Improving the Yields of Blood Cell Extractions from *Botryllus schlosseri* Vasculature

Andy Qarri, Yuval Rinkevich and Baruch Rinkevich

Abstract: The tunicate *Botryllus schlosseri* belongs to the Vertebrata's closest living invertebrate group. This colonial species represents an invertebrate model system that maintain high capacity of adult stem cell activity, where various blood cell types, expressing multipotent or totipotent phenotypes, circulate in vasculature throughout life. While isolated *Botryllus* blood cells may serve as indispensable tools for studying stem cells biology, up to date, no single cell line is available. The major bottle-necks for established cultures include the lack of cell division under in vitro conditions as from 24 to 72 h post isolation and enhanced contami-nation rates by bacteria and protists. Moreover, low yields of blood cells are of significant hindrance to the development of long-term cultures since lower numbers of cells eventually lead to poor results. Tackling these two critical technical obstacles, we present here methodologies for improved aseptic conditions and for higher yields of cells extracted from colonial vasculature. This study was performed on two colonial stocks (Israel, laboratory stocks; Helgoland, Germany—field collected stocks) which resulted with a significant difference in the numbers of cell extrac-tions between the two stocks and significantly different blood cell yields between various blastogenic stages (laboratory stocks), further revealing differences between field/laboratory-maintained colonies.

1. Introduction

The cosmopolitan tunicate *Botryllus schlosseri* belongs to a taxonomic taxon considered as the closest living invertebrates to the Vertebrata (Delsuc et al. 2006) and is used as an important model species in a wide range of biological disciplines (Ben-Hamo and Rinkevich 2021), such as ecotoxicology (Gregorin et al. 2021; Rosner et al. 2021), immunobiology and allorecognition (Magor et al. 1999; Rinkevich 2004), developmental biology including colony astogeny (Manni et al. 2019; Rosner et al. 2006; Rosner et al. 2019), regeneration (Voskoboynik et al. 2007), senescence (Rabinowitz and Rinkevich 2004a; Rinkevich 2017), evolutionary biology (Rinkevich 2002) and above all—stem cell biology (Ballarin et al. 2021; Voskoboynik et al. 2008). *B. schlosseri* colonies express two modes of reproduction, sexual and asexual (Manni et al. 2019). Sexual reproduction cycles occur weekly, each starting with the

fertilization of eggs and progressing through embryonic stages into a tadpole larva featuring chordate characteristics that includes striated musculature, neural tube, notochord and tail (Voskoboynik et al. 2007). The tadpole larva swims for a short period of time and then attaches to a substrate near the mother colony, loses the tail through apoptosis, and then develops into the first zooid (the colonial module), called an oozooid (Berrill 1950). Colonies develop from the oozooids through weekly cycles of growth and death (Manni et al. 2019; Rinkevich 2019) and form several typical star-shaped groups of zooids, each called a system, that are embedded within the tunic, the transparent gelatinous extra cellular matrix (ECM) of the colony which contains cellulose cross-linked with proteins as well as the colonial circulatory system (Figure 1). Colonial systems are connected to each other via common blood vessels, which carry at the periphery of the colony sets of blind vasculature termini, called ampullae (spherical to elongate in structure). Each zooid in the colony possesses an oral siphon (branchial siphon) and an atrial siphon is shared for all zooids in each system (Berrill 1950).

