lating hormone, agouti-related protein, and cocaine-amphetamine–regulated transcript) that are implicated in the control of feeding and energy balance (*23, 24*). The perifornical LH includes neurons that express the long form of the leptin receptor (*25*). Orexin or melanin-concentrating hormone (*23, 26*), neuropeptides that promote food intake and weight gain (*27*), have been found in LH neurons, and LH neurons containing corticotropin-releasing hormone have been implicated in dehydration-induced anorexia (*28*). Working out the contribution of such cells to the rewarding effects of electrical brain stimulation and feeding could prove important to understanding energy balance. Conversely, progress in understanding the neural control of food intake and energy expenditure may shed light on the structure and function of brain reward circuitry.

References and Notes

1. Y. Zhang *et al.*, *Nature* **372**, 425 (1994).
2. M. Maffei *et al.*, *Nature Med.* **1**, 1155 (1995).
3. M. A. Pelleymounter *et al.*, *Science* **269**, 540 (1995); L. A. Campfield, F. J. Smith, Y. Guisez, R. Devos, P. Burn, *Science* **269**, 546 (1995); J. L. Halaas *et al.*, *Science* **269**, 543 (1995); R. J. Seeley *et al.*, *Horm. Metab. Res.* **28**, 664 (1996).
4. J. Olds and P. M. Milner, *J. Comp. Physiol. Psychol.* **47**, 419 (1954); M. E. Olds and J. Olds, *J. Comp. Neurol.* **120**, 259 (1963).
5. J. E. Blundell and L. J. Herberg, *Nature* **219**, 627 (1969); K. D. Carr and T. Wolinsky, *Brain Res.* **607**, 141 (1993).
6. B. Stanley, W. Magdalin, A. Seirafi, W. Thomas, S. Leibowitz, *Brain Res.* **604**, 304 (1993); E. Gillard *et al.*, *J. Neurosci.* **18**, 2646 (1998); S. Leibowitz and C. Rossakis, *Brain Res.* **172**, 115 (1979); S. Leibowitz, *Brain Res.* **98**, 529 (1975).
7. G. Abrahamsen, Y. Berman, K. D. Carr, *Brain Res.* **695**, 186 (1995).
8. With skull landmarks bregma and lambda positioned on the same horizontal plane, bilateral monopolar electrodes were aimed at the perifornical LH (3 mm posterior to bregma, 1.6 mm lateral to the midsagittal sinus, and 7.8 mm below the dura mater), and a 24-gauge stainless-steel guide cannula (Plastics One, Roanoke, VA) was aimed at the right lateral ventricle (0.4 mm posterior to bregma, 1.6 mm lateral to the midsagittal sinus, and 4 mm below the dura mater). After recovery from surgery and before leptin administration, cannula placement was verified by injecting 2 μg of angiotensin II and determining whether vigorous drinking began within 5 min. For further details concerning electrodes and surgery, see A. Arvanitogiannis, L. Riscaldino, P. Shizgal, *Physiol. Behav.* **65**, 805 (1999).
9. The current was held constant throughout testing and ranged from 200 to 450 μA across subjects. The stimulation frequency was decreased from trial to trial by 0.033 $\log_{10}$ units. All testing was conducted toward the end of the dark phase of the light/dark cycle.
10. Broken-line functions, with a horizontal lower asymptote, a rising linear segment, and a horizontal upper asymptote [C. R. Gallistel and G. Freyd, *Pharmacol. Biochem. Behav.* **26**, 731 (1987)], were fit to each of the six rate-frequency curves collected daily. The frequency required to maintain a half-maximal rate of reward delivery (M-50) was derived from each of the broken-line functions. An M-50 value was retained for further analysis when the broken-line function accounted for at least 75% of the variance of the rate-frequency data. Use of this goodness-of-fit criterion eliminated <3% of the data.
