# Invasion, and short- and long-term survival of *Babesia divergens* (Phylum Apicomplexa) cultures in non-bovine sera and erythrocytes

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SUMMARY

In order to explore the feasibility of producing a *Babesia divergens* live vaccine free of bovine material contaminants the parasite's ability to grow in human, sheep and horse erythrocytes and serum and serum-free medium was investigated. *B. divergens* was successfully maintained in bovine erythrocytes overlaid with serum-free HL-1 medium. Supplementation of the culture medium with bovine or sheep serum improved parasite growth (monitored by measuring parasitaemia and uptake of tritiated hypoxanthine) whereas horse and human sera reduced parasite growth. As assessed by Giemsa's stained and FITC-labelled blood smears, the parasite invaded all erythrocyte types. Polyparasitism was less common in sheep and horse erythrocytes than in bovine and human erythrocytes. Accole stages were observed in bovine, human and sheep but not in horse erythrocytes. Proliferation following invasion was higher in human but lower in horse and sheep erythrocytes compared with bovine erythrocytes. Long-term cultures of *B. divergens* reached similar peak parasitaemias in human, sheep and bovine erythrocytes. Attempts to establish long-term cultures in horse erythrocytes failed. These results suggest that *B. divergens* is not host specific at the level of host cell attachment and invasion. Instead, parasite survival appears to be decided once the organism has gained access into the cell.

Key words: *Babesia divergens*, *in vitro* culture, erythrocyte invasion, short-term survival, long-term survival.

## INTRODUCTION

*Babesia divergens*, the main agent of bovine babesiosis in Europe, is an intra-erythrocytic protozoan parasite, transmitted by the tick *Ixodes ricinus*. In Ireland the parasite is widespread and of considerable economic importance (Gray & Harte, 1985). Gray *et al*. (1995) vaccinated cattle with live *B. divergens* parasites raised in gerbils (*Meriones unguiculatus*), highly susceptible laboratory hosts. In combination with imidocarb pre-treatment, the vaccination proved very effective. However, large-scale use of the vaccine would require the transfer of parasite production to a scaled-up *in vitro* system using large animal erythrocytes. While disease transmission and autoimmunity are calculated risks when vaccinating Australian cattle against the more serious pathogen *Babesia bovis*, approval of a vaccine contaminated with bovine material in Europe is highly unlikely. Particularly in the light of recent disease outbreaks, vaccine safety will have to be ensured by the use of non-bovine host cells and serum substitutes.

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Vayrynen & Tuomi (1982) were the first to culture *B. divergens* continuously in bovine erythrocytes using bovine serum supplemented M199. Later workers reported that for unknown reasons sera from cattle vary considerably in their ability to support the growth of the parasite *in vitro* (Canning & Winger, 1987; Neves *et al*. 2001). The parasite appears to be less host specific than other babesias and has been successfully cultured in human (Gorenflot *et al*. 1991; Grande *et al*. 1997; Pudney, 1984) and rat erythrocytes (Ben Musa & Phillips, 1991). No other suitable host erythrocytes have so far been reported. In order to explore the feasibility of a live *B. divergens* vaccine raised *in vitro* in non-bovine erythrocytes, this study investigates the ability of the parasite to invade and survive in non-bovine host cells. By varying the amount and type of serum added to the culture medium, more robust, alternative culture systems for *B. divergens* were developed.

## MATERIALS AND METHODS

### B. divergens in vitro *culture*

A *B. divergens* isolate, designated Tulla Malone, or TM, was established *in vitro* from a wild-type

