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COMMISSIONED REVIEW

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Blood feast: Exploring the erythrocyte-feeding behaviour of the myxozoan *Sphaerospora molnari*

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Abstract

Aims: As the most abundant cell population in the blood, erythrocytes represent an attractive source of nutrients and a protective niche to a number of pathogens. Previously, we observed the attachment of the myxozoan parasite *Sphaerospora molnari* to erythrocytes of its host, common carp (*Cyprinus carpio*), raising a number of questions about the nature of this interaction.

Methods and results: We elucidated the impact of *S molnari* on the number of erythrocytes in healthy and immunocompromised fish, over a period of 6 weeks. While we observed only a mild decrease in RBC numbers in healthy individuals, we witnessed gradual and finally severe haemolytic anaemia in immunosuppressed fish. Accompanying this overt loss was increased erythropoiesis as represented by an increase of erythroblasts in the blood. In vitro, we demonstrated the uptake of host proteins from CFSE-labelled erythrocytes, ultimately inducing death of host RBCs, likely for nutrient gain of the parasite. Nevertheless, the results do not exclude a possible role of erythrocyte-derived proteins in immune evasion.

Conclusion: Overall, the obtained data provide first evidence for the previously unknown appetite of myxozoan parasites for host erythrocytes and create an important framework for future investigations into the molecular mechanisms underlining this interaction.

KEYWORDS
common carp, erythrocytes, flow cytometry, haemolytic anaemia, hematophagy, Myxozoa

1 | INTRODUCTION

Blood is a nutrient-rich mixture of proteins, essential minerals and vitamins, which is relatively consistent in composition and continuously renewed, and hence represents an ideal source of nutrients for parasites. While haematophagous species found amongst monogenean, digeneans, nematodes, annelids, insects, acarines, crustaceans and even vertebrate groups (fish, birds, reptiles and mammals) consume blood nonselectively, there are abundant examples of blood-dwelling protists and microorganisms exploiting various niches including leucocytes and erythrocytes.[1] Infectious agents can interact with these host cells directly via invasion, or affect them indirectly by accelerated clearance of altered red blood cells (RBCs) in spleen and liver.[2] Both scenarios result in a decrease in the total number of erythrocytes and in haemolytic anaemia. At least 39 human pathogens inducing haemolytic anaemia have been described, including protist parasites, *Plasmodium falciparum* and *Babesia* spp., bacterial agents, *Bartonella* or *Mycoplasma*, and a number of viruses.[2] In 2000, Davies et al.[3] compiled an extensive list of intraerythrocytic parasites of mammals and lower vertebrates,

including fish. However, unlike human pathogens, the majority of fish parasites does not receive much attention and research is often based on punctual observations.[3]

Sphaerosporids represent one of five major clades of a highly diverse group of ancient cnidarian parasites, the Myxozoa (reviewed in ref. [4]). Proliferative blood stages of sphaerosporids were initially named unidentified blood objects (UBOs)[5] and only later were these pluricellular organisms associated with myxozoans belonging to the *Sphaerospora* clade. *Sphaerospora molnari*, like other members of the clade, proliferates in the blood of common carp and exhibits a peculiar dancing or twitching movement, an actin-based motility mechanism, which allows the parasites to escape attachment by host immune cells.[6] High motility together with high proliferation rates suggest elevated metabolic requirements of *S molnari* blood stages. Recently, we observed attachment of *S molnari* to erythrocytes in vivo and in vitro, suggesting a possible feeding behaviour of the parasite on RBCs.[7] As RBCs represent a rich source of iron and amino acids exploited by other parasites,[8] we aimed to further elucidate the interaction of *S molnari* with carp erythrocytes. In the present study, we investigated the effect of parasitemia on host RBC quantities and morphology; the cellular interaction between erythrocytes and sphaerosporid blood stages; and we discuss the consequences of *S molnari* exploitation of these host cells in the context of nutrient acquisition and molecular mimicry.

