

ECOLOGY OF MEDITERRANEAN
CRYPTOBENTHIC FISH FAUNA: LESSONS
LEARNT FROM CLINGFISHES
(GOBIESOCIDAE)

Domen Trkov

Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia

Supervisor: Prof. Dr. Lovrenc Lipej, Jožef Stefan International Postgraduate School
and Marine Biology Station Piran, National Institute of Biology, Ljubljana, Slovenia

Evaluation Board:

Asst. Prof. Dr. Al Vrezec, Chair, Jožef Stefan International Postgraduate School and
National Institute of Biology, Ljubljana, Slovenia

Assoc. Prof. Dr. Marcelo Kovačić, Member, Natural History Museum, Rijeka, Croatia

Prof. Dr. Milena Horvat, Member, Jožef Stefan Institute and Jožef Stefan International
Postgraduate School, Ljubljana, Slovenia

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL



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EKOLOGIJA SREDOZEMSKJE KRIPTOBENTOŠKE
RIBJE FAVNE: PRIDOBMLJENA SPOZNANJA NA
PRIMERU RIB PRISESNIC (GOBIESOCIDAE)
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*To all the fish and other animals that lost their lives so that we
could study their ecology and thus contribute to knowledge and
understanding of the environment on which we depend.*

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Abstract

Cryptobenthic fishes are an important part of the fish community whose role has often been overlooked in the past. Due to the lack of knowledge, cryptobenthic fish species have been considered as rare and not numerous in the past. However, recent studies show quite the opposite, as due to their high abundance in the environment, they are very important and they are able to shape the ecosystem. Despite their importance, the cryptobenthic fish fauna of the Adriatic, as well as the Mediterranean Sea in general, is poorly known, especially regarding their ecology. The purpose of the thesis was to review the occurrence of the cryptobenthic fish fauna in the Slovenian part of the Gulf of Trieste (North Adriatic) and to deepen the knowledge on the ecology of the Gobiesocidae family which is one of the least known fish families in the Mediterranean Sea.

In the present study, a total of 12 cryptobenthic fish species belonging to four fish families were confirmed in the Slovenian sea: Gobiidae, Gobiesocidae, Blenniidae and Clinidae. Three species (*Chromogobius quadrivittatus*, *Odondebuenia balearica*, *Thorogobius ephippiatus*) were observed for the first time in Slovenian territorial waters. The family Gobiesocidae was represented by three species: *Lepadogaster lepadogaster*, *L. candolii* and *Apletodon incognitus*.

We have shown that the studied species have different habitat preferences, as well as depth distribution. Species of the genus *Lepadogaster* occurred mainly on the rocky bottom, where *L. lepadogaster* was found in the mediolittoral and upper infralittoral, and *L. candolii* occurred mainly deeper in the infralittoral. Both species occurred beneath the stones under which pebbles appear as a substrate. *A. incognitus* occurred deeper than the other two species, and its occurrence is related to the seagrass meadows where it inhabited mostly empty shells of oysters. Seashells were occupied also by *L. candolii*, which in small proportion occurred in seagrass meadows too.

Fish nests were also found in shelters, which were observed in *L. lepadogaster* from the end of March to the end of May and in *A. incognitus* from mid-May to mid-July. The nest of *L. candolii* was observed only once in May.

For the purpose of studying clingfish diet, a method based on faecal pellets has been developed. Diet study has shown that all three species are opportunists whose most important prey are crustaceans. Benthic copepods, amphipods and decapods were the most important prey groups.

Clingfishes are also important intermediate and final hosts of different parasites. In total, three groups of external parasites were found on the clingfishes: parasitic larvae of the family Gnathiidae (Isopoda) and parasitic copepods of the families Caligidae and Lernaepodidae. Internal parasites were represented mainly by flatworms (Trematoda).

The research provides important information on the occurrence of cryptobenthic fish species of the Slovenian sea and basic data on the ecology of the three clingfishes. With this knowledge we would like to contribute to the understanding of the ecology of neglected species whose role in the ecosystem is far from negligible but of utmost importance.

Povzetek

Kriptobentoške ribe so pomemben del ribje združbe, katerih vloga je bila v preteklosti mnogokrat spregledana. Zaradi pomanjkljivega znanja so bile kriptobentoške ribje vrste v preteklosti obravnavane kot redke in maloštevilne. Novejše raziskave pa kažejo ravno obratno, saj so kriptobentoške vrste zaradi svoje velike številčnosti v okolju zelo pomembne in posledično lahko vplivajo na ekosistem. Kljub njihovi pomembnosti pa je kriptobentoška ribja favna v Jadranskem kot tudi v Sredozemskem morju slabo poznana, predvsem kar se tiče njihove ekologije. Namen raziskave je bil pregled kriptobentoških vrst rib, ki se pojavljajo v slovenskem delu Tržaškega zaliva (Severni Jadran), in poglobitev znanja o ekologiji rib priselnic iz družine Gobiesocidae, ki so ena izmed najslabše poznanih ribjih družin v Sredozemskem morju nasploh.

V pričujoči raziskavi je bilo v slovenskem delu tržaškega zaliva skupno potrjenih 12 vrst kriptobentoških vrst rib, ki so pripadale štirim družinam: Gobiidae, Gobiesocidae, Blenniidae in Clinidae. Tri vrste (*Chromogobius quadrivittatus*, *Odondebuenia balearica*, *Thorogobius ephippiatus*) so bile v slovenskih teritorialnih vodah opažene prvič. Družina Gobiesocidae je bila zastopana s tremi vrstami: *Lepadogaster lepadogaster*, *L. candolii* in *Apletodon incognitus*.

Študija ekologije treh vrst priselnic je pokazala, da imajo vrste različne habitatne preference kot tudi globinsko razporeditev. Vrsti iz rodu *Lepadogaster* se tako pojavljata predvsem na kamnitem dnu, kjer najdemo vrsto *L. lepadogaster* v mediolitoralu in zgornjem infralitoralu, medtem ko se vrsta *L. candolii* pojavlja predvsem globlje v infralitoralu. Obe vrsti se pojavljata pod kamni, pod katerimi se kot podlaga pojavljajo prodniki. Vrsta *A. incognitus* se pojavlja najgloblje, njeno pojavljanje pa je vezano na morske travnike, kjer poseljuje predvsem prazne lupine ostrig. Poleg nje se v morskih travnikih v manjšem številu pojavlja tudi vrsta *L. candolii*, ki za skrivališča prav tako izkorišča lupine mrtvih školjk.

V skrivališčih rib priselnic so bila najdena tudi gnezda, ki so bila pri vrsti *L. lepadogaster* opažena od konca marca do konca maja, pri vrsti *A. incognitus* pa od sredine maja do sredine julija. Gnezdo *L. candolii* je bilo opaženo le enkrat v maju.

Za namen preučevanja prehrane rib priselnic smo razvili nedestruktivno metodo, ki temelji na proučevanju vsebine ribjih iztrebkov. Analiza prehrane je pokazala, da so vse tri vrste oportunisti, katerih najpomembnejši plen so raki. Bentoški kopepod, postranice in raki deseteronožci so bile najpomembnejše skupine rakov, najdenih v prehrani priselnic.

Analiza prisotnosti zajedalcev je pokazala, da so priselniki tudi pomembni vmesni in končni gostitelji različnih vrst zajedalcev. Skupno so bile na priselnikih najdene tri skupine zunanjih zajedalcev: zajedalske ličinke iz družine Gnathiidae (Isopoda) ter zajedalski kopepod iz družin Caligidae in Lernaepodidae. Notranji zajedalci so bili zastopani predvsem s strani ploskih črvov (Trematoda).

Raziskava tako prinaša pomembne informacije o pojavljanju kriptobentoških vrst rib slovenskega morja in podatke o ekologiji treh vrst priselnic. S pridobljenim znanjem želimo tako doprinesti k poznavanju in razumevanju ekologije spregledanih vrst, katerih vloga v ekosistemu še zdaleč ni zanemarljiva, temveč izrednega pomena.

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Abbreviations

3D	...	three-dimensional
B	...	relative gravimetric composition
<i>e.g.</i>	...	exempli gratia
<i>etc</i>	...	et cetera
F	...	frequency of occurrence
H'	...	Shannon-Wiener diversity index
ind.	...	individuals
IRI	...	index of relative importance
ITD	...	index of trophic diversity
MBS	...	Marine Biology Station
MPA	...	marine protected area
n	...	number of specimens
N	...	numerical abundance
RDA	...	redundancy analysis
SA:V	...	surface area to volume ratio
TROPHs	...	trophic level of species

Glossary

“Cryptobenthic” species – species whose “small body size permits exploitation of restricted habitats, where food and shelter are obtained in, or in relation to, conditions of substrate complexity and/or restricted living space, with a physical barrier likely to be interposed between the small fish and sympatric predators (Miller, 1979)”

“false” faecal pellets – faecal pellets which were made only of peritrophic membrane but with no content

anaemia – a condition marked by a deficiency of red blood cells or of haemoglobin in the blood, resulting in pallor and weariness

bioconstructor – organisms that create 3D structure (*e.g.* limestone structures) and offer potential living niches to other organisms. Such structures remain after the death of bioconstructor

biocoenosis – an assemblage of different organisms forming a closely integrated community

biometry – the process of measuring the external shape and dimensions of living organisms

boulder field – arrangement of sandstone boulders and mostly rectangular shaped stones normally in one layer on bedrock

carotenoids – any of a class of mainly yellow, orange, or red fat-soluble pigments

circalittoral – it extends from the end of the infralittoral to a depth where at least 0.01% of the incident light is still present and where autotrophic multicellular algae still thrive

coralline ground – the ground consisting of dead coral parts or limestone remains of algae

cryptic – kept out of sight; concealed

destructive sampling technique – technique which removes the organism from a particular area and has a harmful effect on the investigated organism

epibenthic fishes – fish species which occur on the surface of the substrate where they are more or less passive

eurycious – having a wide range of habitats

haemorrhagia – bleeding

hypertrophy – the enlargement of an organ or tissue from the increase in size of its cells

hypoxia – deficiency in the amount of oxygen

ichthyocide – a chemical that is toxic to fish and causes their death

immunosuppressive effect – effect suppressing the immune response of an individual

infralittoral – a true submerged sea belt that reaches to a depth where 1% of the incident light is still present and coincides with the boundary where seagrass or green algae still thrive. In the Slovenian sea, this boundary varies between 8 and 12 m depth

larvae – the active immature form of an organism

lesion – a region in an organ or tissue that has suffered damage through injury or disease

macrohabitat – a habitat of a sufficient extent to present considerable variation of environment, contain varied ecological niches, and support a large and usually complex flora and fauna

mediolittoral – sea belt under the influence of waves. In addition to the tides, the water level is also affected by winds and air pressure. Organisms inhabiting the tidal belt are subject to periodic alternation of emersion and immersion

meiofauna – benthic organisms which pass through 0.5 mm sieve and are retained on a 45 µm sieve

melanin – a dark brown pigment (= substance that gives colour) found in skin

melanocytes – a mature melanin-forming cell, typically in the skin

meristic – area of ichthyology which relates to counting quantitative features of fish, such as the number of fins or scales

biometrical – technical term for body measurements and calculations

microhabitat – a small area which differs somehow from the surrounding habitat. Its unique conditions may be home to unique species that may not be found in the larger region

nectobenthic fish – fish which live above the sea bottom

non-destructive method – a method that does not cause the death of the studied organism

peritrophic membrane – a semi-permeable, non-cellular structure which surrounds the food bolus in an organism's gut

pycnocline – a layer in an ocean or other body of water in which water density increases rapidly with depth

sciaphilic – shade-loving organism that grows/lives only in shady habitats

sliding dredge – a type of trawl for catching demersal organisms

supralittoral – it is the first sea belt, which is never submerged below the water surface, but is moistened by the spray formed during the breaking of the waves. Living conditions in this zone are very extreme, so it is inhabited by quite a few organisms