 
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bovine infection from Co Clare, Ireland, in June 2000. The procedure followed was a slight modification of that described by Holman *et al*. (1994*a*) for the culture-adaptation of *Babesia equi*. Infected bovine blood with a parasitaemia of 1·5% was collected into EDTA, washed once in Dulbecco's phosphate-buffered saline, pH 7·2, with 15 mM EDTA, and twice in PBS without EDTA (each centrifugation at 200 ***g*** for 10 min at 4 °C). The buffy layer was removed after each centrifugation. Sera and uninfected erythrocytes for continuous culture were obtained from adult cattle (20 dairy cows ⩾ 18 months of age) reared in a babesiosis-free environment (Lyons Research Farm, Newcastle, Co Dublin, Ireland). Donor blood collected by jugular venepuncture was defibrinated by shaking with glass beads and centrifuged at 500 ***g*** for 10 min at 4 °C. The serum was heat-inactivated and stored at −20 °C for later use in culture media. The upper third of the cell pellet including the buffy layer was discarded. The lower half of the remaining pellet was washed 3 times to remove all residual serum. Subsequently the erythrocytes were resuspended in Puck's saline with 2% extra glucose (PSGG) to a final packed cell volume (PVC) of 50% and stored at 4 °C. Cultures were set up in 24-well plates with 1·1 ml of HL-1 medium (BioWhittaker UK) supplemented with 2 mM L-glutamine and 15% adult bovine serum, 0·1 ml of donor erythrocyte suspension and 0·05 ml of infected erythrocytes per well (resulting in 5% PCV and a medium depth of 0·62 cm). Initially, cultures were incubated at 37·5 °C in a special gas mix consisting of 5% $CO_2$, 2% $O_2$ and 93% $N_2$ (BOC gases). After several successful subcultures the gas mix was changed to 5% $CO_2$ in air. The supernatant was replaced with fresh medium daily. Every 2 days at average parasitaemias of 5% subcultures were carried out by diluting 1 in 5. At this point cultures had reached optimum exponential growth. Cultures allowed to grow beyond this point, reached peak parasitaemias of up to 20% within 3–5 days.

### *Non-bovine sera and erythrocytes*

Pooled male human AB sera were purchased commercially (Quest Biomedical). Discard units of human erythrocytes of type 0, rhesus +, provided by the National Blood Centre, Ireland, were washed 3 times in PSGG (180 ***g***, 10 min, 4 °C). The buffy layer was removed after each centrifugation. Finally, the lowest third of the cell pellet was resuspended in the same volume of PSGG. Equine (Lyons Research Farm) and ovine blood (Faculty of Veterinary Medicine, Dublin, Ireland) was defibrinated, centrifuged at 350 ***g*** and 600 ***g*** respectively (10 min at 4 °C) and prepared in the same way as described above for bovine erythrocytes and serum. All sera were heat inactivated and stored at −20 °C.

### *Monitoring of* in vitro *growth*

Proliferation assays were carried out during the exponential growth phase i.e. from 24 to 48 h after subculture. The assays were run in 96-well plates with 200 $\mu$l of resuspended culture per well. For proliferation assays comparing parasite growth in different sera, 50 $\mu$l of HL-1 medium containing various concentrations of heat-inactivated sera and 2·5 $\mu$Ci tritiated hypoxanthine (Amersham Pharmacia Biotech UK Ltd) were added to each well (PCV: 4%; final concentration of [$^3$H] hypoxanthine: 10 $\mu$Ci/ml). Twenty wells were set up with each serum type. For assays investigating invasion into non-bovine erythrocytes, parasites grown in bovine erythrocytes were subcultured 1:5 into the various erythrocyte types ($n = 24$ per group). The following day, the supernatant was changed, and 50 $\mu$l of complete culture medium containing 2·5 $\mu$Ci/well tritiated hypoxanthine were added to each well. After 24 h incubation in the presence of radioactive hypoxanthine, the cells were harvested onto glass-fibre filters (Packard UK) with a cell harvester (Packard Filtermate™). Incorporation of radioactivity as cpm was determined using a direct beta-counter (Packard Matrix™ 9600). Negative control wells contained uninfected erythrocytes.

In addition, parasitaemias were assessed by visual examination of thin Giemsa's stained blood smears based on a total count of 1000 erythrocytes. For short-term survival assays, 6 replicate wells were set up with each serum type and 7 replicate wells with each erythrocyte type. Long-term survival in non-bovine erythrocytes was monitored in 4 replicates per group.

### *Identification of infected equine erythrocytes with FITC-labelled anti-erythrocyte antibodies*

Blood smears fixed in 50% methanol: 50% acetone were blocked with 0·5% BSA in PBS (30 min). Subsequently, they were incubated with 1:100 dilutions of FITC-conjugated rabbit anti-horse erythrocyte IgG fraction (Research Diagnostics, Inc., USA) in BSA/PBS (60 min). The parasites were visualized using 10 $\mu$g/ml propidium iodide (1 min), a bright orange fluorescent stain for nucleic acids. The slides were washed 3 times in PBS after each incubation. All incubations were carried out at room temperature in a humidified atmosphere. The slides were mounted in Dako fluorescent mounting medium. Smears of bovine erythrocytes were used as negative controls.

