

to females with oestrous vaginal smears (sexually receptive) than females with anoestrous smears ( $\chi^2 = 4.8$ ,  $P < 0.05$ ). Male home ranges overlapped with SFHRs to a greater extent (Fig. 2) than would have been expected if there was no spatial association between them; on average 42% of male home ranges overlapped with SFHRs although SFHRs covered less than 25% of the total island area ( $t = 4.1$ ,  $P < 0.001$ ). By contrast, female home ranges are random with respect to SMHRs (Fig. 2); percentage overlap between female home ranges and SMHRs (39%) was not different from the expected 38% ( $t = 0.2$ , not significant). This result together with the fact that females were located more often close to males than expected from random (2.4 times,  $\chi^2 = 37.1$ ,  $P < 0.0001$ ) indicate that a female's position within her home range was affected by the presence of caged males, but that the position of her home range was unaffected by the opposite sex. Males tended to aggregate on SFHRs that contained groups of females (Fig. 2), but not where females were spatially dispersed. As a consequence of this, males shared significantly more space on SFHRs when females were clumped than when females were spatially dispersed (Table 1). Overall, males avoided sharing space, whereas female home ranges overlapped more than expected: ( $n - 1$ ) male home ranges covered on average 35% of the island area whereas the home range of a male on average overlapped 22% with other male home ranges ( $t = 2.3$ ,  $P < 0.05$ ), whereas the corresponding figures for females were 25% and 37% ( $t = 2.5$ ,  $P < 0.05$ ).

Three main conclusions can be drawn from the results presented here. First, the spacing of female home ranges in *C. rufocanus* is not affected by the spatial distribution of males. This result supports the conclusions of earlier experimental<sup>13,14</sup> and theoretical studies<sup>6</sup> showing that habitat characteristics (for example, food distribution) are the main determinants of the spacing system of females. Second, the spatial distribution of males reflects the spatial distribution of receptive females. This result is consistent with the prediction that receptive mates, rather than food or habitat characteristics, are the key factors determining male fitness<sup>15-17</sup>. Third, in contrast to the predictions of theoretical studies, spatial clumping of females was not sufficient to induce territoriality in males<sup>4,7</sup>. On the contrary, I found most space sharing among males when females were clumped. Elsewhere I have shown that *C. rufocanus* males have the capacity to switch to territoriality when the density of competitors is low<sup>13</sup>. Sometimes an increased intruder pressure may be the inevitable consequence of spatial clumping of females because competitors are attracted to patches where the critical resource is abundant<sup>18,19</sup>. Hence, spatially clumped females may in fact be more difficult to monopolize than females that are spatially dispersed. In particular, this may be true for small species like

*C. rufocanus* which live in a complex habitat, where the probability of detecting intruders is low<sup>20</sup>. These results caution against making over-general predictions about determinants of male spacing systems. Different species may respond differently to equivalent conditions depending on other features of their biology. Field experiments of the kind reported here can be very useful in revealing these important species-specific features.

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## Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20

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Immunization of male and female animals with extracts of whole sperm cells is known to cause infertility<sup>1-5</sup>. Also, men and women who spontaneously produce antisperm antibodies are infertile but otherwise healthy<sup>6</sup>. Although the critical sperm antigens are unknown, these observations have led to the proposal that sperm proteins might be useful in the development of a contraceptive vaccine<sup>7-9</sup>. The guinea pig sperm surface protein PH-20 is essential in sperm adhesion to the extracellular coat (*zona pellucida*) of the egg, a necessary initial step in fertilization<sup>10</sup>. Here, we report that 100% effective contraception was obtained in male and female guinea pigs immunized with PH-20. Antisera from immunized females had high titres, specifically recognized PH-20 in sperm extracts, and blocked sperm adhesion to the egg *zona pellucida* *in vitro*. The contraceptive effect was long-lasting and reversible: immunized females, mated at intervals of six to fifteen months after immunization, progressively regained fertility.