Chapter 1

Introduction

1.1 Definition of the Problem

Ichthyofauna is an important part of coastal marine biodiversity, which affects the ecosystem functions and is also an important marine resource for humans (Holmlund & Hammer, 1999). An important part of coastal fish communities is also cryptobenthic fish fauna. “Cryptobenthic” (Miller, 1979) species are those whose “small body size permits exploitation of restricted habitats, where food and shelter are obtained in, or in relation to, conditions of substrate complexity and/or restricted living space, with a physical barrier likely to be interposed between the small fish and sympatric predators” (Figure 1.1). The qualitative definition of cryptobenthic fishes (Depczynski & Bellwood, 2003) and the recent quantitative definition of cryptobenthic reef fishes (Brandl, Goatley, Bellwood, & Tornabene, 2018) were based exclusively on fish size (smaller than 5 cm), while Kovačić, Patzner & Schliewen (2012) suggested that cryptobenthic refers to benthic positioning, since it is ecologically meaningful. Lipej, Orlando-Bonaca & Mavrič (2016) divided cryptobenthic fish species into real cryptobenthic species (that are always hidden) and large cryptobenthic fish species (large-sized fish species that occasionally leave their hiding places, but more or less permanently use them). Exhibiting such cryptic life style affords them protection against predators (Hofrichter & Patzner, 2000). Therefore, they are hardly observed by divers and are usually not detected in conventional ichthyofaunal surveys (Henriques et al., 2002). Smith-Vaniz, Jelks & Rocha (2006) concluded that around 64% of fish fauna can be overlooked by visual census due to their hiding habits. Therefore, knowledge about the occurrence and ecology of cryptobenthic fishes remains very fragmented at a global scale. However, it is very difficult to carry out sampling on mobile animals such as fish in heterogenic rocky habitat, therefore the use of anaesthetic in such studies is very much needed (Gibson, 1967). Use of anaesthetic enables to expel cryptobenthic fishes from hiding places and catch them (Gibson, 1967).



Figure 1.1: Cryptobenthic fishes are small and exploit restricted habitats, so they are rarely observed. The photo shows a specimen of *Lepadogaster lepadogaster* hiding among the stones (Photo: D. Trkov).

Recent studies have shown that cryptobenthic fish species are not rare as previously assumed (Miller, 1986), but rather common (Glavičić, Paliska, Soldo, & Kovačić, 2016; Hofrichter & Patzner, 2000; Patzner, 1999). Ackerman & Bellwood (2000) proved that cryptobenthic fishes from tropical reefs, due to their high abundance, have the potential to influence the ecosystem. Recently many authors report the importance (*e.g.* energy transfer) of cryptobenthic fish species in tropical seas (*e.g.* Ackerman & Bellwood, 2000; Ackerman & Bellwood, 2002; Depczynski & Bellwood, 2003), while there is still a lack of knowledge of cryptobenthic fish species from the Adriatic Sea and their ecology (Hofrichter, 1995; Hofrichter & Patzner, 2000).

Cryptobenthic fish species in the Adriatic Sea have attracted scientific interest in the last two decades, as evidenced by various studies (*e.g.* Brandl, Wagner, & Patzner, 2012; Glavičić et al., 2016; Kovačić, 1997, 2005; Kovačić et al., 2012; Santin, 2008; Santin, Willis, & Abbiati, 2005). Some papers highlighting the occurrence of cryptobenthic species have also been published for the Slovenian part of the Adriatic Sea, such as those of Lipej & Richter (1999), Lipej, Mavrič & Orlando-Bonaca (2012), Lipej et al. (2016), Lipej, Orlando-Bonaca & Richter (2005), Orlando-Bonaca & Lipej (2008), Orlando-Bonaca & Lipej (2007) and Orlando-Bonaca & Trkov (2016). Moreover, knowledge about cryptobenthic fish assemblages is increasing due to the development of new approaches and techniques associated with SCUBA diving and suitable fish sampling (Glavičić & Kovačić, 2016). However, knowledge of cryptobenthic fish species in the Slovenian sea is still very insufficient.

Cryptobenthic fish species mostly occur in the costal part of the sea and are consequently affected by environmental disorders (the influence of the tide, wave, *etc.*), which is also reflected in their ecology (Hofrichter, 1995). Understanding of ecological factors is essential for the development of an effective protection and species management programme. Knowledge of species habitat preferences is essential, since it gives us basic information about the suitability of the environment for a particular species (Cañadas, Sagarminaga, De Stephanis, Urquiola, & Hammond, 2005).

Besides knowing the habitat preferences, feeding habits are also a very important part of animal ecology. Knowledge of feeding habits gives us information on ecological processes at the individual, population and community level (Sánchez-Hernández, Servia, Vieira-Lanero, & Cobo, 2013). Studies on closely related sympatric fishes are often difficult, especially when dealing with the feeding ecology of coastal species. Understanding such feeding relationships is very important, since it could reveal different strategies to reduce competition such as resource partitioning (Velasco, Gómez-Cama, Hernando, & Soriguer, 2010). Factors related to ingestion and assimilation can affect the condition, growth, survival and dispersal of species (Nunn, Tewson, & Cowx, 2012). This is why a successful protection and management plan is dependent on basic knowledge of ecological factors known to affect feeding habits.

The great majority of diet-related studies in fish are based on the examination of stomach content; however, the major deficiency of this method is that the studied animals have to be killed and dissected. Thus, for many studied species, a high number of animals have to be sacrificed (Kamler & Pope, 2001; Sánchez-Hernández et al., 2013), which in some areas can cause major changes in the population structure of fish species (Hartleb & Moring, 1995; Light, Adler, & Arnold, 1983). The possible negative impacts of overcollection on populations of *L. lepadogaster* were highlighted already by King, (1989). In the case of endangered species, species of high commercial value or species with low population density, the sacrifice of specimens might be disapproved by the public (Baker & Fraser, 1976; Crossman & Hamilton, 1978; Haley, 1998; Light et al., 1983). At the same time, such methods are banned in marine protected areas (MPAs) and for species protected by law it is hard and not likely to get the license to perform studies using lethal methods. Taking into account that more than half of the natural Slovenian coastline with 200 m of coastal sea belt is protected and belongs to MPAs (Vidmar & Turk, 2011), the use of non-destructive sampling methods is essential. However, in order to deal with these issues, researchers are trying to discover new non-lethal techniques. The most commonly used methods are based on the collection of stomach content samples, such as the use of gastroscopy (Dubets, 1954), tubes (White, 1930), suction of stomach content (Robertson, 1945), stomach flushing (Hyslop, 1980), emetics (Markus, 1932), removal of stomach content using forceps (Wales, 1962), and X-ray analysis (Stobbs, 1980). The efficiency of non-lethal methods strongly depends on the species, age and size, and also the size of the prey (Kamler & Pope, 2001). The development of a new non-destructive method for the study of fish diet is therefore essential in terms of ethics to use in protected areas, as well as outside of them.

Besides knowing ecological factors, meristic and biometrical analyses are also very important, which give us direct information about the fish specimens. Biometrical analyses enable us to compare different populations, identification of gender and age structure, and also other aspects of population dynamics (Kohler, Casey, & Turner, 1996). Relation between length and weight is very useful for population studies, since it enables us to get information on the population structure, sexual maturity, longevity, mortality and reproduction (Beyer, 1987; Bolger & Connolly, 1989; Diaz, Roa, Garcia, Acero, & Navas, 2000; Fafioye & Oluajo, 2005; King, 1996).

The aims of the study are:

- To present all available information about the occurrence of cryptobenthic fish species (including new unpublished data), their habitat preferences and depth distribution in Slovenian coastal waters.

- To provide information on ecology, with special emphasis on habitat preferences, depth distribution and diet habits, of clingfish (family Gobiesocidae), which is one of the poorest studied cryptobenthic fish families in the Mediterranean Sea (Hofrichter & Patzner, 2000; Patzner, 1999), and also in the Adriatic Sea.
- To explore reproductive ecology of *L. lepadogaster*.
- To test the diet of the *L. lepadogaster* clingfish with a non-destructive method, which does not necessitate the sacrifice of fish specimens. Application of a non-destructive method on other clingfish species to test their feeding habits.

The littoral coastal clingfish *L. lepadogaster* was used to test this diet assessment method because: a) there are some available data of *L. lepadogaster* feeding ecology published by Gibson (1972), King (1989), Mazé (2007), Velasco et al. (2010) and Wilson (1981), b) this clingfish species inhabits shallow coastal waters, usually above 1 m of depth (Hofrichter & Patzner, 2000), consequently it is possible to collect them by snorkelling and not only by SCUBA diving, c) the species has a short digestive tract (Hofrichter, 1993) and consequently rapid digestion (*personal observation*), which is important since the method is based on faecal pellets.

1.2 Cryptobenthic Fish Species in the Adriatic Sea

As different definitions of cryptobenthic fish species are available (Brandl et al., 2018; Depczynski & Bellwood, 2003; Kovačić et al., 2012), different authors state different numbers of cryptobenthic fish species in literature. In this study, cryptobenthic fish refers to benthic positioning as proposed by Kovačić et al. (2012). Among the cryptobenthic fishes in the Adriatic Sea, we mainly include fish species from four families: Gobiidae, Gobiesocidae, Blenniidae and Clinidae. Gobiidae is the most numerous fish family in the Adriatic Sea, which contains 47 species (Kovačić, Lipej, & Dulčić, 2020), followed by Blenniidae with 16 marine species (Lipej, Orlando-Bonaca, & Makovec, 2008). The number of cryptobenthic species of these two fish families varies depending on different studies (*e.g.* Kovačić et al., 2012; Santin, 2008; Santin et al., 2005). Gobiesocidae contain eight species in the Adriatic Sea, while Clinidae only one and all of them are considered as cryptobenthic (Kovačić et al., 2020; Wagner et al., 2017).