2 | METHODS

2.1 | Fish

Specific pathogen-free (SPF) common carp (*Cyprinus carpio*) were reared from peroxide-treated fertilized eggs (700 mg/L for 15 min) in an experimental recirculation system in the animal facility of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences. The system holds a total of 2300 L water, passing through 40 L aquaria, has biological and mechanical filtration, standard ultraviolet filtration and ozone treatment, and we performed a weekly exchange of 25% of water. Water quality (oxygen, pH, ammonia, nitrite and nitrates) is monitored on a weekly basis using probes and titration tests. Ammonia levels never surpassed 0.02 mg/L. The fish were kept at the constant light:dark cycle (14 hours:10 hours), at constant temperature of 21 ± 1°C. During the experiment, fish were fed a commercial carp diet (Skretting) at a daily rate of 2% of biomass. Larvae were tested for *S molnari*-free status at 2 weeks and 1.5 months post-hatching using a specific PCR assay[7] (100 larvae tested at each date, all tested PCR negative).

2.2 | Isolation of parasites

Motile blood stages of *S molnari* were obtained from the blood of donor fish at the peak of infection using an adapted protocol for DEAE-cellulose isolation.[9] Briefly, whole blood was drawn from the caudal vein of infected fish using heparinized syringes. Full blood was then loaded on a cellulose column and separated by ion-exchange chromatography. The enriched parasite (purity >95%) was pelleted (400 g, 5 min), washed once with 50 mL of RPMI and adjusted to 1 x 10^6 parasites/mL.

2.3 | Parasite infection

Prior to the experiment, 26 fish (weight = ±15 g) were individually tagged with glass transponders (AEG). One day before the experiment, 14 fish were treated with triamcinolone acetonide (immunosuppressant), at a dose of 200 µg/g of body weight. At the start of the experiment, fish were divided into four groups; control (CO) (n = 5) fish were intraperitoneally (ip) injected with 100 µL of sterile PBS, immunosuppressed fish (IS) (n = 7) ip injected with 100 µL of sterile PBS, immunosuppressed and infected fish (IS + INF) (n = 7), and naïve infected fish (n = 7) (INF). Infection was achieved by ip injection with 100 000 motile blood stages of *S molnari*. All fish were anesthetized with 0.1 mL clove oil/L water clove oil before bleeding or injection. Sampling of fish was performed on a weekly basis until 6 weeks post-infection. On each sampling date, 20 µL of blood was drawn with a heparinized syringe and analysed by flow cytometry and light microscopy. Animal procedures were performed in accordance with Czech legislation (section 29 of Act No. 246/1992 Coll., on Protection of animals against cruelty, as amended by Act No. 77/2004 Coll.), and animal handling complied with the relevant European guidelines on animal welfare (Directive 2010/63/EU on the protection of animals used for scientific purposes) and the recommendations of the Federation of Laboratory Animal Science Associations.

2.4 | Flow cytometry

The number of erythrocytes was estimated using a protocol for full blood analysis described previously.[10] Briefly, 2 µL of full blood was washed with cold RPMI and resuspended in 200 µL of RPMI. Each sample was acquired for 20 seconds on BD FACSCanto II (BD Biosciences) with a flow rate of 60 µL/min. Erythrocytes were identified based on the forward scatter-width (FSC-W)/ side scatter-area (SSC-A) profile as described earlier.[10] To elucidate the identity of the erythroblasts, we employed ER-tracker; a cell-permeant stain that binds to the sulphonylurea receptors of ATP-sensitive K+ channels prominent in the endoplasmic reticulum. To estimate the amount of erythrocyte death, cells were labelled with propidium iodide (PI). Both markers were analysed by flow cytometry,

2.5 | Erythrocyte attachment and uptake assay

To visualize the attachment of parasites to erythrocytes, DEAE-isolated blood stages of *S molnari* were exposed to erythrocytes isolated from SPF carp by centrifugation (Ficoll, 400 g, 15 minutes, followed by 2 washes in RPMI), in a ratio of 1:10. Parasite stages were left to interact with erythrocytes for 3 hours, and then, the

mixture of cells was pipetted onto poly-L lysine coated slides, left to settle, fixed with 2.5% glutaraldehyde and prepared for scanning electron microscopy (SEM), following a protocol established for myxozoan spores.[11] Sputter- gold coated cells were viewed in a JEOL 7401-F field emission SEM microscope.

