Growth and fecundity of benthic freshwater nematode *Tobrilus* sp. (Andrássy, 1959) transplanted in snail-sediment extract medium

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Summary. *Tobrilus* sp. is an ecologically important benthic nematode. The current study aimed to study its growth and reproduction rates within snail-sediment extract (cheap and locally available culturing medium) compared with that in a control medium (only sediment extract). The former medium was an autoclaved mixture of the control medium and the snail (*Lanistes carinatus* Olivier, 1804) extract in the ratio of (1:1). The control medium was an autoclaved sediment extract of the nematode's natural freshwater habitat. In the snail-sediment extract medium, results showed significantly greater growth rate of both adults and juveniles through greater average body length, width, biomass and reproduction rate. The significantly higher nitrogen and magnesium concentrations in the snail-sediment extract medium may explain those results. It is recommended to use this medium for the mass production of *Tobrilus* sp.

Key words: aquatic, biomass, culture, development, *Lanistes carinatus*.

Rearing nematodes has emerged as a new horizon for using these promising organisms in a variety of ways. For example, nematodes are relevant food source for fish juveniles and prawn larvae (Bruun, 1949; Brüggemann, 2012; Majdi & Traunspurger, 2015). Others aimed at using these animals as very promising biological control agents like Smart (1995), Shapiro-Ilan *et al.* (2006) and Peters *et al.* (2008). Furthermore, nitrogen mineralisation has been studied by Ferris *et al.* (1998) and Chen & Ferris (1999). Therefore, the ways of rearing and growing these animals are very important. The effect of food densities on the growth and reproduction of *Plectus palustris* was considered by Schiemer *et al.* (1980). Traunspurger *et al.* (1997) highlighted the dependence of growth and development of *Caenorhabditis elegans* on the concentration of bacterial food. Similarly, Höss *et al.* (2001) pinpointed the importance of dissolved organic matter on *C. elegans* growth and/or reproduction.

Nematode rearing media varied considerably. For example, for entomopathogenic nematodes production, Glaser *et al.* (1940) and Somwong & Petcharat (2012) used *in vitro* solid media, whereas, Bedding (1981) developed a semisolid culture method. Radwin & Rouse (1990) studied the yield characteristics of a free-living nematode (*Panagrellus redivivus*) in different culture media (wheat flour, oatmeal, cornmeal, cottonseed meal, ground shrimp feed and yeast). Santos *et al.* (2012) compared two *in vitro* methods for multiplication of *Rodopholus similis* and *Pratylenchus brachyurus* in carrot cylinders. In their study, Buecher *et al.* (1970) showed that variation in the proteinaceous supplements in chemically defined media could result in an obvious variation of the *Aphelenchoides* sp. populations. Boisseau & Sarah (2008) reared phytoparasitic Pratylenchidae nematode on carrot discs. Bedding (1984) cultured insect-parasitic nematodes within autoclavable plastic bags on crumbed polyether polyurethane sponge coated with sterilised chicken offal homogenate and inoculated with the primary form of the appropriate symbiotic bacteria. Tabassum & Shahina (2004) used the same method as Bedding (1984) for *in vitro* mass rearing of four virulent nematode species of the genera *Steinernema* and *Heterorhabditis*. In their determination of the optimum physical and chemical components of the medium for maximum production of *Neoaplectana carpocapsae* and *Heterorhabditis heliothidis*, Dunphy & Webster (1989) specified the conditions and nutrients that enhanced their growth and production; they were temperature, pH, lipids, yeast extract, D-glucose, D-

fructose, D-galactose, D-sorbose, D-mannitol, carbon source, magnesium chloride, potassium chloride and potassium nitrate. Buecher & Popiel (1989) reared the entomopathogenic nematode *Steinernema feltiae* in a liquid media containing its bacterial symbiont with the yeast or cholesterol.

Environmental purification in the form of sulfur detoxification by *Tobrilus* sp. has been noted by Nuss & Trimkowski (1984), Bird *et al.* (1991) and Roccuzzo & Ciancio (1991). Nuss (1984) and Nicholas *et al.* (1987) pinpointed that insoluble metal sulfides were mostly deposited in the somatic muscles rather than H_2S oxidation to elemental sulfur as a part of sulfide ions detoxification.