Clingfishes (family Gobiesocidae) are typical representatives of coastal fish assemblages. They are one of the poorest known groups of cryptobenthic fish species in the Mediterranean Sea (Hofrichter & Patzner, 2000; Patzner, 1999), therefore the main part of this research is dedicated to this family. Clingfishes are poorly known due to their small size, cryptic way of life, good camouflage and a small number of ichthyologists working on this family (Brandl et al., 2011). Furthermore, specimens are not possible to collect with common methods (*e.g.* nets or baited traps) (Brandl et al., 2011). Consequently, biological and ecological studies of clingfishes are scarce (Hofrichter, 1995).

Clingfishes are small fish, most of them smaller than 10 cm, with a flattened body which is scaleless (*sensu* Brandl et al., 2011; Hofrichter, 1993). They are characterized by an adhesive sucking disc and reduced swim bladder (Brandl et al., 2011; Briggs, 1955). Adhesive sucking disc, as one of the most characteristic features, is formed by pelvic fins on which papillae are located, and it allows specimens to cling on smooth as well as on rough surfaces (Wainwright, Kleinteich, Kleinteich, Gorb, & Summers, 2013). In addition, clingfishes are not adapted to constant swimming, as their major locomotor muscles are white myotomes, which serve for short but high speed moments (Davison, 1985). The

sucking disc therefore acts as a morphological aid in resisting dislodgment among strong water movements and could serve also during spawning (Davison, 1985; Hofrichter & Patzner, 2000). However, the sucking disc develops in the larval stage and its development is in correlation with the passage to the sea bottom (Russel, 1976). As larvae are free swimming, they have also developed a swim bladder (Allen, 1983). Another characteristic of the family Gobiesocidae is that they do not have a stomach (Harder, 1975).

Three sympatric species of clingfishes have been recorded to date in the Slovenian coastal sea, namely, *Lepadogaster lepadogaster* (Bonnaterre, 1788), *L. candolii* (Risso, 1810) and *Apletodon incognitus* Hofrichter & Patzner, 1997 (Lipej & Orlando-Bonaca, 2020; Lipej et al., 2005). However, none of them had been appropriately investigated in the studied area in terms of their ecology.

L. candolii can reach up to 8 cm. It looks like flatted tadpole with somewhat triangular head. From related species *L. lepadogaster* can be distinguished by a long-based dorsal fin which is well separated from the tail (Louisy, 2015). The body colour is mostly greenish-brown, sometimes purplish with variable markings. Smaller specimens often have a white band between eyes (Hofrichter & Patzner, 2000). The species is present in the Eastern Atlantic coast from England to Senegal, in Madeira and Canary Islands and throughout the Mediterranean (Henriques et al., 2002).

L. lepadogaster is a small clingfish (less than 8 cm) with a long snout and “duck-billed” appearance. The body colouration is green-brown with dark spots and two blue eye spots on the top of the head (Hofrichter & Patzner, 2000; King, 1989). *L. lepadogaster* is distributed in the Mediterranean, the Eastern Atlantic, at least as far north as the extreme north-west of Galicia, south to north-west Africa, the Canary Islands and Madeira Islands (Henriques et al., 2002). It is the most common species of European clingfishes (Wheeler, 1969).

A. incognitus can reach up to 4.1 cm in total length. For the species it is characteristic that the dorsal fin is short-based, with four to seven rays, and first anal fin ray is located under the first or second dorsal fin ray (Hofrichter & Patzner, 1997; Louisy, 2015). The body colour is very variable and depends on the substrate. Specimens are usually green-brown or reddish with a crisscrossed irregular pattern (Hofrichter & Patzner, 2000). It is distributed in the northern part of the Mediterranean Sea including the Adriatic Sea and in the eastern Atlantic at the Azores islands (Bilecenoglu & Kaya, 2006; Hofrichter & Patzner, 1997).

Hypotheses:

- In the Slovenian part of the Gulf of Trieste, there are different cryptobenthic fish species, which are widespread along the entire Slovenian coast.
- Three cryptobenthic fish species from the family Gobiesocidae *L. lepadogaster*, *L. candolii* in *A. incognitus* have different habitat preferences and depth distribution range.
- By using non-destructive methods, it is possible to effectively investigate the diet habits of clingfish species.
- Due to their cryptic way of life and restricted area of distribution, clingfishes (family Gobiesocidae) are considered as opportunistic predators.
- Three species of clingfishes, *L. lepadogaster*, *L. candolii* and *A. incognitus*, are segregated in ecological niches in terms of their diet and habitat preferences.

Chapter 2

Material and Method

2.1 Study Area

The study was conducted in the Slovenian part of the Gulf of Trieste, the northernmost part of both the Adriatic and the Mediterranean Sea. The Gulf of Trieste is a shallow gulf (20-25 m) isolated from the rest of the northern Adriatic and restricted by transect from Grado to Savudrija peninsula. It occupies an area of about 500 km² (Ogorelec, Mišič, & Faganeli, 1991). The Gulf of Trieste lies on the junction of Istrian carbonate platform and Karst, and Friuli plain in the west. It is mostly influenced by freshwater inflow, bottom sediment resuspension and increasing pollution (Ogorelec et al., 1991). The coast of the Gulf of Trieste consists of abyssal flysch cliffs, with deeper rocky undersea and submerged valleys, which are distinctively flat. In the southern part of the Gulf of Trieste, there are Bays of Piran, Koper and Muggia, which are wide submerged valleys of small rivers Dragonja, Rižana and Rosandra, respectively. In northern part of the gulf, there is Panzano Bay, which is formed by the River Isonzo (Soča). Rivers are an important source of detrital material in the sediment, which originates from hinterland. In the aforementioned bays, surficial sediments consist of clayey slit with approximately 30% carbonate, while the western part of the gulf consists of up to 70% carbonate shells of various marine organisms (Ogorelec et al., 1991). Along the Istrian coast, detrital sediments originate mainly from erosion of Paleogene flysch, while in northern part, River Isonzo is the main source of detrital sediments (Ogorelec et al., 1991). Isonzo river is the main freshwater source, which has a significant impact on seasonal plankton (autotrophic plankton and zooplankton) dynamics. In addition, autotrophic plankton biomass evolution is followed by consumer community patterns (Mozetič, 1998).

Salinity in the gulf is typical marine, but under the influence of freshwater inflows. It ranges from 33 to 38.5 ‰ in surface layer, while at the bottom it ranks from 36 to 38.5 ‰. Water temperature normally varies between 8 and 24° C in the surface and between 8 and 20° C in the bottom layer (Ogorelec et al., 1991). In winter, the water column is characterized by vertical homogeneity. In spring, increased freshwater inflow and heating of the surface layer lead to the phenomenon of pycnocline, which intensifies during summer and is broken down by autumnal cooling and wind mixing (Malačič, 1991). Strong density stratification in late summer is associated with hypoxia in bottom waters (Ogorelec et al., 1991). Freshwater inflows and wind patterns (Bora) affect the circulation of the entire Adriatic. General water circulation in the Gulf of Trieste is affected by the anticlockwise northern Adriatic circulation system, but because of the shallowness, it is mostly under the influence of tides and local dominant winds (Ogorelec et al., 1991). Sea-water level in the gulf can rise up to 1 m because of south-westerly winds and high atmospheric pressure (Ogorelec et al., 1991).

The coast of the Slovenian part of the Gulf of Trieste is approximately 46 km long (Lipej, Turk, & Makovec, 2006). The deepest part is located in front of the Cape of Piran (33 m). Sea-bed of the Slovenian sea is predominantly soft sedimentary bottom of fluvial origin, while the coastal bottom is mostly rocky, consisting mainly of Eocene Flysch layers, while in inside bays, soft sedimentary bottom prevails (Ogorelec et al., 1991). In the past decades, the Slovenian coastal area has suffered from many anthropogenic impacts such as new infrastructure, intensive fishing, sewage outfalls and mariculture (Turk, 1999). Most of the flysch cliffs have retained their natural characteristic, while coastal plains are under high anthropogenic impacts (Lipej et al., 2006). Most degraded is the riparian zone of supralittoral and mediolittoral, while negative impacts are observed also in the upper infralittoral; therefore, only 18% of the coastline is still in its natural state (Turk, 1999). However, 10% of the total length of the Slovenian coastline is included into MPAs, while only 0.4% of the Slovenian sea is covered by MPAs (Vidmar & Turk, 2011).

2.2 Data Collection

The information about cryptobenthic specimens' occurrence was obtained from: a) existing published literature sources (Lipej et al., 2005; Lipej & Richter, 1999; Orlando-Bonaca, 2006; Orlando-Bonaca & Lipej, 2007, 2008a; Orlando-Bonaca & Trkov, 2016), b) targeted field sampling of cryptobenthic fish fauna performed from July 1998 to March 2019, and c) targeted field sampling of clingfish (Gobiesocidae) fauna performed from October 2016 to March 2019.

2.2.1 Field work

Sampling of cryptobenthic fish specimens was performed from July 1998 to March 2019 at different localities in the Slovenian part of the Gulf of Trieste. All sampling surveys were carried out in coastal waters. Specimens were collected mostly by snorkelling or SCUBA diving in mediolittoral and infralittoral belts. Additionally, some specimens were also collected in shallow waters and tide pools during low tide. The main types of habitat, in which sampling was performed, were (Figure 2.1): a) boulder field – arrangement of sandstone boulders and mostly rectangular shaped stones normally in one layer on bedrock extending from mediolittoral to around 10 m; b) banks of pebbles – occurring mostly in protected bays, where two main types of pebbles were present. Sandstone pebbles were more flat, while limestone pebbles were rounded. Pebbles were arranged in more layers, where larger pebbles (3-10 cm) were on the surface, while smaller pebbles (1-3 cm) were in deeper layers. Banks of pebbles were present mostly in mediolittoral and upper infralittoral; c) seagrass meadow – meadows of *Cymodocea nodosa* (Ucria) Ascherson, 1870 and *Posidonia oceanica* (Linnaeus) Delile, 1813 where fish were searched in rhizomes (matte) and seashells in particular; d) sandy-muddy bottoms.

Fish were searched in different hiding places such as under stones, boulders, shells, empty date mussel (*Lithophaga lithophaga*, (Linnaeus, 1758)) burrows or inside natural cavities, such as caves, cavities, holes, clefts etc. After sampling, stones and rocks, which have been checked for the presence of cryptobenthic fishes, were placed in their former position, in the sense that we did not cause damage to the natural habitat.