To demonstrate uptake of erythrocyte proteins by *S molnari*, erythrocytes from SPF fish were labelled with carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer's instructions. After a 10-min incubation with RPMI containing 10 µM CFSE, cells were washed twice with RPMI containing 5% FBS and resuspended in RPMI medium. CFSE-labelled erythrocytes were mixed with 100 000 freshly isolated motile blood stages at parasite:erythrocyte ratios of 10:1, 1:1, 1:5 and 1:10, respectively, and incubated for 14 hours (5% CO_2; 25°C). After 14 hours, the mean fluorescence intensity of the *S molnari* population was estimated by flow cytometry. *S molnari* only was used as a negative control while the labelled erythrocytes were used as a positive control.

2.6 | Haematology

Two replicate blood smears per fish, fixed with methanol, were stained with Kwik-Diff (Richard Allen Scientific), according to the manufacturer's recommendations and permanently mounted with Eukitt (Sigma). On the stained slides, the ratio of erythroblasts to erythrocytes was evaluated in 100 red blood cells, using light microscopy.

2.7 | Parasite quantification

DNA was extracted using a modified phenol/chloroform extraction protocol, including RNAse A treatment. *S molnari* was quantified using a TaqMan-based qPCR assay targeting the small subunit ribosomal (SSU) rDNA sequence of the parasite. Parasite SSU rDNA copy numbers were normalized to host β-actin.[7]

2.8 | Statistics

The Kolmogorov-Smirnov test of normality was used to confirm the normal distribution of all datasets. The differences between groups were analysed using the paired or unpaired Student's *t* test (Excel version 14.0; Microsoft). *P* values of .05 or less were considered statistically significant.

3 | RESULTS

3.1 | Infection with *S molnari* reduces the number of circulating erythrocytes

Our laboratory fish-to-fish transmission infection model using parasite blood stages allows us to study the impact of *S molnari* on the health status of its fish host, *C carpio*. We have recently shown that parasite proliferation in the circulatory system induces considerable changes in the composition of blood cells, particularly affecting the population of lymphocytes.[7] However, despite having observed attachment to erythrocytes in our previous study, their numbers did not seem to be significantly influenced by the infection.[7] We believe this can be attributed to the capacity of the adaptive immune response to limit parasite growth. To remove this variable and to focus on the impact of *S molnari* infection on RBCs, we performed an infection experiment involving immunosuppression (IS).[12] Throughout the 6-week experiment, the number of RBCs in the blood of control fish remained stable at approximately 1100 × 10^6 erythrocytes per mL of blood, with a mild decrease on days 28 and 42 post-infection (approximately 900 × 10^6 RBC/mL) (Figure 1A). The number of RBCs in the infected groups followed a similar trend, with a mild decrease on the same two days. Overall, the infection decreased the number of RBCs in the blood by approximately 10% (100 × 10^6 RBC/mL), with significant differences observed only 14 days post-infection (*P* = .05). However, under

