998).
24. Sunda, W. & Huntsman, S. Effect of pH, light, and temperature on Fe-EDTA chelation and Fe hydrolysis in seawater. *Mar. Chem.* **84,** 35–47 (2003).
25. Mehrbach, C., Culberson, C. H., Hawley, J. E. & Pytkowicz, R. N. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* **18,** 897–907 (1973).
26. Dickson, A. G. & Millero, F. J. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Res.* **34,** 1733–1743 (1987).
27. Stoll, M. H. C., Bakker, K., Nobbe, G. H. & Haese, R. R. Continuous flow analysis of dissolved inorganic carbon content in seawater. *Anal. Chem.* **73,** 4111–4116 (2001).
28. Candelone, J.-P., Hong, S., Pellone, C. & Boutron, C. F. Post-Industrial Revolution changes in large-scale atmospheric pollution of the northern hemisphere by heavy metals as documented in central Greenland snow and ice. *J. Geophys. Res.* **100,** 16605–16616 (1995).
29. Hong, S., Candelone, J.-P. & Boutron, C. F. Changes in zinc and cadmium concentrations in Greenland ice during the past 7760 years. *Atmos. Environ.* **31,** 2235–2242 (1997).
30. Hong, S., Candelone, J.-P., Turetta, C. & Boutron, C. F. Changes in natural lead, copper, zinc and cadmium concentrations in central Greenland ice from 8250–149,100 years ago: their association with climate changes and resultant variations of dominant source contributions. *Earth Planet. Sci. Lett.* **143,** 233–244 (1996).

Acknowledgements We thank A. Terbrüggen, K.-U. Richter and B. van der Wagt for laboratory assistance, and M. Lohan, K. W. Bruland and R. E. Zeebe for discussions during the preparation of this manuscript. This work was partly funded by the German Research Foundation (DFG).

Competing interests statement The authors declare that they have no competing financial interests.

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Aggression by polyembryonic wasp soldiers correlates with kinship but not resource competition

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Kin selection theory predicts that individuals will show less aggression and more altruism towards relatives[1,2]. However, recent theoretical developments suggest that with limited dispersal, competition between relatives can override the effects of relatedness[3–9]. The predicted and opposing influences of relatedness and competition are difficult to approach experimentally because conditions that increase average relatedness among individuals also tend to increase competition. Polyembryonic wasps in the family Encyrtidae are parasites whose eggs undergo clonal division to produce large broods[10]. These insects have also evolved a caste system: some embryos in a clone develop into reproductive larvae that mature into adults, whereas others develop into sterile soldier larvae that defend siblings from competitors[11–14]. In a brood from a single egg, reproductive altruism by soldiers reflects clone-level allocation to defence at the cost of reproduction, with no conflict between individuals. When multiple eggs are laid into a host, inter-clone conflicts of interest arise. Here we report that soldier aggression in *Copidosoma floridanum* is inversely related to the genetic relatedness of competitors but shows no correlation with the level of resource competition.

Polyembryonic encyrtids are small (1 mm) parasitoid wasps that oviposit into moth (host) eggs[10,11]. After parasitism the host egg hatches and the larva develops to its final (fifth) instar (Fig. 1). During this period, the wasp egg proliferates clonally, producing multiple embryos in an assemblage called a polymorula. Several species, including *C. floridanum*, produce thousands of embryos per egg. Some embryos develop during the host's early instars into obligately sterile soldier (precocious) larvae with fighting mandibles and an elongate body[10–12]. The remaining embryos develop during the host's final instar into reproductive larvae with tiny mandibles and rounded bodies. These reproductive larvae consume the host, pupate and emerge as adult wasps. Soldier larvae always die after the reproductive larvae consume the host[10].