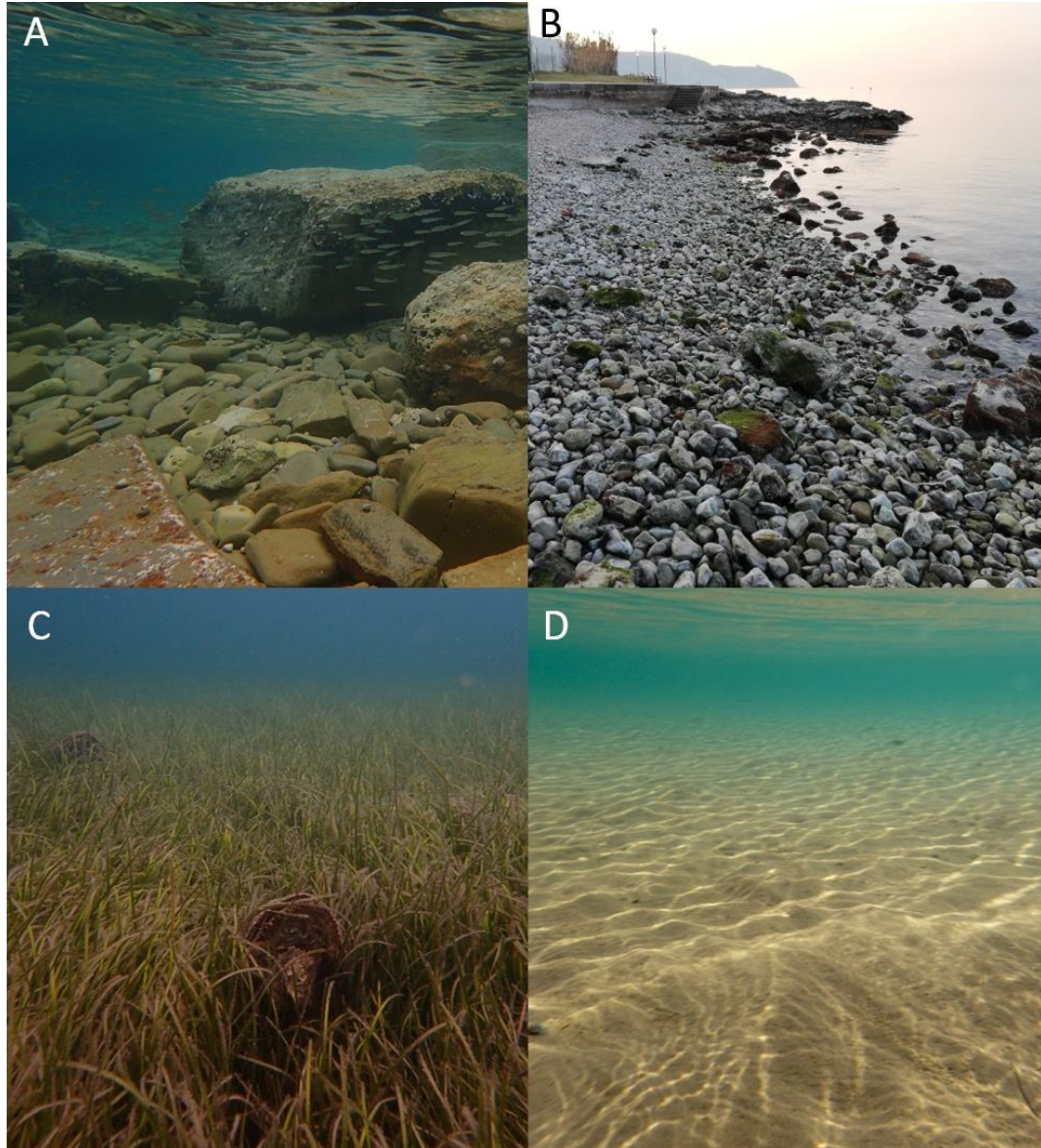


Figure 2.1: Four main types of habitat in which sampling was performed: A – boulder field, B – bank of pebbles, C – seagrass meadow of *Cymodocea nodosa*, D – sandy-muddy bottom (Photos: D. Trkov).

2.2.1.1 Fish collection

All specimens belonging to the family Gobiesocidae were collected to study their diet and biometry. Other cryptobenthic fish specimens, which could not be identified on the field, were taken for further analysis, while others were recorded and then released. Cryptobenthic fish specimens were caught and stored in 100 ml plastic chambers, with small holes on the cover that provided fresh water with oxygen, and small enough to prevent defecated faecal pellets to pass through. To facilitate the collection of fish, the narcotic Quinaldine (Sigma-Aldrich) was used (Lipej et al., 2005, for specific sampling of cryptobenthic fish fauna and clingfish fauna). Quinaldine was diluted to 1:15 solution with alcohol (Gibson, 1967; Patzner, 1999). The narcotic was sprayed into the hiding place using a laboratory wash bottle. The anaesthetized fish were then caught with a hand net. Specimens of *L. lepadogaster* were collected by lifting stones and catching them with the hand net ($d = 40$ cm). Specimens, which were attached to the lower surface of the stone,

then dropped themselves right into the net. Specimens of *A. incognitus*, which were spotted in the oyster shells, were caught by placing a plastic bag over the oyster shell (Figure 2.2). In case of clingfish species, hiding places were also checked, if there were nests inside them, which were subsequently also photographed with an Olympus TG-4 camera. Endolithic specimens were expelled from the burrows with the help of a thin stick, and caught using a plastic bag placed over the burrow opening, as proposed by Kotrschal (1988). This method was used to assess blennioid fish assemblages in Slovenian coastal waters by Orlando-Bonaca (2006) and Orlando-Bonaca & Lipej (2007, 2008a). In this way, the use of anaesthetic was not necessary. We also performed sampling of banks of pebbles in the mediolittoral and upper infralittoral, where pebbles were sampled with a bucket and checked for fish on sieve (Wagner et al., 2019), while seagrass meadows were sampled with a small sliding dredge and a cube method (Hofrichter & Patzner, 2000). By using a cube method, a top- and bottomless cube of 50 x 50 x 50 cm was placed on the bottom of seagrass meadow and quinaldine was squirted inside the cube, while anaesthetised animals were then caught with a hand net.

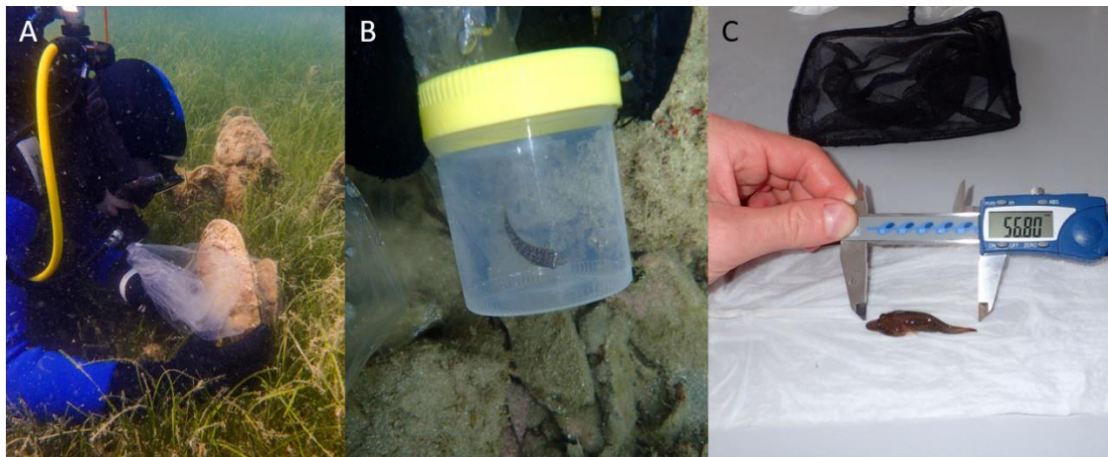


Figure 2.2: A – fish collecting with a plastic bag (Photo: L. Lipej); B – collected fish in a plastic chamber, with small holes on the cover that provided water with oxygen (Photo: D. Trkov); C – measuring the clingfish on a wet paper towel that kept the fish moist (Photo: D. Trkov).

2.2.1.2 Habitat census

Basic ecological data on the sampling locality were obtained at each site. We collected the following data: date, location, macrohabitat (*e.g.* seagrass meadow, rocky area, sedimentary bottom), microhabitat (*e.g.* cluster of stones, noble pen shell with dead oysters), hiding place (*e.g.* under stone, shell of dead oyster) and depth of finding. In case where the hiding place was a stone, we measured its length, width and height and collected data on algal and sponge overgrowth, and substrate under the stone. When sampling was done in mediolittoral, the depth was calculated based on the depth of the sampling point and zero point from tide amplitude calendar (Ličer, Fettich, & Jeromel, 2017, 2018, 2019).

2.2.1.3 Fauna sampling

For the purpose of the diet study, samplings of benthic macroinvertebrates were carried out in 2018 and 2019. We sampled most frequently used shelters by particular fish species. Under stones inhabited by *L. lepadogaster* 10 sample squares (25 x 25 cm) were sampled in cold season and 10 sample squares in warm season, while under stones inhabited by *L. candolii* 10 sample squares were collected only in warm season, as they were caught mostly

at that time. Macroinvertebrates were collected together with the substrate (*e.g.* pebbles, sand) with a hand net (20 x 20 cm, mesh size 500 µm) in the fish shelter within a square 25 x 25 cm. Substrate and macroinvertebrates were then transferred in plastic bags to the Marine Biology Station (National Institute of Biology). As shelters of *A. incognitus* are associated with *Pinna nobilis* Linnaeus, 1758, which is critically endangered species and its populations are declining (Kersting et al., 2019), faunistic sampling in shelters of *A. incognitus* was not performed.

2.2.2 Laboratory work

After sampling, fish were transferred as soon as possible (in less than one hour after the sampling was completed) to the Marine Biology Station (MBS) where they were photographed with Olympus TG-4, cautiously measured to the nearest 0.01 mm with a calliper and weighed (wet weight) to the nearest 0.01 g using a Sartorius TE 1502S balance. Measurements of body parts, such as length of nasal outgrowths and urogenital papillae, were done under Olympus SZx16 stereomicroscope with an Olympus DP74 camera. Special attention was devoted to the condition of clingfishes that were always kept moist during the measurements. Specimens were also checked for external parasites, such as parasitic copepods. Twenty-two specimens of *A. incognitus* from the cryptobenthic collection of the MBS were dissected and examined under stereomicroscope in order to examine the presence of internal parasites.

Specimens of the family Gobiidae, which were impossible to be identified in the field, were euthanized with an overdose of Quinaldine and preserved in 70% EtOH. In order to study fish scales and head lateral line system of Gobiidae and Gobiesocidae (from ichthyological collection), specimens were reversibly stained in 2% solution of Cyanine Blue and distilled water, as proposed by Saruwatari, López & Pietsch (1997). After 120 seconds of staining, excess dye was washed away with distilled water and lateral line system was checked under stereomicroscope. Fish were identified to species level by using identification keys of Jardas (1996), Kovačić (2008) and Marčeta (1999). All collected specimens were labelled and stored in the cryptobenthic collection of the MBS.

In case of clingfishes, *L. lepadogaster* and *A. incognitus* specimens were carefully checked since there are other related species (*Lepadogaster purpurea* (Bonnaterre, 1788), *Diplecogaster bimaculata* (Bonnaterre, 1788) and *Apletodon dentatus* (Facciola, 1887)) which are closely related and very similar based on overall appearance. *L. lepadogaster* is very similar to a broadly sympatric species of *L. purpurea*, which is also present in the Adriatic Sea (Wagner et al., 2017). For proper identification of the species, special attention has to be given to morphological traits such as size and number of papillae on the sucking disc, size and head-marks and eyespots and body colouration as proposed by Henriques et al. (2002) and Wagner et al. (2017).

