Occurrence of two distinct lineages of the freshwater jellyfish *Craspedacusta sowerbii* (Hydrozoa: Limnomedusae) in Italy

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ABSTRACT

The freshwater jellyfish *Craspedacusta sowerbii* Lankester 1880 is a cryptic cosmopolitan invasive species, which occurs in all continents except Antarctica. Recent molecular studies suggest the existence of at least three very different genetic lineages of *Craspedacusta*: the "*sowerbii*", the "*kiatingi*", and the "*sinensis*" lineages. We report the presence of both medusae and polyps of this alien taxon in the Large Lake of Monticolo / Montiggl, a meso-eutrophic natural lake in the Province of Bolzano / Bozen in Northern Italy. Molecular analyses of mitochondrial 16S sequences showed that this population belongs to a different lineage than that recently described for Sicily (Southern Italy). Therefore, there are two different genetic lineages of *C. sowerbii* in Italy. In the Large Lake of Monticolo / Montiggl medusae were observed in 6 consecutive summers (2015 – 2020), from July to September. All the examined medusae were males. The stomach content analyses showed that zooplanktonic copepods and cladocerans with size range between 0.3 and 0.8 mm were the preferred prey of medusae. Polyps of *C. sowerbii* were recorded in the lake on the zebra mussel *Dreissena polymorpha* in shallow water and on the underside of artificial substrates. The analyses of zebra mussels would therefore be a simple method to check for the presence of the polyp stage of *C. sowerbii* in various aquatic environments.

INTRODUCTION

The first description of the freshwater jellyfish *Craspedacusta sowerbii* (Cnidaria: Hydrozoa: Limnomedusae) was published after its discovery in a water lily tank in Regent's Park, London, England in 1880 (Lankester, 1880). The number of species in the genus *Craspedacusta* is still disputed (Jankowski *et al*., 2008). As in other hydromedusae, their high morphological plasticity makes it difficult to identify diagnostic traits that can be reliably used to differentiate species. Molecular markers like 16S is thus an essential tool to analyze in the study of jellyfish invasions. Recent molecular studies suggest the existence of at least three very different lineages of *Craspedacusta*: the "*sowerbii*", the "*kiatingi*", and the "*sinensis*" lineages (Fritz *et al*., 2009; Zou *et al*., 2012; Karaouzas *et al*., 2015; Schifani *et al*., 2018; Oualid *et al*., 2019). The hydromedusa reported as *C. sowerbii* has been asserted to be native to the Yangtze River system in China (Kramp, 1961), but a conclusive, global phylogeographic study has never been made. It is a cosmopolitan invasive taxon, occuring in all continents except Antarctica (Dumont, 1994; Jankowski, 2001; Jankowski *et al*., 2008). After its discovery in England, the taxon was recorded in numerous other European countries (Arbačiauskas and Lesutienė, 2005; Lundberg *et al*., 2005; Fritz *et al*., 2007; Karaouzas *et al*., 2015; Minchin *et al*., 2016; Ciutti *et al*., 2017; Medina–Gavilán and González–Duarte, 2018), in North America (Acker and Muscat, 1976; DeVries, 1992), in Central America (Moreno-Leon and Ortega-Rubio, 2009), in South America (Failla Siquier *et al*., 2017; Caputo *et al*., 2018), in Asia (Bekleyen *et al*., 2011; Gasith *et al*., 2011; Lewis *et al*., 2012); in Africa (Rayner, 1988; Oualid *et al*., 2019), in Australia (Thomas, 1950) and in New Zealand (Boothroyd *et al*., 2002).

In this paper, we report biological observations on *C. sowerbii* made in a Northern Italian lake, including genetic data showing that this population belongs to a different lineage than one from Sicily (Southern Italy) recently described in Schifani *et al*. (2018).

