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A temperature-controlled, circular maintenance system for studying growth and development of pelagic tunicates (salps)

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Abstract

Salps have attracted attention as zooplankton organisms that may be able to expand their habitat range and increase their ecological importance in the face of ongoing global warming. Due to their gelatinous nature, unique feeding strategy, and reproductive ecology such changes could have profound impacts on regional marine ecosystems. While their role in the regional carbon cycle is receiving attention, our knowledge of their physiology and life cycle is still limited. This knowledge gap is mainly due to their fragile gelatinous nature, which makes it difficult to capture and maintain intact specimen in the laboratory. We present here a modified kreisel tank system that has been tested onboard a research vessel with the Southern Ocean salp *Salpa thompsoni* and at a research station with *Salpa fusiformis* and *Thalia democratica* from the Mediterranean Sea. Successful maintenance over days to weeks allowed us to obtain relative growth and developmental rates comparable to in situ field samples of *S. thompsoni* and *S. fusiformis*, and provided insights into previously unknown features of their life cycle (e.g., testes development). Our results show that traditional methods of estimating growth, such as cohort analysis, may lead to a general overestimation of growth rates and neglect individual strategies (e.g., shrinkage), which can affect the results and conclusions drawn from population dynamic models. By providing a starting point for the successful maintenance of different species, comparable experiments on the physiology of salps is made possible. This will contribute to refining model parameters and improving the reliability of the predictions.

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Additional Supporting Information may be found in the online version of this article.

Over the past few decades, there has been a growing focus on gelatinous zooplankton, which often emerges as a dominant presence in marine zooplankton communities globally. This trend is arguably influenced by factors such as overfishing and anthropogenic climate change (Richardson et al. 2009). Research has mainly focused on true jellyfish (cnidaria) and comb jellies (ctenophores), while pelagic tunicates, salps in particular, have generally been overlooked (Condon et al. 2012; Henschke et al. 2016). The main reason for such historical underestimation is that salps have been mistakenly associated with jellyfish due to similar traits, suggesting that they are unimportant in food webs and biogeochemical cycles (Henschke et al. 2016).

Salps are found in all open ocean waters with the exception of the Arctic (Madin and Deibel 1998). They are known as

efficient filter feeders and frequently occur in dense aggregations, when food is abundant (Madin et al. 1996; Andersen 1998). The development of these so-called blooms is facilitated by the high reproductive capacity of salps during their asexual stage (i.e., oozoids, solitaries), which alternates with their sexual reproductive stage (i.e., blastozooids, aggregates) (Foxton 1966; Madin et al. 1996; Madin and Deibel 1998). Oozoids have a stolon that produces chains of genetically identical clones (blastozooids). The individuals that comprise the chains are released as females. So far it is believed that blastozooids are protogynous hermaphroditic, that is, the ovary develops first, and the testes later (Foxton 1966; Godeaux et al. 1998; Daponte et al. 2001). Recently, however, an overlap in the expression of male and female genes in blastozooid embryos suggests alternative hypotheses and challenges our current view of salp protogynous hermaphroditism (Castellano et al. 2023). Due to their high fecundity, which is considered to be the primary cause of explosive blooms, salps are able to outcompete other zooplankton, play a significant role in regional biogeochemical cycling (Lee et al. 2010; Gleiber et al. 2012; Böckmann et al. 2021; Pauli et al. 2021), and serve as food sources for higher trophic levels (Harbison 1998; Henschke et al. 2016).

Global climate change has altered the abundance and distribution of salps, which has profound implications for local ecosystems (Loeb et al. 1997; Atkinson et al. 2017). Currently, the role of salps in marine ecosystems is increasing, but only little is known about their ecology, physiology, and life cycle (Henschke et al. 2016). This restricts our understanding of marine carbon export and, therefore, its adequate implementation in regional and global biogeochemical models. In addition, the lack of knowledge of salp biology and sensitivity to future climate change scenarios makes it difficult to predict future population dynamics and assess the potential impact of a salp-dominated zooplankton community on an ecosystem. This knowledge gap can be mainly explained by the seasonal and spatially patchy occurrence of salps, which allows only narrow time windows for research. Furthermore, their fragile gelatinous nature restricts field sampling of intact specimens with nets or trawls and makes them delicate organisms for long-term maintenance in aquaria for experimental studies (Henschke et al. 2016).

In first attempts, individuals of the pelagic tunicate *Thalia democratica* were kept in the laboratory in jars filled with seawater replenished daily (Braconnot 1963). This method resulted in high mortalities because salps became attached to the surface film. This issue was resolved in a subsequent study by Heron (1972) using small tanks with lids. A detailed description of the cultivation technique is lacking and the initial phytoplankton concentration was grazed down during the experiment, likely influencing the growth rates obtained (Heron 1972). A cultivation technique introduced by Paffenhöfer (1970) used a gentle stirrer and was successfully tested on the appendicularians *Oikopleura dioica* and *Fritillaria borealis*, two small (trunk length of ~ 1–2 mm) pelagic tunicates (Paffenhöfer 1973; Paffenhöfer and Harris 1979). However, the most useful aquarium for maintaining pelagic gelatinous zooplankton to date is the planktonkreisel, first introduced as a horizontal device by Greve (1968) and modified by Greve (1975) and Hamner (1990). The unique circular shape of the planktonkreisel and position of the inlet and outlet prevent organisms from coming into contact with any of the tank's surfaces (Raskoff et al. 2003). The cultivation of gelatinous zooplankton in the planktonkreisel was successfully tested and described for true jellyfish and ctenophores (Greve 1970, 1975; Raskoff et al. 2003; Courtney et al. 2020; Lechable et al. 2020; Ballesteros et al. 2022). For salps, the first maintenance attempts in kreisel tanks were published in recent years (Lüskow et al. 2020; Stukel et al. 2021). However, these studies only involved short-term incubations of salpsand lacked detailed maintenance descriptions (Lüskow et al. 2020; Stukel et al. 2021). This limits implementation of long-term studies to qualify and quantify salp life strategies and obtain reproducible physiological rates for parameters such as growth, oxygen consumption, and development under controlled experimental conditions. In addition, studies on the response of salps to changing environmental conditions are also limited, which is particularly important in the light of ongoing climate change.

The objective of our study was to construct a temperature-controlled circular and flow-through aquarium system for salps to facilitate future studies on salp physiology and response to changing environmental conditions. We tested our system by maintaining three salp species from two different habitats: *Salpa thompsoni* from the Southern Ocean, and *Salpa fusiformis* and *T. democratica* from the Mediterranean Sea. The experiments with *S. thompsoni* were conducted during a ship-based expedition onboard the German research ice breaker *Polarstern* in the Southern Ocean, whereas the experiments with *S. fusiformis* and *T. democratica* took place at the Institut de la Mer de Villefranche (France). In addition to a detailed description of our aquarium system, we highlight limitations, improvements, and advice for field sampling and future experimental studies on salps. We present results on growth and developmental rates of *S. thompsoni* and *S. fusiformis* maintained in our experimental kreisel tank system, validated by in situ rates using cohort analysis and published data.

