bration curve, the L observed at a known concentration of free calcium in a solution approximating the intracellular milieu (140 mM KCl, 2 mM Mg^{2+}, pH 7.4, 25°C) is divided by L_{max}, the maximum signal obtained when the same amount of aequorin is briskly injected into a solution containing a saturating concentration of calcium (10 mM). The ratio L/L_{max} is called the fractional luminescence. The titration curve is sigmoidal when drawn as a double logarithmic plot with pCa ($-\log Ca^{2+}$) as the abscissa and pF ($-\log L/L_{max}$) as the ordinate. The coefficient of variance is 1.6 percent. Similar aequorin luminescence titration curves have been published (*4*, *7*). In our experiments, L/L_{max} was determined from the observed signal minus background (L) divided by L_{max} (which is an estimate of the amount of aequorin incorporated in the cells) obtained when the cells were lysed at the end of the experiment with a solution containing 10 percent Triton-X, 10 mM $CaCl_2$, 140 mM KCl, 3 mM Hepes (pH 7.4), and 2 mM $MgCl_2$. The membrane detergent Triton-X does not interfere with the aequorin-calcium reaction, and, since the cell surface membrane is destroyed chemically, the optical geometry of the system is not disturbed. The luminescence evoked by Triton-X (L_{max}) was five to six orders of magnitude larger than the signal L, an indication that the consumption of aequorin during the experiments was negligible. An estimate of Ca_i^{2+} was obtained by interpolation of the experimental values on the calibration curve obtained with 2 mM Mg^{2+} in the solution, the assumed concentration of intracellular free Mg^{2+}. Table 1 shows the results of ten separate cell preparations where Ca_i^{2+} ranged between 16 and 90 nM with a mean of 57 nM. The validity of these estimates rests on several assumptions regarding the ionic composition of the cytosol. Although the activities of ions in the cytosol of monkey kidney cells are unknown, those chosen for the calibration curves (that is, 140 mM KCl, pH 7.4, 2 mM Mg^{2+}, 3 mM adenosine 5′-triphosphate) are close enough to provide an initial approximation for Ca_i^{2+}.

We investigated whether the HOST method altered the viability and the physiological properties of the cells. We found that HOST cells excluded the dye trypan blue as well as control cells. Their O_2 consumption was not stimulated by succinate (*8*). There was no difference between HOST cells and controls with respect to their total calcium or the size of calcium pools and fluxes measured by ^{45}Ca steady-state kinetics (*9*). The HOST cells could reestablish their normal ionic gradients for sodium and potassium even if loaded with 140 mM NaCl instead of KCl during the resealing after aequorin incorporation, and, if reinoculated into culture, HOST cells grew at a rate identical to that of control untreated cells.

We have developed a method by which a sufficient amount of aequorin can be incorporated into small mammalian cells to provide a measurable signal which reflects changes in Ca_i^{2+}. Its chief advantages are the following: (i) the method is relatively simple; (ii) the procedure is relatively inexpensive; (iii) with this method, a system is provided in which extracellular solutions may be perfused and changed without disturbing the cells; (iv) the viability and the physiological properties of the cells appear to be left unaltered; and (v) aequorin can be incorporated into large quantities of isolated cells, which produce an adequate signal and a good statistical representation of a cell population. The method can be applied to other cells: obelin has been similarly incorporated into pigeon erythrocytes (*10*) and in hybrid cells (*3*). In our laboratory, aequorin has been successfully incorporated into freshly isolated rat hepatocytes; their resting Ca_i^{2+} has been estimated to be 50 nM.

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8 March 1982; revised 20 April 1982

Antigenic Diversity in the Human Malaria Parasite *Plasmodium falciparum*

Abstract. *Monoclonal antibodies against blood forms of* Plasmodium falciparum *were used to demonstrate considerable antigenic diversity in this species. Different isolates were distinguished by their ability to react with certain antibodies, and most of the antibodies reacted specifically with merozoites, schizonts, or both. The distribution of different antigenic types appeared not to be related to geographic origin. Serological typing with monoclonal antibodies extends the range of methods for identification of different strains of this malaria parasite.*

Knowledge of antigenic diversity among populations of malaria parasites is important in research aimed at the development of a vaccine against malaria. Immunity to the blood forms of the parasite is, at least in part, directed against antigens of merozoites and schizonts (*1–4*), and since the immunity to *Plasmodium falciparum* may be strain-specific (*5–7*), these antigens seem likely to vary. The soluble S antigens present in the serum of patients infected with *P. falciparum* show extensive serological diversity (*8*), although the role of these antigens in protection is not clear and it is not known whether they are associated with different developmental stages of the parasite. The existence of stage-specific antigens associated with blood forms of *P. falciparum* has been shown by the use of monoclonal antibodies, some of which inhibit the growth of the parasite in culture (*3*). We have used monoclonal antibodies to investigate antigenic heterogeneity in *P. falciparum* isolated in different parts of the world, and here demonstrate that some antigens of merozoites and schizonts exhibit considerable diversity.