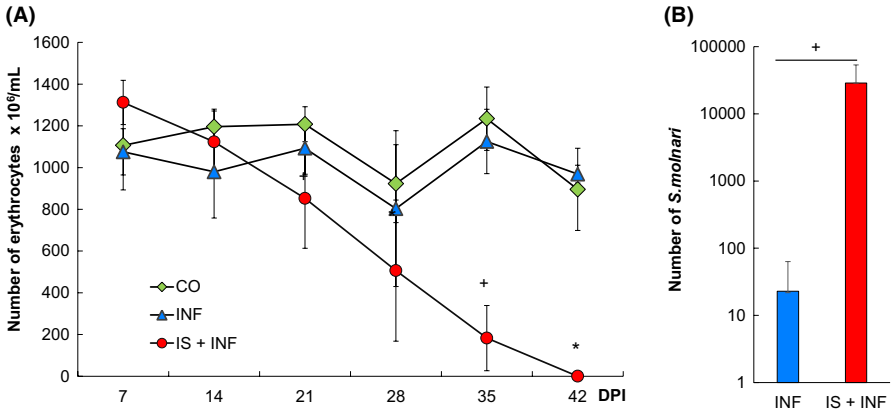

FIGURE 1 A, Impact of the *S molnari* infection on the number of erythrocytes. Healthy carp (n = 19) were divided in three groups. The CO group (♦; n = 5) was ip injected with 100 µL PBS, the INF group (▲; n = 7) was ip injected motile *S molnari and* the IS + INF group (●; n = 7) was treated with an immunosuppressive drug a day prior to infection. The number of erythrocytes was measured weekly and evaluated from 2 µl of full blood by flow cytometry. Each symbol represents the mean value at a given time point, and error bars represent the standard deviation in the group. B, Number of *S molnari* in the blood at 35 dpi as analysed by quantitative PCR. Symbols represent the mean value with standard deviation shown as error bars. (+ *P* < .05; * *P* < 0.01)

conditions of immunosuppression, we observed a steep decline in erythrocyte numbers, from day 21 onward, and resulting in the loss of 84% of erythrocytes two weeks later (35 dpi) (Figure 1A). Notably, fish treated with immunosuppressant but without infection exhibited mild but significantly increased numbers of erythrocytes when compared to the control group (Figure S1A). Simultaneously, the number of parasites in the blood of infected fish was analysed at the peak of infection (35 dpi), using qPCR. Our results revealed a thousand-fold increase in the number of parasites in the IS + INF group (28 704 parasites/µL), compared with infected fish (22 parasites/µL) (Figure 1B). *S. molnari* was not observed in the control group.

3.2 | Infection with *S molnari* induces release of erythroblasts

The decrease in the number of the erythrocytes during late stage infection was further accompanied by changes in the morphology observed by flow cytometry and light microscopy. While the erythrocytes in the control and infected group had a typical biconvex and elliptical shape, creating a 'donut-like' population in the FSC-SSC dot plot, ranging from FSC^{low} to FSC^{hi}, with an arch of SSC^{hi} events, the RBCs in the IS group appeared as an uniform population of round cells with medium size and reduced complexity ($FSC^{med}SSC^{low}$) (Figure 2A). In contrast to erythrocytes in homoeostasis which display an elliptic morphology, an agranular cytoplasm and a condensed nucleus, these cells were more spherical, with enlarged nuclei and a basophilic cytoplasm, characteristic for erythroblasts (Figure 2B).[13] While in healthy fish only 1.5% of erythroblasts were found in circulation, the infection significantly increased their frequency to 5.5% ($P = .01$). In the IS group, they represented approximately 75% of all RBCs at 28 days post-infection ($P = .00001$) (Figure 2C).

Using ER-Tracker, we identified approximately 8% of ER^{hi} cells in the erythrocyte population isolated from control and infected fish on day 28 post-infection. The frequency of ER^{hi} cells in the IS group ranged between 20% and 77%, suggesting an increased translational activity and synthesis of proteins in the erythroblasts, further solidifying their identity as immature RBCs.

3.3 | Blood stages of *S molnari* interact with erythrocytes

The reduction in the number of erythrocytes observed in vivo raised questions about the nature of the interaction between *S molnari* and the RBCs. Microscopic analysis of blood smears from infected fish at the peak of infection (28 dpi), revealed a high rate of attachment of *S molnari* to erythrocytes.[7] This interaction was successfully replicated in vitro after only 3-hour exposure, and parasites were readily