Materials and procedures

Kreisel tank sets and modifications

The kreisel tank is a well-established aquarium for the maintenance of gelatinous zooplankton (Greve 1970, 1975; Raskoff et al. 2003; Courtney et al. 2020; Lechable et al. 2020; Ballesteros et al. 2022). To maintain the different salp species (*S. thompsoni*, *S. fusiformis*, *T. democratica*) in our study, two

sizes of water-running breeding kreisel tanks (Schuran Seawater Equipment, Jülich Germany) were used:

I. $V = \sim 73.10$ liters, diameter = 610 mm, depth = 250 mm (hereafter big kreisel tanks, $n = 6$)
II. V = ~ 14.14 liters, diameter = 300 mm, depth = 200 mm (hereafter small kreisel tanks, $n = 6$)

The big kreisel tanks (I) were connected in pairs, whereas the small kreisel (II) tanks were connected in a set of three, each of the two setups sharing one outflow (Fig. 1a,b). However, the inflow of the individual kreisel tanks could be controlled independently by valves attached to each inlet pipe. The larger salp species, *S. thompsoni*, was maintained only in the bigger kreisel tanks (I), while the smaller species *S. fusiformis* and *T. democratica* were housed in both kreisel sets (I, II) (Table 1; Fig. 1c–f).

Although the kreisel tanks differed in size, the general technical functionality was similar (Fig. 2): the inlet pipe (spray bar, Fig. 2a) in each kreisel tank created a gentle, uniform circular flow that prevented organisms from any contact with the water surface or tank walls (Greve 1968; Raskoff et al. 2003). The water flowed in a circular stream and drained through a centrally positioned filter (pore size: 2 mm, Fig. 2b) located in the back of the tank into the external filter chamber (Fig. 2c). A sponge located behind the filter reduced the effect of suction from the kreisel into the filter chamber. For our studies we implemented a number of additional modifications: filter holes were deburred to prevent the salp tunics from catching on sharp edges. The water level was raised in the small tanks to the level of the spray bar by installing elbow tubes in the outflow pipe (Fig. 2d). All kreisel tanks were equipped with air connection valves (Fig. 2e) to optionally switch to an air-running system. An aluminum frame and a

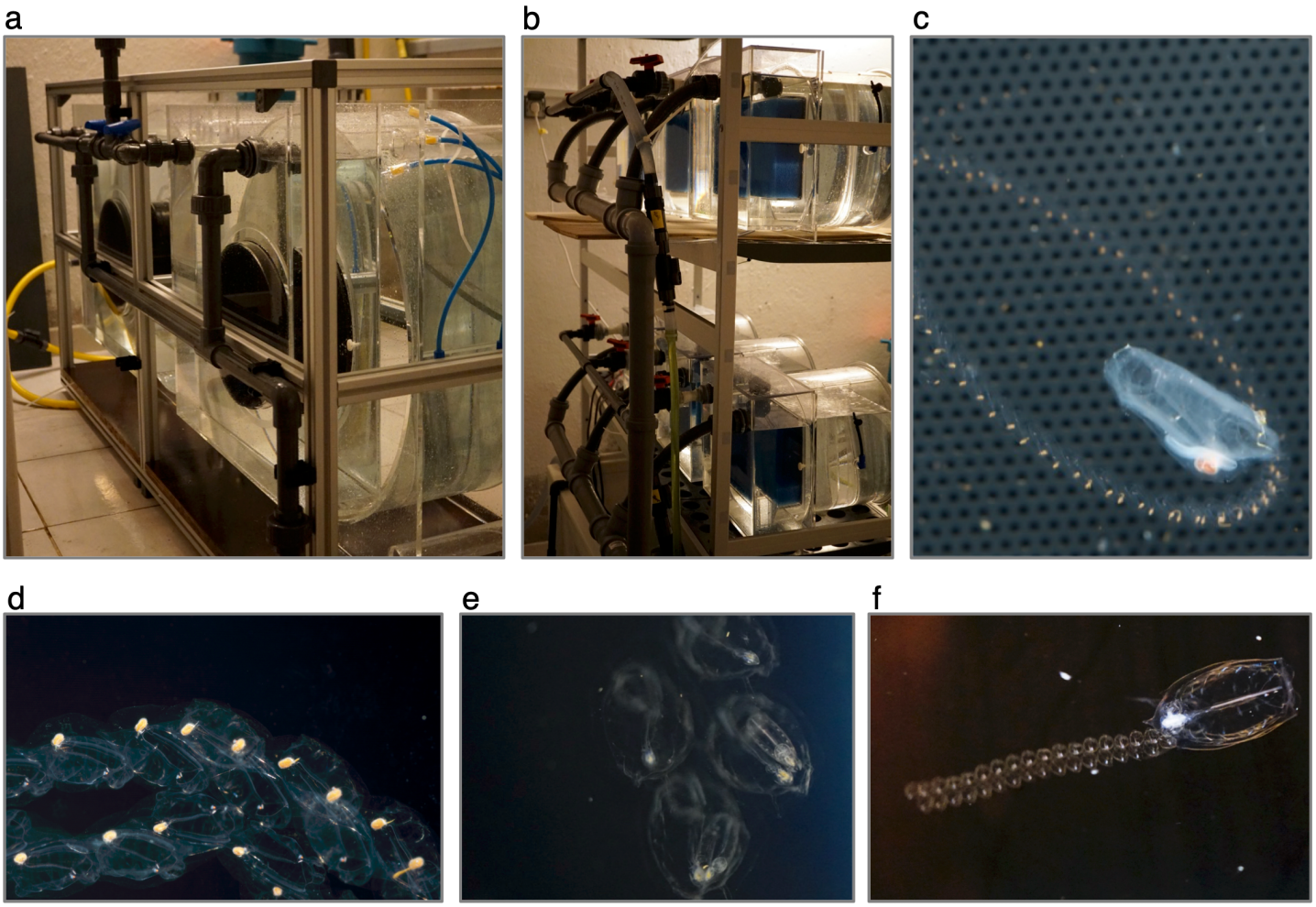

Fig. 1. Experimental kreisel tank setup and salps maintained. (**a**,**b**) Setup with big kreisel tanks, each connected in pairs (**a**, $V = 73.10$ liters) and small kreisel tanks each connected in a set of three (**b**, $V = 14.14$ liters). (**c**,**d**) Chain of blastozooids and a single oozoid (**c**) of *Salpa fusiformis* maintained in the kreisel tank system. Chain of blastozooids (**e**) and an oozoid in the progress of releasing a chain of blastozooids (**f**) of *Thalia democratica* in our kreisel tank system. Photo credit: (**a**,**b**,**e**,**f**) S. J. Mueller, (**c**,**d**) A. Jan; 2021, Institut de la Mer de Villefranche, France.

Table 1. Overview of the maintenance of *Salpa thompsoni*, *Salpa fusiformis*, and *Thalia democratica* in kreisel tanks. Information is presented species- and form-specific (Bz = blastozooids, Oz = oozoids). Breeding kreisel set refers to the (I) big kreisel tanks ($V = \sim 73$ liters) and (II) small kreisel tanks ($V = \sim 14$ liters). All data refer to maintenance settings during the experiments determining growth and development rates, with number of replicates (chains of blastozooids/oozoids) in brackets. Settings for additional successful cultivation attempts outside of these experiments are also shown.