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METHODS

Study area

The Large Lake of Monticolo/Montiggl (from 46°25’16” to 46°25’37”N and from 11°17’16” to 11°17’41”E) is located at 492 m asl in the Bolzano Province, Italy (Fig. S1A). It has a surface area of 178,400 m^2 and a volume of about 1,490,000 m^3. Its maximum length is 757 m, its average width is 236 m and its maximum water depth is about 12.5 m (Thaler and Tait, 1981a) (Fig. S1B). It is a natural lake of glacial origin. The unique surface tributary of the lake is the effluent of the overlying Small Lake of Monticolo/Montiggl (22 m altitude difference), which is located about 400 meters away (Fig. S1C). The tributary is only active after heavy rainfall. The water supply of the Large Lake depends mainly on rain and underground springs. The unique effluent (Angelbach) is located on the southern shore. The Small Lake of Monticolo/Montiggl (from 46°25’41” to 46°25’50”N and from 11°17’38” to 11°17’50”E) is a natural glacial lake and is located at 514 m asl. The lake has a surface area of 52,100 m^2 and a volume of about 517,000 m^3. Its maximum length is 316 m, its average width is 165 m and its maximum water depth is 14.8 m (Thaler and Tait, 1981b). Tributaries are absent and the water supply depends on rain and underground springs.

The Large Lake and the Small Lake of Monticolo/Montiggl are classic dimictic lakes (Thaler and Tait, 1987). Normally, from the end of December until the end of February or beginning of March the surfaces of the lakes are covered by ice. In summer, there is thermal stratification of the water with a thermocline at a depth of about 5 m and the temperature of the epilimnion reaches 24-26°C.

Tab. 1 contains the most important chemical and physical water parameters of the first five meters of depth from 2015 to 2019 of Large Lake of Monticolo/Montiggl. Chemical data classify the lake as a meso-eutrophic water body.

The Large Lake of Monticolo/Montiggl has a huge number of different rotifer species. The most common species in the period from 2010 to 2015 with an average individual density above 10 individuals per liter include: *Ascomorpha ecaudis*, *Filinia terminalis*, *Gastropus stylifer*, *Hexarthra mira*, *Kellicottia longispina*, *Keratella cochlearis* and *Polyarthra dolichoptera*. The following crustaceans were found during the same period: *Alona affinis*, *Bosmina longirostris*, *Bosmina longispina*, *Ceriodaphnia quadrangula*, *Cyclocypris ovum*, *Cyclops strenuus*, *Cyclops vicinus*, *Daphnia longispina*, *Diaphanosoma brachyurum*, *Eudiaptomus gracilis* and *Mesocyclops leuckarti* (source database of the Biological Laboratory, Agency for Environment and Climate Protection of the Autonomous Province Bolzano South Tyrol, Italy, 2020).

The alien invasive zebra mussel *Dreissena polymorpha* (Pallas, 1771) has been present in the lake for at least 20 years (Morpurgo and Thaler, 2002) and is currently very abundant.

Tab. 1. Main chemical and physical water parameters of the first 5 m of depth in Large Lake of Monticolo/Montiggl in the years 2015-2019.

Parameter	Unit	2015	2016	2017	2018	2019
max Temp	°C	26.1	24.6	25.9	25.8	25.2
max pH		8.59	8.64	8.59	8.54	8.58
Conductivity	μS cm^{-1} 20°C	317	308	303	297	300
MRP	μg L^{-1}	1	2	2	3	3
TP	μg L^{-1}	20	33	17	16	14
TOC	mg L^{-1}	5.9	6.0	6.1	5.7	5.7
NNO_3	μg L^{-1}	21	22	17	14	20
NNH_4	μg L^{-1}	123	170	110	65	109
TFe	μg L^{-1}	27	23	35	30	30
Chl a	μg L^{-1}	4.1	2.7	1.4	4.2	2.6
SO_4	mg L^{-1}	8.90	9.35	10.78	10.71	10.65
min O_2	mg L^{-1}	7.33	5.32	7.82	7.57	4.70
max O_2	mg L^{-1}	9.43	12.33	9.71	9.89	10.31
TN	μg L^{-1}	537	602	547	467	510

max Temp, maximum temperature; max pH, maximum pH; MRP, mean molybdate reactive phosphorus; TP, mean total phosphorus; TOC, mean total organic carbon; NNO_3, mean nitrate nitrogen; NNH_4, mean ammonium nitrate; TFE, mean total iron; Chl a, mean chlorophyll a; SO_4, mean sulfate; min O_2, oxygen minimum; max O_2, oxygen maximum; TN, mean total nitrogen.