Hamilton's rule specifies that altruism is favoured when $rb - c > 0$, where c is the fitness cost to the altruist, b is the fitness benefit to the recipient and r is their genetic relatedness[1,2]. Conditions favouring the most extreme form of altruism, that is, sterile castes, include high relatedness and/or factors that make $b > c$. Key elements in the evolution of sterile soldier larvae by polyembryonic wasps are thus likely to be: (1) clonal development in a confined space (high r) and (2) the benefits, b, of defending a nutrient-rich but limiting resource (the host) from competitors relative to the costs, c, in lost reproduction[10,11]. Previous studies indicate that soldiers attack and frequently sacrifice themselves in defence of their clone-mates against other species of parasitoids[13,14]. In laboratory experiments, 96% of females with no previous oviposition experience ($N = 50$) oviposited into hosts that were parasitized by another wasp (superparasitism). Females superparasitized hosts irrespective of when the first female oviposited or the relatedness of the resident clone. Screening individual wasps from broods (56 broods with 20 same sex wasps per brood) collected in Georgia, using polymorphic allozymes such as glucosephosphate isomerase, further indicated that some broods contain progeny of at least two genotypes (D.G., K. G. Ross and M.R.S., unpublished results). Given that females also often lay two eggs per host (see below), these data collectively indicate that: (1) different kin classes of *C. floridanum* frequently compete within the same host, and (2) relatedness of potential competitors ranges from full siblings to distant relatives. This provides an opportunity for experimentally separating the effects of relatedness and competition under conditions of limited dispersal while using soldier behaviour to quantify conflicts of interest.

Like most Hymenoptera *C. floridanum* is haplodiploid, with unfertilized eggs producing males and fertilized eggs producing females. Mothers produce all-male or all-female broods by laying one egg per host, and mixed broods by laying two eggs (one male and one female)[15]. Almost 1,400 wasps per host emerge from each brood type[16,17]. A developmental asymmetry, however, exists between males and females: female broods produce about 30–50 soldiers by the host's fourth instar and male broods produce almost none[15–18]. Correspondingly, soldiers in mixed broods are almost exclusively female. Female soldiers have been shown to attack male embryos which results in mixed broods having strongly female-biased sex ratios[16,19]. The resolution of conflict in mixed broods in favour of females could be due to relatedness asymmetries, because soldiers are predicted to value reproductive, clone-mate sisters ($r = 1$) more than brothers ($r \sim 0.25$), particularly as only a few of the latter are needed for sib mating[16]. The indirect pay-off (inclusive fitness) to soldier larvae through a brother's post-dispersal matings may, however, provide some check on siblicidal behaviour[19].

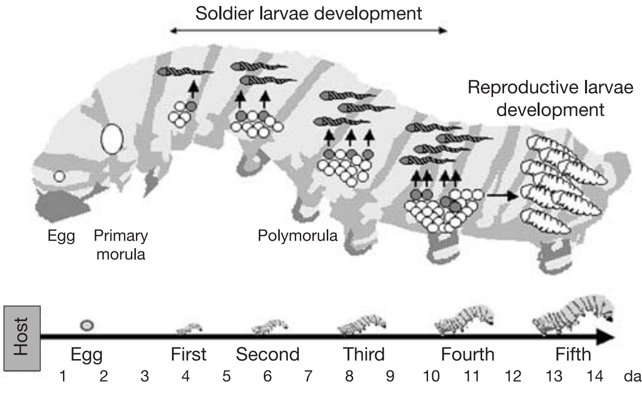

Figure 1 Life cycle of *C. floridanum* in its host *T. ni*. The schematic shows a host larva and the developmental stages of *C. floridanum*. Below the schematic are the host's life stages in relation to time (days). After parasitism, the host egg hatches and the larva develops to a fifth instar over 14 days. The *C. floridanum* egg develops initially into a primary morula. The primary morula then clonally proliferates to form a polymorula of more than 1,000 embryos during the host first to fourth instar period. Soldier larvae eclose during this period. Reproductive larvae eclose in the host fifth instar.