Species	Form	Sampling region	Experiment	Temperature (°C)	Kreisel set	No. of individuals L^{-1}	Flow rate (mL min^{-1})	Diet/food added	Chl *a* (μg L^{-1})	Maintenance in kreisel tanks (days)	Survival rate (%)
S. thompsoni	Bz	Region Elephant Island, Southern Ocean	Growth and development *(n = 19)*	~ 1.5	I	0.32 (± 0.1)	350	*Isochrysis*, *pavlova* sp., *Thalassiosira weissflogii*	~ 2	5	—
	Oz		Cultivation attempt		I	—	350			21	—
S. fusiformis	Bz	Villefranche-sur-mer, Mediterranean Sea	Cultivation attempt	14.91 (± 0.07)	I	1.31	1300	*Chaetoceros gracilis*, *Tisochrysis lutea*	0.49 ± 0.3	12	66.67
			Growth and development *(n = 6)*		II	1.64 (± 0.12)	250			9	69.48 (± 11.59)
	Oz		Growth and development *(n = 8)*		I	0.29	1300			9	85.71
T. democratica	Bz		Cultivation attempt	14.95 (± 0.04)	I	—	1300		0.3 ± 0.14	9	—
					II	0.90 (± 0.14)	300				79.58 (± 7.15)
	Oz				I	1.03	1300				~ 80.0
					II	—	300				—

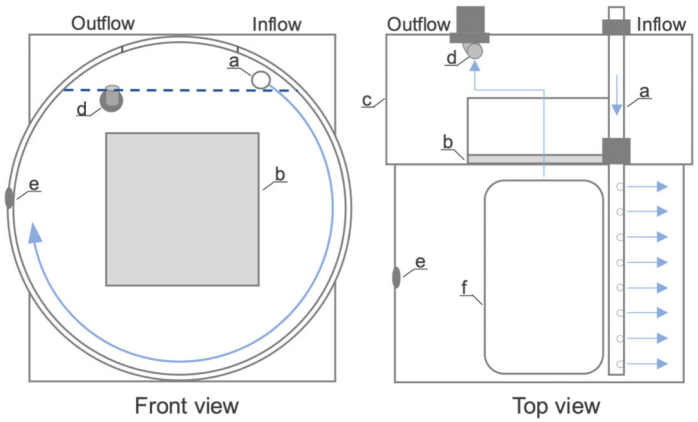

Fig. 2. Schematic representation of the functionality of the kreisel tank used in front and top view. a = spray bar, b = filter (pore size: 2 mm), c = external filter chamber, d = elbow tubes in outflow pipe, e = air connection valves, f = lid. The light blue arrows indicate the direction of water flow. The dashed horizontal line marks the water level.

floor mount were built for the large kreisel tanks to provide protection against ship motion when used at sea (Fig. 1a). Lastly, the position of each spray bar was carefully adjusted to prevent bubble formation. A full list of the equipment used can be found in Supporting Information Table S1. Further details on the specific experimental setup for *S. thompsoni* and *S. fusiformis* that resulted in the growth and development rates obtained are given below.

Field sampling and laboratory maintenance of *S. thompsoni*

Sampling

For onboard experiments, *S. thompsoni* were caught during expedition PS112 with R.V. *Polarstern*, close to Elephant Island (60°54.777′S 054°51.036′W) on 24 April 2018, using an Isaacs-Kidd mid-water trawl (IKMT 1.8 m^2 mouth opening, 550 μm mesh size) (Supporting Information Fig. S1a). To obtain individuals in healthy condition, still pumping and swimming, we sampled the upper 50 m of the water column at a maximum speed of up to 2.0 knots using a custom-built, bucket-shaped, closed cod end for the IKMT, in which the salps were retained in 25 liters of seawater. Back on deck, the cod end was removed from the net and transferred to the laboratory, where the salps were immediately sorted by form (blastozooids and oozoids), and chains of blastozooids. Chains ($n = 19$) of different sizes and length (3–9 individuals $chain^{-1}$) were carefully transferred into big kreisel tanks (I, $n = 5$) shortly after they were caught. Sea surface temperature (SST) during sampling was ~ 0.51°C.

In the vicinity of Deception Island (~ 62°34′S–59°55′W), *S. thompsoni* were collected at a multiday station, 27 March 2018 to 01 April 2018 using oblique IKMT tows in the top 0–170 m water layer in order to obtain field sample abundance and growth (*see* Wessels et al. 2018).

Maintenance

Animals were kept in a temperature-controlled room for 5 d under natural/local light conditions (13 h : 11 h/day : night) and fed three times a day with a mix of different instant algae (*Isochrysis*, *Pavlova* sp., *Thalassiosira weissflogii*) at a chlorophyll concentration of ~ 2 $\mu g\ L^{-1}$ (Iso 1800™, PAV 1800™, TW 1800™; Reed Mariculture, CA, USA), determined using a handheld fluorometer (AquaFlour, Turner Designs, CA USA). Filtered (5 μm) ambient seawater was pumped through the kreisel tanks at a constant flow rate of ~ 350 mL min^{-1} using Eheim pumps (Eheim, Universal 1200, Germany) to ensure constant food supply. Due to the passage through the vessel's pump system, the temperature of the filtered seawater was ~ 1.5°C when arriving at the kreisel tank setup. A turnover rate (defined as the time needed to fully replace the water per tank) of ~ 6.5 h ensured sufficient distribution of food and prevented accumulation of harmful bacteria. Coarse impurities were removed daily by suction with a glass tube (inner diameter 8 mm) attached to a hose. Salinity and temperature were checked regularly using a conductivity meter (Cond 3110, WTW, Germany).

Field sampling and laboratory maintenance of *S. fusiformis*

Sampling

Field sampling was conducted by snorkeling during a salp bloom period in spring (March/April 2021) at sunrise, when salps were most abundant at the surface, using ziploc-bags or a beaker (Supporting Information Fig. S2). Collected salps were stored in a cooler to keep water temperature constant during transport. After checking for parasites (amphipods, copepods), healthy specimens were transported to the kreisel setup in the temperature-controlled room (16°C) at the Institut de la Mer de Villefranche (IMEV) France, within 1.5 h after capture. During the same bloom period, salps were sampled onboard R.V. *Sagitta* at regular intervals at Point B at the mouth of the bay of Villefranche-sur-Mer (Supporting Information Fig. S1b). This was to estimate the abundance and growth rates of field samples using a Regent net (1 m^2 mouth opening, 680 μm mesh size, towed obliquely) in the upper 80 m. The filtered volume was estimated by attaching a flow meter placed close to the center of the net. Upon retrieval of the nets, subsamples were preserved in 4% formaldehyde for further analyses at the Alfred-Wegener-Institute Helmholtz Centre for Polar and Marine Research, Bremerhaven. The in situ conditions at Point B were characterized by a mean chlorophyll *a* (Chl *a*) concentration of $0.24 \pm 0.1\ \mu g\ L^{-1}$ and temperature of 14.82 ± 0.30°C throughout the whole sampling period (Service d'Observation en Milieu Littoral; www.somlit.fr).