11. By means of within-subject, one-way analyses of variance, a set of 12 M-50 values from the food-restriction condition was compared to 12 values obtained after return to normal body weight, as determined by comparison to the body weights of age-matched control subjects. The leptin test intervened between the gathering of the M-50 values during food restriction and the onset of the period of refeeding.
12. S. Fulton, B. Woodside, P. Shizgal, unpublished data.
13. A Dunnett test for multiple comparisons was employed to compare the 12 M-50 values from two vehicle treatment days to the six values obtained on each day after leptin administration.
14. In a subsequent test of the restriction-sensitive placements under free-feeding conditions, leptin again produced significant increases ($P < 0.005$) in M-50 values in rats L38 and L51. Smaller increases seen on 2 of 4 days in rat L28 fell short of the statistical criterion, and little change was seen in the remaining two rats (data available at www.sciencemag.org/feature/data/1044048.shl). It is not surprising that, in several rats, the effect of a fixed amount of exogenous leptin was stronger during restriction than after refeeding. Body weight overshot prerestriction levels during refeeding, and hence, circulating levels of leptin were probably much higher.
15. The cannula in rat L47 became clogged before leptin testing.
16. With the stimulating electrode serving as the anode, a 100-μA current was applied for 15 s. Rats were then injected intraperitoneally with a lethal dose of sodium pentobarbitol (100 mg/kg) and perfused intracardially with phosphate-buffered saline followed by a mixture of 10% formalin (100 ml), trychloroacetic acid (0.5 g), potassium ferrocyanide (3 g), and potassium ferricyanide (3 g). Brains were removed and stored in 10% formalin. After a 24-hour immersion in a 20% sucrose-formalin solution, the brains were frozen, sliced on a cryostat into 30-μm coronal sections, and mounted on gelatin-coated slides (Fisher Scientific). Sections were stained for Nissl substance, using formal thionin. The location of stimulation sites was identified with the aid of a stereotaxic atlas (*29*).
17. A. Arvanitogiannis, M. Waraczynski, P. Shizgal, *Physiol. Behav.* **59**, 795 (1996).
18. Acute-deprivation data for rat L28 did not meet the stability criterion, and rat L51 died before acute-deprivation testing.
19. S. Cabeza de Vaca, S. Holiman, K. D. Carr, *Physiol. Behav.* **64**, 251 (1998).
20. K. L. Conover and P. Shizgal, *Behav. Neurosci.* **108**, 559 (1994).
21. I. Cusin, F. Rohner-Jeanrenaud, A. Stricker-Kongrad, B. Jeanrenaud, *Diabetes* **45**, 1446 (1996).
22. R. Herrnstein, *J. Exp. Anal. Behav.* **13**, 243 (1970); P. Shizgal, *Curr. Opin. Neurobiol.* **7**, 198 (1997).
23. C. Elias *et al.*, *J. Comp. Neurol.* **402**, 442 (1998).
24. T. Horvath, S. Diano, A. N. van den Pol, *J. Neurosci.* **19**, 1072 (1999).
25. J. G. Mercer *et al.*, *FEBS Lett.* **387**, 113 (1996); J. K. Elmquist, C. Bjorbaek, R. S. Ahima, J. S. Flier, C. B. Saper, *J. Comp. Neurol.* **395**, 535 (1998);
26. C. Broberger, L. de Lecea, J. G. Sutcliffe, T. J. Hokfelt, *J. Comp. Neurol.* **402**, 460 (1998).
27. D. Qu *et al.*, *Nature* **380**, 243 (1996); T. Sakurai *et al.*, *Cell* **92**, 573 (1998).
28. A. G. Watts, G. Sanchez-Watts, A. B. Kelly, *J. Neurosci.* **19**, 6111 (1999).
29. G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates* (Academic Press, New York, ed. 4, 1998), figures 30, 33, and 34.