If soldiers are able to assess their relatedness to other individuals in the host, we might expect the aggressive behaviour of soldiers to be negatively correlated with relatedness to a competing clone. The highest levels of inter-clone aggression should thus be observed when unrelated mothers lay eggs into the same host. The completely local level of competition for limiting host resources could, however, override effects of relatedness[3–9], in which case soldier behaviour towards other clones is predicted to correlate with the severity of resource competition. This will increase with the number of competitors and/or reductions in host size, irrespective of relatedness. An additional consideration is that female clones might benefit from the presence of future mating partners if mating opportunities after dispersal are scarce. If so, soldiers might exhibit lower levels of aggression towards non-relative males than females.

To evaluate the importance of relatedness asymmetries, resource competition and sex of the competitor on soldier aggression, we conducted two complementary experiments. In the first experiment, we injected a labelled polymorula that was either a full sibling (brother or sister) or a non-relative (male or female) into a host containing an all-female brood (see Methods). Note that introducing different kin classes into hosts containing a female brood varied relatedness (and sex) of the potential competitors. However, the intensity of resource competition among treatments was approximately the same because each host was almost identical in size and host resources for the resident clone would be similarly reduced by injection of a second polymorula regardless of its relatedness. Hosts containing a female brood were also injected with a labelled tissue (gonad) collected from a non-parasitized host larva. This tissue would have no impact on relatedness, resource competition or future mating opportunities, and controlled for the possibility that soldiers attack any foreign entity introduced into the host. In all treatments, attack of the target by resident soldiers was scored as: (1) the proportion of hosts from each treatment that contained at least one soldier with fluorescent tracer in its gut, and (2) the mean proportion of soldiers per host with tracer in their gut. Both measures revealed a strong association between soldier aggression and relatedness of the competitor (Table 1). Attack rates were highest towards non-relative female or male clones, intermediate towards brothers and lowest towards full sisters or host tissue. Resource competition did not seem to affect attack rate because a sister clone would reduce available host resources whereas host tissue (gonad) would not, but resident soldiers rarely attacked either. In contrast, soldiers attacked non-relative clones much more frequently than sisters even though all introduced competitors would similarly reduce host resources for the resident clone. Soldier aggression also did not correlate with the sex of the competitor and future mating opportunities, given that soldiers attacked males as frequently as non-relative females. Direct observation of soldier larvae *in vitro* corroborated our *in vivo* results by showing that non-relative clones and brothers were attacked significantly more often

Table 1 **Proportion of labelled competitors attacked by soldiers *in vivo***

Relatedness of the competitor (*N*)	Resource competition	Future mating opportunities	Proportion of hosts with labelled soldiers	Mean proportion of soldiers labelled per host
Sister (40)	Yes	No	0.12	0.001^{a}
Brother (31)	Yes	Yes	0.52	0.09^{b}
Unrelated female, Georgia (40)	Yes	No	0.68	0.16$^{b, c}$
Unrelated female, Wisconsin (30)	Yes	No	0.76	0.21^{c}
Unrelated male, Wisconsin (31)	Yes	Yes	0.77	0.24^{c}
Gonad (control, 31)	No	No	0.07	0.001^{a}

The proportion of hosts containing at least one labelled soldier varied significantly with relatedness of the competitor, resource competition and future mating opportunities (Full model, $G^2 = 75.43$; d.f. = 7; $P < 0.0001$). Calculation of likelihood ratios for each effect revealed that relatedness of the competitor was of greatest significance ($G^2 = 48.33$; d.f. = 5; $P < 0.0001$). Attack rates towards full sisters did not differ significantly from controls (host gonads) ($G^2 = 0.75$; d.f. = 1; $P > 0.40$), suggesting that resource competition did not affect attack behaviour. Soldier attack rates towards males and unrelated females did also not significantly differ ($G^2 = 2.75$; d.f. = 1; $P > 0.25$), indicating that soldier aggression was unaffected by the sex of the competitor and potential future mating opportunities. A similar association between relatedness of the competitor and soldier aggression was obtained when the mean proportion of labelled soldiers per host was determined. A mean (±s.e.m.) of 56.6 ± 16.2 soldiers were present per host with no significant differences between treatments ($F_{5,203} = 1.72$; $P > 0.50$). Almost no soldiers were labelled when challenged by sisters or host gonads, whereas an increasing proportion of the soldiers per host contained label in their gut when the competitor was a brother or less-related clone ($F_{5,203} = 27.6$; $P < 0.0001$; arcsin transformed data; means with the same superscript letter (a, b, c) were not significantly different (Tukey–Kramer method)).