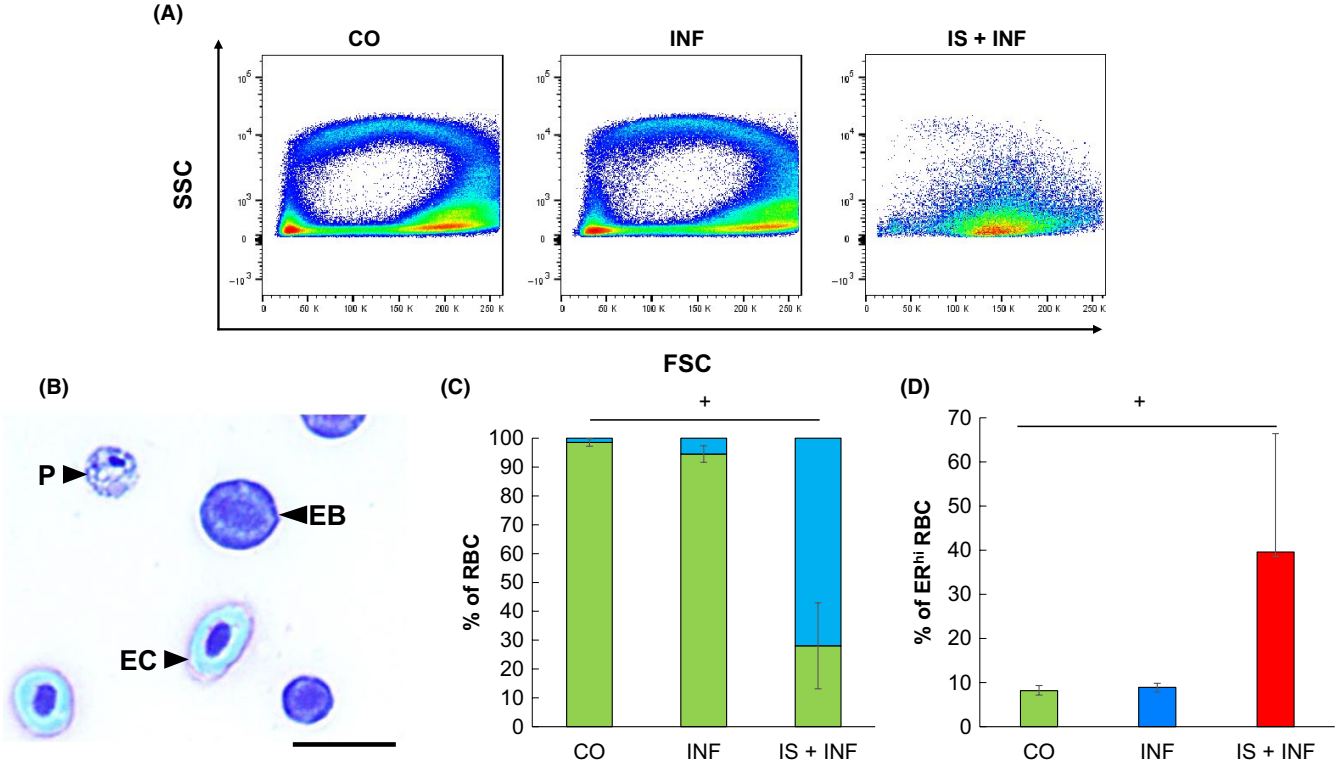

FIGURE 2 Infection with *S molnari* induces the release of erythroblasts into the blood stream of infected fish. A, Comparison of the FSC-A to SSC-A profile of erythrocytes obtained from experimental fish at 28 dpi. B, Blood smear showing the parasite (P), erythrocyte (EC) and erythroblasts (EB). C, Proportion of erythrocytes and erythroblasts in the blood of experimental fish, 28 dpi. The ratio was estimated from 100 RBC counted on stained blood smears (n = 5 in each group). Bars represent mean values with error bars showing standard deviation. D, Frequency of ER^{hi} RBCs present in the blood of experimental fish, 28 dpi. The cells were stained with the ER-tracker and the frequency of ER^{hi} RBC was measured by flow cytometry

observed attached to and gnawing on the surface of erythrocytes (Figure 3C).