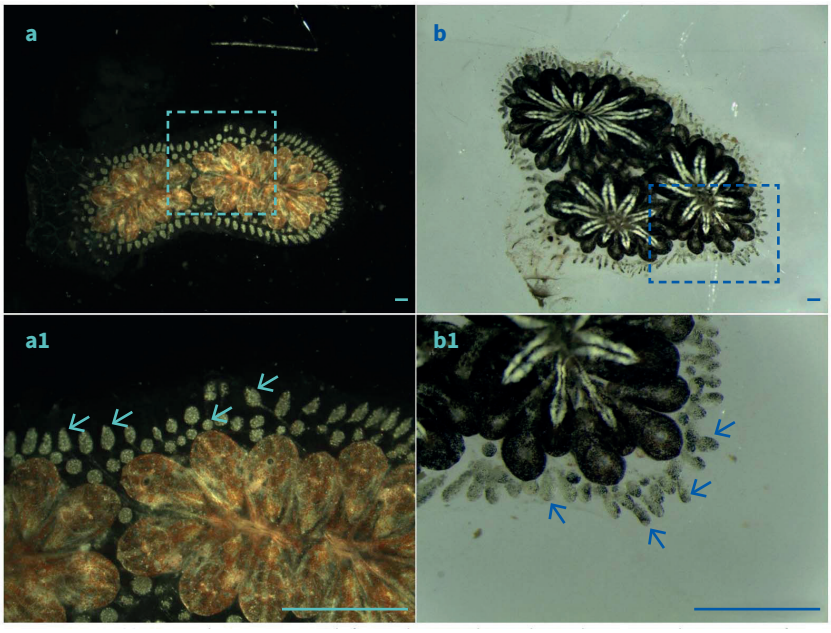

Figure 1. A *B. schlosseri* colony originated from the Israeli stock at the National Institute of Oceanography, Haifa (**a**,**a1**), at blastogenetic stage A, and (**b**,**b1**) a colony from Helgoland Island, Germany in blastogenetic stage C. Cells extracted from the marginal ampullae (arrows in a1, **b1**) of the colonies. Dotted squares represent the enlarged area of a1 and b1, respectively. Bars = 0.1 mm (**a**,**b**) and 1 mm (**a1**,**b1**). Source: Graphic by authors.

The asexual mode of development in *B. schlosseri* is expressed as weekly developmental cycles called blastogenesis, where each blastogenic cycle is composed of four major stages (marked by the letters A to D (Mukai and Watanabe 1976), during which the primary buds mature to adult zooids in concert with the development of the secondary buds from the body wall of each primary bud. A massive apoptotic event concludes each blastogenic cycle with the morphological resorption of all parental zooids, concurrently followed with the development of primary buds to functioning zooids (Lauzon et al. 1993). Thus, the blastogenesis process can be characterized by somatic self-renewal and vasculature regeneration, which demonstrate a model organism that carry out continuous somatic proliferation throughout the organism life span. In other words, the weekly budding process of somatic self-renewal and high vasculature regeneration capacity suggests an invertebrate model organism that maintain high capacity of stem cell activity throughout life (Ben-Hamo and Rinkevich 2021; Qarri et al. 2020; Rinkevich 2019).

Blood cell isolation and culturing are essential tools in the study of stem cells and regeneration in this model organism. Various cell types from *B. schlosseri* possess extensive potentialities such as multipotency and totipotency (Laird et al. 2005; Rinkevich and Rabinowitz 1994; Rinkevich and Rabinowitz 1997; Rosner et al. 2009; Rosner et al. 2021) and may serve as important tools in studying immunology, developmental biology, apoptosis and regeneration (Ballarin et al. 1994; Lauzon et al. 1993; Rosner et al. 2009; Rosner et al. 2021; Voskoboynik et al. 2007). Studies that attempted to develop primary cultures and permanent cell cultures from *B. schlosseri*, commonly used to extract blood cells that are directly collected from the blood vessels (Ballarin et al. 2008; Rinkevich and Rabinowitz 1993). Other studies used cells originated from epithelial layers (Rinkevich and Rabinowitz 1997), which show de novo stemness signatures (Rabinowitz et al. 2009; Rabinowitz and Rinkevich 2011) and cells originating from embryos (Rinkevich and Rabinowitz 1994). However to date, no single *Botryllus* cell line is available and it has repeatedly shown that extracted cells stop dividing in vitro within 24–72 h after their isolation. Moreover, many of the cultures are contaminated with opportunistic organisms including bacteria and protists, such as thraustochytrids (Qarri et al. 2021; Rabinowitz et al. 2006; Rinkevich and Rabinowitz 1993; Rinkevich and Rabinowitz 1994; Rinkevich 1999; Rinkevich 2011.)

The above studies indicate that, in order to establish long-term cell cultures, attempts should approach two critical technical statuses prior cell cultivation, (a) approved methodologies for aseptic conditions and (b) high yields of cell extraction. High yields of cells are of significant importance since lower numbers of cells eventually lead to poor results and fast senescence of extracted cells, primarily when dealing with blood cells that represent a rapid turnover and survival of only several weeks (Raftos et al. 1990; Rinkevich 1999; Rinkevich 2011). These

limitations have led to attempts of pooling of blood cells originated from several colonies. For example, Ballarin et al. (2008) extracted 10^6 cells from more than three colonies, and Kamer and Rinkevich (2002) obtained the same cell concentrations (10^6 cells) by cutting the tunic matrix and the zooids without specifying the number of used colonies.