than sisters ($P < 0.0001$) (Table 2). Combined, these results clearly indicate that *C. floridanum* soldiers differentially attack competitors as a function of relatedness. Recent studies further reveal that soldiers distinguish kin from non-kin by cues from the extraembryonic membrane that surrounds each individual[20]. This membrane is also essential for protecting *C. floridanum* from the host's immune response, suggesting that the cues used in kin recognition may be maintained in part by selection for resistance against the host.

In our second experiment, we simultaneously assessed the effect of resource competition and relatedness on soldier aggression by comparing attacks towards competitors of different relatedness in large versus small hosts. Large hosts were naturally parasitized larvae (unstarved) as used in experiment 1, whereas small hosts were created by depriving parasitized larvae of food for 48 h. Hosts always survived transient starvation but their average maximum size (286 mg) was reduced by almost 50% relative to unstarved hosts (507 mg) (see Methods). Host starvation clearly intensified resource competition among progeny of the resident clone, because the number of adult progeny produced in starved hosts (485 ± 74 (mean ± s.e.m.), $N = 20$) was significantly smaller than in unstarved hosts (mean = 1,206 ± 117, $N = 20$; $P < 0.0001$ (*t*-test)) (see Methods). By extension, the intensity of resource competition after introduction of a second polymorula would likewise be much higher between kin classes in a starved host compared with an unstarved host. We therefore injected labelled sister, brother, non-relative female (Wisconsin) or non-relative male polymorulae into starved or unstarved hosts containing a Georgia female clone ($N = 30$ for each treatment). When we compared the outcomes among only starved hosts, we found that soldier aggression remained strongly associated with the relatedness of the competitor ($G^2 = 53.87$; d.f. = 4; $P < 0.0001$). This indicates that soldiers differentially attacked competitors as a function of relatedness even when host resources were more limited than in experiment 1. Moreover, we found no differences in soldier attack rates towards sisters (unstarved, 0.18; starved, 0.12; $P > 0.5$), brothers (unstarved, 0.52; starved, 0.5; $P > 0.8$), non-relative females (unstarved, 0.76; starved, 0.80; $P > 0.9$) or non-relative males (unstarved, 0.77; starved, 0.76; $P > 0.9$) when we compared outcomes in starved hosts versus unstarved hosts. This indicated that levels of soldier aggression are negatively correlated with relatedness of the potential competitor, but do not change with the severity of resource competition.

Table 2 **Proportion of competitors attacked by soldiers *in vitro***

Competitor	*N*	Proportion of competitors attacked
Sister	30	0.16
Brother	30	0.57
Unrelated female (Georgia)	30	0.60
Unrelated female (Wisconsin)	29	0.76
Unrelated male (Wisconsin)	31	0.71

Sister clones are attacked less than brothers or non-relative clones ($G^2 = 24.63$; d.f. = 1; $P < 0.0001$).