### *Statistical analysis*

Parasite proliferation in cultures supplemented with various sera is presented as a percentage of parasite growth in the absence of serum. In assays comparing

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parasite growth in the presence of different erythrocyte types, proliferation in bovine erythrocytes was regarded as 100%. To compensate for the difference in erythrocyte volume between species results were expressed as cpm/$10^6$ erythrocytes. Parasitaemias and cpm were compared using one-way ANOVA followed by Scheffe's Post Hoc Test. The null-hypothesis was rejected at $P < 0{\cdot}05$.

## RESULTS

### *Parasite growth in culture medium supplemented with different sera*

Parasitaemia in *B. divergens* cultures in bovine erythrocytes overlaid with serum-free HL-1 medium rose from about 1% to an average of 4·2% (±0·6 S.D., $n = 8$) within 2 days after subculture. Hypoxanthine uptake indicated that addition of bovine serum to the culture medium led to an increase in parasite growth by up to 40% (Anova: F = 27·4; D.F. (error, group) = 89, 3; $P \leqslant 0{\cdot}0001$) (Fig. 1A). This improvement in parasite growth was most pronounced at a serum concentration of 10%. The level of parasitaemia was also improved in the presence of bovine serum but this difference was not significant (F = 2·19; D.F. = 22, 3; $P = 0{\cdot}12$) (Fig. 1B). Inclusion of heat-inactivated human sera in the culture medium caused a decrease in hypoxanthine uptake of up to 60% (F = 18·4; D.F. = 99, 3; $P \leqslant 0{\cdot}0001$) and a decrease in parasitaemia of approximately 70% (F = 63·1; D.F. = 22, 3; $P \leqslant 0{\cdot}0001$). The detrimental effect of human serum was evident even at 5% serum concentration. Five to 10% heat-inactivated sheep serum in the culture media resulted in a significant increase in hypoxanthine uptake (40%; F = 19·37; D.F. = 97, 3; $P \leqslant 0{\cdot}0001$) and parasitaemia (up to 60%; F = 8·44; D.F. = 22, 3; $P = 0{\cdot}0006$). When the concentration of sheep serum was increased to 20%, parasite growth was similar to that in the absence of serum. Heat-inactivated horse serum caused a 20–35% decrease in hypoxanthine uptake (F = 9·9; D.F. = 96, 3; $P \leqslant 0{\cdot}0001$) and parasitaemia. The reduction in parasitaemia due to horse serum was not significant (F = 1·69; D.F. = 22, 3; $P = 0{\cdot}2$).

### *Invasion and polyparasitism in bovine and non-bovine erythrocytes*

*In bovine erythrocytes.* After 3 subcultures, storage in liquid nitrogen and 7 further subcultures in bovine red blood cells, the percentage of infected erythrocytes with more than 2 intracellular parasites ranged between 7 and 21·5% (average: 14% ± 5·3 S.D.; $n = 1140$, 8 culture wells). Most red blood cells carried 4 (59%, $n = 141$) or 3 parasites (38%), and very occasionally 5 and 6. Uneven numbers of intracellular parasites generally consisted of 1 or 2 dividing stages plus 1 ring stage. Numbers of multiply-invaded erythrocytes were not dependent on parasitaemia. Parasites in the typical peripheral or accole position were common. With successive subcultures the rate of proliferation increased and multiply-infected erythrocytes became less common.

*In human erythrocytes.* In infected human erythrocytes the percentage of multiply-infected red blood cells ranged between 4 and 30% (average: 14% ± 7·7 S.D.; $n = 1099$, 14 culture wells). Generally these erythrocytes carried 3 (48%), 4 (40%), more rarely 5 parasites (9%, $n = 129$). In some instances cells with up to 8 parasites were observed. Parasites in the accole position were present. Ring stages were characterized by large vacuoles.