Maintenance

For the maintenance of blastozooids and oozoids of *S. fusiformis* in the laboratory we used a kreisel-tank system that consisted of one set of big kreisel tanks (I, total $n = 2$) and two sets of small kreisel tanks (II, total $n = 6$), and a

reservoir ($V = \sim 200$ liters). While oozoids of *S. fusiformis* were only maintained in big (I) kreisel tanks, blastozooids of *S. fusiformis* were maintained in big (I) and small (II) kreisel tanks (Fig. 1a,b; Table 1). Animals were kept under a natural day : night rhythm of 11 h : 13 h and were fed with an algal mix of 50 mL cultured *Chaetoceros gracilis* and 50 mL *Tisochrysis lutea*, which was added to the reservoir daily. This experimental setup resulted in a constant water temperature of ~ 15°C and a Chl *a* concentration of 0.49 ± 0.3 μg L^{-1}, similar to the in situ conditions at sampling Point B (outlined above). To ensure a stable water temperature of 15°C, an inline aquarium cooler (TK3000, TECO) was deployed that circulated the water in the reservoir by a submersible pump (Eheim, Universal 2400, Germany). Filtered seawater (5 μm cartridge filter) was continuously supplied into the ~ 200 liters reservoir, with a flow rate of 4200–4800 mL min^{-1}, to ensure constant food supply from on-site. Flow from the reservoir to each big and small kreisel tank was maintained with pumps (Eheim, Universal 1200) at a flow rate of 1300 and 250 mL min^{-1}, respectively. Water from the small kreisel tanks flowed back into the reservoir, while water from the large tanks was discharged directly and was replaced by new incoming filtered seawater. In this way, a turnover rate of approx. 3 h (V = approx. 430 liters) was achieved at constant temperature. Water temperature was monitored by a logger (HOBO pedant temp/light logger [MX2202]), placed into the overflow chamber of a randomly selected kreisel tank system. Water level was controlled by an overflow pipe connected to the reservoir. After recording the condition of the salps (e.g., actively swimming, dead) and sampling, coarse impurities and dead individuals were carefully removed daily by suction with a glass tube (inner diameter 8 mm).

Determination of relative growth and developmental rates

Salpa thompsoni

Immediately after field sampling, one individual salp from each chain ($n = 19$) was carefully separated by a spring-steel tweezer. Oral-atrial length (OAL) was measured and each specimen was staged according to Foxton (1966) before transferring the rest of each chain to the kreisel tanks ($n = 5$). All individuals in a chain remained connected and were maintained under constant feeding conditions at ~ 1.5°C for 5 d. On the 5th day after capture, all individuals (individuals chain^{-1} n = 3–8) of the respective chains were sampled and OAL was measured.

In situ growth of blastozooids during the same cruise was determined over 5 d using cohort analysis conducted by Wessels et al. (2018). A subsample of > 200 salps was taken during a multiday station and all individuals were measured for OAL to an accuracy of 1 mm (Wessels et al. 2018).

Salpa fusiformis

After field sampling, six chains of blastozooids were separated into halves by gently twisting a spring steel tweezer back and forth. Only half of each chain was placed in the kreisel tank system, the other half was used for purposes not relevant to this study. A total of six chain halves were distributed among the six small kreisel tanks (1 chain kreisel^{-1}). There were no oozoids of *S. fusiformis* found during any of the morning samplings in the bay of Villefranche-sur-mer, France. As an alternative of tracking growth of oozoids, a more developed chain of blastozooids, almost all carrying a fully developed embryo (oozoid), was placed in a big kreisel tank. After 3, 7, and 9 d spent in kreisel tanks, individuals (n = 6–7) of *S. fusiformis* of both forms were sampled from each kreisel tank. Based on the similarity of embryonal and stolon development to *S. thompsoni*, *S. fusiformis* was staged according to Foxton (1966) under a microscope (Nikon C-LEDS, No. 224659, China) equipped with a digital camera (Dino-Eye AM7025X) followed by measurements of OAL and total length (TL) at each sampling.

In situ growth of blastozooids of *S. fusiformis* from the field was measured from subsamples of paraformaldehyde field samples (*see* above), removed by a Henson-Stempel pipet (50 mL). Samples were counted, and OAL and TL of both forms of *S. fusiformis* were measured and staged according to Foxton (1966). A 10% shrinkage correction was applied due to effects of formalin (Heron et al. 1988). Estimation of growth rates using cohort analysis were calculated under the assumption of sampling the same salp population.

Statistical analysis and data visualization

Relative growth rates (RGR, % d^{-1}) in the kreisel tanks were calculated by combining the OALs of all individuals measured during 1st sampling (OAL_0) with OALs of all individuals measured at the end of the experiment (OAL_t) within each chain (blastozooids) or within siblings (oozoids). The natural logarithm was used to linearize the growth model to examine how body length changes over time (days). Each individual RGR (% d^{-1}) based on body length (mm) was then determined as:

$$\mathrm{RGR} = \ln\left(\frac{\mathrm{OAL}_t}{\mathrm{OAL}_0}\right) \cdot t^{-1} \cdot 100\%$$

This cross-combination of individuals within chains/siblings was done to account for the uncertainty in initial OAL of the salps sampled at the end of the experiment. The approach allowed us to obtain growth rate ranges instead of point estimates, providing a more robust metric for comparison of the effects of initial stage and initial length on the rate. A linear regression model was used to explore the effect of mean initial OAL on RGR for each chain of blastozooids/group of oozoids.

R package mixtools (Benaglia et al. 2009) were used to identify distinct subgroups (cohorts) of the field samples by finite mixture analysis (estimation maximization-based algorithms) for each sampling time point. Using the *normalmixEM* and *mixEM* function provided by the R package mixtools (Benaglia et al. 2009), mean OAL ± SD were extracted and RGRs were estimated according to the equation stated above. To compare

RGRs from the present study with published data of *S. thompsoni* (Loeb and Santora 2012; Pakhomov and Hunt 2017; Wessels et al. 2018; Lüskow et al. 2020; Henschke et al. 2021), and to account for methodological differences (cohort analysis, kreisel tank maintenance), we standardized growth rates of *S. thompsoni* to 5°C using the following temperature coefficient (Q_{10}) equation, assuming a Q_{10} value of 2:

$$Q_{10} = \left(\frac{R2}{R1}\right)^{10/(T2-T1)}$$

To estimate developmental rates in the kreisel tanks (i.e., the time [in days] required to develop to the next stage) the mean stage of all individuals within a chain/all oozoids was calculated during the 1st sampling ($Stage_0$) and at the end of the experiment ($Stage_t$).

The mean development rate for each chain/oozoids was defined as:

$$a = \frac{1}{(Stage_t - Stage_0) \cdot t^{-1}}$$

Blastozooids of stage "X" (i.e., stage not clearly identified, for example, due to the absence of an embryo, placental remnants, or sperm channels) were neglected in this calculation. Mean and standard deviation (SD) of development rates were obtained by grouping variable initial stage.

The environmental data (temperature, Chl *a*) in Villefranche-sur-Mer (Point B) were extracted from the SOMLIT database (Service d'Observation en Milieu Littoral; www.somlit.fr) on 30 June 2022. All data were processed and visualized with help of *tidyverse v*. 1.3.2 (Wickham et al. 2019).

Assessment

Suitability of the kreisel tank system for maintenance of salps

Kreisel tanks have been used for the culturing of true jellyfish and ctenophores (Greve 1970, 1975; Raskoff et al. 2003; Courtney et al. 2020; Lechable et al. 2020; Ballesteros et al. 2022). However, the suitability of the circular kreisel tank for maintaining salps has never been described. In our study, the circulation of the kreisel tank gently directed salps away from the tank surface by a circular flow, preventing collision. We observed no evidence of damage due to wall contact. By adjusting the water level in the kreisel tanks (*see* Kreisel tank sets and modifications section), we reduced harmful bubble formation and did not observe any specimens getting caught at the water surface. The flow rate of each kreisel was adjusted to meet the different needs of each salp species and form without causing them to tumble (Table 1).