30. The authors thank D. Richard for his insightful comments on an earlier draft and the "Fonds pour la Formation de Chercheurs et l'Aide à la Recherche du Québec" (grant 97ER0124) and the Natural Sciences and Engineering Research Council of Canada (grant OGP0000308) for research support.

29 July 1999; accepted 16 November 1999

Sex Determination in Malaria Parasites

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A century ago, W. G. MacCallum identified distinct male and female forms in malaria parasites of both birds and humans. Since then, scientists have been puzzled by the high female-to-male ratios of parasites in *Plasmodium* infections and by the mechanism of sex determination. The sex ratio of malaria parasites was shown to become progressively more male as conditions that allow motility and subsequent fertilization by the male parasites become adverse. This resulted from an increased immune response against male gametes, which coincides with intense host erythropoietic activity. Natural and artificial induction of erythropoiesis in vertebrate hosts provoked a shift toward male parasite production. This change in parasite sex ratio led to reduced reproductive success in the parasite, which suggests that sex determination is adaptive and is regulated by the hematologic state of the host.

Malaria parasites are transmitted from the vertebrate host to the mosquito vector by sexual blood stages (gametocytes). When taken up in the bloodmeal by the engorging female mosquito, male gametocytes (microgametocytes) undergo exflagellation, producing up to eight male gametes; a female gametocyte (macrogametocyte) produces only one female gamete, which is fertilized by a single male gamete. The gametocyte sex ratio tends to be female-biased in all species of malaria parasites (*1*) and several authors have considered that the theory of local mate competition, which successfully explains many other cases of biased sex ratios (*2*), determines the gametocyte sex ratio of malaria parasites (*3*). According to this theory, when an infection consists of a few parasite clones, whose offspring will mate among themselves, a

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female-biased sex ratio is favored by natural selection, because it will reduce competition among brothers for mates (*4*). However, empirical data are conflicting (*3*, *5*) and the mechanism of gametocyte sex determination in malaria parasites remains unknown.

Gametocyte sex is not determined by segregation of sex-determining genes or chromosomes, because malaria parasites are haploid in the vertebrate host and a single clone can produce both male and female gametocytes (*1*). Here, to evaluate the adaptive significance of gametocyte sex ratio in parasite transmission, we examine the gametocyte sex ratio of the avian malaria parasite *Plasmodium gallinaceum* and the murine malaria parasite *Plasmodium vinckei* during the course of infection and after induction of erythropoiesis (*6*).

Most *P. gallinaceum* infections are cleared by the host in a predictable manner. From the time the infection becomes apparent, parasitemia increases to a peak about 4 days later, when there is an increase in the presence of reticulocytes followed by infection clearance. Peak parasitemia is highly variable and unpredictable, but it never exceeds 50% of erythrocytes. Peak infectiousness to mosquitoes occurs the day before peak parasitemia (*1*, *7*), and the day of infection with respect to peak parasitemia is the strongest predictor of parasite infectiousness to mosquitoes (*1*, *7*–*9*). At peak parasitemia, the host either dies or mounts an effective immune response, which is accompanied by intense erythropoietic activity. The days after peak parasitemia are characterized by intense hematologic activity, in which most erythrocytes are replaced by reticulocytes. In lethal infections, there is no, or minimal, reticulocyte production and the host dies as a result of very high parasitemia (>60% of erythrocytes parasitized) and hence extreme anemia. Parasite gametocytemia, by contrast, increases more predictably whatever the infection method (mosquito or injection) or outcome (lethal or cured) and reaches a maximum of 1% to 2% at peak parasitemia. In all infections in which the host recovered, the sex ratio was found to be initially very female biased (10% to 20% males), became less so during the course of infection, and approached equality at peak parasitemia. By contrast, in cases of lethal infection, the sex ratio remained highly female biased until death (10% to 30% males). We found a strong correlation between the presence of reticulocytes and the gametocyte sex ratio ($r^2 = 0.65$; $P < 0.00001$) (*10*).

The blood environment of the malaria parasite changes considerably during an infection, with an increase in both hematologic and immune factors that adversely affect gamete fertilization efficiency of the malaria parasite in the mosquito midgut (*1*, *7*). Although *Plasmodium falciparum* sex ratio data in vivo and in vitro suggest that there is no immune-mediated sex-biased gametocyte mortality (*3*), it is possible that a frequency-dependent immune response might selectively act against female gametocytes (*11*). To test for sex-selective gametocyte removal, we vaccinated chickens with either irradiated purified male gametes or an irradiated mixture of female gametes and microgametocytes; then we infected the chickens with parasitized blood (*12*). There was no difference from control infections in either the course of infection (the duration of infection from the appearance of parasites to peak parasitemia, the maximum parasite and gametocyte densities) or the sex ratio for any of the vaccination treatments (male, $\chi^2 = 0.04$; female, $\chi^2 = 2.1$) (*13*). Mosquito infection rates were very low (*14*), which indicates successful vaccination, and male gamete immunization was more effective than female gamete/microgametocyte immunization at transmission blocking ($\chi^2 = 24.9$; $P < 0.001$) (*15*), as found in (*8*). These results suggest that immune factors do not affect the gametocyte sex ratio.

Plasmodium gallinaceum normally invades mature erythrocytes and invades reticulocytes only at exceedingly high parasitemias (*6*). We observed no increase in reticulocyte invasion by *P. gallinaceum* after induction of erythropoiesis. The asexual erythrocytic cycle of *P. gallinaceum* lasts 36 hours and gametocytes mature in 40 hours (*16*), although the exact stage at which sex is determined is unknown for any *Plasmodium* spp. It is therefore unlikely that the observed shift to males was a result of the gametocyte developing in reticulocytes instead of in erythrocytes. However, to control for this eventuality, we injected reticulocytes into infected phlebotomized chickens to maintain hematocrit and suppress erythropoiesis while creating a reticulocyte-rich environment (*17*). There was no change in the sex ratio ($\chi^2 = 3.4$), which suggests that the association between the presence of reticulocytes and the proportion of male gametocytes was not a result of the parasites becoming male from developing within a reticulocyte. Reproductive success was not different from controls as measured by infection rates in mosquitoes ($\chi^2 = 0.5$).