*In sheep erythrocytes.* Multiply-infected erythrocytes were less common than in the other two erythrocyte types (range: 0–16%, average: 6% ± 4·7 S.D.; $n = 1196$, 10 culture wells). Multiply-invaded erythrocytes contained either 3 (43%) or 4 parasites (55%, $n = 67$), while cells simultaneously infected with 5 or more parasites were extremely rare. Accole stages were observed.

*In horse erythrocytes.* Horse erythrocytes were distinguished from similarly sized bovine erythrocytes using FITC-conjugated antibody which was specific for horse erythrocytes and did not cross-react with bovine erythrocytes. Intracellular parasites, visualized using propidium iodide, were observed within horse red blood cells. Most erythrocytes carried 1 parasite. What appeared to be dividing stages were observed in 32% of infected erythrocytes ($n = 79$). Multiple infections with 3 parasites per cell were rare (approximately 5%), and infections with more than 3 merozoites seemed to be absent. Parasites in the accole position were not observed. *B. divergens* in what were thought to be horse erythrocytes on Giemsa's stained smears had a very granular appearance.

### *Short-term survival in non-bovine erythrocytes*

During the course of this experiment, cultures maintained in medium supplemented with 15% heat-inactivated bovine serum and bovine erythrocytes reached average parasitaemias of 4·5% (±1·2 S.D, $n = 14$) within 2 days after subculture. By 48 h after transfer into human erythrocytes uptake of radioactive hypoxanthine exceeded that in bovine red blood cells (124·0% ± 39·4 S.D.), but the difference was not significant (F = 30·51; D.F. = 116, 3; $P = 0{\cdot}06$) (Fig. 2A). Relative cpm/$10^6$ red blood cells was lowest in sheep (44·8% ± 4·2 S.D., $P \leqslant 0{\cdot}0001$) and horse erythrocytes (51·6% ± 10·5 S.D., $P \leqslant 0{\cdot}0001$). Hypoxanthine uptake in uninfected erythrocytes ranged between 0·07 and 3·1 cpm/$10^6$ red blood cells.

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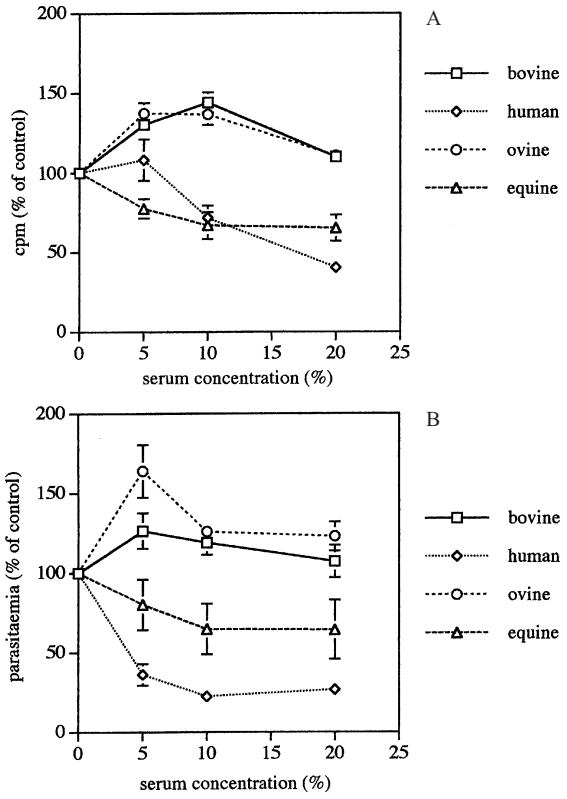


Fig. 1. Relative hypoxanthine uptake per well (±S.E.) (A) and relative percentage parasitaemia (±S.E.) (B) in *Babesia divergens* cultures in bovine erythrocytes incubated with various serum types.