Responding to the above challenges, here we present a general aseptic approach with higher yield for blood cell (including stem cells) extractions employed on *B. schlosseri* colonies originating from two colonial stocks, the long-term established laboratory colonial cultures from Israel and from newly collected colonies originated from Helgoland Island, Germany. The essence of this approach is to improve blood cells yields from a single colony for in vitro applications.

2. Materials and Methods

2.1. Botryllus schlosseri Husbandry

Twenty-two colonies originated from Israeli cultures (long-term cultures maintained at the National Institute of Oceanography, Haifa, Israel) and freshly collected colonies from Helgoland Island, Germany, were selected for cell extraction experiments. Thirteen colonies (blastogenic stages A = 4, B = 3, C = 2 and D = 4) were derived from laboratory stocks reared in the Israeli facility for several years and originated from several USA west coast marinas (Monterey, Half Moon Bay and Moss Landing, California), as from Nelson Marina, New Zealand. The colonies were kept vertically on 5 × 7.5 cm^2 glass slides in slots of glass staining racks at 20 °C, in a 21-Liter plastic tank under a 12:12 h light:dark regimen, in a standing seawater system, as described (Rinkevich and Shapira 1998). Air stones were continuously used and the seawater was changed twice a week. Colonies were fed daily with freeze-dried rotifers, green unicellular algae and commercial powdered plankton. Nine colonies (blastogenic stages A = 5, B = 2 and C = 2) were collected from the rocky intertidal zone in Helgoland. These colonies were reared at the Biological Institute Helgoland (BAH) of the Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research—house C and maintained as the Israeli stock colonies under running seawater system and temperatures between 15.7 and 23.4 °C. Colonies were fed daily with dried algae powder. Colonies of both stocks were gently cleaned twice a week using small and soft brushes to remove trapped food particles, fouling organisms and debris. All experimental colonies were in good health and well adapted to their maintenance conditions.

2.2. Aseptic Solution, Instruments and Environment

Washing solution (WS) was used in order to reduce contaminations during the process of cell extraction. Artificial seawater (ASW) was prepared as described in

Rabinowitz and Rinkevich (Rabinowitz and Rinkevich 2004b), autoclaved, sterilized by a 0.2 μm filter membrane (Millipore) and stored at room temperature. For each 50 mL of WS, we used 44 mL of ASW, supplemented with 3 mL of PSA (Biological Industries; Penicillin 10,000 units/mL, Streptomycin sulphate 10 mg/mL and Amphotericin B 25 μm/mL; Cat. 03-033-1B. MP Biomedicals; Cat. 091674049) and 3 mL of Gentamycin Sulfate (Biological Industries; 50 mg/mL; Cat. 03-035-1. Gibco; 50 mg/mL; Cat. 15750037). Only sterilized plasticware was used. In addition to ASW sterilization, glassware was routinely autoclaved. Cell extraction protocols were carefully observed to maintain pathogen-free conditions. Additionally, prior to cell isolation colonies were kept under sterile conditions in a biosafety cabinet within a 20 °C cool room (Israel) and in an incubator of 20 °C (Helgoland).