To investigate whether host erythropoietic activity could affect the gametocyte sex ratio, we induced erythropoiesis in the vertebrate host at the beginning of the infection before the normal appearance of reticulocytes and before development of the immune response in control infections. Erythropoiesis was induced by hemorrhage (by phlebotomy of 20% to 30% total blood volume) and by mild hypoxia (by exposure to 15% oxygen for 16 hours after the appearance of parasites in the blood) (*18*, *19*). In both treatments, the male-to-female sex ratio significantly increased after treatment up to, but not including, the day of peak parasitemia, when the control infections became similarly less female-biased (Fig. 1) (phlebotomy: $\chi^2 = 11.8$, $P < 0.001$; hypoxia: $\chi^2 = 26.5$, $P < 0.001$). Apart from an increase in male-to-female sex ratios, no other difference was observed in parasitemia, gametocytemia, and reticulocyte levels between control and treatment groups (Fig. 2). Mosquito infection rates were significantly lower in both treatment groups compared with controls (Fig. 3) (phlebotomy: $\chi^2 = 75.9$, $P <$

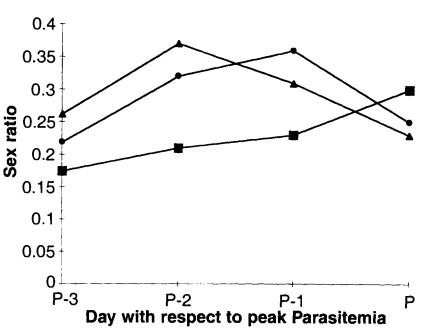

Fig. 1. Effect of erythropoietic treatment on mean gametocyte sex ratio (proportion male) of the avian malaria parasite *P. gallinaceum*. The *x* axis is scaled according to the day of the infection with respect to infection peak *P* (9). Circles, phlebotomy on the first day parasites appeared in the blood [*x*-axis equivalent: four chickens treated on day peak − 3 (*P* − 3), two were treated on day peak − 2 (*P* − 2)]; triangles, hypoxia on the first day parasites appeared in the blood (*x*-axis equivalent: six chickens treated on day *P* − 3, two treated on day *P* − 2); squares, control ($n = 30$).

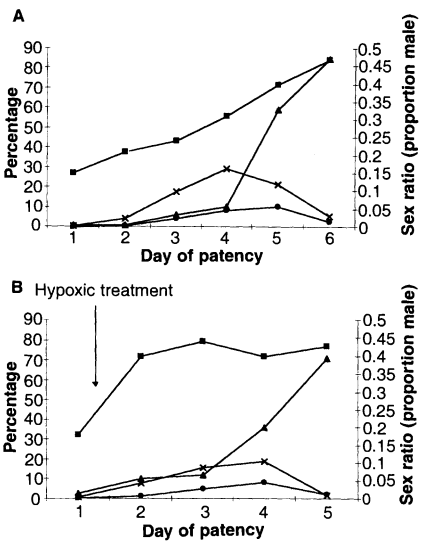

Fig. 2. Examples of infection parameters during the course of a single *P. gallinaceum* infection in its vertebrate host in control (**A**) and hypoxic treatment (**B**) infections. Infected vertebrate host was subjected to hypoxic treatment (*19*) during the night after the first appearance of parasites in the blood. The following infection parameters were considered: crosses, parasitemia (%); circles, gametocytemia (for clarity, shown as % observed × 10); triangles, reticulocytes (%); and squares, gametocyte sex ratio (proportion male). The course of infection runs from the first day parasites appeared in the blood (patency) until infection clearance. Phlebotomy treatment infections gave a similar course of infection profile to hypoxic treatment.