Tobrilus sp. presence in drinking water (WHO, 2008) and in freshwater habitats (Heyns, 2002), as well as being one of the constituents of juvenile fish food (Majdi & Traunspurger, 2015) highlighted its value. Moreover, it has been reported that *Tobrilus* sp. is frequently infested by microsporidian spores (Poinar, 2001). Only recently, microsporidia have been documented to parasitise humans (https: //web. stanford.edu/group/parasites/ParaSites2006/Microsporidiosis/microsporidia1.html). For all these reasons, detailed studies on *Tobrilus* sp. are recommended.

The current study aimed to evaluate *Tobrilus* sp. growth expressed as biomass (µg) (adult and juvenile) as well as reproduction as a result of rearing in the snail-sediment extract medium (1:1).

MATERIAL AND METHODS

Extraction, identification and acclimation of the worms. The worms were extracted using the Whitehead tray extraction method (Hodda & Abebe, 2006). The worms were identified according to Ferris *et al.* (1973), Tarjan *et al.* (1977) and Abebe *et al.* (2006). Two sets (three replicates each) were prepared. Each replicate comprised 25 adult females and 15 adult males (40 individuals in total). These individuals were transferred using a fine needle to the acclimation media (renewable water from the natural habitat) for 1 week in 9-cm-diameter glass Petri dish for the acclimation of the worm prior their transplantation in the culturing media.

Preparation of the culturing media. Medium 1 (sediment extract). 50 g of sediment from the nematode's natural habitat were boiled in 1 l distilled water, then cooled and filtered. The filtrate was autoclaved at 120°C for 30 min.

Medium 2 (snail extract (*Lanistes carinatus*): sediment extract) (1:1). 15 g of the snail *Lanistes carinatus* (Class: Gastropoda, family: Ampullariidae) soft tissues were homogenised in 1 l distilled water. The supernatant was collected and autoclaved as for medium 1. The composition of medium 2 was in the ratio of sediment extract:snail extract (1:1).

The snail was identified according to Brown (1994). It was chosen because it is among the most abundant snails in the Egyptian fauna (Hussein *et al.*, 2011; Abd El-Wakeil *et al.*, 2013). It flourishes in areas of agricultural activity (Lange & Van Damme, 2010). Its prevalence has encouraged the use of its tissue extract as cheap local nutritional source for culturing and rearing nematodes.

Three sample replicates of the culturing media were measured for nitrogen, phosphorous, sodium, potassium, calcium and magnesium, using atomic absorption spectrophotometer (Model Avanta E A5616).

Worm's inoculation. The first and the second nematode sets were inoculated in 30 ml (per replicate) of medium 1 (control) and medium 2, respectively. The two media were kept at room temperature with a 12-h photoperiod, and were supplemented regularly (every 2-3 days) by additional amounts of the same medium to keep the media volumes constant.

The produced larvae were transferred to new media with the same components of their original medium. These new media were regularly supplemented (every 2-3 days), exactly as in their original media. The body length, width and biomass of adults and juveniles were measured every 3-4 days (to be able to process the data for all replicates), over a period of 1 month, according to Ramsay *et al.* (1997). Essentially, this method is for producing benthic biomass size spectra relying on subsampling; calculations depend mainly on the individual's body dimensions using image analysis measurements. Nematodes were approximated to cylindrical shape; their volumes were converted to dry weights assuming a specific gravity of 1.05 and a dry- to wet-weight ratio of 0.15. The average number of the measured adult nematodes in both control (sediment extract medium) and snail-sediment medium (three replicates each) was 13 individuals. The average numbers of the measured juveniles were 10 and 11 for control and snail-sediment media respectively in each replicate.

Statistical analysis. STATGRAPHICS Centurion XVI software package was used. Differences between the elemental composition of the two media as well as the differences between the two media (parametric data) in terms of adult and juvenile growth as biomass (µg) and juvenile production were evaluated using the t-test.