Sampling

This study was carried out between August 2015 and September 2020. In this timeframe, 30 sampling and visual census scuba dives were performed in the Large Lake of Monticolo / Montiggl for collection and abundance estimation of *C. sowerbii* medusae. In total, 63 specimens were gently collected by hand with wide-mouth glass jars with screw-on lids or with transparent plastic bags by a scuba diver during daylight hours from the water-column between 1 and 6 m of depth. During the scuba dives, water temperature in Celsius degrees (±1°C) and depth in meter (± 0.1 m) were measured by a scuba dive computer Suunto Vyper. A medusa of *C. sowerbii* voucher specimen, collected on the 30th of July 2017 and fixed in ethanol 70%, was deposited in the zoological collection of the Museum of Nature South Tyrol, Bolzano / Bozen, Italy (*C. sowerbii* collection number NMS EVV5). Two specimens of *C. sowerbii* medusae were collected on the 22nd of July 2018 and were fixed in ethanol 96% for molecular analyses. A total of 30 medusae were collected for sex determination on the 9th, the 11th and the 18th of August 2019. In the laboratory they were maintained alive in aquarium at 24-25°C for about three weeks. The jellyfish were fed live and frozen cyclopoid copepods. Within three days of their collection, tiny biopsies of fresh gonad tissue were taken from live medusae. The sex of medusae was determined by observing these tiny fresh gonadal tissues with the use of a microscope at magnification 100 × and 400 × as reported in the literature (Pérez-Bote *et al*., 2006; Lundberg *et al*., 2007; Xu and Wang, 2009). Photographs of medusae gonads were taken under a Leica DMR microscope with objective HCX PL Fluotar 40x/0.75, PH 2.

For the analyses of stomach contents, 30 medusae were collected: 15 specimens on the 13th of September 2019 and 15 specimens on the 19th of September 2019. Immediately after sampling they were fixed in ethanol 70%. In the laboratory, the medusae were dyed with pink colored ethanol 90% to facilitate the visual search for prey. The umbrella diameter of the medusae was measured putting graph paper under the petri dish containing the medusae and stomachs were dissected using tweezers under a stereo microscope Olympus SZX 12 at a 90-fold magnification. Prey was identified and counted under a bright-field microscope Olympus BX40 at magnification 400 ×.

To estimate medusae abundance, the scientific diving visual census technique for pelagic organisms was used, in accordance with Heine (2011). Underwater timed transects were performed swimming with scuba diving equipment at a constant depth (between 2 and 3 m) and a constant speed for a specific length of time (60 minutes). All counted medusae were recorded underwater on a slate with indication of depth and water temperature of observation point. During the visual census dives the underwater visibility was variable between approximately 2 and 5 m.

According to the literature (Stanković and Ternjej, 2010), we searched for polyps of *C. sowerbii* on zebra mussels *D. polymorpha* in the lake. On the 14th August 2017, in total 24 zebra mussels were collected randomly by hand by a scuba diver in the Large Lake of Monticolo/Montiggl. The zebra mussels were between 15 and 25 mm long. Twelve specimens were collected at a depth of 1 m. The zebra mussels were in the shade, attached to bottom side of a step of the ladder of a wooden pier. Twelve zebra mussels were collected from a rock at a depth of 5 m.