Monoclonal antibodies were prepared in mice against two *P. falciparum* isolates, K1 and PB1, from Thailand. The parasites to be used for immuniz tion were grown in vitro (*9*) and includ d all stages that occur in the blood, th t is, ring forms, trophozoites, schizonts, mer-ozoites, and gametocytes. Hybrid my-elomas were made by using a moc fica-tion of the protocol of Perrin *et a* . (*3*) (see legend to Table 1). Those prod cing antibodies against parasites were i entified by indirect immunofluorescen e as-say (IFA) (*10*) on acetone-fixed s ears of cultured blood forms. In seven f sion

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experiments we obtained 856 hybrid cultures of which 247 secreted antibodies to the parasite. We cloned 32 hybrids and classified the antibodies that they produced into five groups, according to the stage-specificity of their reactions and to the characteristic patterns of IFA-staining of mature segmented schizonts and merozoites (Table 1 and Fig. 1).

The antibodies were tested by IFA on 27 isolates of *P. falciparum* from eight countries. Eighteen monoclonal antibodies reacted equally well with all isolates (data not shown), indicating that the respective antigenic determinants were either ubiquitous or that variant forms were rare. The remaining 14 monoclonal antibodies revealed antigenic differences between isolates (Fig. 2). On reaction with a given antibody some isolates contained no fluorescing organisms, and these were scored as negative. In other isolates we could detect a minority of positively reacting parasites among a majority of nonreactive ones, and these were considered to be mixtures of more than one antigenically distinct organism (isolates NF58, T17, SK16, K36, and T21). Isolates where the majority of parasites stained were scored as positive; it is not excluded that these may have contained some nonreactive organisms, but in practice these would have been difficult to detect.

The IFA staining patterns indicated that antibodies in groups I to V (Table 1 and Fig. 1) reacted with different antigens distinguished by their stage or subcellular distributions, but we do not yet know whether antibodies within a single group recognize one or several antigens. Most of the strain-specific monoclonal antibodies belonged to group IV, which reacted with determinants of the merozoite surface. The distribution of some of these specificities among the isolates provided further clues to their relationships (Fig. 2). The K1 monoclonal antibodies 6.1, 7.3, and 7.6 reacted identically with all isolates and in a mutually exclusive manner with PB1 monoclonal antibodies 9.2, 9.7, and 10.3; the most notable exception was isolate S118, which reacted positively with both sets of antibodies. It is possible that these two sets of monoclonal antibodies recognize alternative forms of one antigen, most of the isolates containing parasites expressing one or the other form, and S118 containing a mixture of the two (in this isolate approximately 50 percent of parasites reacted with either set of monoclonal antibodies). By cloning a similar mixed population (*11*) two types of clones have been isolated, and while each type has its own distinct combina-

Table 1. Panel of monoclonal antibodies to *P. falciparum*. The *P. falciparum* isolates K1 and PB1 for immunizations were grown in vitro (*9*) and contained all stages of the blood cycle, that is, ring forms, trophozoites, schizonts, merozoites, and gametocytes. Female Balb/c mice were immunized intraperitoneally with organisms released from host red cells by saponin lysis (*12*) and incorporated in Freund's incomplete adjuvant (100 to 200 μg of protein) two to three times at 2-week intervals. At various times (2 to 16 weeks) after the last injection, the mice were challenged intravenously with parasites in saline (100 μg of protein), and 3 days later their spleen cells were fused (*15*) with the P3-X63-NS/1 (NS-1) myeloma cells at ratios of 2:1 to 10:1. Two weeks after the fusion, hybrid cell cultures producing antibodies to plasmodia were identified by IFA (*10*) on acetone-fixed smears of infected blood, and selected hybrids were purified by limiting-dilution cloning with thymocyte feeders. Spent medium from cloned cultures and, in some experiments, ascitic fluids from mice bearing the hybrid myelomas, were used as sources of monoclonal antibodies.