2.3. Cell Extraction Approach under Aseptic Conditions

Before cell extraction procedure (Figure 2) colonies were taken out from aquaria and photographed under stereomicroscope (SMZ1000, Nikon equipped with DeltaPix digital camera Invenio 3SII, S/N: 3648213012. Leica M125 equipped with a camera Leica IC80 HD). Then colonies were meticulously cleaned by soft brushes, and the glass slides on which they were grown were comprehensively cleaned and wiped with 70% ethanol. Using razor blades under a biosafety cabinet, the colonies were carefully pulled off from the slides and placed in the centers of sterile 60 mm Petri dishes (Greiner bio-one, CELLSTARR 628160, Petri-dish 60×15 mm^2) for approximately 20 min in a humidity chamber, containing ASW, to actively attach to the dish substrates (detailed procedure in Rinkevich and Weissman (1987)). Then, 6 mL of WS (Washing solution) was added, and animals were left under sterile conditions for 48 h without food (starvation with antibiotic supplements significantly reduced contaminations of cell cultures; Rinkevich and Rabinowitz 1993). Then, the WS was changed 12 times (every hour for the first 6 h, left for 12 h and then, from the 18th hour, the WS changes protocol was repeated for the next 6 h; total of 12 washes). Following the above, all peripheral ampullae of each colony in a plate were punctured with an insulin syringe needle (28-Gauge) and the WS containing *B. schlosseri* blood cells was dropped into a 15 mL tube, pursued by centrifugation (2000 rpm for 10 min) using Eppendorf (Hamburg, Germany). The plates were then supplemented with WS and left for an addition 1.5 h in a biosafety cabinet, following which the WS from each plate was collected into a tube and cell extraction procedure (described above) was performed again. *B. schlosseri* blood cells in the tubes were centrifuged and the pellets were suspended in 1 mL of WS for further investigations.

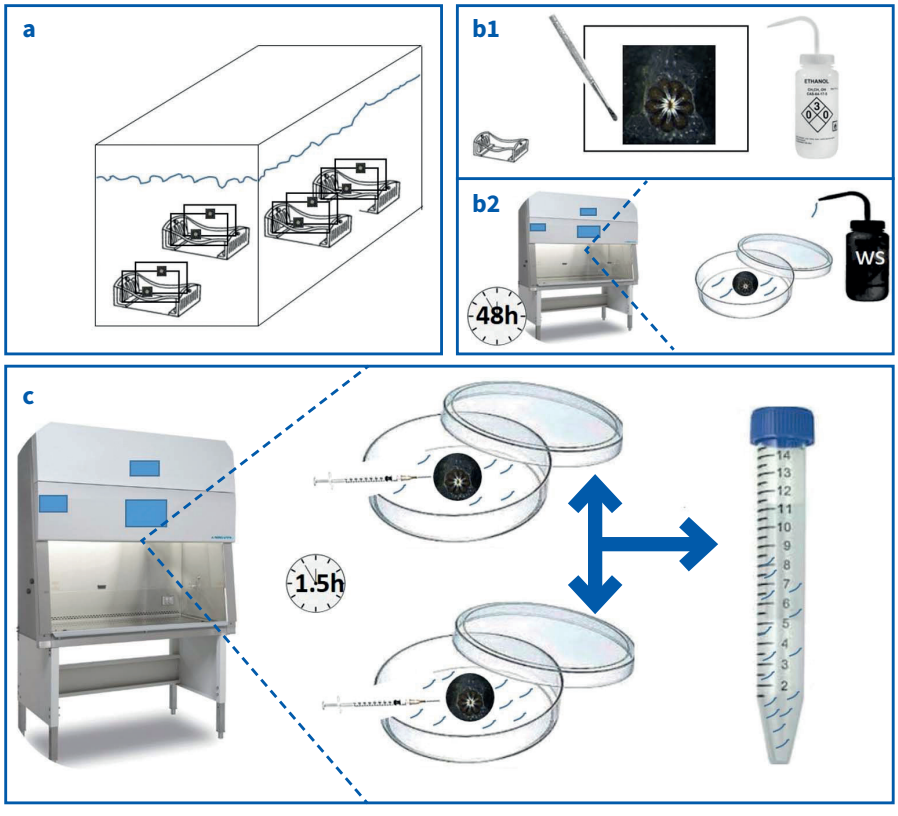

Figure 2. Schematic illustration of the cell extraction approach. (**a**) Colonies maintained under laboratory conditions, in tanks. (**b1**) Prior to cell extraction, colonies with their glass substrates are removed from their growth system, cleaned meticulously by soft brushes and the glass slides are wiped with 70% ethanol. (**b2**) Working in biosafety cabinet, each colony is removed, using a razor blade, from the glass substrate and is transferred to a sterile 60 mm Petri dish until actively attached. Then the Petri dish is being filled with 6 mL of WS. The plates are left in a biosafety cabinet for 48 h following 12 changes of WS. (**c**) Cell extraction is performed within a biosafety cabinet by puncturing the ampullae of each colony with the insulin syringe needle. This procedure is repeated after 1.5 h. Then the WS containing *B. schlosseri* blood cells are collected into a tube for further investigation. Source: Graphic by authors.