The growth data (expressed as biomass (µg)) of the adults as well as juveniles in the two media were

Table 1. Elements concentrations (mean ± SD) in the two media as mg l^{-1}.

Element concentration (mg l^{-1})	Medium 1. Sediment extract	Medium 2. Sediment extract and snail extract (1:1)	*P*
Nitrogen*	32.03 ± 3.11	46.65 ± 1.86	0.0000
Phosphorous	1.72 ± 0.05	1.70 ± 0.02	0.1963
Sodium	33.53 ± 1.59	33.47 ± 3.01	0.9369
Potassium*	23.53 ± 1.41	20.61 ± 1.64	0.0043
Magnesium*	4.14 ± 0.62	6.25 ± 1.26	0.0030
Calcium	16.53 ± 1.25	17.24 ± 1.36	0.1761

* – Significant difference between the two media at $P = 0.05$.

Table 2. Mean adult and juvenile body length (mm), width (mm) and biomass (µg) in the two media.

Body dimensions and biomass	Age	Mean ± SD		*P*
		Sediment extract	Snail-sediment extract	
Length (mm)*	adult	1.80 ± 0.13	2.11 ± 0.22	0.0000
Width (mm)*	adult	0.04 ± 0.006	0.06 ± 0.007	0.0000
Biomass (µg)*	adult	0.5 ± 0.1	1 ± 0.3	0.0000
Length (mm)*	juvenile	0.76 ± 0.28	1.21 ± 0.50	0.0000
Width (mm)*	juvenile	0.02 ± 0.005	0.03 ± 0.01	0.0000
Biomass (µg)*	juvenile	0.08 ± 0.05	0.2 ± 0.1	0.0000

* – Significant difference between the two media at $P = 0.05$.

tested for normality for regression analysis. Comparison of regression lines were performed to determine whether there were significant differences between the intercepts and the slopes at the different levels of growth for both adults and juveniles.

RESULTS

Media mean nutritional constitutions. Nitrogen and magnesium were significantly higher in snail extract medium. Potassium was significantly higher in sediment extract medium. However, phosphorous, sodium and calcium were the same in both media (Table 1).

Adult and juvenile nematode body length (mm), width (mm) and biomass (µg) within the two media. The mean body length, width and biomass values of both adult and juvenile worms over a 1 month period were significantly higher ($P = 0.0000$) in snail-sediment extract medium than those in sediment extract medium (Table 2; Figs 1 & 2). Adult worms were, on average, 1.2× longer, 1.5× wider and had 2× more biomass in snail-sediment extract medium compared with those in sediment extract medium. Juvenile worms were, on average, 1.6× longer, 1.5× wider and had 2.5× more biomass in snail-sediment extract medium compared with that in the sediment extract medium.

Reproduction rate in the two media. Cumulative reproduction rate varied significantly ($P = 0.0005$) within the two media (Fig. 3). The reproduction rate in the snail-sediment extract medium reached more than 250 worms within a week, whereas the juvenile number in the sediment extract medium did not reach 20 worms within the same time. However, the reproduction rate had a steady pattern in both media after a month.

Regression analysis. Regression of the adult biomass (µg) in the time intervals (3 days) varied significantly between the two media in terms of time, intercepts and slopes ($P = 0.0000$ each) with R-Squared = 68.5492%. The regression equations were:

Adult biomass (µg) = 0.601116 + 0.103962×time (snail-sediment extract medium)

Adult biomass (µg) = 0.43673 + 0.0253631×time (sediment extract medium)

The incremental increase of adult biomass within the 3-day intervals in both media was not the same; it was 10% and 3% in snail-sediment extract and sediment