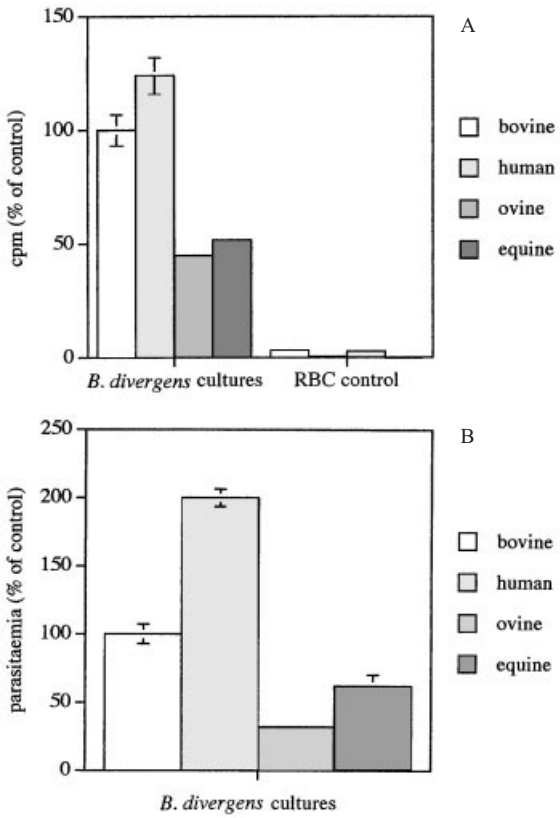


Fig. 2. Relative hypoxanthine uptake per $10^6$ cells (±S.E.) (A) and relative percentage parasitaemia (±S.E.). (B) in *Babesia divergens* cultures transferred into various erythrocyte types and maintained in medium supplemented with 15% heat-inactivated bovine serum.

Average parasitaemias in human red blood cells rose to twice the level reached in bovine erythrocytes (mean: 200·2% ± 16·8 S.D. of growth in bovine red blood cells) (Anova: F-ratio = 82·68; D.F. = 31, 3; $P \leqslant 0{\cdot}0001$) (Fig. 2B). Parasitaemias in ovine (32·2% ± 4·9 S.D.) and equine erythrocytes (61·8% ± 22·0 S.D.) were significantly lower than in bovine erythrocytes ($P = 0{\cdot}006$ and $P \leqslant 0{\cdot}0001$ respectively).

### *Long-term survival in non-bovine erythrocytes*

In bovine erythrocytes the TM isolate typically reached between 4 and 9% parasitaemia within 2 days after subculture. This culture was subcultured into human, sheep and horse erythrocytes. Following transfer into human erythrocytes parasitaemias rose to between 7 and 17% in the first two subcultures and then adjusted to peak levels similar to those in bovine erythrocytes (3–10%). Following an adaptation period of 4–6 days, parasites transferred from bovine erythrocytes into sheep erythrocytes proliferated at the same rate as in bovine red blood cells. Upon transfer of parasites from bovine into horse erythrocytes cultures reached peak parasitaemias of up to 7%. Following subculture, however, parasites failed to proliferate. All cultures were maintained in HL-1 medium supplemented with 15% heat-inactivated bovine serum.

## DISCUSSION

Although, according to the literature, only certain cattle are suitable donors of serum and erythrocytes for *B. divergens* cultures (Canning & Winger, 1987; Neves *et al.* 2001) all the bovine sera used in this study supported parasite growth if they were used in combination with HL-1 medium. Originally HL-1 medium was designed as a serum-free, chemically defined medium for *in vitro* cultivation of hybridoma cells. It was found to support *in vitro* cultures of *B. caballi* (Holman *et al.* 1993), *B. equi* (Holman *et al.* 1994*a*) and *Babesia* spp. from North American elk (Holman *et al.* 1994*b*) and woodland caribou (Holman *et al.* 1994*c*). The only other bovine species so far cultured in HL-1 medium has not yet been classified (Zweygarth *et al.* 1995). However, it is likely that the medium will also prove suitable for other bovine *Babesias*.

Grande *et al.* (1997) adapted *B. divergens* to growth in serum-free medium with routine peak parasitaemias of 3%. Supplementation of the medium with serum substitutes raised parasitaemias to over 30%. The TM isolate could be maintained in serum-free HL-1 medium without adaptation or supplementation. The growth rate, however, was higher in the presence of bovine or sheep serum. The advantageous effects of both serum types were most