**Table 1** Characteristics of space use of males in relation to the dispersion pattern of SFHRs

Male space-use characteristics	Female dispersion pattern		Wilks' $\lambda$ †
	Clumped (N = 11)	Dispersed (N = 13)	
% Overlap with males on SFHR*	55 ± 12	18 ± 6	0.72
% Overlap with males outside SFHR*	10 ± 6	17 ± 7	0.56
% Overlap with SFHR	47 ± 6	36 ± 5	0.47
Home range size of males (m <sup>2</sup> )	605 ± 183	898 ± 175	0.43

Values are presented as mean ± standard error of the mean for pooled data from the two periods (Fig. 2). All estimates are based on minimum convex polygons of home ranges drawn on the basis of coordinate points obtained from radiotelemetry (Fig. 2). Variables are listed in the order in which they were entered into a step-wise discriminant function analysis<sup>21</sup>, used to determine which space-use characteristics were significantly affected by female dispersion pattern.

\* Significant variables.

† Wilks'  $\lambda$  is an inverse measure of the discriminating power of the discriminant function after the corresponding variable entered the function.

An integral membrane protein, PH-20, with a relative molecular mass ( $M_r$ ) of 64,000, identified by monoclonal antibodies (mAbs), is present on both the plasma membrane and inner acrosomal membrane of guinea pig sperm<sup>10-13</sup>. To determine whether immunization with affinity-purified PH-20 (ref. 13) would affect fertility, four female guinea pigs were each immunized with different PH-20 doses (10, 20, 30 and 50  $\mu$ g) which were given in a first injection and again in a second injection one month later. Control animals received injections lacking PH-20 at the same times. Approximately two months after the initial injection, control-injected and PH-20-injected females were housed with the same males and allowed to mate. None of the four PH-20-immunized females had litters. Twelve of 13 (92%) of control animals had litters; the 13 controls gave birth to an average of 3.5 progeny per litter (Table 1, group 1).

A second group of 21 females were immunized with PH-20 (5, 10 and 20  $\mu$ g) using the same schedule as the first group. None of these animals had litters, although 22 out of the 23 (96%) control animals did (Table 1). In total, 34 out of the 36 (94%) control-injected animals had litters and the 36 controls gave birth to an average of 3.3 young. Based on this average, the 25 PH-20 immunized females would have been expected, if fertile, to give birth to 82 progeny; that there were none indicates 100% effective contraception in these animals (Table 1).

Antisera, obtained from injected females one week before

mating, were titred in a solid-phase assay using a detergent extract of whole sperm as antigen. Antisera from the infertile females showed high binding levels that began to decrease significantly at dilutions greater than  $10^{-4}$  and remained measurable even at dilutions of  $10^{-8}$  (Table 2).

Like anti-PH-20 mAbs<sup>10</sup>, the anti-PH-20 polyclonal sera from the infertile females blocked sperm binding to the egg *zona pellucida in vitro*. The relative inhibitory potency of the antiserum provided a second, perhaps more physiologically significant, measure of antiserum titres. In the *in vitro* sperm-zona binding assay<sup>10</sup>, antisera from the infertile females inhibited sperm-zona binding by 90-94% at a  $10^{-2}$  or  $10^{-3}$  dilution. The extent of inhibition decreased at  $10^{-4}$  dilution, but remained substantial even at  $10^{-5}$  dilution (Table 2).

The antisera from the infertile, PH-20-immunized females specifically recognized the PH-20 protein. The immunofluorescence-staining patterns obtained with sera ( $10^{-3}$  dilution) from females immunized with 5-50  $\mu$ g PH-20 were the same as those previously reported with anti-PH-20 mAbs<sup>10-12</sup>: surface-staining was restricted to the posterior head region on the plasma membrane of acrosome-intact sperm and to the inner acrosomal membrane of acrosome-reacted sperm. Sera from control-injected animals showed no fluorescent staining of sperm. In addition, antisera from infertile females recognized a single band in immunoblots of SDS extracts of whole sperm (Fig. 1,

**Table 1** Contraceptive effect of PH-20 immunization

	Amount of PH-20 injected ( $\mu$ g)	No. of animals	No. with litters (% with litters)	No. of progeny	Average no. progeny in control
Group 1 females	50	1	0	0	3.5
	30	1	0	0	
	20	1	0	0	
	10	1	0	0	
	0 (control)	13	12 (92%)	46	
Group 2 females	20	14	0	0	3.2
	10	4	0	0	
	5	3	0	0	
	0 (control)	23	22 (96%)	74	
Total PH-20 immunized females		25	0 (0%)	0	
Total control females		36	34 (94%)	120	3.3
Females with fetuses					
Mated females					
Group 3 males	50	1	0/2	0	4.4
	30	1	0/2	0	
	20	1	0/2	0	
	10	1	0/2	0	
	5	1	0/2	0	
	2.5	1	0/2	0	
	0 (control)	7	14/14	62	
Total PH-20 immunized males		6	0/12 (0%)		
Total control males		7	14/14 (100%)		