A. incognitus is very similar to related species of *A. dentatus* and *D. bimaculata*, which are also present in the northern Adriatic (Brandl et al., 2011). In order to properly identify the species, morphological traits, such as the number of lacrymal pores and mandibular canals, were checked as proposed by Brandl et al. (2011), Hofrichter (1995) and Hofrichter & Patzner (1997). For identification purpose meristic counts of fin rays on preserved specimens of *A. incognitus* were done as well.

A few specimens of *L. lepadogaster*, *L. candolii* and *A. incognitus*, were placed in aquarium at MBS where they were observed to facilitate the understanding of their feeding habits and behaviour patterns. The aquariums dimensions were 150 x 50 x 50 cm (length x width x height) and they were arranged in order to mimic the natural habitat of the fishes.

2.2.2.1 Diet study

2.2.2.1.1 Pre-experiments

For the study of the clingfish diet, we tested a new method which is based on the analysis of faecal pellets. The method was first tested on the species *L. lepadogaster*. Previously, we observed some specimens of *L. lepadogaster* in buckets (10 l) with aerators to verify the process of defecation. Specimens were fed and then observed in order to assess how much time they need to produce the faecal pellets.

On 16 specimens of *A. incognitus*, it was also checked how many faecal pellets they produce within 24 hours if they have food available all the time. As food, three days old nauplia of *Artemia* sp. were used, which were bred from purchased eggs (Artemio Pur, JBL). Specimens were kept in black chambers (11 x 13 x 14 cm) with aerators used for the process of defecation (Figure 2.3).

We also carried out some tests to verify if all ingested prey can be identified in faecal pellets. While specimens of *L. lepadogaster* did not feed normally under experimental conditions, we tried to ascertain it on six specimens of closely related clingfish species *L. candolii*. Specimens were kept in chambers used for defecation and feed with two specimens of four different crustacean taxa (*Pisidia* sp., *Athanas nitescens* (Leach, 1813), *Eualus* sp. and Gammaridea). Faecal pellets were then collected within 24 hours after feeding and analysed.

2.2.2.1.2 Method used for the diet study

The method was firstly tested on *L. lepadogaster* and was then used also on *L. candolii* and *A. incognitus*. After measurements and weighing, each freshly caught clingfish was placed into a 11 x 13 x 14 cm chamber with filtered seawater (125 µm) supplied by an aerator (Figure 2.3). The aerator was placed above the bottom to prevent defragmentation of faecal pellets. After every use, the chambers were disinfected with 70% EtOH in order to avoid the transmission of diseases and parasites.

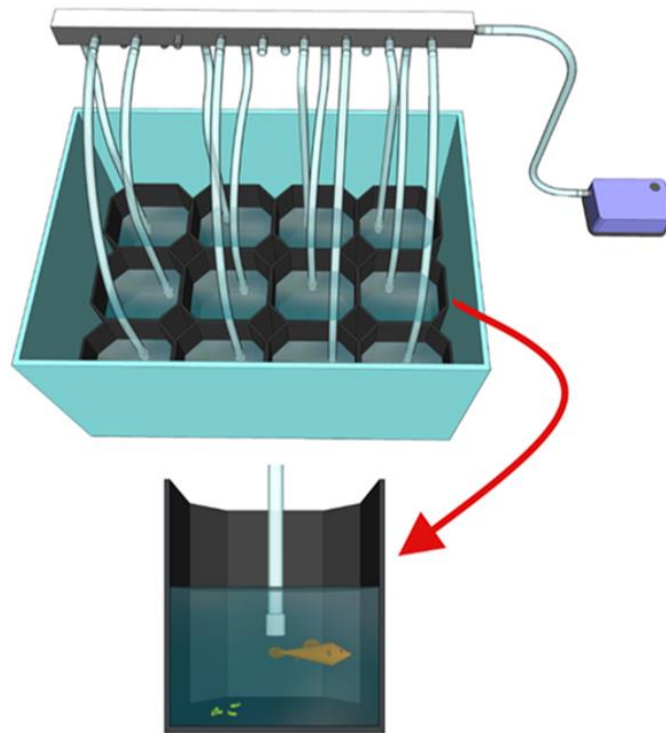


Figure 2.3: Box designed for collecting faecal pellets. It contains 12 smaller chambers in which fish were placed, each equipped with filtered water and an aerator. Faecal pellets were subsequently collected from the bottom of the chamber (Illustration: T. Makovec).

Freshly caught clingfishes were left in the chambers for 24 hours. On every three to four hours, specimens were checked if they had produced faecal pellets. The pellets were carefully removed from the bottom of the chambers with a modified pipette and were fixed in 70% alcohol solution. Pellets consist of a peritrophic membrane and the internal part with undigested prey items. In certain cases, the pellets were broken and the content of the whole chamber was filtered through 125 μm mesh size plankton net and checked. After defecation, the clingfish specimens were released at the site where they were collected. Due to a small sample of freshly caught *A. incognitus* examined with a non-destructive method, we include also eight specimens from MBS collection whose intestine (as they do not have a stomach) content was searched by dissection.

The contents of the faecal pellets and intestines were analyzed under an Olympus stereomicroscope and photographed with a camera. Some faecal pellets were also weighed (70% alcohol wet weight) with Sartorius CP 225D scale. Prey items were determined to the lowest taxa possible to be identified and counted. A large proportion of digested prey items were whole or almost whole, while digested prey broken up into smaller parts was recognized by typical body parts such as carapace. The most digested prey items, such as polychaetes and amphipods, were identified by their jaws. The wet weight (from specimens conserved in 70 % ethanol) of the most important prey (*e.g.* crabs) was calculated from length-weight correlation curves obtained from animals collected in the sampling area. Undigested prey and prey that only occasionally appeared in the faeces, or prey whose weight could not be calculated from the correlation curves, were weighed directly from the faecal pallets, or was used the average weight of it. Average weights of the animals were obtained from animals collected in the sampling area (*e.g.* harpacticoid copepods).

Substrate collected in fish shelters was checked for macroinvertebrates which could be potential prey for clingfishes. Macroinvertebrates were determined to major taxa and

counted. The most important prey taxa were also measured and weighed (Olympus SZx16 stereomicroscope with an Olympus DP74 camera; Sartorius CP 225D scale; wet weight from 70 % ethanol) for the purpose of obtaining length-weight correlation curves used in the diet study.

2.3 Data Analysis

Most of the data were processed with the Microsoft Excel programme, while also some statistical programmes were used to obtain important statistical information. As data of fish findings were collected with different samplings, some data were incomplete and were not included in analyses.

2.3.1 Sampling locations and occurrence of cryptobenthic fish species

Sampling locations of cryptobenthic fish species were grouped based on the sampling method: locations investigated with SCUBA diving equipment (infralittoral) and locations investigated with snorkelling and manual collecting (upper infralittoral and lower mediolittoral). All samplings of a particular sampling method within 100 m radius were grouped together in one sampling location.

In order to present the data on cryptobenthic fish occurrence, the Slovenian sea was divided into 73 quadrants (2 x 2 km) according to the approach of Lipej et al. (2018). The coastal area considered in this study comprised 24 quadrants. On the maps for single species, increasing dot size denotes the increasing number of specimens found within a quadrant.

In order to assess the habitat preference of fish, the redundancy analysis (RDA) method was performed. This method is a directional gradient analysis based on linear models with a short length gradient. For each fish specimen was determined in which microhabitat it was found and as environmental variables were used depth and microhabitats. In case of all cryptobenthic fish species environmental variables were: A – artificial objects, Al – algae, C – cavity with sand, D – depth, Gr – seagrass, P – pebbles, R – artificial wall, S – stone, Sh – shell, Te – under roof of boulder/sandstone terrace, W – vertical wall/boulder wall; while in case of Gobiesocidae species were used: P – pebbles; S_1 – stones with sandstone terrace as substrate; S_2 – stones with sandstone terrace and pebbles as substrate; S_3 – stones with pebbles as substrate; S_4 – stones with pebbles and sand as substrate; S_5 – stones with sand as substrate; Te – under sandstone terrace; Pi – on *P. nobilis*; Gr – seagrass; O_s – other seashells; Oy – oyster shells; A – artificial objects. The diagram shows the main pattern of variation in a fish community in relation to environmental factors, as also the approximate distribution of species along each environmental variable (Liu, 1997).

In order to compare the shape of stones, which are considered as hiding places, the surface-area-to-volume ratio (SA:V) was calculated:

$$SA:V = S/V$$

where S is surface (in cm²) and V is volume (in cm³). The higher the SA:V, the flatter the stone.

In order to test differences in depth distribution between species analysis of variance (ANOVA) was used. Linear regression t-test was used to test linear regression of depth distribution and size of the fish, microhabitat preferences and size of the fish and flatness (SA:V) of the occupied stone and depth. Furthermore, potential differences in depth distribution between species were tested with Tukey's post-hoc test, while potential

differences in microhabitat between species were tested with chi-square (χ^2) statistics ($\alpha = 0.05$).

2.3.2 Sex structure and biometry

Measurements of fish were analyzed and compared in Microsoft Excel (Microsoft Corporation, 2016). Sex of the clingfish specimens in *L. lepadogaster* and *L. candolii* was determined based on the length and size of urogenital papillae, which is in males larger and more elongated than in females (Hofrichter, 1993, 1995), while in *A. incognitus*, sex was determined based on gonads (specimens from MBS collection), length and shape of urogenital papilla and the size of caniniform teeth (Hofrichter, 1995). Potential differences between sexes were tested with chi-square (χ^2) statistics ($\alpha = 0.05$). In order to test linear relations of biometrical measurements of the specimens the analysis of covariance (ANCOVA) was used. Fish age was estimated based on fish size and age-size classes as proposed by Hofrichter (1995; Table 2.1). However, age estimation from size could have potential substantial error, as cohorts of fishes usually have overlapping ranges, so the age estimate is only approximative.

Table 2.1: Age classes of three clingfish species as proposed by Hofrichter (1995).

Age (years)	<i>L. candolii</i>	<i>L. lepadogaster</i>	<i>A. incognitus</i>
0+	< 25 mm	< 22 mm	< 16 mm
1+	26–39 mm	23–37 mm	17–24 mm
2+	40–51 mm	38–50 mm	25–34 mm
3+	52–70 mm	51–63 mm	> 35 mm
4+	> 71 mm	> 64 mm	-

2.3.3 Diet

In order to assess the feeding habits of the studied fish, the non-parametric permutational multivariate analysis of variance (PERMANOVA) based on prey abundance data and the Bray-Curtis dissimilarity measure matrix was performed. Additional tests for multivariate dispersions and prey importance were also calculated by using the R statistical environment (R Core Team, 2016) and the Vegan package (Oksanen, Blanchet, Friendly, & Kindt, 2017). Furthermore, linear regression t-test was used to test linear dependence between size of the fish and size of prey. However, to obtain the complexity of the studied fish diets, three different quantitative methods were used: frequency of occurrence (F%; Hynes, 1950), numerical abundance (N%; Macdonald & Green, 1983) and gravimetric composition (B%; Macdonald & Green, 1983). In addition, the modified index of relative importance (IRI) (Pinkas, Oliphant, & Iverson, 1971; Simenstad, 1979) was calculated from the formula:

$$IRI = F\% (N\% + B\%)$$

where F% is the frequency of occurrence, N% is numerical abundance and B% is gravimetric composition. To facilitate comparisons between different types of food, Cortes (1997) recommended the expression of IRI as a percentage. The IRI% for a given food category f is:

$$IRIf \% = (IRIf / \sum IRIf) \times 100$$

Diet diversity of prey was expressed through the index of trophic diversity (ITD), which is a modified Shannon-Wiener diversity index (H'; Shannon & Weaver, 1963):

$$ITD = 1 - H'$$

ITD's values range from 0 (no diversity) to 1 (full diversity).