Hamilton originally proposed that the evolution of altruism could arise through either direct kin recognition or limited dispersal (population viscosity) which would have the effect of elevating local relatedness sufficiently to favour generalized altruism towards neighbours[1,2]. Our results with *C. floridanum* indicate that altruism extends beyond clone-mates to close relatives like non-identical sisters. This response is unlikely to reflect generalized altruism due to high neighbour–neighbour relatedness because females do not benignly share resources with brothers or non-relatives. Recent theory further suggests that altruism is unlikely to evolve in purely viscous populations because viscosity increases neighbour–neighbour competition for resources as much as it elevates relatedness[3–9]. Potential benefits of sharing a host with future mating partners also seem to be relatively unimportant in *C. floridanum* because soldiers usually attack males. Instead, our data support the alternative conclusion that altruism in *C. floridanum* operates by means of kin recognition[1,2]. Theory suggests that the evolution of kin-recognition based altruism may be suppressed in viscous populations unless periodic dispersal (alternating viscosity) occurs, which allows the beneficiaries of altruism to compete for resources against non-relatives[3,4,6]. Within the Georgia field population that was used in this study, the frequency of homozygous broods for the polymorphic allozyme glucosephosphate isomerase depart significantly from Hardy–Weinberg expectations ($P < 0.002$; Fisher's exact test) suggesting a high level of inbreeding (D.G., K. G. Ross and M.R.S., unpublished results). However, adult dispersal and subsequent resource competition between non-relatives is also likely because winged adult females must disperse to find new hosts and winged adult males are likewise able to disperse and can potentially mate with females from other hosts[15,16]. Our observations of sister–sister ($r \sim 0.75$) altruism but sister–brother ($r \sim 0.25$) conflict within hosts further suggest that opportunities exist for surviving males from mixed broods to disperse and mate with non-sisters[16]. In contrast, a comparative study of fig wasps suggests that local competition between males for mates is severe enough to obviate the benefits of kin selection[8].

The critical interplay between relatedness and competition in the evolution of altruism is potentially best understood in organisms that develop in non-standard ways, like clonal species that form assemblages of potentially competing relatives and non-relatives[21]. Like *C. floridanum*, several other clonally developing animals show a strong ability to recognize close relatives[22–24]. In contrast, examples of one clone exploiting another when clones mix are rare, and may reflect either the inability of some species to suppress conflict, or that a sufficiently large benefit to cost ratio ($b > c$) favours cooperation in the absence of high relatedness[25,26]. In *C. floridanum*, soldier attack clearly depends on relatedness, even under severe resource competition. ☐

Methods

Two populations of *C. floridanum* were established in the laboratory from field-collected broods obtained in the southern (Georgia) and northern (Wisconsin) US. Each culture was maintained separately as large randomly mating populations on the host *Trichoplusia ni*. Host larvae were reared on an artificial diet at 27 °C and in a 16 h light/8 h dark photoperiod as previously described[27]. Experimental conditions were manipulated to favour production of all-male and all-female broods. Ovipositions were observed to determine that wasps laid one male or female egg to produce all-female or all-male broods[17]. Identifying the composition of broods in advance was possible, because females exhibit different behaviours when laying a fertilized or an unfertilized egg[14]. Hosts containing full sisters were produced by mating clonal females from one brood with a male from another. Hosts containing brothers were produced by allowing unmated females from the same brood to oviposit.

Male or female polymorulae containing on average 500 embryos were collected from fourth instar hosts and labelled with carboxyfluorocein diacetate succinimidyl ester (CFSE) using previously established methods[14,16]. These previous studies confirmed that soldiers that attack CFSE-labelled tissues ingest the label which is then clearly visible when larvae are examined by epifluorescent microscopy. In experiment 1, CFSE-labelled sister, brother, non-relative Georgia female, non-relative Wisconsin female and non-relative Wisconsin male polymorulae were injected into fifth instar hosts containing a female brood from the Georgia population using a glass needle mounted on a micromanipulator. As a control, testes from fourth instar *T. ni*, which are similar in size to a polymorula, were labelled and individually injected. Hosts were then dissected 24 h after injection and the number of soldiers with label in their gut were determined. *In vitro* assays were conducted in 1-ml culture wells containing TC-100 medium (Sigma)[14,16]. Soldiers and polymorulae from relative and non-relative broods were collected from fourth instar hosts immediately before the experiment. One soldier larva was placed in the culture well with a full sister, brother, non-relative female or non-relative male polymorula. The proportion of each type of polymorula that was attacked during a 2-h bioassay period was then recorded. An attack was defined as the larva gripping a polygerm with its mandibles for more than 1 min. When this occurred, consumption of tissue by the soldier was readily visible.