To determine the presence of *C. sowerbii* polyps by qualitative analysis, on the 2nd of June 2018 and on the 25th of April 2019, zebra mussels were also collected in the Small Lake of Monticolo/Montiggl at a depth between 1 and 1.5 m from the underside of the steps of a ladder attached to a wooden pier. All zebra mussel samples were gently placed into 0.5 L glass jars filled to approximately 70% of capacity with lake water. In the laboratory, the samples were kept in the jars without lids at room temperature (24-25°C) and with a cycle of 14 hours of light and 10 hours of darkness. Within 10 days after the sampling date, the live zebra mussels were then examined on all sides under a stereomicroscope, at magnifications between 20 × and 45 ×, to ascertain whether there were polyps present, as reported in the literature (Stanković and Ternjej, 2010; Duggan and Eastwood, 2012). The polyps were not fed during the days in the laboratory. The microscopic photographs of polyps were taken under a stereomicroscope Leica M205 A.

Molecular analyses

The methods for obtaining about 600 bp of the mitochondrial 16S gene as well as maximum likelihood analyses are given in Schuchert (2014, 2018, 2019). In addition to the two medusae samples from Large Lake of Monticolo/Montiggl, we obtained a tissue sample of a female medusa *Craspedacusta sowerbii* from Sicily kindly provided by Federico Marrone and Luigi Naselli-Flores, University of Palermo (Italy). The animal originated from the same pool and the same population described by Schifani *et al*., (2018). The resulting partial 16S sequences have been deposited in GenBank under the accession numbers MT107153 to MT107155. For our comparisons we retrieved aligned further 16S sequences of *Craspedacusta* species from GenBank and ran a maximum likelihood analysis using the substitution model HKY+G proposed by Modeltest. As outgroup we choose a sequence of *Limnocnida tanganjicae* (EU293972), the most similar known sequence not derived from a *Craspedacusta* species (comp. Oualid *et al*., 2019). The origin of the material used to determine the sequences in GenBank is unfortunately not always given in the metadata. Where possible, we tried to obtain the missing ones by contacting the authors (sequences EU293971, EU293974, and KY077294) or inferred them from the publications they were used in (see also Oualid *et al.*, 2019).

RESULTS AND DISCUSSION

Molecular analyses

The two medusae from the Large Lake of Monticolo/Montiggl had two identical mitochondrial 16S sequences (Fig. 1), while the one from Sicily was quite different from them and clustered in a separate clade (clade 2 in Fig. 1). The medusae from Lake of Monticolo/Montiggl had the same sequences as hydroids from two locations found in Switzerland and differed only in one position from two other sequences, one from Germany and one from Morocco. The medusa sample from Sicily (see Material and Methods) clustered in a second clade (Fig. 1), together with geographically widely separated samples originating from the Americas to China. This separation for the mitochondrial 16S (Fig. 1) into two separate clusters has also been observed for the mitochondrial COI gene and the nuclear ITS marker (Fritz *et al.*, 2009; Zhang *et al.*, 2009; Karaouzas *et al.*, 2015; Schifani *et al.*, 2018; Fuentes *et al.*, 2019; Oudalid *et al.*, 2019). Our clade 1 (Fig. 1) corresponds to the "*kiatingi*" cluster, clade 2 to the "*sowerbii*" cluster of Fritz *et al*. (2009) and Schifani *et al*. (2018). Some of the taxonomic implications are discussed in Oudalid *et al.* (2019) and also the other works mentioned above. The new evidence on the potential presence of more than one invasive lineage in the genus *Craspedacusta* is an emergent aspect which has been little discussed until now and which deserves more attention. Although the concordance of mitochondrial and genomic lineages suggests that two distinct *Craspedacusta* species could be present in Europe and also that they may be spreading to new freshwater bodies around the world (Karaouzas *et al*., 2015; Fuentes *et al*., 2019), the available data are still preliminary and not conclusive. A similar situation was observed in Schuchert 2014 for the marine hydroid *Plumularia setacea.* As discussed in that work, it is desirable to search for places were both lineages occur in sympatry, allowing the application of a biological species concept. As there are two lineages present in Italy, one in the very north and the other in Sicily, it is possible that there is a contact zone anywhere between these two localities where both lineages co-occur.