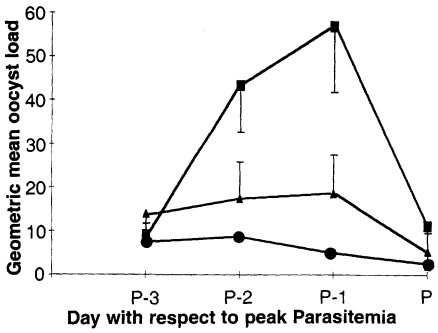

Fig. 3. Effect of erythropoietic treatment on infection rates of mosquitoes (geometric mean oocyst load) by the avian malaria parasite *P. gallinaceum*. *Aedes aegypti* mosquitoes (30 per chicken per day) fed on *P. gallinaceum*–infected chickens that had been subject to erythropoietic treatment on the first day parasites appeared in the blood. The *x* axis is scaled according to the day of the infection with respect to infection peak in the vertebrate host (*9*). The 99% confidence intervals are shown for the treatment group with mean oocyst loads the least different from the controls (hypoxia). There were no differences in daily gametocytemia or parasitemia between control and treatment groups (Table 1). Circles, phlebotomy (n = 3); triangles, hypoxia (n = 4); and squares, controls (n = 4).

Table 1. χ^2 test statistics (*13*) for the effect of erythropoietic treatment (phlebotomy, n = 3; hypoxia, n = 4), by comparison with control infections (n = 4), on the gametocyte sex ratio (male/female) and vertebrate host infection parameters for the *P. gallinaceum* infections used for the mosquito infection studies (Fig. 3).

	Sex ratio	Gameto-cytemia	Para-sitemia	Reticu-locyte (%)
Phlebotomy	19.9*	0.16	2.1	6.8†
Hypoxia	29.9*	3.5	1.9	9.9†

*$P < 0.001$. †$P < 0.01$.

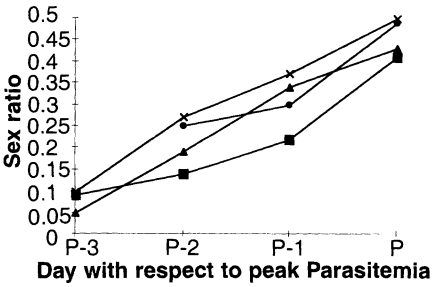

Fig. 4. Effect of erythropoietic treatment on mean gametocyte sex ratio (proportion male) of the murine malaria parasite *P. vinckei*. The *x* axis is scaled according to the day of infection with respect to infection peak *P* (*9*). Triangles, hypoxia on the first day parasites appeared in the blood [*x*-axis equivalent: four mice treated on day peak − 3 (*P* − 3), one mouse treated on day peak − 4 (*P* − 4)]; circles, injection of low-dose rM-Epo 3 days after the first day parasites appeared in the blood (*x*-axis equivalent: all five mice treated on days $P - 4 \rightarrow P - 2$); crosses, injection of high-dose rM-Epo 3 days after the first day parasites appeared in the blood (*x*-axis equivalent: two of five mice treated on days $P - 4 \rightarrow P - 2$; three mice on days $P - 3 \rightarrow P - 1$); and squares, control, n = 10. Gametocytemia was too low on day $P - 4$ to give reliable sex ratios.

0.001; hypoxia: $\chi^2 = 38.2$, $P < 0.001$), despite the lack of differences between the control and treatment groups in daily gametocytemia and parasitemia (Table 1). In contrast to phlebotomy, which affects other hematologic parameters, mild hypoxia stimulates only erythropoiesis (*20*). Other than the expected increase in reticulocyte levels after erythropoietic treatment (Table 1), which does not affect mosquito infection rates (concluded from the reticulocyte transfusion experiment described above), parasite sex ratio was the only infection parameter to change (Table 1). Therefore, the decrease in infectiousness to mosquitoes is probably a result of the change in sex ratio. Such a decrease in reproductive success (low mosquito infection rates) implies that the natural sex ratio is adaptive; gametocyte sex allocation optimizes zygote production. Here, manipulation of the sex ratio resulted in a suboptimal allocation of gametocytes into males and females and reduced reproductive success (*21*).

Erythropoietin (Epo) is a glycoprotein hormone that is responsible for erythropoiesis. In mammals, reticulocytosis usually occurs 3 to 4 days after an acute increase in plasma Epo (*22*). Attempts to isolate erythropoietin from birds have been unsuccessful. However, mouse recombinant erythropoietin (rM-Epo) is readily available; therefore, we tested the hypothesis that erythropoietin itself causes a shift in sex ratio by either injecting rM-Epo into mice infected with *P. vinckei* or subjecting infected mice to hypoxic conditions (*6*, *23*). In control infected mice, there was a strong relationship between sex ratio and reticulocyte levels (r^2 = 0.70; $P < 0.00001$), which was similar to the relationship observed in the avian malaria model. We injected two doses (70 and 700 units per liter) into infected mice each day for 3 days starting on the day the parasites appeared in the blood. All three erythropoietic treatments resulted in significantly elevated sex ratios (Fig. 4) (hypoxia: $\chi^2 = 5.1$, $P < 0.025$; rM-Epo-70: $\chi^2 = 13.3$, $P < 0.001$; rM-Epo-700: $\chi^2 = 16.5$, $P < 0.001$), despite the lack of differences from control infections in reticulocyte levels and in other infection parameters.