Female or male Hartley guinea pigs (about 300 g at the time of the first injection) received two injections of the stated amount of PH-20. PH-20, purified from sperm by mAb-affinity chromatography, showed no detectable contaminants using silver-staining of high loads (5  $\mu$ g) of protein on SDS gels<sup>13</sup>. Purity of each PH-20 preparation used for immunization of females or males was verified by SDS polyacrylamide gel electrophoresis and silver staining. The affinity-purified PH-20, in 0.375 ml phosphate-buffered saline (PBS) containing 3 mM octylglucoside (OG), was emulsified with 0.375 ml complete Freund's adjuvant (CFA). Each animal received 0.5 ml the emulsion subcutaneously in the back and 0.25 ml intramuscularly in a rear leg. About one month later the same amount of PH-20 in PBS and 3 mM OG, emulsified with incomplete Freund's adjuvant (IFA), was injected in the same sites in each animal. (A single exception was the male that received 30  $\mu$ g PH-20 and had only the primary injection.) Control females and males received the same injections on the same schedule and containing PBS and 3 mM OG and CFA or IFA, but lacking PH-20. To allow the injected females to mate, about two months after the initial injection they were housed with males for three weeks. Each cage contained one male (575-600 g), two or three PH-20 immunized females and from two to four control-injected females. After three weeks, the females were separated from the males, pregnant females had litters and progeny were counted. Control-injected females that failed to become pregnant had been in cages where the other controls did become pregnant, indicating that all the males mating with immunized females were fertile. To allow the injected males to mate, about two months after the initial injection, each injected male was housed with two females (about 600 g) for three weeks. The females and males were then separated and after an additional five weeks females were killed and fetuses counted.

lanes 1-5). This band co-migrated with purified PH-20, of relative molecular mass ( $M_r$ ) 64,000 (64K) when immunoblotted with antiserum from the 50  $\mu\text{g}$ -immunized female (Fig. 1, lane 7). The production of high levels of anti-PH-20 antibodies by immunized females indicates that the PH-20 protein may be a non-self antigen in females. To determine whether PH-20 is found on tissues other than sperm, we assayed a spectrum of other tissue types from female guinea pigs to see if they could bind the anti-PH-20 antiserum from the 50  $\mu\text{g}$ -immunized female. The antiserum showed high binding to a sperm extract, but no binding was detectable to extracts of other tissues (Fig. 2). Addition of 50  $\mu\text{g ml}^{-1}$  sperm protein to 5  $\text{mg ml}^{-1}$  tissue protein resulted in antiserum binding, indicating that the assay was sensitive enough to detect a 1% level of sperm extract in the tissue extract. We have also found that antiserum from the 50  $\mu\text{g}$ -immunized female immunoblotted the PH-20 band in

sperm and testis extracts, but blots of the other tissues showed either no bands or bands that were also seen with control sera (data not shown). Thus, PH-20 may have one of the suggested theoretical advantages of sperm contraceptive immunogens<sup>5,9</sup>: that it would not raise potential problems of auto-immunity when used in females.

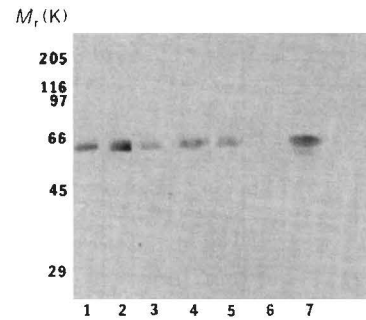
To test for reversal of the contraceptive effect, the PH-20-immunized females were mated at intervals. The immunized animals progressively regained fertility: about six months after the initial injection, four of 23 tested (17%) were fertile; by 9-11 months 11 of 24 (46%) had regained fertility; and by 15 months, all four in the longest-studied (group 1) females had delivered litters. About a fourfold decrease (relative to initial antiserum titres) was found at six months for titres from group 1 females immunized with 10, 20 or 30  $\mu\text{g}$  of PH-20. The 10- and 20  $\mu\text{g}$ -immunized females were fertile at six months,