Polyps

In August 2017, polyps of *C. sowerbii* were recorded in the Large Lake of Monticolo/Montiggl on the shells of zebra mussels *D. polymorpha* collected at a depth of 1 m (Tab. S1). The polyps were between 0.6 and 1.0 mm high (Fig. S2-A). On the zebra mussels collected at a depth of 5 m no polyps of *C. sowerbii* were found. In June 2018, 15 polyps (7 individual polyps and 4 colonies of two polyps) were recorded on one 20 mm long zebra mussel collected in the Small Lake of Monticolo/Montiggl at 1 m depth at 21°C water temperature. In April 2019, in this lake polyps of *C. sowerbii* were found again on zebra mussels collected at a depth of 1.5 m at 14°C water temperature (Tab. S1). From the year 1946 (Stefanelli, 1948), the occurrence of *C. sowerbii* has been documented in at least 40 freshwater ecosystems in Italy, but almost exclusively based on observations of the medusa stage and not of the polyp stage (Ramazzotti, 1962; Rossi and Lodi, 1971; Badino and Lodi, 1972, Cotta Ramusino, 1972; Trentini, 1993; Stefani *et al.*, 2010; Groppali, 2013; Morpurgo and Alber, 2015; Ciutti *et al*., 2017; Schifani *et al*., 2018). Probably, the species may have a far wider distribution in Italy (Ciutti *et al*., 2017) than previously thought due to often-overlooked polyps (Duggan and Eastwood, 2012).

Stanković and Ternjej (2010) described the association between polyps of *C. sowerbii* and the zebra mussel *D. polymorpha* as commensalism. The above-mentioned authors believe that the polyps of *C. sowerbii* benefit from *D. polymorpha,* as the water current created by the bivalve feeding brings food directly to the polyps. Polyps of *C. sowerbii* prefer areas without heavy sedimentation and surfaces without heavy algal growth (Acker and Muscat, 1976; Duggan and Eastwood, 2012). In the present study, polyps of *C. sowerbii* were detected on shells of *D. polymorpha* without algae or detritus, attached in shade to the underside of the steps of submerged stairs (this position prevents sediment accumulation) in shallow water (between 1 and 1.5 m depth). The polyps were found in the two lakes on zebra mussels in April, June and August with water temperature from 14°C to 24°C. Considering the wide distribution of *D. polymorpha* in Italy (Cianfanelli *et al*., 2010), the analyses of these bivalves could be a good method to check for the presence of polyps of *C. sowerbii* in various aquatic environments. The search for the polyps on zebra mussels can be carried out even when the water temperature is below 19°C, while medusae are usually observable when the water temperature is between 19°C and 30°C (Acker and Muscat, 1976; DeVries, 1992).

Underwater visual census of medusae

In August 2015, medusae of *C. sowerbii* were recorded for the first time in the Large Lake of Monticolo/Montiggl (Morpurgo and Alber, 2015) (Fig. S2B). In the following five years 2016-2020, from mid-July to the beginning of October with water temperature from 19°C to 26°C medusae were sighted in the lake. The number of observed medusae in 60 minutes of scuba diving ranged from 1 to a maximum of 36 specimens in September 2019 (Fig. 2). The increase in jellyfish numbers in summer 2019 compared to other years could be related to the prolonged period of epilimnion water temperature at 25-26°C. Experiments in laboratory have proven that 26°C is the optimal temperature for medusae growth (Folino-Rorem *et al*., 2016). The meteorological summer 2019 in the Bolzano Province, Italy, was characterized by particularly high temperatures (3°C more than the average of the temperatures of the century 1901-2000), ranking third in the historical series that dates back to 1850, just behind the record summers of the years 2003 and 2015 (Peterlin *et al*., 2019). Production of medusae is related to increased water temperatures, although other environmental factors may also be important such as abundance of food and water quality (Acker and Muscat, 1976; DeVries, 1992).