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pronounced at 10% serum concentration. Addition of either human or horse serum to the growth medium, on the other hand, resulted in a significant decline in parasitaemia. Human serum has been used very successfully in *B. divergens* cultures in the past (Gorenflot *et al.* 1991; Grande *et al.* 1997; Pudney, 1984). It is unclear whether the failure of TM to grow in the presence of human serum was due to the serum source, the serum blood group or the parasite isolate itself. There are no previous reports on the use of ovine or equine sera in *B. divergens* cultures. Detrimental sera may contain deleterious factors or lack necessary components. By comparing parasite growth in the presence of various sera to background levels observed in serum-free medium, our results indicate that there are serum factors in bovine and ovine sera which are beneficial to parasite growth while the human and equine sera contain factors which are detrimental to *B. divergens*. Interestingly, the opposite has been reported for *B. bovis* and *B. bigemina* cultures in the presence of horse serum. While Yunker, Kuttler & Johnson (1987) observed that *B. bovis* cultures declined within days after substituting all bovine for equine serum, Neves *et al.* (2001) found that instead of containing putative inhibitory molecules horse (and foetal calf) serum supported both *Babesias* once the media were supplemented with hypoxanthine.

Our study shows that *B. divergens* is able to invade human, sheep and horse erythrocytes *in vitro*. In the bovine host the parasites are typically situated in the accole position (Garnham & Bray, 1959). In *B. divergens* cultured in human and bovine cells, on the other hand, Pudney (1984) reported peripheral piroplasms to be absent. In our cultures, parasites were generally observed in an off-centre position, while typical accole stages were only observed in bovine, human and ovine erythrocytes.

Polyparasitism in the bovine host *in vivo* is infrequent (Gorenflot *et al.* 1991). In unnatural hosts, however, such as gerbils (Gorenflot *et al.* 1991), rats, hamsters (Canning, Killick-Kendrick & Monk, 1976) and chimpanzees (Garnham *et al.* 1959) and in *in vitro* culture (Pudney, 1984; Ben Musa *et al.* 1991), multiply-infected erythrocytes are frequently observed. In the present study polyparasitism was most common in *Babesia* cultures in bovine and human red blood cells, less common in sheep and rare in horse red blood cells. It is not clear whether polyparasitism is due to multiple infections or repeated multiplication in the same host cell. The low incidence of multiple infections in infected sheep erythrocytes and in fast growing 'older' bovine cultures indicates that polyparasitism is not related to high parasitaemia. While most erythrocytes contained 3 or 4 parasites, maximum numbers of parasites per cell appear to depend on host cell size with up to 8 merozoites in the largest, human red blood cells.

In agreement with previous work by Pudney (1984) and Gorenflot *et al.* (1991) human erythrocytes were found to be highly suitable for culturing *B. divergens*. No adaptation period was required with parasitaemias reaching very high levels within 24 to 48 h after transfer. Following a short adaptation period, growth in sheep red blood cells was comparable to that in bovine erythrocytes. *B. divergens* has never before been reported to infect sheep red blood cells either *in vitro* or *in vivo*. Although there are several ovine *Babesia* spp. (Kakoma & Mehlhorn, 1994), confusion with *B. divergens* is unlikely: even though sheep are parasitized by the vector, *Ixodes ricinus*, ovine babesiosis has never been reported in Ireland. The initial rise in parasitaemia indicates that *B. divergens* transferred into equine erythrocytes was able to invade horse erythrocytes and proliferate to some extent. However, following subculture, parasites gradually disappeared. Thus all erythrocyte types were invaded, while only the 2 ruminant and the human erythrocytes supported long-term cultures of *B. divergens*. These results imply that this *Babesia* species is not specific at the level of host cell attachment and invasion. Instead, parasite survival appears to be determined by factors in the erythrocyte cytoplasm or permeable serum factors, which affect the organism once it has gained access into the cell. The fact that equine serum was detrimental to parasite growth supports this hypothesis.

The study identified a new suitable host erythrocyte for *B. divergens* and a new culture medium, which supports *in vitro* growth even in the absence of serum. Unfortunately, due to obvious risks of disease transmission, neither ovine nor human erythrocytes are suitable for vaccine production. However, further research into alternative host erythrocytes is strongly encouraged by the parasite's low host specificity observed in *in vivo* experiments (Canning *et al.* 1976; Garnham *et al.* 1959; Gorenflot *et al.* 1991) and confirmed by our study. In this context, our results indicate the importance of long-term culture experiments, as early behaviour in culture is not necessarily a good indicator for long-term growth.

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