2.4. Cell Observation and Counting

All cell extractions were counted, using a hemocytometer, and photographed under the microscope (Olympus inverted system microscope, model I $\times$ 70, equipped with DP73 camera. Leica ICC50 HD). Cell viability was determined using Trypan

Blue solution (Biological Industries; Cat. 03-102-1B. Gibco; Cat. 15250061). Obtained values of *B. schlosseri* blood viable cells were between 93.6 and 98.5%.

2.5. Statistical Analyses

Statistical analyses were applied on extracts of two *B. schlosseri* colonial stocks originated from Israeli laboratory cultures and Helgoland Island, Germany using an SPSS V16. An independent-samples T test was performed on two stock cell yields. One-way ANOVA test using post hoc comparison (Bonferroni and Tukey HSD) was applied on blastogenesis of cell extracts of each *B. schlosseri* colonial stocks. Pearson correlation test was performed on zooid numbers of each colony with respect to cell yields.

3. Results

Cell Yields

The cell extraction protocol was performed on the 22 *Botryllus schlosseri* colonies originating from the Israeli stock of colonies (Figure 1a; 13 colonies in blastogenic stages A-D) and from Helgoland, Germany newly established stock (Figure 1b; 9 colonies in blastogenic stages A–C). Zooid numbers of the two stocks varied between 8 and 32 per colony for the Israeli colonies and 8 and 31 for the Helgoland colonies, yet no correlation (Figure 3) was recorded between the number of zooids per colony (of the colonial sizes used in this experiment) and cell yields for each stock ($r_{pearson} = -0.097$, $p > 0.05$; $r_{pearson} = 0.503$, $p > 0.05$; for Israeli/Helgoland stocks, respectively). Cells were extracted (Figure 4) from the marginal ampullae (Figure 1(a1,b1)) and numbers of cells and viability were studied on yields upon cell collections with respect to donors' blastogenic stages. Comparing between the two stocks of colonies, the results revealed a significant difference ($p < 0.0001$; independent-sample T test) in the numbers of cell extractions between the two stocks, where more cells were extracted from the freshly collected Helgoland stock. Within stock analyses revealed significant blastogenic-associated differences in cell yields from the Israeli stock colonies ($p < 0.05$; one-way ANOVA) but not in the freshly collected colonies from Helgoland ($p > 0.05$; one way ANOVA).

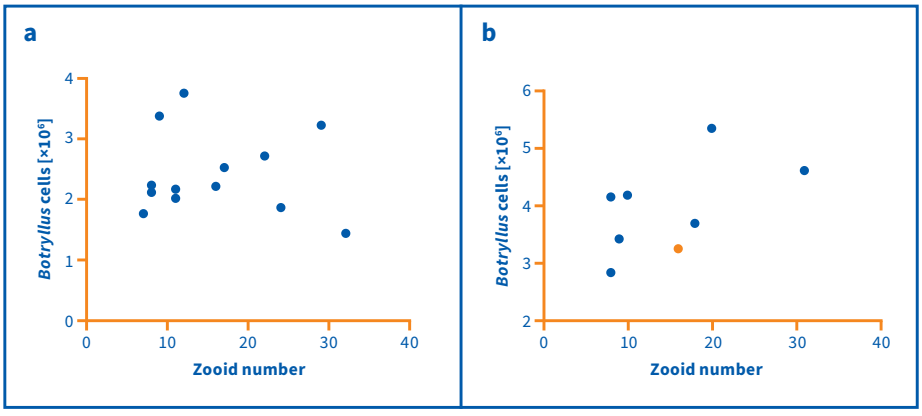

Figure 3. The correlation for cell yields vs. zooid numbers. (**a**) Cell yields vs. zooid numbers for the Israeli stock. (**b**) Cell yields vs. zooid numbers for the Helgoland stock. The red dot in b represents two different colonies with 16 numbers of zooids and cell yields of 3.28 and 3.27 × 10^6 cells. Source: Graphic by authors.