Although in 2019 up to 36 medusae were observed in

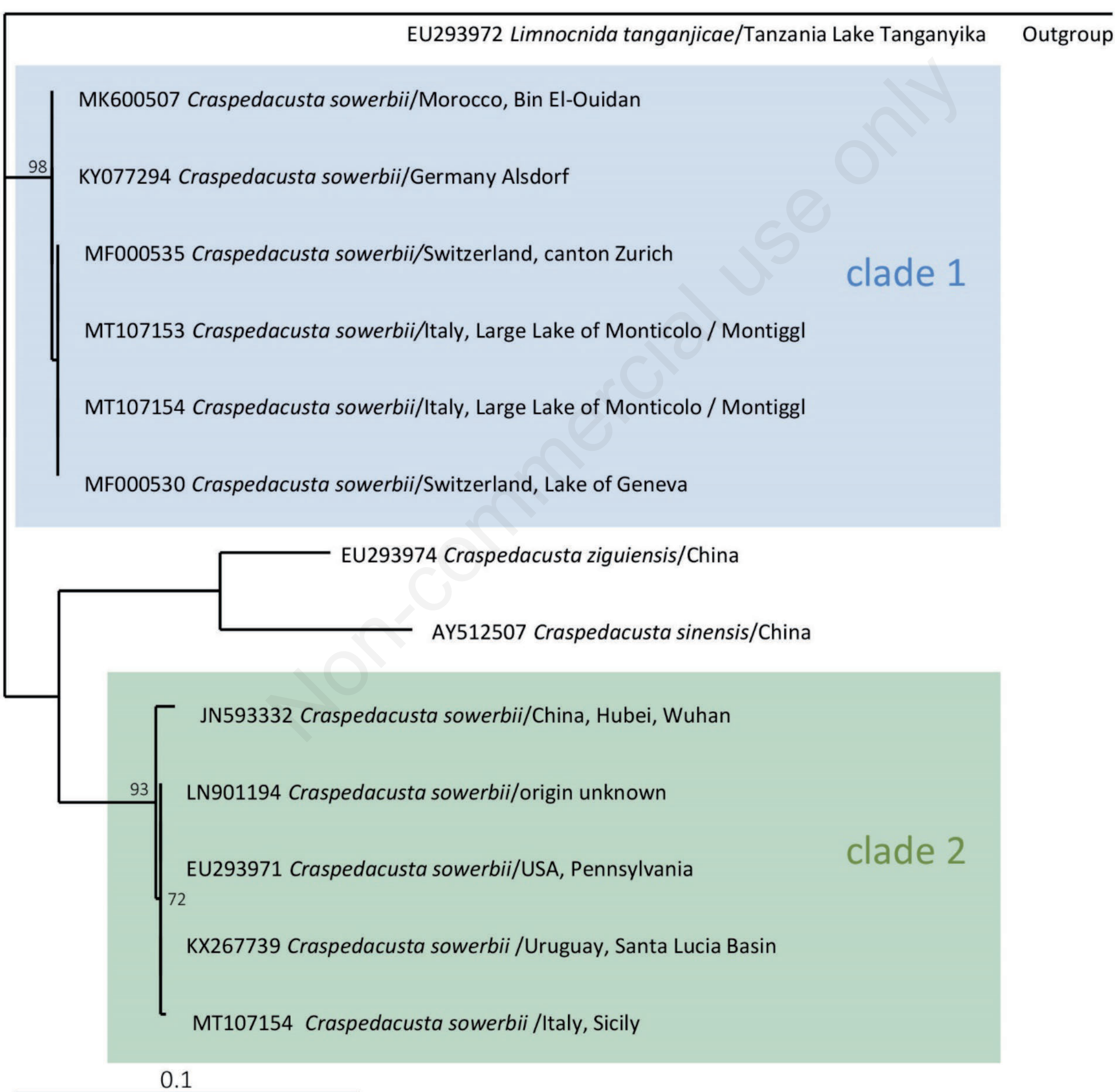

Fig. 1. 16S maximum likelihood phylogenetic tree of *Craspedacusta* species (medusae or polyps) obtained with PhyML (HKY+G model) and based on 610 bp positions of the mitochondrial 16S gene. Node-support values are bootstrap values of 100 pseudoreplicates (shown only if >70%). For more review, see text. The taxon legend consists of the EMBL accession code followed by the species name and specimen origin.