Group	Reactive blood stages*	Number of monoclonal antibodies against		Total
		Isolate K1	Isolate PB1	
I	All asexual and gametocytes	4 (0)†	1 (0)	5 (0)
II	Schizonts, merozoites, rings	7 (0)	2 (0)	9 (0)
III	All asexual	2 (1)	0	2 (1)
IV	Schizonts, merozoites	5 (4)	9 (8)	14 (12)
V	Trophozoites, schizonts	2 (1)	0	2 (1)

*See Fig. 1 for the morphology of IFA-stained mature schizonts and merozoites. †Numbers in parentheses show the numbers of monoclonal antibodies that distinguished between isolates (see Fig. 2).

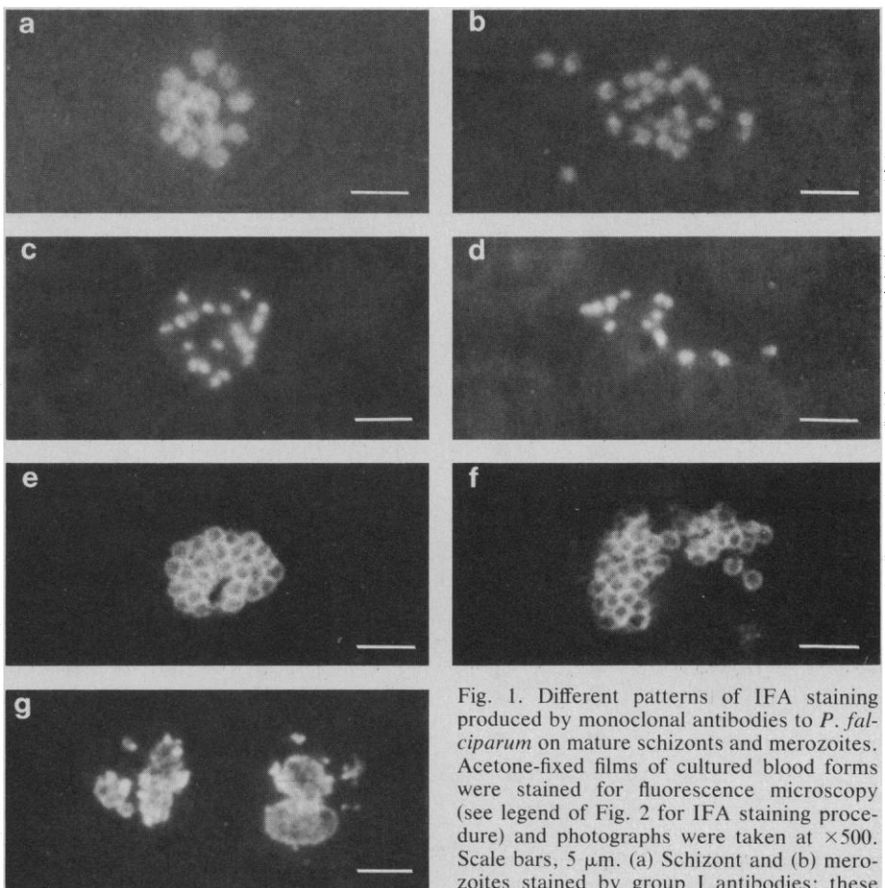

Fig. 1. Different patterns of IFA staining produced by monoclonal antibodies to *P. falciparum* on mature schizonts and merozoites. Acetone-fixed films of cultured blood forms were stained for fluorescence microscopy (see legend of Fig. 2 for IFA staining procedure) and photographs were taken at ×500. Scale bars, 5 μm. (a) Schizont and (b) merozoites stained by group I antibodies; these monoclonal antibodies also reacted with all other asexual blood forms and gametocytes (not shown). (c) Schizont and (d) merozoites stained by group II antibodies. Note localization of the most intense staining to a small area of the merozoites, indicating that the antigen or antigens concerned may be associated with an organelle. These monoclonal antibodies also reacted weakly with ring forms where this characteristic localization was not seen (not shown). (e) Schizont and (f) merozoites stained with group III and group IV antibodies, respectively. Note the concentration of staining around the perimeter of individual merozoites. Since monoclonal antibodies of group IV produced a similar pattern of IFA staining on unfixed parasites released from saponin-lysed red cells, they probably react with the merozoite surface. Monoclonal antibodies of group III also reacted with all other asexual stages (not shown). (g) Morphologically ill-defined staining of pigment-containing parasites with group V antibodies.

tion of other antigenic specificities, the alternative sets of antigenic markers do not occur together. The mutual relationship between the postulated alternative forms could be further tested by biochemical analysis and by genetic studies.