As erythrocytes represent a rich source of nutrients, we aimed to elucidate, whether the attachment results in any transfer of proteins from the erythrocytes to the parasite. To answer this question, the freshly isolated parasite was incubated with CFSE-labelled erythrocytes in various densities. We observed a significant increase in the staining intensity of the parasite population with a positive correlation with the number of CFSE-labelled erythrocytes (Figure 3A). The greatest increase of parasite CFSE intensity of at least 15-fold over background was achieved with an erythrocyte:parasite ratio of 10:1 (Figure 3B). The transfer of the fluorescent material from RBCs to *S molnari* was further supported by fluorescence microscopy: we identified lightly stained *S molnari* cells, though specific localization of the transferred dye was not possible in or on the parasite cells.

Additionally, we evaluated whether the interaction with parasites influences the viability of the erythrocyte population. Analysis with propidium iodide (PI) revealed a significant increase in the proportion of dead cells in the cells incubated with parasites for 12 hours. Thus, while we observed only 5% of dead erythrocytes in the control group, the exposure to the parasite significantly increased the proportion of PI^+ cells to 20% (Figure S1B). Overall, we observed that the interaction of the parasite with host erythrocytes results in acquisition of host proteins, likely to the detriment and lysis of RBCs.

4 | DISCUSSION

Erythrocytes comprise the most abundant population of cells in the blood, ranging in 1–1.8 × 10^6 cells a microlitre of blood. From the perspective of a pathogen, RBCs represent a rich source of iron and amino acids,[8] as well as a unique niche protecting the microorganisms from the wide array of humoral and cellular defense mechanisms present in the blood. It is therefore not surprising that many viral, prokaryotic and eukaryotic pathogens evolved unique strategies to exploit erythrocytes as a source of nutrients or for immune evasion.[3,14] The observation of motile stages of *S molnari* attached to erythrocytes is novel and raises a number of questions about the nature of this interaction in myxozoans and their hosts.