**Table 2** Titres of antisera from females

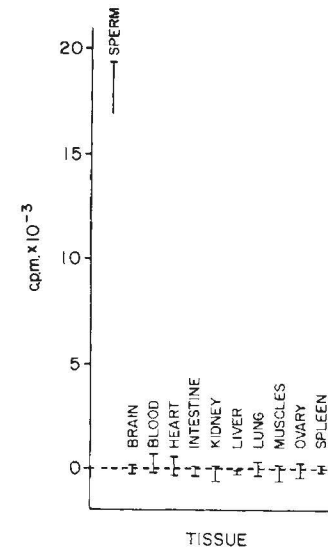
Antiserum from animals injected with PH-20 ( $\mu\text{g}$ )		Antisera binding in radioimmune assay							
		$^{125}\text{I}$ bound in microtitre well (c.p.m. $\times 10^{-2}$ )							
		Antiserum dilution							
		$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	$10^{-7}$	$10^{-8}$	
0 (control)		27	5	2	1	1	1	1	
5		156	152	120	44	14	5	2	
10		150	149	130	66	21	8	4	
20		150	147	128	66	24	10	5	
30		155	148	113	43	13	4	2	
50		142	140	130	64	18	6	2	
Antiserum from animals injected with PH-20 ( $\mu\text{g}$ )		Percentage inhibition of sperm-zona binding							
		Antiserum dilution							
		$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	$10^{-7}$	$10^{-8}$	
0 (control)		0	0						
5		92	94		84		58		
10		94	91		63		48		
20		94	94		67		26		
30		92	92		57		50		
50		92	90		72		37		
Antiserum from animals injected with PH-20 ( $\mu\text{g}$ )		Antisera binding before first mating compared with second mating							
		$^{125}\text{I}$ bound in microtitre well (c.p.m. $\times 10^{-2}$ )							
		Antiserum dilution							
		Mating	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	$10^{-7}$	$10^{-8}$
0 (control)	1st		26	6	2	1	1	1	1
	2nd		25	5	2	1	1	1	1
10	1st infertile		144	145	110	40	9	5	3
	2nd fertile		139	128	59	13	4	4	2
20	1st infertile		142	139	105	33	10	6	3
	2nd fertile		144	129	59	16	6	4	3
30	1st infertile		155	144	109	42	14	6	3
	2nd infertile		152	132	60	14	5	4	2
50	1st infertile		142	144	125	59	18	8	3
	2nd infertile		145	139	104	37	12	8	3

Binding of antisera, obtained one week before the first mating and diluted as indicated, was measured in a radioactive solid-phase (microtitre plate) assay using an OG extract of live sperm as antigen and  $^{125}\text{I}$ -protein A as the second reagent<sup>26</sup>. Non-specific binding was blocked with 3% non-fat dry milk in PBS. Values shown were determined in duplicate in one experiment and were from one immunized female (50  $\mu\text{g}$  and 30  $\mu\text{g}$ ) or the averages of four females (0  $\mu\text{g}$ ), two females (5  $\mu\text{g}$ ), one group 1 and two group 2 females (10  $\mu\text{g}$ ) and one group 1 and three group 2 females (20  $\mu\text{g}$ ). Group 2 females, immunized with 10 or 20  $\mu\text{g}$ , had somewhat higher titres than group 1 females immunized with 10 or 20  $\mu\text{g}$ . The ability of antisera, diluted as indicated, to inhibit sperm binding to the *zona pellucida* of guinea pig eggs was measured as previously described for anti-PH-20 mAbs<sup>10</sup>. Sperm, capacitated and allowed to acrosome-react in modified Tyrode's medium, were pre-incubated with the diluted antiserum for 15-20 min and then added to eggs in the continuing presence of diluted antiserum. After 30 min, eggs with bound sperm were washed into sperm-free drops, fixed, and the number of sperm bound to the *zona* counted in one plane of focus. The mean number of sperm per egg was  $16.4 \pm 3.3$  in the absence of antiserum in the 13 experiments in the Table. Percentage inhibition is  $(1.0 \text{ minus the number of sperm bound in the presence of antiserum/number of sperm bound in the absence of antiserum in the same experiment}) \times 100\%$ . Antiserum from one female injected with each stated amount of PH-20 was tested one time at each dilution to determine percentage inhibition. Antiserum from the control-injected female was tested twice at  $10^{-2}$  dilution. Binding of antisera, obtained one week before the first mating or one week before the second mating, diluted as indicated, was measured as in the top panel. Values shown are averages determined in duplicate in two experiments. Specific binding of c.p.m. at  $10^{-3}$  dilution is maximal binding. Antiserum titre is defined as the dilution at which c.p.m. bound is half-maximal.