Thus, stimulation of erythropoiesis is sufficient to provoke a significant shift in sex ratio toward males. This strongly suggests that the sex of malaria parasites is determined by host hormonal cues that the parasite normally confronts when transmission conditions become adverse. Sex ratio adjustment in response to hormones has been documented in rats, where overcrowding stimulates production of adrenocorticotropin hormone and results in more female births (*24*). Such adjustments occur in the face of chromosomal sex determination of rats.

Our results demonstrate that transiently varying host cues can adjust sex ratio, thereby maintaining reproductive success. Many animals adjust the sex ratio of their offspring according to the prevailing environmental conditions or those predicted to be experienced by their offspring (*2*). Malaria parasites appear to be a special case in which reproductive success is constrained by the effect of the immune system of the host directly on the process of fertilization. Parasites maintain their transmission success during the course of an infection by (i) increasing overall gametocytemia as parasitemia rises and (ii) adjusting the gametocyte sex ratio in response to the changing host environment. Such an adaptive transmission capacity not only provides insight into the hitherto unknown mechanism of sex determination in malaria parasites, but also may have pertinent ramifications for the epidemiology of human malaria. Epo concentrations are suppressed during acute *P. falciparum* infection and the antimalarial drug chloroquine inhibits Epo synthesis (*25*). Therefore, both the disease and the treatment may influence the gametocyte sex ratio and the transmission efficiency.

References and Notes

1. R. Carter and P. M. Graves, in *Malaria: Principles and Practice of Malariology*, W. H. Wernsdorfer and I. McGregor, Eds. (Churchill Livingstone, London, 1988), vol. 1, p. 253.
2. E. L. Charnov, *The Theory of Sex Allocation* (Princeton Univ. Press, Princeton, NJ, 1982).
3. A. F. Read, A. Narara, S. Nee, A. E. Keymer, K. P. Day, *Parasitology* **104**, 387 (1992); R. E. L. Paul *et al.*, *Science* **269**, 1709 (1995); A. F. Read, M. Anwar, D. Shutler, S. Nee, *Proc. R. Soc. London Ser. B.* **260**, 359 (1995); D. Shutler and A. F. Read, *Oikos* **82**, 417 (1998).
4. W. Hamilton, *Science* **156**, 477 (1967).
5. J. J. Schall, *Parasitology* **98**, 343 (1989); D. Shutler, G. F. Bennett, A. Mullie, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6748 (1995).
6. *Plasmodium gallinaceum*: *Gallus gallus*, the jungle fowl, is the natural host of the avian malaria parasite *P. gallinaceum*. We used the domestic race of the natural host, 3- to 4-week-old White Leghorn chickens (*G. gallus domesticus*) (INRA, France), and *P. gallinaceum* strain 8A (obtained from D. Kaslow, NIH, Bethesda, MD). All experimental animals were maintained according to European Union guidelines. Control infections were either mosquito or parasitized-blood induced (intravenous injection of 1 ml of parasitized blood, 15% to 30% parasitemia). Experimental manipulations were carried out on blood-induced infections. *Plasmodium vinckei*: *P. vinckei petteri* (strain 106HW) (obtained from I. Landau, Museum d'Histoire Naturelle, Paris, France) was inoculated intraperitoneally into 8- to 10-week-old female Swiss albino mice (IFFA-CREDO, France). Parasitemias were obtained by Giemsa staining of daily blood smears. Parasitemias and reticulocytes were calculated as percentages observed in a minimum of 100 red blood cells (RBCs); gametocytemias were calculated as percentages observed in 10,000 RBCs. Mature male and female gametocytes are distinguishable after Giemsa staining: *P. gallinaceum* males stain a pale rose color with no distinct nucleus, and females stain blue with a distinct red nucleus (*16*). *Plasmodium vinckei* males stain pink, and

females stain blue with a distinct red nucleus [P. C. C. Garnham, *Malaria Parasites and Other Haemosporidia* (Blackwell Scientific, Oxford, 1966)]. Sex ratios based on counts of 50 to 75 gametocytes were found to be representative. We calculated sex ratios from either 50,000 RBCs or 100 gametocytes, whichever was less. Sex ratios are given as proportion of males.