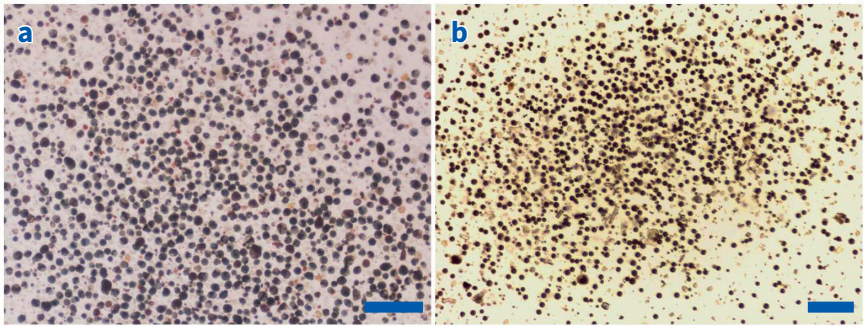

Figure 4. *B. schlosseri* cells under in vitro conditions. (**a**) A primary culture of blood cells from a blastogenesis stage B colony originated from the Israeli stock. (**b**) A primary culture of blood cells from a blastogenesis stage A colony from Helgoland, Germany. Bars = 100 µm. Source: Graphic by authors.

Cell yields from the Israeli blastogenic stages A and C colonies (Figure 5) composed of two significant groups, shared by the cell yields of blastogenic stages B and D colonies (Tukey HSD comparison). Cell yields from blastogenic stage A colonies (n = 4) varied between (2.12 ± 0.3) × 10^6 and (3.75 ± 0.2) × 10^6 ($p < 0.05$) in the Israeli stocks. For Helgoland colonies (n = 5), cell numbers varied between (2.9 ± 0.08) × 10^6 and (4.6 ± 0.69) × 10^6 ($p > 0.05$). Cell yields from blastogenic stage B colonies (n = 3) varied between (2.2 ± 0.13) × 10^6 and (2.7 ± 0.3) × 10^6 ($p > 0.05$) in the Israeli stocks, and for Helgoland colonies (n = 2) cell numbers varied between (2.2 ± 0.21) × 10^6 and (5.35 ± 0.3) × 10^6 ($p > 0.05$). Cell yields from blastogenic stage

C colonies (n = 2) varied between (1.76 ± 0.12) × 10^6 and (1.87 ± 0.34) × 10^6 ($p < 0.05$) in the Israeli stocks, and for Helgoland colonies (n = 2) cell numbers varied between (3.28 ± 0.08) × 10^6 and (3.72 ± 0.83) × 10^6 ($p > 0.05$). Cell yields from blastogenic stage D colonies ($n = 4$) varied between (1.44 ± 0.11) × 10^6 and (2.17 ± 0.48) × 10^6 ($p < 0.05$) in the Israeli stocks.

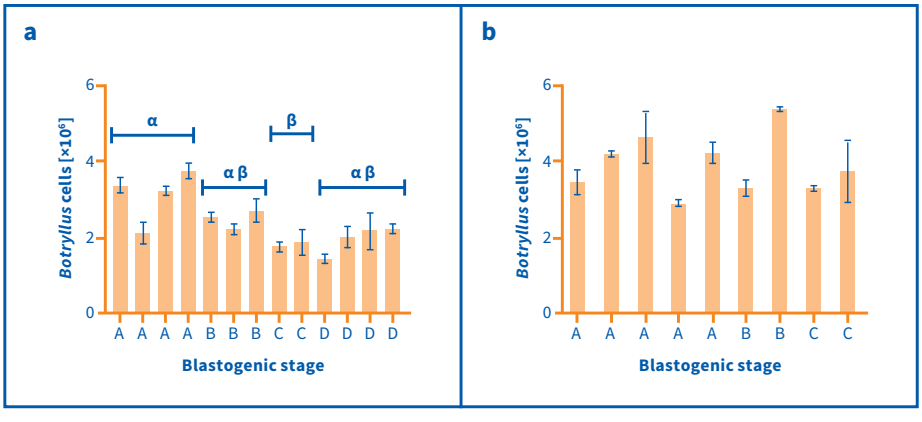

Figure 5. *B. schlosseri* cell yields. (**a**) Cell yields (×10^6) of colonies at blastogenic stages A–D originated from the Israeli stock. (**b**) Cell yields (×10^6) of colonies at blastogenic stages A–C originated from Helgoland Island. Each column represents an average of two extractions per colony (±S.D.). α and β symbolize statistical group differences between the tested blastogenesis stages obtained by Tukey HSD. Source: Graphic by authors.