While some jellyfish species require different breeding tanks for different stages (Lechable et al. 2020; Ballesteros et al. 2022), our kreisel tanks appeared to be suitable for culturing almost all stages of salps. We were able to maintain *S. thompsoni* and *S. fusiformis* in several trials ranging from days to weeks by adjusting the system to the needs of the respective species and form (Table 1). The system was also successfully tested with the smaller species, *T. democratica*, at the Institut de la Mer de Villefranche (IMEV), France, in spring 2021. Both forms (blastozooids and oozoids) were maintained up to 9 d in the kreisel tank system, underlining the versatility of our system (Fig. 1e, f). Summarized information on maintenance of *Thalia democratica* is given in Table 1. However, due to a small number of individuals sampled and a low number of buds per chain, *Thalia democratica* were excluded from the growth and developmental rate analysis here. Although the kreisel tanks appeared to be suitable for all salps and forms tested, two limitations should be noted at this point and considered in future experiments: With the exception of *S. thompsoni* and oozoids of *S. fusiformis*, individuals of the different species were maintained during culturing attempts in both kreisel tank sizes (I, II, Table 1). Due to the larger size of *S. thompsoni* and the strong swimming behavior of the oozoids of *S. fusiformis*, large kreisel tanks (I) are recommended for future maintenance experiments for both. Newly released chains of the smaller species, *T. democratica*, with a total body length of ~2 mm tended to break and were occasionally sucked through the 2-mm pores of the filter. It is therefore advisable, to use a filter (Fig. 2b) with 1-mm pores and reduce the flow rate, at least until the animals have grown beyond a minimum size of 3–4 mm. During maintenance, successful releases of chains by asexual reproduction and of young oozoids by sexual reproduction of both species from the Mediterranean Sea were observed (personal observation, Fig. 1e,f; Supporting Information Video S1). This is strong evidence of the favorable rearing conditions in our kreisel tanks.

Using a flow-through system, we ensured high water quality and a continuous natural food supply through filtered seawater (5 μm) combined with instant algae and phytoplankton cultures representing local phytoplankton species for *S. thompsoni* and both Mediterranean species (*S. fusiformis*, *T. democratica*), respectively. The described kreisel tanks and their modifications (*see* Kreisel tank sets and modifications section) would also allow switching to a closed system, in combination with biological filtration and UV disinfection. In terms of experimental studies, our system is fully adjustable and allows for different experimental designs (e.g., temperature manipulation), that will be crucial for improving predictions on the performance of salps in the face of global climate change. Consequently, different temperature regimes were tested at the IMEV in Villefranche-sur-Mer in spring 2021: By using a titanium heater (500 W, Aqua Medic, Germany) in combination with a temperature controller (Aqua Medic T-controller twin, Germany) placed in the reservoir, we were able to perform a stable temperature increase from 15°C to 17°C and 15°C to 18°C by a steady temperature increment of 1°C d^{-1} and 1.5°C d^{-1}, respectively. Target temperature was

kept stable at ± 0.2°C for several days, indicating a reliable system for future experimental designs.

Growth and development of *S. thompsoni* and *S. fusiformis*

Most studies examining growth and development of salps are based on modeling approaches (Henschke et al. 2018; Groeneveld et al. 2020) and cohort analyses (Loeb and Santora 2012; Pakhomov and Hunt 2017; Lüskow et al. 2020). Published rates are therefore primarily from short-term observations and vary between regions and/or studies. By providing a circular kreisel tank system as a potential future tool for monitoring salp performance, growth and development, we were able to gain novel insights into life history events of salps. Growth and developmental rates of blastozooids of *S. thompsoni* and both forms (blastozooids and oozoids) of *S. fusiformis*, which were maintained in our kreisel tank system, were calculated and evaluated based on the rates determined in situ.

Blastozooids of S. thompsoni

Chains ($n = 19$) of blastozooids of *S. thompsoni* were maintained in big (I) kreisel tanks ($n = 5$) onboard R.V. *Polarstern* during PS112 for 5 d (Fig. 1a). The focus of this experiment was on comparing growth and development within chains (representing clones). The duration of the experiment was therefore limited by the number of individuals per chain and number of samplings.

The size of blastozooids appeared to vary within a chain (± SD range 0–1.7 mm; Supporting Information Fig. S3a; Table S2), so mean RGRs (% d^{-1}) over 5 d were determined by combining the OAL of all sampled individuals within a chain. The mean RGRs estimated from change in body length ranged from −1.64 to 3.15% d^{-1}(−0.36 to 0.36 mm d^{-1}) (Fig. 3a). In comparison, in situ growth rates assessed near Deception Island using cohort analysis were ~ 5% d^{-1} (~ 0.4 mm d^{-1}) for 8-mm blastozooids (Wessels et al. 2018). This growth rate corresponds to the upper end of the growth estimates in our experiments, probably due to the neglect of negative growth in cohort analyses. Upper growth rates in this study (0–3.15% d^{-1}) were within the range calculated for *S. thompsoni* caught near the Antarctic Peninsula of 0.3–4.6% d^{-1} (Loeb and Santora 2012), but in the lower range of in situ rates for blastozooids from the Antarctic Polar Front zone (3.7–9.1% d^{-1}) applying cohort analysis (Pakhomov and Hunt 2017). In a study by Lüskow et al. (2020), a newly released (~ 5 mm OAL) chain of blastozooids from the Chatham Rise region, New Zealand, was held in a kreisel tank for 5 d at 11°C and high growth rates of 8.78% d^{-1} were observed. These were comparable to in situ growth rates (9–12% d^{-1}) of ~ 10-mm OAL blastozooids obtained using cohort analyses in the same study (Lüskow et al. 2020). The differences in growth rates between studies can be explained by differences in temperature,