In experiment 2, hosts containing female broods were starved by removing them on the first day of the fifth instar from the food source to cups containing moist cotton wool. Each larva was returned to food 24 or 48 h after starvation and reared until completion of *C. floridanum* development. The average maximum size of hosts treated in this manner declined linearly from 507 ± 56 mg (mean ± s.e.m., $N = 20$) for unstarved controls to 398 ± 42 mg ($N = 20$) and 286 ± 38 mg ($N = 20$) for hosts starved for 24 or 48 h, respectively. Previous studies indicated that the total number of wasp progeny produced per polymorula is established at the end of the host fourth instar[11,18]. Thus, any reduction in the number of progeny produced in starved, fifth instar hosts is due to mortality caused by increased competition for more limited host resources rather than a change in the development of the polymorula itself. To assess the effect of host starvation on the resident clone, parasitized hosts containing a female brood were starved for 48 h, returned to food and then reared until completion of development. The total number of wasp progeny per host was then counted. For comparisons of soldier aggression in starved and unstarved hosts, one cohort of hosts was starved for 48 h, returned to food and then injected 24 h later with a CFSE-labelled sister, brother, non-relative female (Wisconsin) or non-relative male polymorula. The second, unstarved cohort was treated identically except that hosts were continuously provided with food. Hosts in both cohorts were dissected 24 h after injection and the number of hosts containing soldiers with label in their gut was determined as described in experiment 1.

The association between soldier aggression, relatedness, resource competition or gender of the competitor were analysed by likelihood ratio chi-square tests using the JMP, v.3.0 statistical package.

Received 30 March; accepted 7 June 2004; doi:10.1038/nature02721.