In order to study trophic level of species, the TROPH's value was calculated for all three species of clingfishes. The trophic level was calculated as (Pauly & Froese, 2000; Pauly et al., 2000; Pauly & Palomares, 2000):

$$TROPH_i = 1 + \sum DC_{ij} \times TROPH_j$$

where DC_{ij} represents the proportion of prey j in the diet of the consumer species i ; $TROPH_j$ represents the fractional trophic level of prey j . Program TrophLab (a stand-alone Microsoft Access routine for estimating trophic levels was downloaded from www.fishbase.org; Pauly et al. (2000) and used to calculate the TROPH's index of the studied species.

For diet comparison between species we used the Bray–Curtis dissimilarity index, which gives us information of the ecological or nutritional niche overlap (Bray & Curtis, 1957; Ricotta & Podani, 2017):

$$BC_{ij} = 1 - (2C_{ij} / (S_i + S_j))$$

where C_{ij} is the sum of the lesser values for only those prey taxa in common between both clingfishes. S_i and S_j are the total number of prey taxa counted in each species diet. Values range from 0 (no overlap) to 1 (full overlap).

To show the importance of each prey taxa among different depths, the SIMPER function was used (Clarke, 1993):

$$d[ijk] = \text{abs}(x[ij] - x[ik]) / \text{sum}(x[ij] + x[ik])$$

where x is the abundance of prey taxa i in sampling units j and k . The overall index is the sum of the individual contributions over all S prey taxa $d[jk] = \text{sum}(i=1..S) d[ijk]$. The SIMPER function performs pairwise comparisons of groups of sampling units and finds the average contributions of each prey taxon to the average overall Bray-Curtis dissimilarity (<https://www.rdocumentation.org/packages/vegan/versions/2.4-2/topics/SIMPER>; assessed 14 May 2020). By applying the summary function (ordered = TRUE), the results include the cumulative contribution and are ordered by prey taxon contribution to the potential difference in prey composition and abundance.

2.3.4 Analysis of clingfish nests

Data of occurrence (*e.g.* season, nest place, depth) of the nests were analyzed with the Microsoft Excel programme. Nests photographs taken in the field were analyzed with the ImageJ2 programme (Rueden et al., 2017). All present eggs were counted and divided into different batches, depending on the level of embryonal development.

Chapter 3

Results

3.1 Sampling Locations

Altogether, 72 sampling locations were studied, of which 31 were investigated with a SCUBA diving equipment (infralittoral) and 41 locations were investigated with snorkelling and collecting with a hand net in the mediolittoral and upper infralittoral during low tide (Figure 3.1). A single sampling location can include up to 29 samplings over different time periods, which are shown in the Appendix A. Altogether 206 samplings were performed within quadrants (Figure 3.2). Samplings were performed in three main habitats; rocky bottom, seagrass meadow and sedimentary bottom, differently depending on the individual 2 x 2 km quadrant (Figure 3.3). Samplings were mostly carried out close to the coast, since the rocky bottom is restricted close to the coast.

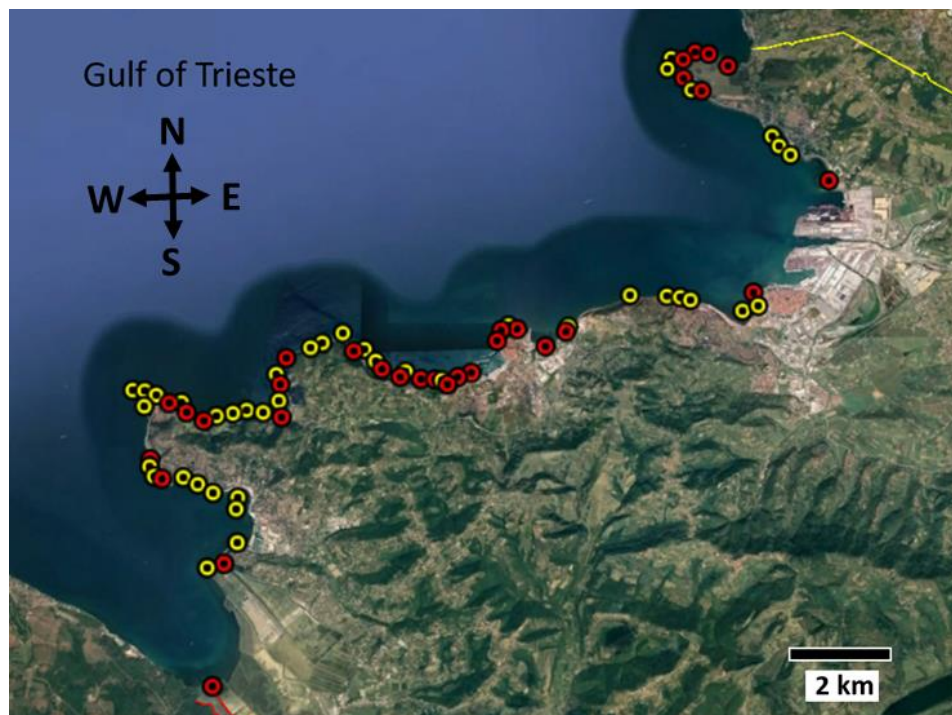


Figure 3.1: Sampling locations along the Slovenian coastline where cryptobenthic fish and clingfish specimens were collected. Yellow circles represent sampling locations where SCUBA diving equipment was used for investigation (infralittoral) and red circles represent locations sampled with snorkelling and manual collecting (mediolittoral and upper infralittoral).

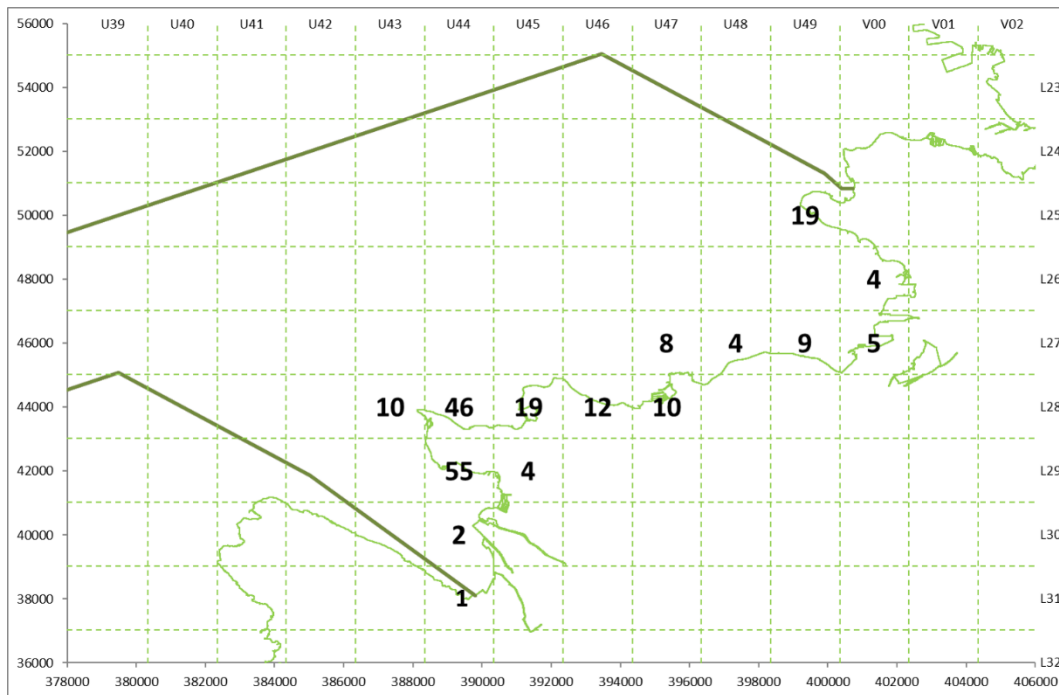


Figure 3.2: Number of samplings performed within 2 x 2 km quadrants.

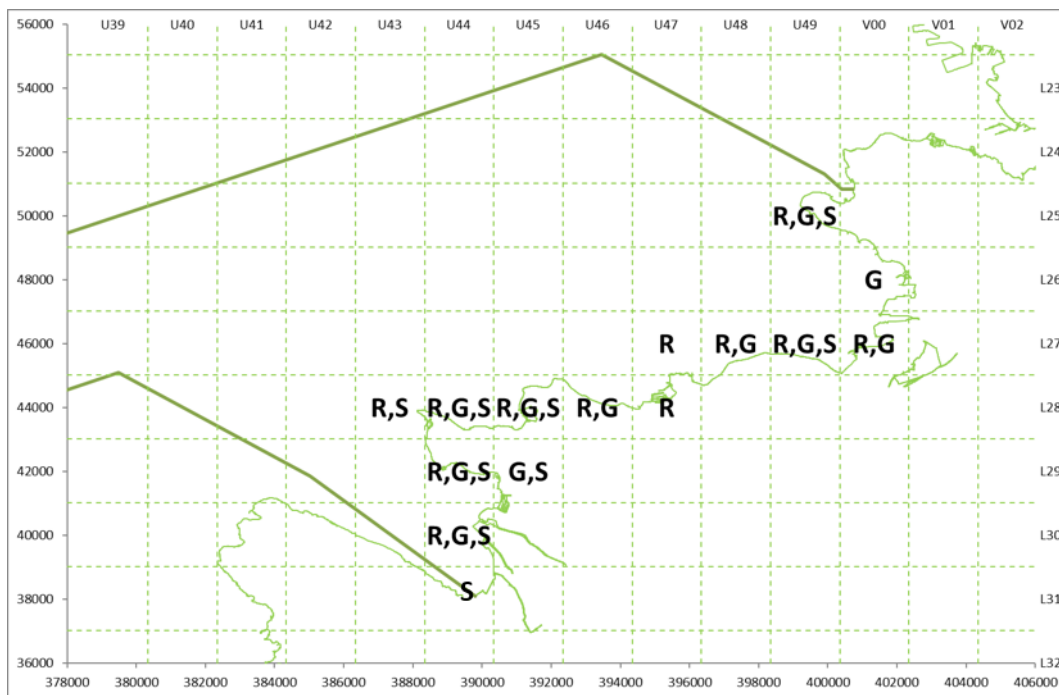


Figure 3.3: Occurrence of habitats in which sampling were performed within 2 x 2 km quadrants: R – rocky bottom; G – seagrass meadow; S – sedimentary bottom.