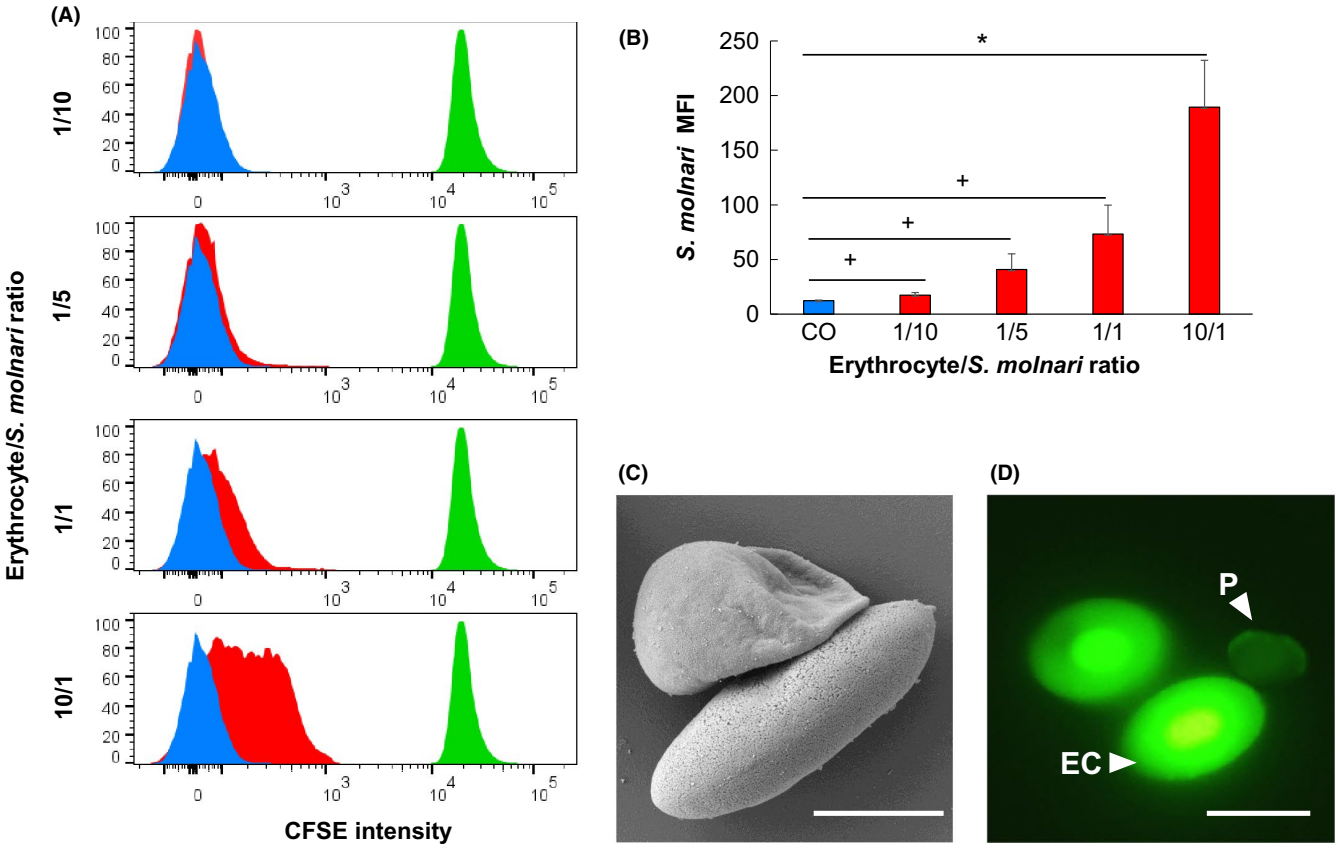

FIGURE 3 Feeding behaviour of *S molnari* in vitro. Freshly isolated *S molnari* (purity >95%) was co-incubated for 12 hours with the CFSE-labelled erythrocytes in four different ratios (parasite to erythrocyte ratios of 10:1, 1:1, 1:5 and 1:10). Following the incubation, the mean fluorescence intensity (MFI) of parasites was assessed by flow cytometry. In the histograms (A) the unlabelled parasite is visualized in blue, the CFSE-labelled erythrocytes in green and the population of parasites following the co-incubation in red. MFI values were plotted in the graph (B). Each bar represents a mean value (n = 3) with error bars of standard deviation. (+ $P < .05$; * $P < .01$) (C) SEM image demonstrating the attachment of the parasite to the erythrocytes. Bar = 10 µm. (D) Uptake of the green fluorescence from CFSE-labelled erythrocytes (EC) by the motile *S molnari* (P). Bar = 10 µm

Though the impact of the infection on the host is difficult to assess, it is worth mentioning that in contrast to the many intraerythrocytic parasites of mammals, only few infections in fish have proven to be pathogenic.[3,15] This notion is confirmed in the experimental infection with *S molnari*, which causes only a 10% reduction in the number of erythrocytes in infected fish. However, under natural conditions, infections show much higher intensities and result in considerable reduction of haematocrits and anaemia in infected fish,[16] with oxygen being limited in carp culture ponds, especially during algal blooms in the summer months. This has a negative impact on fish health and results in important mortalities. Use of a corticosteroid treatment that impairs the adaptive immune response[12] resulted in uninhibited parasite growth and haemolytic anaemia. Reduction of mature erythrocytes induced a high rate of erythropoiesis, resulting in the mobilization of immature erythroblasts, characterized by the typical morphology of spherical, basophilic cells with an expanded nucleus, and exhibiting high endoplasmic reticulum activity due to increased protein synthesis.[17] Anaemia is common also in other pathological species of myxozoans that do not proliferate in the blood, for example *Tetracapsuloides brysalmonae* in the hematopoietic organs[18] or *Parvicapsula pseudobranchicola*[19] in the pseudobranch, potentially suggesting common means of erythrocyte exploitation by different myxozoan lineages or their detrimental effect on hematopoietic tissue in their fish hosts.