4. Discussion

The literature reveals that primary cell cultures originated from *B. schlosseri* vasculature stop dividing 24–72 h post isolation (Rinkevich 1999) and further indicates low yields of cells per colony (Table 1) with high contamination rates (Rinkevich 1999; Rinkevich 2011.)

Table 1. Studies on *B. schlosseri* that refer to blood cell yields under laboratory conditions. Abbreviations: AC—Aseptic conditions; C—a colony; LS—laboratory stocks; NS—not specified; ABX—antibiotics; FSW—filtered seawater; FASW—filtered artificial seawater; ?= unknown number of colonies.

No of Colonies	Colony Origin	Cell Extraction under AC	Use of: FSW FASW/ABX	Cell Yields Colony^{-1}	Reference
>3	Italy (Venice)	No	FSW	3×10^5/C	(Ballarin et al. 2008)
NS	Italy (Venice)	No	FSW	5×10^6/?	(Menin and Ballarin 2008)
NS	LS	No	FASW	1×10^6/?	(Kamer and Rinkevich 2002)
NS	Italy (Venice)	No	FSW	8–10 $\times 10^6$/?	(Ballarin et al. 1994)
NS	LS	No	FSW	8–10 $\times 10^6$/?	(Ballarin et al. 2011)
NS	Italy (Venice)	No	FSW	1×10^7/?	(Ballarin and Cima 2005)
NS	LS	No	FSW/ABX	1×10^5/?	(Rinkevich and Rabinowitz 1993)
1	Helgoland	Yes	FASW/ABX	5.3–5.4 $\times 10^6$/C	This study
1	Israel LS	Yes	FASW/ABX	3.6–3.9 $\times 10^6$/C	This study

Source: By the authors.

Here we present an improved approach for blood cell extractions from *B. schlosseri* vasculature, performed under our aseptic conditions, which showed reduced contamination rates as compared to former outcomes. Yet, this issue was not analyzed in the present study. We used two *B. schlosseri* colonial stocks originated from a long-term laboratory cultures (from Israel) and colonies freshly collected from Helgoland Island, Germany. While at the colonial sizes used in this experiment there was no differences between the blood cell numbers obtained per colony, the results of this study clearly revealed, (1) a significant difference ($p < 0.0001$) in the number of blood cells obtained between the two disparate stocks and (2) changes in the numbers of blood cells obtained from various blastogenic stages (recorded only for the Israeli stock colonies). We obtained two significantly different blood cell yields between colonies at blastogenic stage A vs. stage C colonies. These results point to possible differences in numbers of total blood cells between freshly collected colonies from the field and colonies from established stocks, a result which should be taken into consideration when cell yields are an important component in structuring a research. This is also an interesting result regarding the *B. schlosseri* blood cell (and potentially stem cells) biology that should be studied in further experimentation.

The present study is the first that focuses on cell yields from *B. schlosseri* colonies. The literature (Table 1) reveals that past studies used undefined numbers of colonies in the research, or that the yield was lower than levels detailed in this study. Thus, our approach demonstrates potential for improving the extracting of circulating cells, including stem cells, under aseptic conditions, for any in vitro application, without pooling cells from different genotypes, augmenting the importance of *B. schlosseri* as a model organism in the field of cell biology (Ballarin et al. 2011; Ben-Hamo and Rinkevich 2021; Frizzo et al. 2000; Rosner et al. 2021). As a final point, the recognition of this model organism in the field of cell biology and stem cells biology is associated with its circulating blood cells that hold potentialities such as multipotency and totipotency (Ballarin et al. 2021; Laird et al. 2005; Rosner et al. 2009; Rosner et al. 2021).

Author Contributions: A.Q., Y.R. and B.R. conceived and designed the experiments, B.R. and Y.R. contributed reagents/materials/analysis tools. A.Q. performed the experiments, analyzed the data, prepared figures and authored drafts of the paper. All authors have read and agreed to the published version of the manuscript.

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