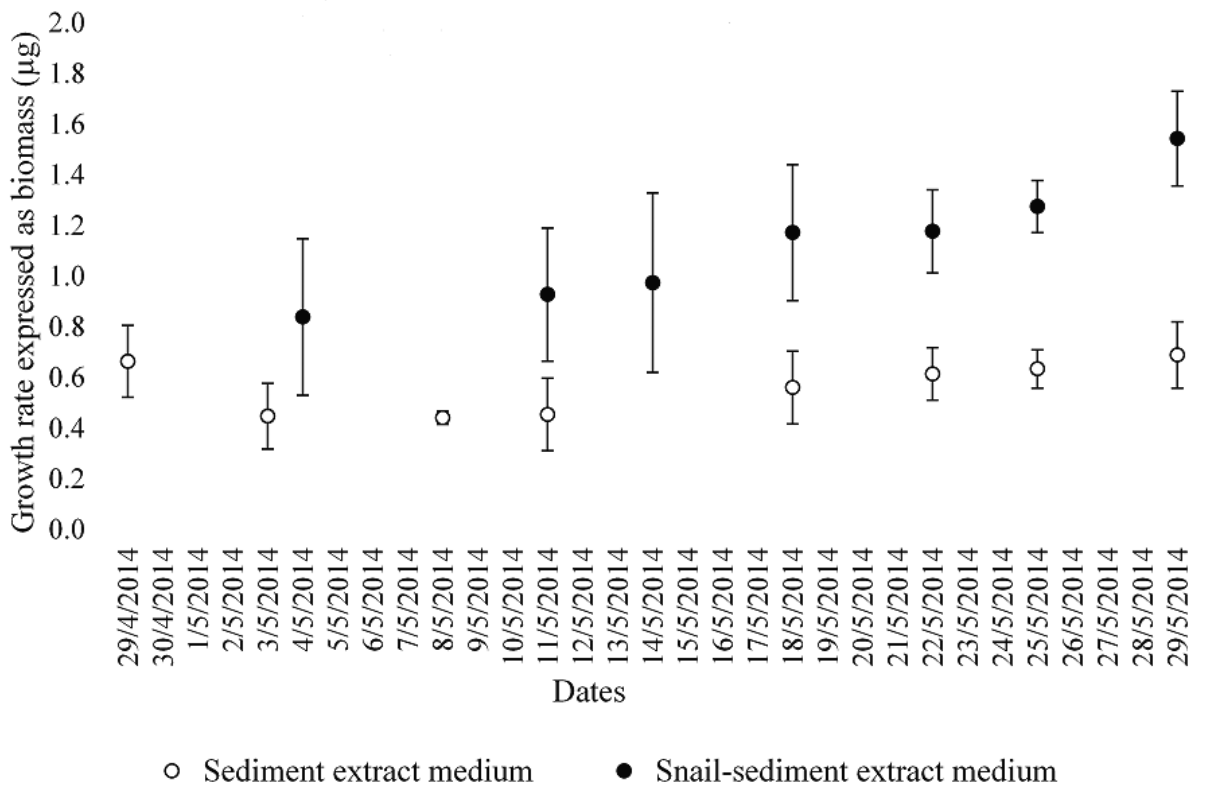

Fig. 1. Mean ± S.D. adult *Tobrilus* sp. growth rate in sediment and snail-sediment extract media over a 1 month period (the 3^{d} and 5th readings of snail-sediment extract and sediment extract media were missed respectively).

extract media, respectively. Similarly, juvenile biomass (µg) regression with time intervals (3 days) showed a significantly different relationship with time, intercepts and slopes (P = 0.0000 each) with R-Squared = 87.604%. The regression equations were:

Juvenile biomass (µg) = –0.00992892 + 0.0683499×time (snail-sediment extract medium)

Juvenile biomass (µg) = 0.000027368 + 0.0191137×time (sediment extract medium)

According to these equations, the incremental increase in juvenile biomass within the 3-day intervals in both media were 7% and 2% in snail-sediment extract and sediment extract media, respectively.

The ratio of biomass increments of the adult worms reared in snail-sediment extract medium to those reared in sediment extract medium was 3.3. On the other hand, the ratio of juvenile biomass reared in snail-sediment extract to those reared in sediment extract was 3.5 (*i.e.*, the growth of adults and juveniles is 3.3 and 3.5 higher in snail-sediment extract medium than that in sediment extract medium, respectively). Interestingly, the average growth ratio of adult to that of the juvenile in the snail-sediment extract medium and sediment extract medium was 1.4 and 1.5, respectively.