3.2 The Occurrence and Depth Distribution of Cryptobenthic Fish Fauna

In the last two decades, altogether 838 specimens of cryptobenthic fish were found at 54 localities within 14 different 2 x 2 km quadrants (58.3% of 24 coastal quadrants; Figure 3.4) along the Slovenian coastline. Specimens belong to 12 cryptobenthic fish species from four different families (Figure 3.5 and Figure 3.6; Table 3.1).

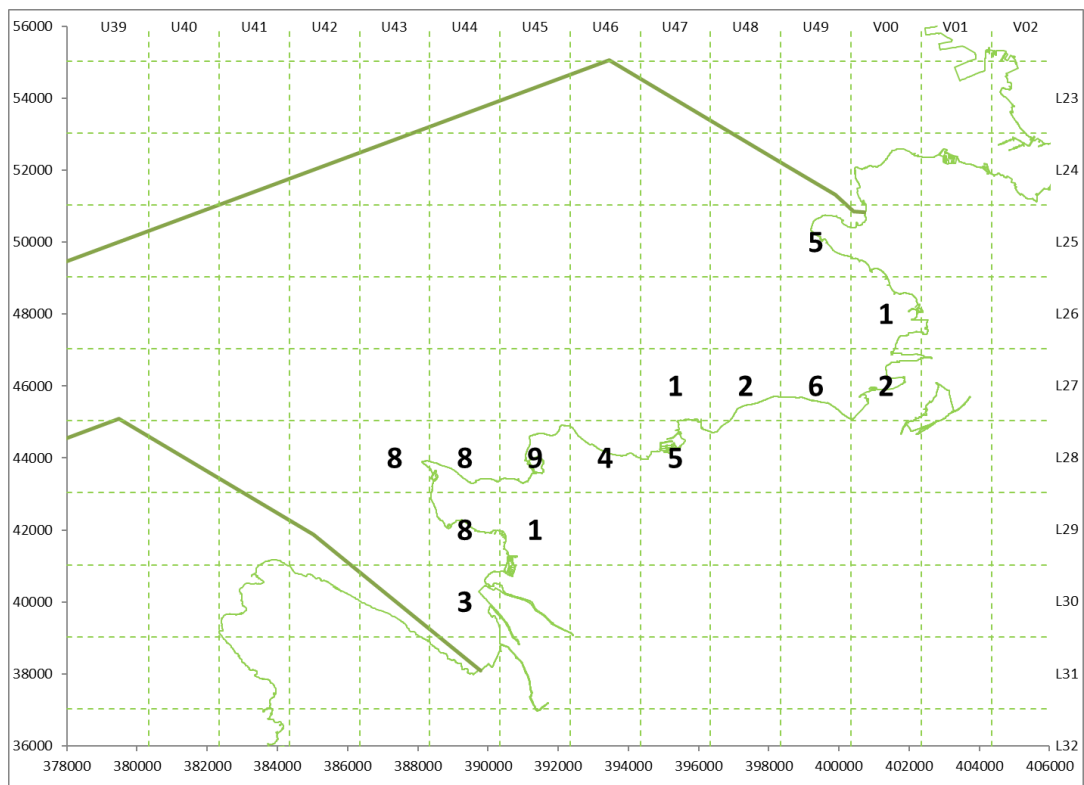


Figure 3.4: Quadrants (2 x 2 km) with numbers of cryptobenthic fish species present within.

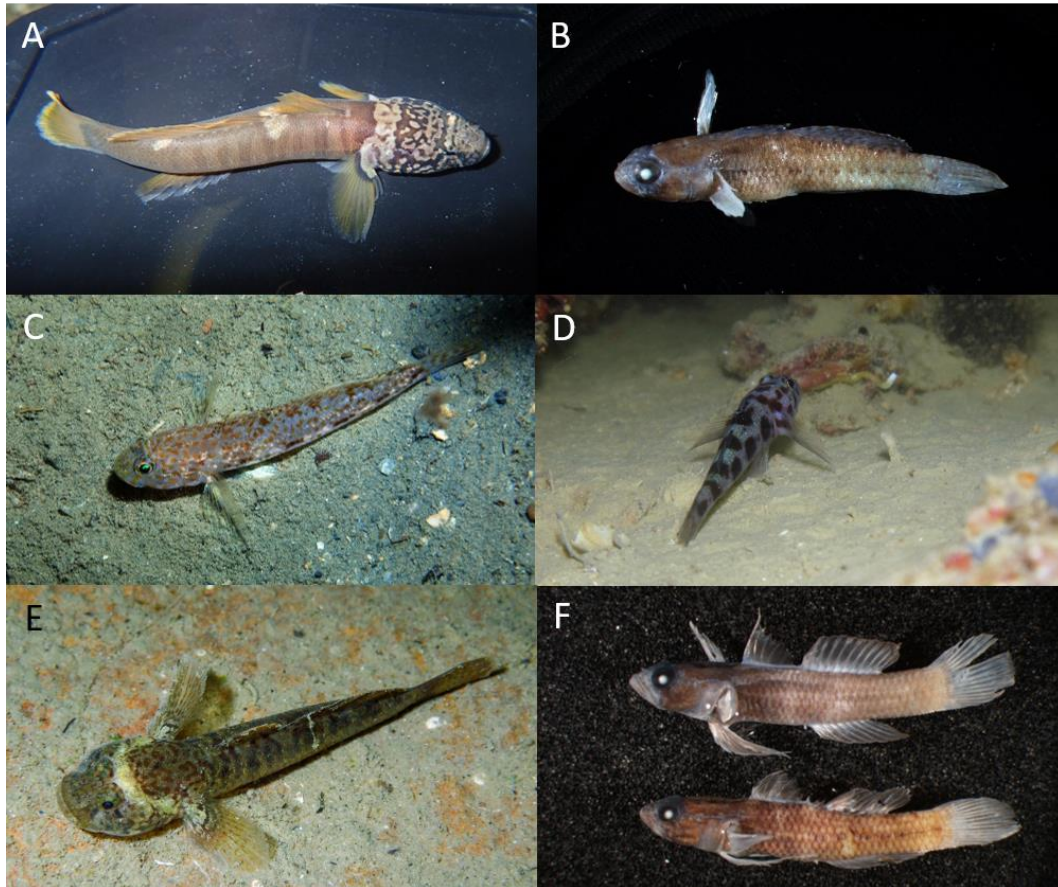


Figure 3.5: Cryptobenthic gobies in the Slovenian sea: A) *Chromogobius quadrivittatus* (Photo: D. Trkov), B) *Millerigobius macrocephalus* (Photo: D. Trkov), C) *Thorogobius macrolepis* (Photo: B. Mavrič), D) *Thorogobius ephippiatus* (Photo: B. Mavrič), E) *Zebrus zebrus* (Photo: D. Trkov) and F) *Odondebuenia balearica* (male – above, female – below; Photo: D. Trkov).

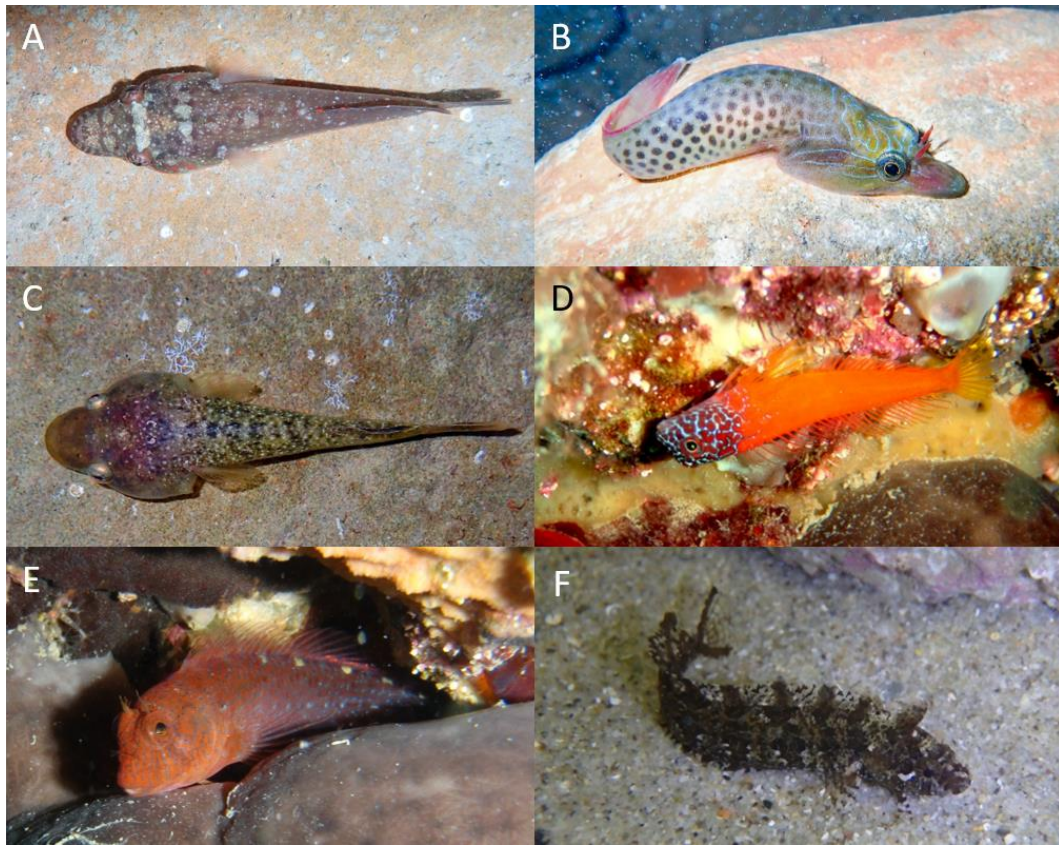


Figure 3.6: Cryptobenthic fish species in the Slovenian sea: A) *Lepadogaster candolii* (Photo: D. Trkov), B) *Lepadogaster lepadogaster* (Photo: D. Trkov), C) *Apletodon incognitus* (Photo: D. Trkov), D) *Microlipophrys nigriceps* (Photo: L. Lipej), E) *Parablennius zvonimiri* (Photo: L. Lipej) and F) *Clinitrachus argentatus* (Photo: D. Trkov).

Table 3.1: Number of specimens per fish species and their proportion according to the number of all cryptobenthic fishes found during field sampling in Slovenian coastal waters.