1. Hamilton, W. D. The evolution of altruistic behaviour. *Am. Nat.* **97,** 354–356 (1963).
2. Hamilton, W. D. The genetical evolution of social behaviour, I & II. *J. Theor. Biol.* **7,** 1–52 (1964).
3. Wilson, D. S., Pollock, G. B. & Dugatkin, L. A. Can altruism evolve in a purely viscous population? *Evol. Ecol.* **6,** 331–341 (1992).
4. Taylor, P. D. Altruism in viscous populations—an inclusive fitness model. *Evol. Ecol.* **6,** 352–356 (1992).
5. Taylor, P. D. Inclusive fitness in a homogeneous environment. *Proc. R. Soc. Lond. B* **249,** 299–302 (1992).
6. Queller, D. C. Genetic relatedness in viscous populations. *Evol. Ecol.* **8,** 70–73 (1994).
7. Grafen, A. in *Behavioural Ecology: An Evolutionary Approach* (eds Krebs, J. R. & Davies, N. B.) 62–84 (Blackwell Scientific, Oxford, 1984).
8. West, S. A., Murray, M. G., Machado, C. A., Griffin, A. S. & Herre, E. A. Testing Hamilton's rule with competition between relatives. *Nature* **409,** 510–513 (2001).
9. West, S. A., Penn, I. & Griffin, A. S. Cooperation and competition between relatives. *Science* **296,** 72–75 (2002).
10. Strand, M. R. in *Encyclopedia of Insects* (eds Carde, R. & Resch, V.) 928–932 (Academic, San Diego, 2003).
11. Strand, M. R. & Grbic', M. The development and evolution of polyembryonic insects. *Curr. Top. Dev. Biol.* **35,** 121–160 (1997).
12. Donnell, D., Corley, L. S., Chen, G. & Strand, M. R. Caste determination in a polyembryonic wasp involves inheritance of germ cells. *Proc. Natl Acad. Sci. USA* (in the press).
13. Cruz, Y. P. A sterile defender morph in a polyembryonic hymenopterous parasite. *Nature* **294,** 446–447 (1981).
14. Harvey, J. A., Corley, L. S. & Strand, M. R. Competition induces adaptive shifts in caste ratios of a polyembryonic wasp. *Nature* **406,** 183–186 (2000).
15. Strand, M. R. Oviposition behavior and progeny allocation by the polyembryonic wasp *Copidosoma floridanum*. *J. Insect Behav.* **2,** 355–369 (1989).
16. Grbic', M., Ode, P. J. & Strand, M. R. Sibling rivalry and brood sex ratios in polyembryonic wasps. *Nature* **360,** 254–256 (1992).
17. Ode, P. J. & Strand, M. R. Progeny and sex allocation decisions of the polyembryonic wasp *Copidosoma floridanum*. *J. Anim. Ecol.* **64,** 213–224 (1995).
18. Grbic', M., Nagy, L., Carroll, S. B. & Strand, M. R. Development and pattern formation in the polyembryonic wasp, *Copidosoma floridanum*. *Development* **122,** 795–804 (1996).
19. Ode, P. J. & Hunter, M. S. in *Sex ratios: Concepts and Research Methods* (ed. Hardy, I. C. W.) 218–234 (Cambridge Univ. Press, Cambridge, 2002).
20. Giron, D. & Strand, M. R. Host resistance and the evolution of kin recognition in polyembryonic wasps. *Proc. R. Soc. Lond.* (Suppl.) *Biol. Lett.* published online 17 June 2004 (DOI:10.1098/rsbl/2004.0205).
21. Queller, D. C. Relatedness and the fraternal major transitions. *Phil. Trans. R. Soc. Lond. B* **355,** 1647–1655 (2000).
22. Crozier, R. H. Genetic clonal recognition abilities in marine invertebrates must be maintained by selection for something else. *Evolution* **40,** 1100–1101 (1986).
23. Buss, L. W. *The Evolution of Individuality* (Princeton Univ. Press, Princeton, 1987).
24. Grosberg, R. K. The evolution of allorecognition specificity in clonal invertebrates. *Q. Rev. Biol.* **63,** 377–412 (1988).
25. Strassmann, J. E., Zhu, Y. & Queller, D. C. Altruism and social cheating in the social amoeba *Dictyostelium discoideum*. *Nature* **408,** 965–967 (2000).
26. Abbot, P., Withgott, J. H. & Moran, N. A. Genetic conflict and conditional altruism in social aphid colonies. *Proc. Natl Acad. Sci. USA* **98,** 12068–12071 (2001).
27. Strand, M. R. Development of the polyembryonic parasitoid *Copidosoma floridanum* in *Trichoplusia ni*. *Entomol. Exp. Appl.* **50,** 37–46 (1989).

Acknowledgements This work was supported in part by the Natural Environment Research Council (UK), the National Science Foundation (US), the University of Georgia Experiment Station, and the Conseil General de la Region (France).

Competing interests statement The authors declare that they have no competing financial interests.

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High mutation rate and predominance of insertions in the *Caenorhabditis elegans* nuclear genome

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Mutations have pivotal functions in the onset of genetic diseases and are the fundamental substrate for evolution. However, present estimates of the spontaneous mutation rate and spectrum are derived from indirect and biased measurements. For instance, mutation rate estimates for *Caenorhabditis elegans* are extrapolated from observations on a few genetic loci with visible phenotypes and vary over an order of magnitude[1]. Alternative approaches in mammals, relying on phylogenetic comparisons of pseudogene loci[2] and fourfold degenerate codon positions[3], suffer from uncertainties in the actual number of generations separating the compared species and the inability to exclude biases associated with natural selection. Here we provide a direct and unbiased estimate of the nuclear mutation rate and its molecular spectrum with a set of *C. elegans* mutation-accumulation lines that reveal a mutation rate about tenfold higher than previous indirect estimates and an excess of insertions over deletions. Because deletions dominate patterns of *C. elegans* pseudogene variation[4,5], our observations indicate that natural selection might be significant in promoting small genome size, and challenge the prevalent assumption that pseudogene divergence accurately reflects the spontaneous mutation spectrum.