The antigenic differences between isolates ranged from minor ones, detected by only one antibody (for example, isolates SK18 and SK19 differ only in reactivity with monoclonal antibody 10.4) to multiple differences recognized by all 14 antibodies (for example, K1 and PB1). No clear regional differences were seen, identical antigenic combinations occurring in isolates from different parts of the world; for example, isolates K1 and T22 from Thailand, SL3 from Sri Lanka, and M23 from Honduras possessed similar antigenic phenotypes.

Most of the strain-specific monoclonal antibodies produced either strongly positive or negative reactions even when high-titer ascitic fluids were used. For example, antibody 7.3 reacted with isolate K1 at dilutions up to 1 in 31,000 but did not react at all with K28, MAD 20, or Palo Alto at 1 in 10. Conversely, monoclonal antibody 9.7, which titrated to 1 in 100,000 with the last three isolates, did not react with K1 at 1 in 10. However, some antibodies produced IFA reactions which varied in intensity between isolates. These differential reactivities were confirmed by using serial dilutions of ascitic fluids. For example, antibody 7.1 reacted strongly with K1 at a 1 in 10,000 dilution but gave consistently dull reactions with K28 or SK18 at all dilutions up to 1 in 100 and failed to react at all with MAD 20. Similarly, antibody 9.4, reactive with K28 and SK18 at 1 in 10,000, reacted with K1 and MAD20 only up to a dilution of 1 in 100. It is not known whether such cross-reactions reflect qualitative differences or differences in quantitative expression of the antigens.

Our results provide evidence of considerable antigenic diversity in the human malaria parasite *P. falciparum*. The antigenic phenotypes of isolates K1 and PB1 have remained stable for 10 and 6 months, respectively, in continuous culture, and the antigens provide markers