**Fig. 1** Immunoblotting of an SDS-extract of whole cauda epididymal sperm and purified PH-20 protein with antisera from immunized females. Antisera were from females injected with the following amounts of PH-20: lane 1, 5  $\mu\text{g}$ ; lane 2, 10  $\mu\text{g}$ ; lane 3, 20  $\mu\text{g}$ ; lane 4, 30  $\mu\text{g}$ ; lane 5, 50  $\mu\text{g}$ ; lane 6, 0  $\mu\text{g}$  (control-injected); lane 7 (50  $\mu\text{g}$ ). The samples in lanes 1–6 were whole sperm extracts; the sample in lane 7 was purified PH-20. Sperm extracts were prepared by solubilizing fresh live cauda epididymal sperm in non-reducing SDS-polyacrylamide gel electrophoresis sample buffer, centrifuging and discarding the insoluble residue, and using the entire supernatant as sample. Immunoblots were performed as described<sup>27</sup> using antisera (1 in 3,000 dilution) and alkaline phosphatase conjugated-goat anti-guinea pig immunoglobulin G as second reagent. Relative molecular masses (K) of marker proteins are shown on the left.



**Fig. 2** Binding of antiserum (from female injected with 50  $\mu\text{g}$  PH-20) to extracts of a female's tissues, compared with binding to extract of sperm. Values shown are the range of four determinations for sperm and ovary and six determinations for other tissues; background binding obtained with control antiserum was subtracted from all values. Fresh tissues from a female and sperm from a male were disrupted in a Brinkmann Polytron in the presence of protease inhibitors<sup>13</sup> and OG was added to 30 mM. Tissue extracts (at 5 mg per ml protein to ensure saturating amounts of antigen were present) and sperm extract (at 0.2 mg per ml protein) were allowed to bind to a microtitre plate overnight. Binding of antiserum ( $10^{-4}$  dilution) from the female injected with 50  $\mu\text{g}$  PH-20 and binding of antiserum ( $10^{-4}$  dilution) from a control-injected female were measured using <sup>125</sup>I-labelled protein A as second reagent.



whereas the 30  $\mu\text{g}$ -immunized female, with an identical titre, remained infertile (Table 2). This suggests that, when serum titres have fallen fourfold, any individual female has a certain probability of being either fertile or infertile.

Although immunization of males with a sperm protein raises the possibility of autoimmune disruption of testicular function, men with anti-sperm antibodies of natural occurrence or following vasectomy do not have autoimmune reactions in the testis<sup>6,14</sup>. In an initial experiment to assess contraceptive effectiveness of PH-20-immunization in males, six male guinea pigs were immunized with 2.5–50  $\mu\text{g}$  PH-20; seven control males received injections without PH-20. About two months after the initial injection, each male was housed with two females for mating. None of the 12 females mated with the PH-20-immunized males carried fetuses; 14 out of 14 of the females mated with control males did (group 3, Table 1). Four of the six immunized males regained fertility by seven months after the initial injection and thus do not show the irreversible sterility associated with autoimmune orchitis.

Purified sperm proteins previously tested as contraceptive immunogens include the sperm enzymes hyaluronidase, acrosin and lactate dehydrogenase C-4. Immunization of female animals with these enzymes had either no effect on fertility<sup>15,16</sup>, or partial effects on fertility<sup>17–19</sup>, which were not large enough to make these proteins suitable for use as contraceptive agents<sup>8,9,20</sup>. The high contraceptive effectiveness of PH-20 seems to depend on several of its specific properties, including its presence on the sperm surface, its strong immunogenicity and its essential role in fertilization. Human sperm are like guinea pig and certain other mammalian sperm in that they can bind to the *zona*

*pellucida* either before or after the acrosome reaction<sup>21–25</sup>. Although the molecular details of these events are unknown with human gametes, a human functional analogue of PH-20 would be a candidate for an effective contraceptive immunogen.

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