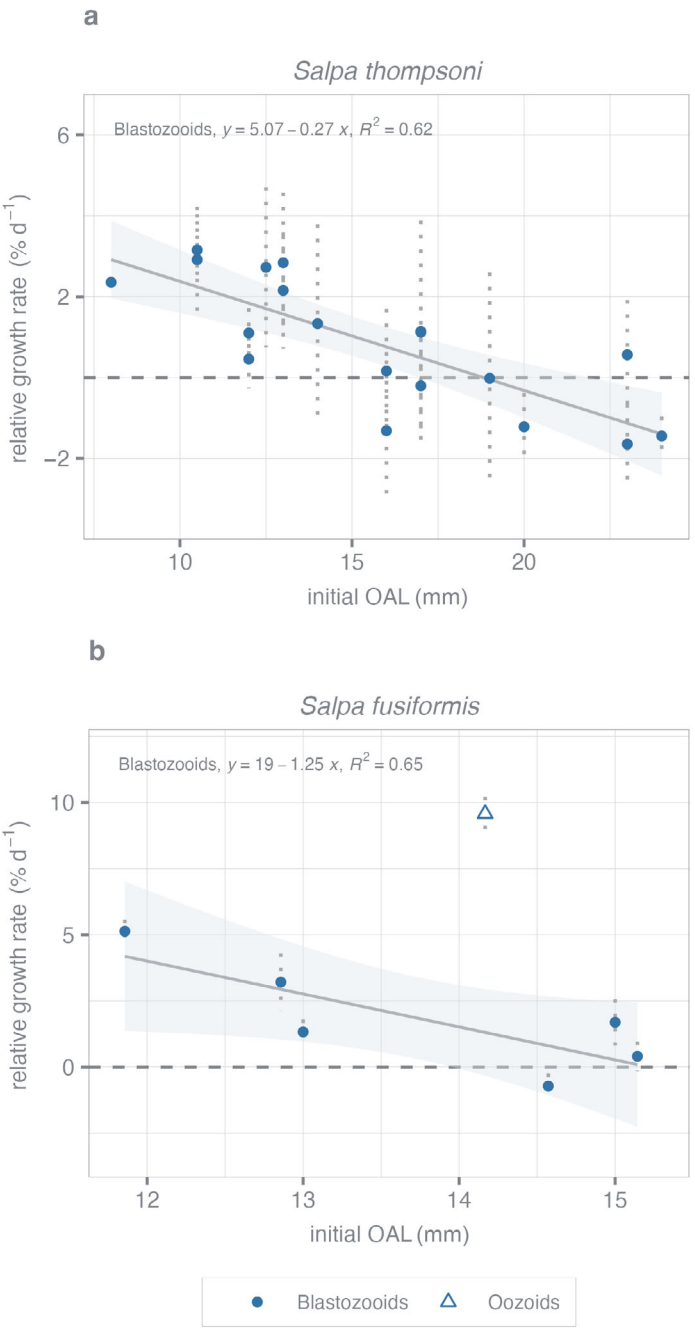

Fig. 3. RGRs (% d^{-1}) of *Salpa thompsoni* and *Salpa fusiformis* maintained in the kreisel tank system. Mean growth rates and confidence intervals of individuals cross-combined within a chain of blastozooids or group of oozoids were calculated as a function of initial mean OAL for (a) *S. thompsoni* over 5 d at ~ 1.5°C and (b) *S. fusiformis* over 4 and 6 d for blastozooids and oozoids at ~ 15°C, respectively. Regression model and 95% confidence interval are shown. The dashed horizontal line marks the putative threshold for the increase/decrease of daily rates. Each data point represents the mean growth rate of a single chain of blastozooids (**a**, $n = 19$; **b**, $n = 6$) or a group of oozoids (**b**, $n = 1$). The different shapes indicate the different reproductive forms (triangle = oozoids, circle = blastozooids).

size and food concentration in the different regions, all of which are known to affect salp growth rates (Heron 1972; Deibel 1982). Therefore, the lower SST of −1°C to 2°C in the study by Loeb and Santora (2012) and of ∼ 1.5°C in our study may explain the lower growth rates compared to those observed for salps from the Chl *a*-rich APF region at ∼ 4–5°C (Pakhomov and Hunt 2017) and in the kreisel tank at ∼ 11°C in the study by Lüskow et al. (2020).

Negative growth rates observed in our study were only found in chains with a larger initial OAL (range of >16 mm). However, it is questionable whether the salps began to shrink after reaching a certain OAL (and/or developmental stage) or merely reduced their growth rates for a short period of time. Nevertheless, the RGR decreased moderately with increasing initial OAL, yielding a regression function of $y = 5.07–0.27x$ ($R^2 = 0.62$, Fig. 3a), a trend that was already observed for blastozooids of *S. thompsoni* using cohort analyses (Pakhomov and Hunt 2017; Lüskow et al. 2020). An increased growth rate at the beginning of life may be beneficial to outgrow the feeding range of most planktonic predators (Heron 1972). Reduced energy investment in somatic growth (and possibly shrinkage) may result from their energetic flexibility to allocate resources to physiological processes such as growth and reproduction depending on their environment (Harbison 2009). Other gelatinous organisms, such as ctenophores and jellyfish, have been observed to shrink under poor conditions in favor of keeping essential metabolic rates unchanged (Harbison 2009; Lilley et al. 2014). The potential ability of blastozooids of *S. thompsoni* to shrink would favor embryonic development at a certain stage, as the embryonic growth depends on a continuous supply of nutrients from the parental blastozooid via the placenta (Chiba et al. 1999). This may represent a strategy to successfully develop their offspring at a maximum rate regardless of external circumstances. Our results suggest that this strategy of energy conservation may begin early after fertilization (stage 1.5; Supporting Information Fig. S3c) and becomes more evident as the embryo continues to develop within the parental blastozooid. About 82% of all individuals that showed potential negative growth rates were of initial developmental stage 2 or higher at the 1st sampling timepoint.

The developmental rate of blastozooids was estimated by calculating the time (in days) required to develop to the subsequent stage within each chain. Chains containing individuals of stage "X" ($n = 2$, 10.53%) were neglected in the calculation. Blastozooids performed between 0 and 0.4 stage transitions d^{-1}. For individuals that continued development (development rate > 0, 88.24%) it may take between 2.5 and 10 d to develop to the next development stage (Supporting Information Fig. S3b,d). It remains unresolved if the developmental rate depends on the initial stage and whether the development between stages not captured in this study would differ. Assuming, however, a general mean developmental time of 5.5 d ± 2.48 (± SD) for completing one stage transition and six necessary transitions from a newly released blastozooid to a functional male (i.e., from stage 0 to stage M) it may take 33.00 d ± 14.86 (range of 15–60 d) to complete the entire developmental process. This is consistent with the average time of 23 d calculated for the development of *S. thompsoni* blastozooids (Lüskow et al. 2020). We were not able to run a similar experiment for oozoids but if we assume the same duration for the complete development of the asexual reproducing oozoids, which is defined by the development of the stolon (= chains), the complete life cycle (including sexual and asexual reproduction) would be 28–110 d, consistent with the 2–3 months proposed by Pakhomov and Hunt (2017) and much shorter than the 9 months assumed by Loeb and Santora (2012). Our results highlight the complexity of potential life strategies of salps. However, further experiments are needed to investigate their "energetic flexibility" during different life stages.

Blastozooids of S. fusiformis

Blastozooids of *S. fusiformis* were maintained in six small kreisel tanks under constant feeding and temperature conditions at the Institut de la Mer de Villefranche (France) for 9 d. During the 9-d incubation period, OAL of sampled blastozooids was measured three times, at 3, 7, and 9 d after field sampling (Supporting Information Table S3). However, due to a low number of replicates on the 9th day, mean RGRs obtained by cross-combining individuals for each chain ($n = 6$) were calculated based on the 1st two samplings over 4 d (sampled individuals $n = 6–7$ sampling^{-1}). OAL varied within chains during both samplings (± SD range 0.38–1.95 mm; Supporting Information Fig. S4a). The mean RGRs for individuals cross-combined within each chain of blastozooids ranged from −0.72 to 5.13% d^{-1} (−0.10 to 0.68 mm d^{-1}) (Fig. 3b). Published growth rates of *S. fusiformis* are rare. A study by Braconnot et al. (1988) estimated a rate of 9–35% d^{-1} for blastozooids of *S. fusiformis* maintained in a large container (V = 700 liters) at 15°C. It has to be considered, however, that these results were based on various experiments and included very small blastozooids (> 2 mm) (Madin and Deibel 1998). As previously observed for *S. thompsoni*, the growth rate of *S. fusiformis* decreased moderately with increasing initial OAL ($y = 19–1.25x$, $R^2 = 0.65$; Fig. 3b). Therefore, the high growth rates (∼ 35% d^{-1}) found by Braconnot et al. (1988) could be related to the very small initial sizes of blastozooids maintained. Two cohorts were detected in the Bay of Villefranche-sur-Mer (2021). Their absolute growth was 0.95 and 0.54 mm d^{-1} and their RGRs were 10.20 and 10.94% d^{-1}, based on initial OAL of 5.8 and 3.3 mm of blastozooids, respectively (Supporting Information Fig. S5). This growth rate is consistent with the upper end of the growth estimates for smaller individuals (∼ 10 mm) maintained in our kreisel system. In contrast to *S. thompsoni*, the growth rates obtained for *S. fusiformis* seemed not to depend on the initial development stage (Supporting Information Fig. S4c). However, this can be due to the fact, that most individuals (52.17%) were not

fertilized (stage 0), limiting the number of fertilized and more advanced stages in our study. Interestingly, individuals of stage 0 showed shrinkage potential from OAL > 14 mm (Fig. 3b), which may indicate that the decrease in RGRs may be independent of fertilization status and is rather an obligate and/or temporal driven strategy.