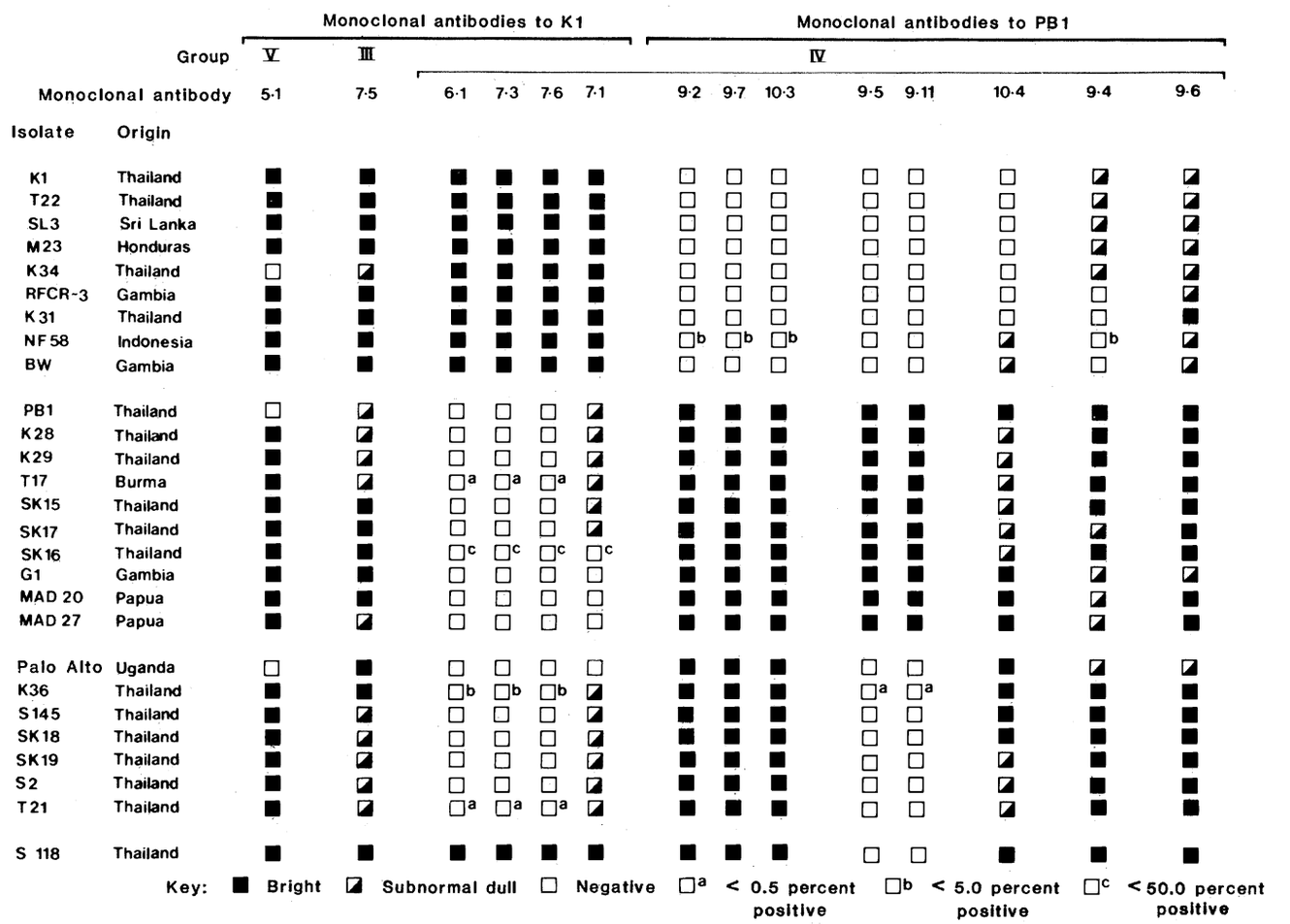

Fig. 2. Reactivities of strain-specific monoclonal antibodies with *P. falciparum* isolates from different regions. Each isolate was tested by IFA (*10*) at least three times using cultured (*9*) parasites harvested at weekly intervals, and the homologous isolates K1 and PB1, which were grown in parallel, were included as controls on all occasions. For testing, infected blood from cultures was washed in RPMI 1640 medium and was diluted in the same medium to contain 1×10^4 to 10×10^4 schizonts (and 10^5 to 10^7 other stages) per 20-μl portion, each portion being dried onto the well of a multispot glass slide. The hybridoma culture fluids were used undiluted, that is, at a 10- to 80-fold excess of the concentration of antibody that still gave optimal reactions with the homologous isolates. Background fluorescence was minimized by the use of a fluorescein isothiocyanate-conjugate of immunoadsorbent-purified rabbit antiserum to mouse immunoglobulin (Miles-Yeda Ltd.) and by counterstaining with Evans blue [0.1 percent in phosphate-buffered saline (PBS)]. The stained slides were mounted in 50 percent glycerol in PBS and the reactions were scored under ×500 magnification as follows. Provided that approximately 50 percent or more of parasites at the appropriate stage of development reacted, the isolate was scored as positive, and the intensity of staining of individual parasites relative to that of the homologous strains was used as an additional criterion to grade the positive reactions as bright or subnormal dull (see key). Reactions where no parasites stained above the background were scored as negative. In some isolates most parasites were negative, but a minority clearly did react and such mixed reactions are indicated by superscripts according to the approximate contents of the positive organisms (see key). To estimate the total number of parasites mature enough to react with the stage-specific monoclonal antibodies, we used two controls: antibodies of similar stage-specificity that were reactive with all isolates and, for group IV antibodies, a 1:1:1 mixture of 6.1, 9.2, and 9.5

which distinguish *P. falciparum* strains in addition to the isoenzyme (*12, 13*), protein variants (*14*), and S antigen (*8*) markers already in use. Since most of the strain-specific determinants identified in this work are associated with the surface of the merozoite, it will be of interest to ascertain the function of the antigens in protective immunity. Should they prove to be important in this respect, it would obviously be necessary to determine the full extent of the antigenic diversity and its genetic basis, in order to assess the potential effectiveness of a vaccine based on such antigens.

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16. We thank S. Thaithong, G. Knowles, J. H. E. Meuwissen, L. H. Perrin, W. Trager, and R. J. M. Wilson for donating the isolates of *P. falciparum*. The project was supported by grants from the Wellcome Trust to H. S. Micklem and from the Medical Research Council of Great Britain to J. G. Scaife and D. Walliker.

17 December 1981; revised 17 March 1982

Hatching of Amphibian Embryos: The Physiological Trigger

Abstract. *Marbled salamander embryos hatch in water if the environmental oxygen pressure is 86 torr or less, but do not hatch if the environmental oxygen pressure is equivalent to that of air. Under hypoxic conditions, embryos hatch in aqueous and nonaqueous media with equal success. Increasing carbon dioxide pressure does not induce hatching, but does decrease the time to hatching by altering environmental* pH.