7. R. E. Sinden, G. A. Butcher, O. Billker, S. L. Fleck,. *Adv. Parasitol.* **38**, 53 (1996).
8. R. Carter, R. W. Gwadz, F. M. McAuliffe, *Exp. Parasitol.* **47**, 185 (1979).
9. Infection classification by day with respect to peak parasitemia is retrospective, where $P - 1$, $P - 2$, and $P - 3$ are 1, 2, and 3 days before peak parasitemia. Erythropoietic treatments were carried out on the first day parasites appeared in the blood (parasite patency) and infection peak was not always the same number of days after parasite patency. Hence, treatment with respect to day of peak parasitemia varied, and that information is supplied in the figure legends. Such variation was taken into account in the statistical analyses.
10. Polynomial regression was performed in SIMSTAT for Windows (Provalis Research).
11. K. P. Day, R. E. Hayward, M. Dyer, *Parasitology* **116**, S95 (1998).
12. Two vaccination procedures were carried out according to protocols previously established (*8*) [R. Carter, R. W. Gwadz, I. Green, *Exp. Parasitol.* **47**, 194 (1979); D. C. Kaushal *et al.*, *J. Immunol.* **131**, 2557 (1983)]. Chickens received intravenous injections with x-irradiated (35 Krad) purified male gametes or a mixture of female gametes and microgametocytes, which are not separable. Each vaccination type was administered three times over 3 weeks per chicken host (n = 3 for both treatments) before intravenous injection of live parasitized blood.
13. Statistical analyses were conducted with the statistical package Genstat 5.4.1. Because each individual vertebrate host was included in the data set many times, we corrected for repeated measures by fitting a generalized linear mixed model (GLMM procedure) with a Poisson error structure with "animal" as the only term in the random model. For both the sex ratio and oocyst analyses, the data were overdispersed and so were corrected for by estimating a dispersion parameter for each analysis [M. J. Crawley, *GLIM for Ecologists* (Blackwell Scientific, Oxford, 1993)]. All analyses of the effects of sex ratio on infectivity were controlled for gametocyte density, infection outcome (live or die), day of infection with respect to day of peak parasitemia, and individual host. Statistical significance was presented as Walds statistics, which are equivalent to a χ^2 analysis. Between-treatment comparisons were performed with respect to peak parasitemia up to, but not including, the day of peak parasitemia, at which time any erythropoietic treatment effects were disguised by the erythropoietic response normally associated with infection control. For clarity, Figs. 1 and 4 show treatment means.
14. Five- to seven-day-old *Aedes aegypti* (Liverpool Blackeye strain) were used for the mosquito infection studies. Oocyst counts in mosquitoes were made 7 days postinfection on midguts dissected from 30 gravid females and then stained with 0.5% mercurochrome.
15. Mosquito infectivity: between-treatment comparisons were done by day with respect to peak parasitemia specifying a Poisson error structure (which gives the same fit as a negative binomial) [K. Wilson, B. T. Grenfell, D. J. Shaw, *Funct. Ecol.* **10**, 592 (1996)] for the distribution of oocyst counts per mosquito per chicken. For clarity, Fig. 3 shows treatment geometric means with 99% confidence intervals.
16. F. Hawking, K. Gammage, M. J. Worms, *Trans. R. Soc. Trop. Med. Hyg.* **65**, 189 (1972).
17. Reticulocyte-rich blood was injected intravenously in exchange for blood (20% of total blood volume) taken from an infected chicken host, thereby increasing the proportion of RBCs that were reticulocytes without stimulating erythropoiesis (n = 6). Reticulocytes were obtained from chickens 72 hours after subcutaneous injection of the hemolytic drug phenylhydrazine hydrochloride (Sigma) at a dose of 6 mg per 100 g of body weight; the blood was washed in 1× phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8,1 mM Na_2HPO_4), and the volume was restored with serum from naive chickens.
18. S. L. Schindler and R. P. Gildersleeve, *Comp. Biochem. Physiol. A Comp. Physiol.* **87**, 533 (1987).
19. Two methods were used to induce erythropoiesis: exsanguination of 20% to 30% of total blood volume by cardiac puncture on the day of parasite patency (>0.1% parasitemia) (n = 6), and 16 hours of exposure to mildly hypoxic conditions on the day of patency (15% oxygen, 85% nitrogen; Carboxyque, France) (n = 8); W. F. Rosse and T. A. Waldmann, *Blood* **27**, 654 (1966).
20. J. S. Milledge and P. M. Cotes, *J. Appl. Physiol.* **59**, 360 (1985); H. Pagel, A. Engel, W. Jelkmann, *Adv. Exp. Med. Biol.* **317**, 515 (1992); M. J. McGuire and J. L. Spivak, in *The Fundamentals of Clinical Hematology*, J. L. Spivak and E. R. Eichner, Eds. (Johns Hopkins Univ. Press, London, 1993), pp. 117–128.
21. R. E. L. Paul, A. Raibaud, P. T. Brey, *Parassitologia* **41**, 153 (1999).
22. W. Jelkmann, *Clin. Invest.* **72**, S3 (1994).
23. Two methods were used to induce erythropoiesis in mice: intraperitoneal injection of mouse recombinant Epo (Boehringer Mannheim, France) at a concentration of 70 or 700 units/liter administered in 100 μl of 0.9% NaCl on the 3 days after parasite patency, and 16 hours of exposure to mildly hypoxic conditions on the day of patency (15% oxygen, 85% nitrogen) (n = 5 for each treatment and n = 10 for control infections).
24. E. Geiringer, *Proc. Soc. Exp. Biol. Med.* **106**, 752 (1961).
25. A. M. el Hassan, A. M. Saeed, J. Fandrey, W. Jelkmann, *Eur. J. Haematol.* **59**, 299 (1997).
26. Supported by Institut Pasteur, Paris. R.E.L.P. was supported by the Medical Research Council (UK)/CNRS, Fondation pour la Recherche Médicale, and a European Commission Training and Mobility of Researchers Marie Curie Research Training Grant. We thank A. Danchin, G. Milon, P. Langlade-Demoyen, C. Roth, P. Druilhe, C. Lazure, K. Kean, and S. Cole for helpful discussions and A. Carmi-Leroy for mosquito rearing.