On the 3rd day after field sampling, most individuals were either not yet fertilized (stage 0, 54.8%) or just fertilized (stage 1/1.5, 45.2%) and continued to develop during experimental maintenance (Supporting Information Fig. S4b). Since most blastozooids were still not fertilized (65%) after 7 d and only some showed advanced stages (stage > 3, 35%), we conclude that no more salps were fertilized in our kreisel tank system. Under the assumption that no more salps were fertilized in our system and neglecting individuals of stage 0 at both sampling timepoints, blastozooids performed between 0.43 and 0.75 stage transitions d^{-1} resulting in a development rate between 1.34 and 2.35 d to develop to a more advanced development stage (Supporting Information Fig. S4d). By assuming a mean developmental rate of 1.82 d ± 0.44 (± SD) for performing one transition, it may take 10.93 d ± 2.69 (range of 8–14.12 d) for full development through six stage transitions. This is consistent with the 14 d proposed by Braconnot et al. (1988) for growth and development of blastozooids of *S. fusiformis* until birth of the embryo.

During the same experiment we observed the timing of testes development in the sexually reproducing blastozooids. Most individuals (85%) sampled in kreisel tanks on the 7th day after field sampling had male characteristics (testes). This included individuals that did not have embryos, that is, not yet fertilized. Blastozooids were proposed to be protogynous hermaphroditic, with the ovary developing first, and the testes later (Foxton 1966; Godeaux et al. 1998; Daponte et al. 2001). It has already been shown, however, that blastozooids can have both, the female (presence of the embryo) and male (development of testes) characteristics simultaneously (Müller et al. 2022), but it was unclear whether fertilization is a prerequisite for the development of male physiology. Our results suggest that blastozooids (whether fertilized or not) continue to develop into males after a certain point in time onwards. Our findings are consistent with recent observations of gene expression of male and female genes in blastozooid embryos of *S. thompsoni*, suggesting alternative hypotheses (synchronous, bidirectional) to sequential hermaphroditism in salps (Castellano et al. 2023). Our study describes, for the first time, the timing of testes development at the whole-animal level, a feat made possible only by our favorable rearing conditions in our kreisel tanks.

Oozoids of S. fusiformis

Oozoids ($n = 18$) were released by a chain of blastozooids ($n = 21$, OAL 18.17 ± 1.94 mm, stage 5) during recovery (2 d) after field sampling in a big kreisel tank and were maintained for 9 d. RGRs of oozoids over 6 d at 15°C were determined (Supporting Information Table S4). At the 1st sampling timepoint (day 3), oozoids were 1–2 d old and had an OAL of 14.17 ± 1.17 mm ($n = 6$; Supporting Information Fig. S6a). All oozoids sampled were at stage 1, suggesting that stolon formation began while they were still within the parent blastozooid or immediately after release. The relative mean growth rates was 9.58% d^{-1} (1.83 mm d^{-1}) (Fig. 3b) over 6 d. Braconnot et al. (1988) reported a wide growth rate spectrum of 5–41% d^{-1} for oozoids maintained in aquaria at 15°C but further comparable data are unavailable (Madin and Deibel 1998). Determining growth rates for oozoids represents a challenge for cohort analyses because very often oozoids are less abundant than blastozooids (Lüskow et al. 2020). Here, oozoids accounted for a maximum of 1.72% of total catch in field samples (mid of March 2021; Supporting Information Fig. S5a). They were completely absent from morning samplings at the surface in the Bay of Villefranche-sur-Mer during spring bloom 2021. As a result, in situ growth rates of oozoids could not be derived from field samplings. After experimental day 7 (~ 6 d after release by parental blastozooids) most (83.3%) and after 9 d (~ 8 d after release by parental blastozooids) all oozoids were stage 2 (Supporting Information Fig. S6b). We can therefore assume a stage transition time between 0.16 and 0.18 stages d^{-1} and a development rate between 4.8 and 6 d. Assuming 5 stage transitions (0–5), according to Foxton (1966), the complete development of oozoids from release by the parent blastozooid to release of the first blastozooid chain itself would take 24–30 d. This is consistent with the 8–22 d proposed by Braconnot et al. (1988).

Kreisel-derived growth vs. field-estimated growth

To account for potential methodological differences (cohort analysis, kreisel tank maintenance), we compared our in vitro blastozooid growth rates ($RGR_{kreisel}$, % d^{-1}) with cohort growth rate estimates (RGR_{cohort}, % d^{-1}) of *S. thompsoni* compiled from four different studies by Lüskow et al. (2020), all standardized to 5°C and assuming $Q_{10} = 2$. Because the Q_{10} equation is not capable of generating growth rates that include negative values, we excluded chains showing potential negative growth ($n = 12$) before fitting the model. The results show that estimating RGRs using cohort analyses systematically yields higher growth rates than those derived from measurements of individual chains ($n = 8$) in kreisel experiments (Fig. 4). Whether these systematic differences are due to methodological differences cannot be said with certainty, as kreisel-derived estimates are limited to only two studies, and the small number of replicates ($n = 1$) in our study at the 1st sampling time point might have led to inaccuracies in calculated growth rates. However, our kreisel-derived growth rates allowed for direct length comparisons within chains (clones). This method may be more accurate for estimating growth rates compared to cohort analyses that consider the average length of cohorts consisting of many chains. Cohort analyses have

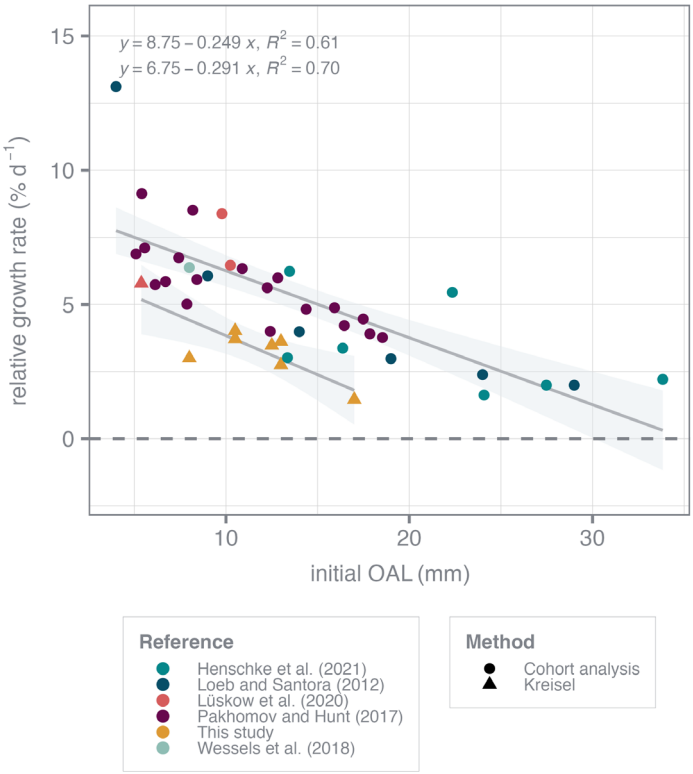

Fig. 4. Standardized RGRs (% d^{-1}) of blastozooids of *Salpa thompsoni* as a function of initial OAL (mm). Growth rate estimates were compiled from four different studies (Loeb and Santora 2012; Pakhomov and Hunt 2017; Lüskow et al. 2020; Henschke et al. 2021) by Lüskow et al. 2020. Mean growth rates (for each chain) determined in this study were also added (chains $n = 7$). All growth rates were standardized to 5°C assuming $Q_{10} = 2$.