Prior to hatching, virtually all anuran and urodele embryos develop numerous unicellular ectodermal glands containing hatching enzymes (*1, 2*). Hatching involves the extracellular release of these enzymes, which digest the vitelline membrane and gelatinous egg capsule. After the egg capsule is partially digested, the embryo completes hatching by muscular activity. Although there are numerous descriptions of hatching (*3*) the environmental or physiological factors that initiate enzyme release and subsequent hatching are unknown.

The marbled salamander *Ambystoma opacum* deposits eggs in terrestrial nests in the depressions of temporary pools or along the margins of reduced pools (*4*). Within a few weeks, the embryos develop to a stage capable of hatching and then remain quiescent within the egg capsule until the nest is flooded by rainwater. Embryos may remain at that stage for months within the egg capsule, but typically hatch within minutes to hours after they are covered by water. This curious phenomenon prompted us to investigate what factors initiate hatching in this species. Earlier workers noted that submerging marbled salamander eggs in water stimulated embryos to hatch and implied that water played an essential role in the hatching process (*5*).

Experiments in which we varied either the medium or the composition of gases surrounding the eggs (*6*) indicate that a reduced oxygen pressure (PO_2), rather than water per se, is the trigger for hatching (Table 1). In water (rows 1 to 4) hatching occurred only when the PO_2 was below that of the atmosphere (about 150 torr). The effects of PO_2 on hatching time were determined by either allowing embryos to consume oxygen in a closed system (row 1) or by lowering ambient PO_2 experimentally (row 4). In both cases embryos hatched. In contrast, hatching did not occur when embryos were maintained in an environment in which PO_2 was at or above atmospheric levels (rows 2 and 3). In an initial trial, 2 of 12 embryos hatched in aerated water; however, this occurred during failure of our aeration system which presumably resulted in a low PO_2. In later trials with more than 200 embryos, hatching did not occur when embryos were maintained in aerated water. Embryos that were placed in water equilibrated with 100 percent O_2 (row 3) did not hatch even after 10.5 hours; however, when transferred to water equilibrated with 100 percent N_2 they hatched rapidly ($\bar{x}$ = 23 minutes, row 4). Embryos also hatched in cooking oil (row 5) and in gaseous nitrogen (row 6). These results demonstrate that external water is not essential for hatching and support our contention that low PO_2 triggers hatching.

Carbon dioxide did not induce hatching when PO_2 was at or above atmospheric levels (rows 2 to 5 in Table 2). Hatching occurred more rapidly when water was equilibrated with 5 or 10 percent CO_2 in nitrogen (rows 9 and 10) than when equilibrated with 100 percent nitrogen (rows 6 to 8). At 23°C, embryos exposed to elevated CO_2 hatched about

Table 1. Effects of hypoxia on the hatching of *A. opacum* embryos. In all experiments, groups of four embryos were placed at room temperature (22° to 24°C) into vials (5.3 by 2.5 cm) fitted with inflow and outflow valves. Eggs in water were either allowed to stand without agitation, or were gently agitated with air, O_2, or N_2. Eggs were allowed to stand in oil without agitation, while those in the gas phase were placed on the bottoms of vials and continuously flooded with either N_2 or air. When a liquid medium was used, eggs were covered to a depth of 3 cm. The number of repetitions of each experiment can be derived by dividing the total number of eggs (column 3) by 4.

Medium	Treatment	Eggs (No.)	Hatched (%)	Hatching (minutes)*	Experimental time (hours)
Water	None	24	100	96 ± 111	4
Water	Air	12	16.6†	225 ± 134	8
Water	O_2	12	0.0		3, 4, 10.5
Water	N_2‡	12	100	23 ± 8	0.7
Oil§	None	24	100	33 ± 8	4
Gas	N_2‖	12	100	44 ± 11	1
Gas	Air	24	0.0		48

*Values are means ± 1.0 standard deviation. †Larvae hatched after the aeration system became clogged for about 2 hours. ‡Eggs were placed in O_2 saturated water for 3, 4, and 10.5 hrs (line 3) before transferring to water equilibrated with N_2. §Partially hydrogenated soybean cooking oil. ‖Gases were bubbled through water before passing into the inflow valve of experimental chambers to prevent egg desiccation.

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