14 September 1999; accepted 17 November 1999

DNA Topoisomerase IIβ and Neural Development

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DNA topoisomerase IIβ is shown to have an unsuspected and critical role in neural development. Neurogenesis was normal in IIβ mutant mice, but motor axons failed to contact skeletal muscles, and sensory axons failed to enter the spinal cord. Despite an absence of innervation, clusters of acetylcholine receptors were concentrated in the central region of skeletal muscles, thereby revealing patterning mechanisms that are autonomous to skeletal muscle. The defects in motor axon growth in IIβ mutant mice resulted in a breathing impairment and death of the pups shortly after birth.

Murine DNA topoisomerase IIβ (IIβ) is a member of the type II DNA topoisomerase subfamily that mediates the passage of one DNA double helix through another (*1*). Yeasts and *Drosophila* possess a single type II DNA topoisomerase, which is indispensable for segregation of intertwined pairs of newly replicated chromosomes (*2*). In yeasts, the enzyme also shares the function of DNA topoisomerase I in relieving torsional and flexural strains in DNA. Simultaneous inactivation of DNA topoisomerases I and II severely affects DNA and ribosomal RNA synthesis and arrests cell growth irrespective of the stage of cell cycle (*3*).

In mammals there are two closely related type II topoisomerases, IIα and IIβ, encoded by distinct genes (*1*). The IIα rather than the IIβ isoform appears to unlink DNA during chromosome segregation. Cell lines expressing IIα but not IIβ have been identified, indicating that IIβ is dispensable in cellular processes (*4*). To determine the role of IIβ in vivo, we disrupted the murine *TOP2*β gene according to standard procedures (*5*). Two adjacent exons in one copy of *TOP2*β in embryonic stem cells, one of which contains the active-site tyrosine codon, were replaced by the neomycin-resistance marker (Fig. 1A) [see supplementary Web material (*6*) for details on targeting vector construction]. Germ line chimeras from blastocysts injected with the mutated cells were then used to obtain heterozygous *top2*$\beta^{+/-}$ mice (*5*). Whereas *top2*$\beta^{+/-}$ mice are phenotypically indistinquishable from their wild-type (WT) littermates, homozygous *top2*$\beta^{-/-}$ embryos from intercrosses of the heterozygotes are dead at birth. Genotypying of a total of 194 progeny from these intercrosses identified 46 *top2*$\beta^{-/-}$ homozygotes among 50 perinatally dead pups, and none among the 144 surviving neonates. Analysis of mRNA from the liver of an embryonic day 18.5 (E18.5) *top2*$\beta^{-/-}$ embryo showed no detectable IIβ transcript [Web figure 1 (*6*)], and antibodies specific to

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