DISCUSSION

Production of large numbers of *Tobrilus* sp. using cheap and locally available resources will definitely promote their use as live food for fish larval stages as an example for ecological application (Weber & Traunspurger, 2014). Furthermore, the use of *Tobrilus* in experimentation will facilitate understanding of the complicated trophic interactions and energy fluxes within the ecosystem. The importance of the nutritional constituents in the nematode culture media has been highlighted by many authors. Buecher & Popiel (1989) pointed out the importance of heme as well as sterol as necessary nutrients for *S. feltiae* in liquid cultures. Similarly, Buecher *et al.* (1970) confirmed that chick embryo extract plus serum was the best supplement for rearing adults and juveniles of *Aphelenchoides* sp. Buck *et al.* (2015) came to the same result, emphasising the important role of nutrients, particularly proteins, in producing a high

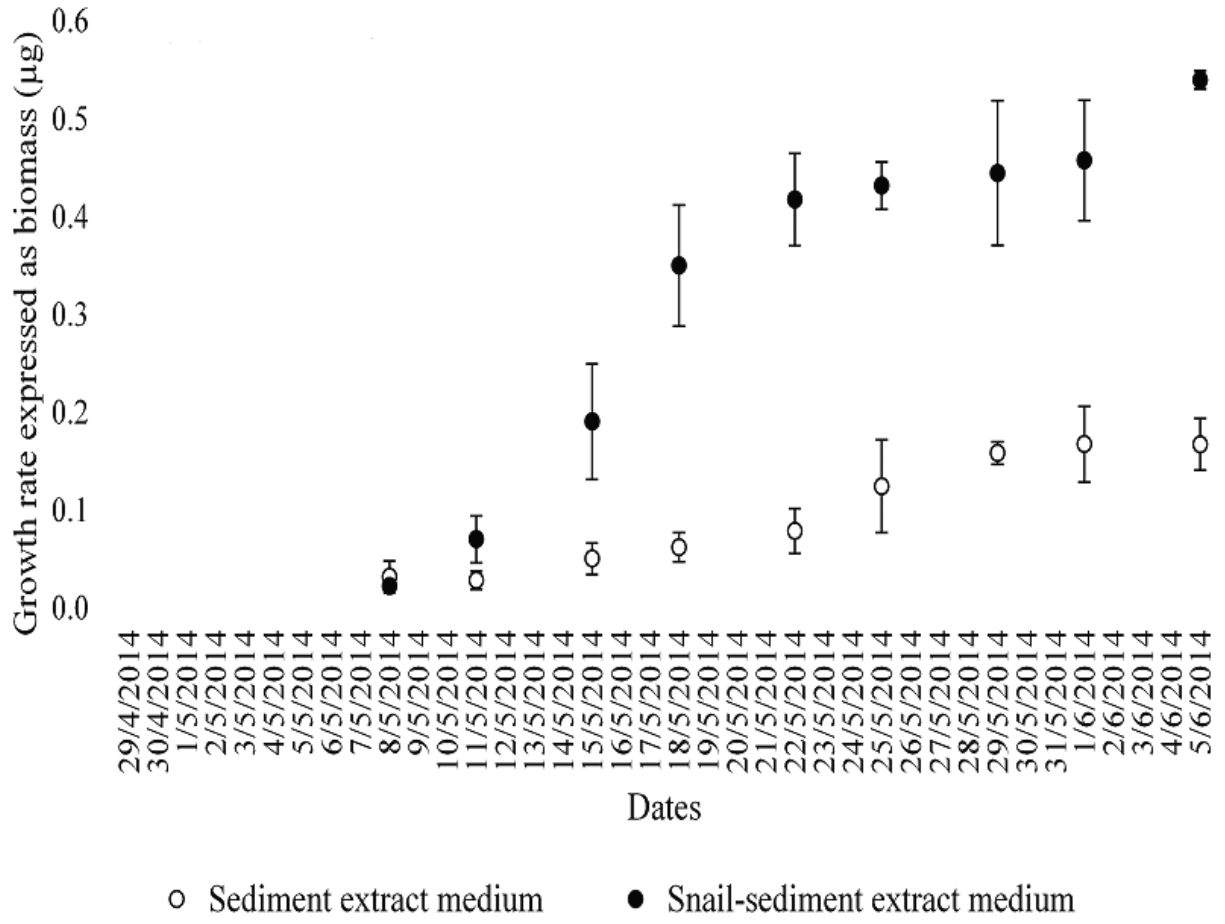

Fig. 2. Mean ± S.D. juvenile *Tobrilus* sp. growth rate in sediment and snail-sediment extract media over a 1 month period.