been used for decades. However, they may already have their shortcomings due to the fact that the distribution of different stages of salps, and therefore the data obtained, may be patchy (Alldredge and Madin 1982; Greer et al. 2015; Henschke et al. 2016), making it difficult to generalize results on growth. In the maintenance system presented here, we exactly know the conditions they are exposed to and can control them. However, while we were able to control many factors (e.g., water temperature, food concentration) that may affect the growth of salps, as with any aquarium-based experiment, the conditions to which salps are exposed in a kreisel tank system may not accurately represent their natural environment (e.g., natural food composition, hydrodynamics). In summary, our results show that traditional methods of estimating growth rates may have their limitations and future research is needed to test this in more detail. Furthermore, the occurrence of negative growth rates in our data suggests that the Q_{10} equation may not be an appropriate function to account for the effects of temperature on growth rates of salps. Due to the limited growth data available for *S. fusiformis*, this methodological comparison is limited to *S. thompsoni*.

Discussion

The delicate gelatinous structure of salps and their irregular seasonal and spatial occurrence make them a difficult subject for experimental research and explain the lack of any physiological data. A reliable system with specific modifications for the maintenance of salps using the well-established kreisel tank (Greve 1968, 1975) is an advance in several respects. First and foremost, it allows to study the behavior of salps and to obtain reproducible physiological data, such as growth and development rates. Salp growth rates are essential components in the development and parametrization of salp life cycle models (Henschke et al. 2018; Groeneveld et al. 2020). Most studies examining growth and development so far, were based on cohort analyses (Loeb and Santora 2012; Pakhomov and Hunt 2017; Lüskow et al. 2020). However, as shown in our study, cohort analyses may lead to a general overestimation of growth rates and neglect individual (and possibly stage-specific) strategies (e.g., shrinkage). Inaccuracies or high variability in input data may affect model results on population dynamics and the respective conclusions drawn, highlighting the need for a laboratory approach. Attempts have been made in the past to maintain salps for longer periods, using aquaria ranging from small jars to large containers (Heron 1972; Braconnot et al. 1988). However, differences in aquarium size, food, temperature, and sampling condition appeared to have significant impact on growth rates (Heron and Benham 1984; Godeaux et al. 1998) further emphasizing the need for a standardized and thus, universal experimental system. Here, we tested and described a temperature-controlled kreisel tank system, suitable for maintaining a variety of salp species for the first time. Our results show that maintained salps have similar growth and development rates as in situ and therefore provide a reliable and reproducible basis for future work. Furthermore, we were able to observe key processes in the life cycle of salps, such as the timing of testes development. The hermaphroditic nature of salps, particularly the male stage, is still an under-explored area of research. A system that allows the salps to be maintained for a longer period of time is key to further study the specific characteristics of their life cycle. In addition, manipulation of different abiotic factors (e.g., temperature) for various experimental designs is possible, which is important to better understand their habitat preferences in the water column. It also improves existing predictions on future performance of salps, especially with respect to the possible impacts of global climate change. So far, only a few studies have examined effects of increased water temperatures on salp performance using pulsation and filtration rates (Harbison and Campenot 1979; Andersen 1986) and oxygen consumption (Trueblood 2019) as proxies.

Overall, we hope to stimulate future experimental research and thus advance the limited field of laboratory studies on salps. We also encourage others to publish detailed descriptions of their efforts and progress based on our descriptions, which is essential for comparison of the physiological data obtained here.

Comments and recommendations

The objective of this study was to test, optimize and describe an approach for the collection and maintenance of different salp species. Our aforementioned experiences with the system may be useful for future experimental designs, and will therefore be summarized in the following:

- *Sampling method*: Successful maintenance requires the collection of samples in healthy condition, as this directly affects the survival rate during cultivation as well as the reliability of the (physiological) parameters assessed. By sampling the upper 50 m at a maximum speed of up to 2.0 knots and a closed cod end, we were able to successfully catch individuals of *S. thompsoni* in very good condition, while smaller specimens from the Mediterranean Sea rather seemed to suffer during towing (sampling depth ~ 80 m). It may therefore be advisable to tow at shallow depths (max. 50 m depth) and at very low towing speeds of 1–2 knots during future sampling campaigns. In this study, the best results with Mediterranean salps were obtained by snorkeling, using beakers and zip-lock bags to catch salps directly within the water column (Supporting Information Fig. S2). A prerequisite for this sampling technique is the close proximity of the sampling site to research facilities and the occurrence of a salp bloom, which is assumed to terminate with the decline of phytoplankton concentrations, temperature change, and stratification of the water column and can last weeks to months (Menard et al. 1994; Licandro et al. 2006; Deibel and Paffenhöfer 2009). In our opinion, the gentlest "sampling" method is obtaining the respective form of interest via its release by the parental salp in the kreisel tanks. This reduces handling time for both generations and allows for focusing on the form of interest. This is especially important when the form is less abundant in the field as it was the case with *S. fusiformis* oozoids in the present study.
- *Number of experimental replicates*: Because even individuals of the same chain (representing clones) showed variability in growth rates in this study, a sufficient number of replicates is recommended to account for inter-individual variability and obtain statistically significant results.
- *Flow rate*: Flow should gently direct salps in a circular motion around the kreisel without causing them to tumble when passing the inflow stream. Also, flow rates should be adjusted in a way that no bubbles are formed. See also Kreisel tank sets and modifications section for an optimal position of the spray bar and Table 1 for appropriate flow rates tested for each species and form.
- *Number of animals per tank*: The number of animals to be kept in the tanks should be carefully considered and will depend on the size and species of salps. If the tank is too crowded, salps can stick to fecal pellets, which could result in bacterial infections. Table 1 gives an indication of an appropriate number of animals per liter for the salps species investigated in this study.
- *Check animals for parasites*: Before placing the animals in the kreisel tanks, the animals should be examined for parasites (amphipods, copepods).
- *Establishment of noninvasive measurements through photography*: Our approach of tracking growth and developmental rate within chains of blastozooids and oozoids (representing siblings) allowed insights into both rates as a function of initial OAL and stage. However, length and developmental measurements were conducted after sampling individual salps. Therefore, the duration of the experiment was limited by the number of individuals available per chain. In addition, the sampling method excluded growth and developmental rate analysis of shorter chains of *T. democratica*. Therefore, future research should focus on optimizing noninvasive measurements such as photography (as in Lüskow et al. (2020)).

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