Generally, haemolytic anaemia is induced either directly by the parasite, which is released from the lysed erythrocyte after intracellular proliferation, or by accelerated clearance of the erythrocytes by splenic macrophages or liver Kupffer cells, due to the absorption of immune complexes, complement activation or development of cross-reactive antibodies.[2] Though *S molnari* was never observed inside erythrocytes, its killing capacity could be mediated by direct contact with erythrocytes. This notion is supported by an increased proportion of dead erythrocytes observed after incubation with the parasite. However, the mechanism underlying the interaction of *S molnari* with erythrocytes remains yet to be elucidated. The attachment of *S molnari* to the host cells in vitro is instantaneous and highly specific, resembling the rapid recognition, attachment and entry of *P falciparum* merozoites into erythrocytes, which can be completed within minutes.[20] Interestingly, while we observed a high number of parasites interacting with erythrocytes, attachment of parasites to parasites is very rare. This suggests a nonpromiscuous process, involving specialized receptors on the surface of the parasite that interact with an unknown but likely monomorphic ligand on the host cells. This represents yet another similarity with *Plasmodium*, whose attachment and penetration cascade involves a number of highly selective adhesins.[21] The host-pathogen ligand-receptor pair remains to be identified. Known erythrocyte receptors include but are not limited to mammalian ICAM-1, CD36 and CR1 bound by *P falciparum* whereas pathogen molecules such as PfEMP1 or the aptly named hemagglutinin are known to be less specific.[21]

Generally, pathogens come into contact with erythrocytes for the purpose of nutrient acquisition. Recently, trogocytosis was observed in *Entamoeba histolytica that* acquires nutrients but also uses host proteins for immune evasion: Host-parasite interaction led to the display of host membrane proteins on the surface of the parasite, preventing it from lysis by the immune serum.[22] To investigate, whether *S molnari* possesses a similar capacity, we aimed at elucidating the acquisition of host material from CFSE-labelled erythrocytes. We witnessed a dose-dependent increase in parasite fluorescence, suggesting possible feeding behaviour. Possibly due to continuous entrance of material and simultaneous degradation of proteins, fluorescence did not increase specifically in vacuoles or on the surface of the *S molnari*, and the method used did not allow a conclusive resolution of the localization and usage of the host material. However, some of the available data allow us to hypothesize that *S molnari* exploits the erythrocytes in feeding behaviour rather than molecular disguise: the presence of host antigens on the surface of *S molnari* would induce production of autoantibodies favouring the clearance of host erythrocytes.[2,23] Nevertheless, we observed no seroreactive IgM binding to the surface of erythrocytes isolated from CO, IFN and IS + IFN fish, nor did we observe any reduction in their numbers (data not shown). Additionally, as the triamcinolone acetonide impairs the antibody response,[12] it is unlikely that the reduction of erythrocytes in the IS + IFN group is mediated by increased erythrocyte clearance induced by autoantibodies.

Taken together, we present first evidence of the strong appetite of the myxozoan *S molnari* for host erythrocytes, which was brought to light in immunocompromised hosts, resulting in severe haemolytic anaemia. We demonstrate attachment of the parasite to host erythrocytes in vitro and the transfer of host material from erythrocytes to parasites. Considering an emerging role of nucleated erythrocytes as important players of the immune system,[15,24] this study provides an important framework for future investigation into the molecular mechanisms driving the interaction of parasites with host erythrocytes and their defense mechanisms on an attractive model shaped by over 400 Myrs of coevolution.

Availability of data and materials: All data supporting the findings of this study are available within the article and its additional file.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTION

TK and ASH planned and designed the study; TK performed the flow cytometry analysis and co-wrote the manuscript; JTHC co-wrote the

manuscript and assisted in the analysis of the data; MV performed SEM methods and imaging; ASH was responsible for haematology, LM + FM images, supervised the findings and analyses of the data and co-wrote the manuscript. All authors read and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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