nematode biomass. The results of the present study agree with this, as there were larger nematode sizes and yield within snail-sediment extract media of significantly higher protein content, compared with control, as represented by nitrogen.

Despite the habitat differences, the results of the present study coincide with the findings of Tahseen (2012) who reported that marine habitat richness of organic matter reflected on the nematode body size, *i.e.*, the higher the amount of organic matter the bigger the body size. Furthermore, low production of small sized eggs is characteristic of the smaller sized nematodes. Similarly, the results of the present study indicated that the low nutritional medium (sediment extract) resulted in low production rate as well as smaller sized juveniles leading to smaller sized adults. Yi-Chang (2014) showed that the yield of *Steinernema abbasi* could be enhanced by adding whole milk powder to the liquid media. This supports the results of the present study in terms of growth and yield propagation as a result of significantly elevated protein content represented by nitrogen in snail-sediment extract medium. However, Radwin & Rouse (1990) argued that the nutritional richness of the culture media is not necessarily a limiting factor for enhanced growth and juvenile yield. They expected that chemical changes in the media may help explaining the differences among the rearing media. Furthermore, it has been reported that magnesium has an enhancing effect on the entomopathogenic nematodes by stimulating their biological activities (Jaworska & Gospodarek, 2009), or enhancing their pathogenic abilities (Jaworska, 2014). The results of the present study also show that the significantly elevated magnesium concentration in snail-sediment extract medium enhanced the nematode biological state as reflected by its growth and reproduction.

Similarly, the results of Somwong & Petcharat (2012) coincide with the present study in terms of the strong differential effects of the culture media on a nematode species. They pinpointed that *S. carpocapsae*