the Large Lake of Monticolo/Montiggl in 1 hour of underwater visual census, this is a relatively small abundance and could not be considered as a real jellyfish bloom. These data correspond to a density of much less than one individual per square meter or per cubic meter. Freshwater jellyfish blooms with very high densities are reported in the literature: Arbačiauskas and Lesutienė (2005) observed up to 20-30 individuals per square meters in an old water-filled gravel pit in Lithuania. In Germany, Dumont (1994) reported medusae densities of up to 30 individuals per cubic meter in an artificial quarry reservoir near Ulm and Jankowski (2000) counted even over 400 individuals per square meter in a eutrophic pond near Aachen.

In the Large Lake of Monticolo/Montiggl, medusae were observed in 6 consecutive summers from 2015 to 2020 with water temperature from 19°C to 26°C, while other studies in Italy typically report the record of a single year or the sightings of 2 or at most 3 years (Ciutti *et al*., 2017). The present study confirms that scuba diving represents a good method for monitoring and sampling of freshwater jellyfish, as reported in the literature (Fritz *et al.*, 2007; Oualid *et al.*, 2019).

Sex determination of medusae

The 30 medusae collected in August 2019 (water temperature 26°C) for sex determination had umbrella diameters between 12 and 20 mm and had four mature gonads. All the examined medusae were males. Under microscope at magnification 400 × the gonad tissue of a medusa male was recognizable as a uniform tissue of small cells. At the borders of the uniform tissue numerous mobile spermatozoa were identifiable (Fig. S2C). The results of gonads analyses indicate that the *C. sowerbii* population of Large Lake of Monticolo/Montiggl most probably consists of males only. Usually, outside China single sexed populations are observed, and the sexual reproduction seems infrequent (Acker and Muscat, 1976; DeVries, 1992). Some studies report unisexual populations of only female medusae (Trentini, 1993; Pérez-Bote *et al*., 2006; Minchin *et al*., 2016), whereas in other aquatic ecosystems only males were recorded (Lankester, 1880; Boothroyd *et al*., 2002; Lundberg *et al*., 2005; Lundberg *et al*., 2007).

Stomach contents of medusae

The analysis of stomach contents showed that crustaceans were the preferred prey of medusae of *C. sowerbii* at the time of investigation (Tab. S2). The 30 examined medusae had umbrella diameters between 10 and 16 mm. The percentage of medusae containing prey was 66.7%. In total, 51 zooplanktonic prey were found in the stomachs. The number of preys for each medusa varied from 1 to 6. The most frequent prey in the stomachs of medusae were the cladoceran *Bosmina longispina* (size: 0.4±0.1 mm, n=11) and cyclopoid copepods (size:

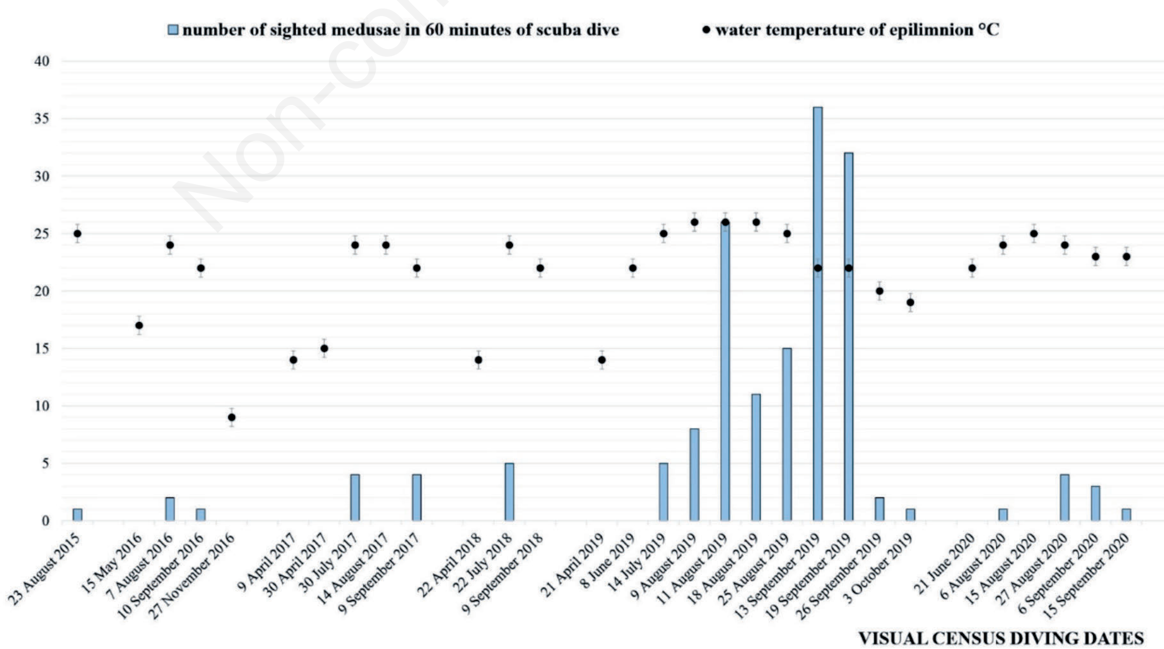

Fig. 2. Underwater sightings of *Craspedacusta sowerbii* medusae in the Large Lake of Monticolo/Montiggl during scuba dives in years 2015-2020. The number of sighted medusae in 60 min of a visual census dive is reported with indication of the water temperature of epilimnion (between 1 and 5 m of depth) for each diving date.

0.6±0.1 mm, n=12). The size range of prey was mostly between 0.3-0.8 mm. The smallest was one single rotifer *Keratella cochlearis* (0.1 mm). In accordance with other studies (Dodson and Cooper, 1983; Spadinger and Maier, 1999; Pérez-Bote *et al.*, 2006; Smith and Alexander, 2008; Stefani *et al.*, 2010), the number of copepods and cladocerans exceeded the number of rotifers in all stomachs of medusae, although the individual density of rotifers in the pelagic zone was always higher than that of crustaceans in the years from 1981 to 2015 (source database of the Biological Laboratory, Agency for Environment and Climate Protection of the Autonomous Province Bolzano South Tyrol, Italy, 2020).

CONCLUSIONS

In the natural Large Lake of Monticolo/Montiggl medusae were observed in 6 consecutive summers from 2015 to 2020 with water temperature from 19°C to 26°C. Polyps of *C. sowerbii* were recorded in the lake on the zebra mussels *D. polymorpha* in shallow water and on the undersides of artificial substrates. The polyp and medusa stages are only rarely reported together in the same aquatic ecosystem (Failla Siquier *et al*., 2017). Considering the commensalism between polyps of *C. sowerbii* and *D. polymorpha* described by Stanković and Ternjej (2010), the analyses of zebra mussels could be a simple method to check for the presence of *C. sowerbii* in various aquatic environments. Even though sightings of jellyfish are rare, this species may be more widespread in Italy than previously thought (Ciutti *et al.*, 2017), because the small polyps tend to go unnoticed (Duggan and Eastwood, 2012). The rather simple collecting method via zebra mussels could also be used to examine a larger number of water bodies in order to identify the genetic lineages present in it. As there are now two distinct genetic lineages known to occur in Italy, perhaps places can be found where both co-occur. This would allow an easier evaluation of their species status. A good sampling strategy would be to investigate first populations in middle Italy and the southern tip of the peninsula. According to the lineages found, the subsequent sampling regions can then be narrowed down.

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Ethical standards

The authors declare that this study complies with the current Italian and Swiss laws.

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