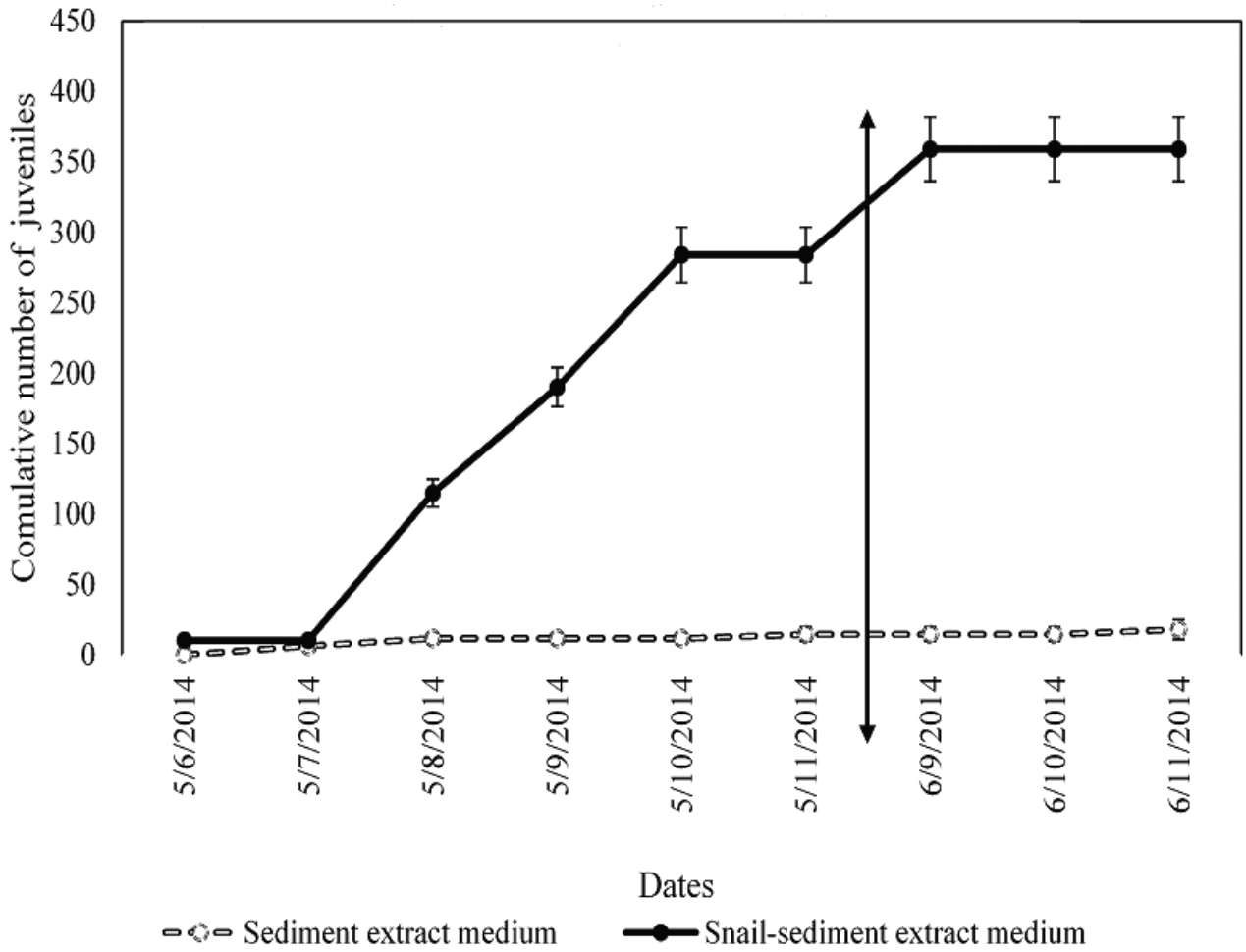

Fig. 3. Cumulative reproduction rate in sediment and snail-sediment extract media. The two-headed vertical arrow indicates about a period of 1 month gap of no more juveniles in both media followed by 19% and 21% juvenile production in sediment extract (control) and snail-sediment extract medium, respectively.

responded differently to the different rearing media in terms of its body length, width, yield and penetration rate of infective juveniles.

It has been reported that the hatching period for *Tobrilus gracilis* is from April to July with one generation a year (univoltine) (Pehofer, 1989). Furthermore, *Tobrilus* sp. has been reported as having a long developmental period which may reach 12 months (Tahseen, 2012). This information coincides with the reported cessation of the juvenile production in the present study. Similar studies (Radwin & Rouse, 1990) reported cessation of production of *Panagrellus redivivus* in a period from 20 to 53 days, depending on the rearing media used. The observed discontinuation of juvenile production in the present study within a period of 1 month (the two-headed vertical arrow in Fig. 3) may indicate that not all nematodes were mature enough to reproduce within this period of time.

An increase in biomass of more than 3× for both adult and juvenile worms in the snail-sediment extract medium is in itself an encouragement to use this culture medium for *Tobrilus* spp.

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A.E.A. Abada. Развитие и плодовитость бентосной пресноводной нематоды *Tobrilus* sp. (Andrássy, 1959) на среде из донных осадков и тканей моллюсков.

Резюме. Бентосные нематоды *Tobrilus* sp. играют существенную роль в экосистемах. Проведено изучение развития и размножения этих нематод на среде, содержащей очищенные донные осадки и ткани пресноводных моллюсков в сравнении со средой, состоящей лишь из автоклавированных донных осадков. Первая среда содержала автоклавированную смесь донных осадков и гомогенат пресноводных улиток *Lanistes carinatus* Olivier, 1804 в соотношении 1:1. На среде с тканями моллюсков рост нематод был более активным, как у взрослых нематод, так и у личинок, что выражалось в большей длине и ширине тела, общей массе и скорости размножения. Предполагается, что такие повышенные показатели роста определяются более высокими концентрациями азота и магния в среде, содержащей ткани моллюсков. Среда рекомендована для массового размножения пресноводных нематод *Tobrilus* sp.