Family	Number of species	Species	Number of specimens	Percentage (%)
Gobiidae	6	<i>Chromogobius quadrivittatus</i>	1	0.1
		<i>Millerigobius macrocephalus</i>	57	6.8
		<i>Odondebuenia balearica</i>	2	0.2
		<i>Thorogobius ephippiatus</i>	1	0.1
		<i>Thorogobius macrolepis</i>	2	0.2
		<i>Zebrus zebrus</i>	42	5.0
Gobiesocidae	3	<i>Apletodon incognitus</i>	222	26.5
		<i>Lepadogaster candolii</i>	177	21.1
		<i>Lepadogaster lepadogaster</i>	242	28.9
Blenniidae	2	<i>Microlipophrys nigriceps</i>	48	5.7
		<i>Parablennius zvonimiri</i>	33	3.9
Clinidae	1	<i>Clinitrachus argentatus</i>	11	1.3

The number of collected specimens was the highest in two quadrants: the first covering the area between Piran and Fiesa (305 specimens), and the second comprising the area between Piran and Portorož, including the waters in front of the MBS (263 specimens). The highest number of species was recorded in four adjacent quadrants ranging from Strunjan to Portorož, including the protected area of the Natural Monument Cape Madonna Piran and the Strunjan Nature Reserve which is a part of the Strunjan Nature Park. The absence of cryptobenthic fishes in some coastal quadrants is due to the lack of sampling surveys in inaccessible areas, such as ports and harbours, rather than to unsuitable habitats for these species. It should also be taken into consideration that the sampling effort and sampling methods were not the same in all 2 x 2 km quadrants. Thus, data on the abundance or on the diversity of the 2 x 2 km quadrants are not comparable and are only informative. On coastal rocky bottoms, 11 cryptobenthic fish species were found, while on sedimentary bottom only four species were observed (three of them were present also on the rocky bottom). Microhabitat preferences of cryptobenthic fish species were also analysed with the RDA method (Figure 3.7) where 780 specimens were included altogether. For species *Chromogobius quadrivittatus* (Steindachner, 1863), *Odondebuenia balearica* (Pellegrin & Fage, 1907), *Thorogobius macrolepis* (Kolombatović, 1891), *Thorogobius ephippiatus* (Lowe, 1839) and *Clinitrachus argentatus* (Risso, 1810), the sample, in terms of the number of specimens, was too small to show any correlation of fish occurrence and microhabitat factors.

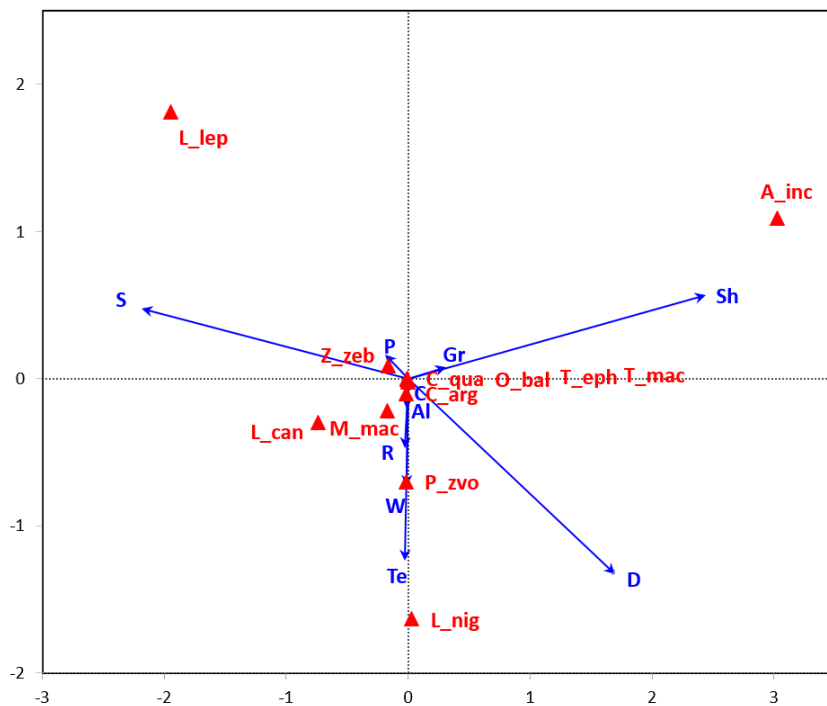


Figure 3.7: RDA ordinate system for microhabitat of 12 cryptobenthic fish species (indicated by triangles). Microhabitat: A – artificial objects, Al – algae, C – cavity with sand, D – depth, Gr – seagrass, P – pebbles, R – artificial wall, S – stone, Sh – shell, Te – under roof of boulder/sandstone terrace, W – vertical wall/boulder wall. Fish species: C_qua – *Chromogobius quadrivittatus*, M_mac – *Millerigobius macrocephalus*, O_bal – *Odondebuenia balearica*, T_eph – *Thorogobius ephippiatus*, T_mac – *Thorogobius macrolepis*, Z_zeb – *Zebrus zebrus*, A_inc – *Apletodon incognitus*, L_lep – *Lepadogaster candolii*, L_lep – *Lepadogaster lepadogaster*, M_nig – *Microlipophrys nigriceps*, P_zvo – *Parablennius zvonimiri*, C_arg – *Clinitrachus argentatus*.

3.2.1 Family Gobiidae

Chromogobius quadrivittatus (Steindachner, 1863)

A single specimen of *C. quadrivittatus* was captured on 26 July 2018 between Salinera and Pacug (Figure 3.8). It was found in a rocky environment (boulder field) where it was hiding under a stone at a depth of 1.5 m. This finding represents the first record of this species in Slovenia. The specimen is stored in the ichthyological collection of the MBS: IC-MBP 334.

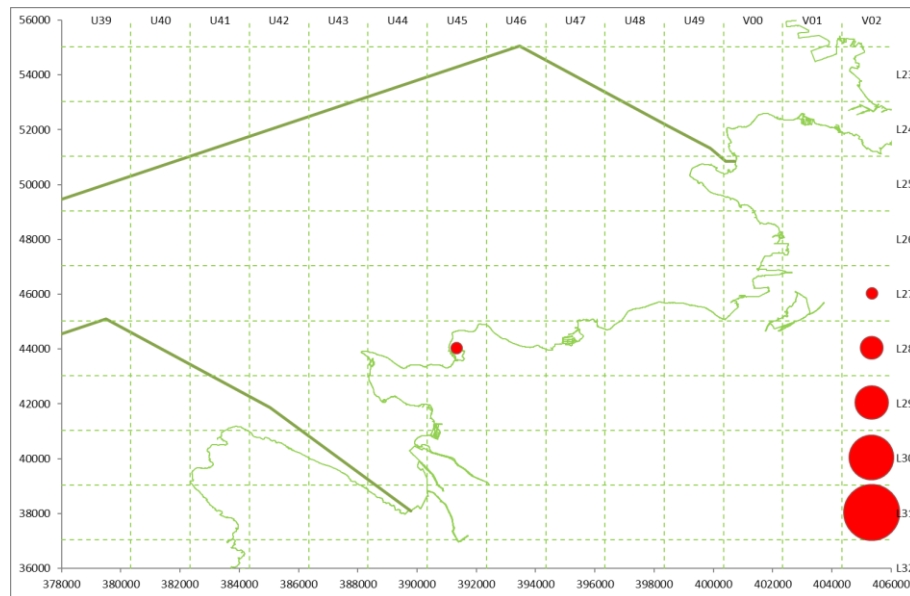


Figure 3.8: Occurrence of *Chromogobius quadrivittatus* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

Millerigobius macrocephalus (Kolombatović, 1891)

Altogether, 57 specimens of *M. macrocephalus* were found within 8 quadrants (Figure 3.9). RDA analysis showed that the species is in relation with shallow waters, stones and vertical walls. The majority of the specimens were found in rocky environments at depths between 1 and 4 m (87.0%; Figure 3.10) where they were hiding mostly under stones or in rocky clefts on vertical walls. A few specimens were also found in a seagrass meadow under isolated stones. Specimens are stored in the ichthyological collection of the MBS: IC-MBP 006 and IC-MBP 023.

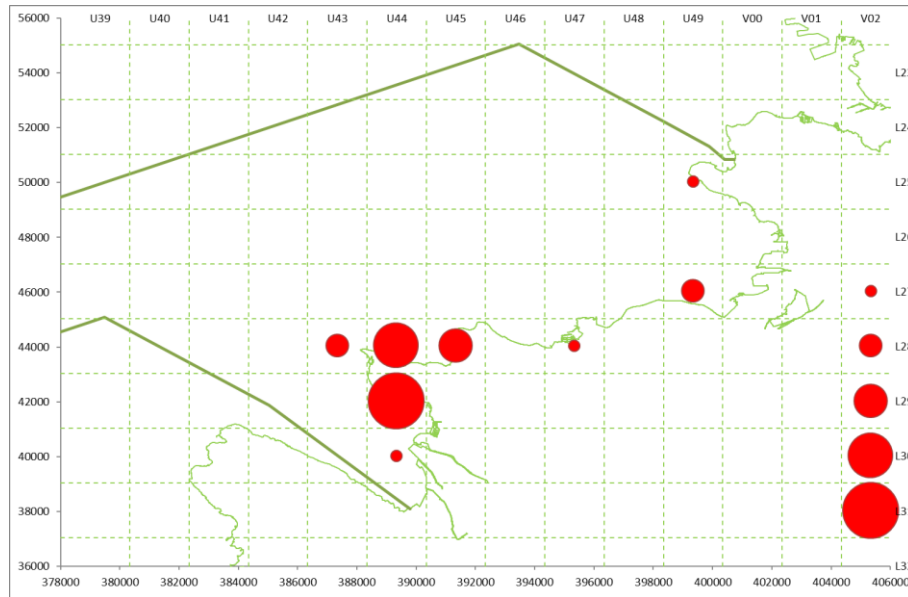


Figure 3.9: Occurrence of *Millerigobius macrocephalus* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

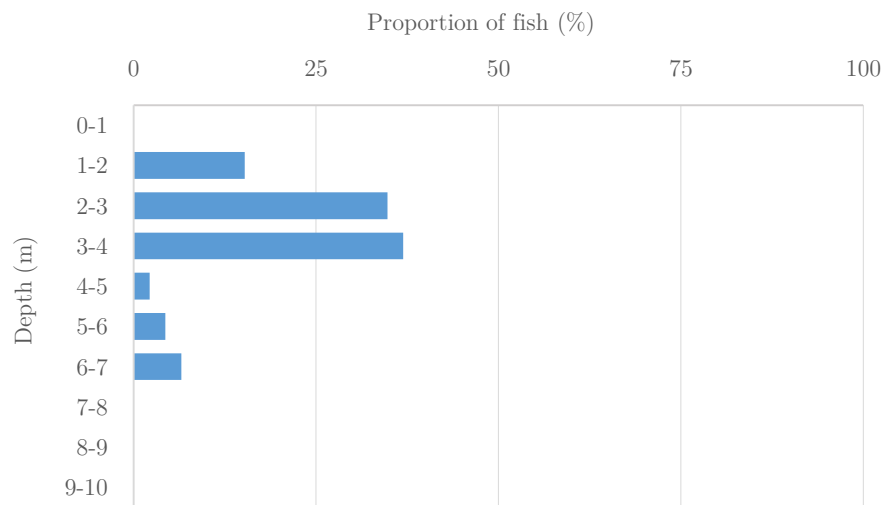


Figure 3.10: Depth distribution of *Millerigobius macrocephalus* (n = 46).

Odondebuenia balearica (Pellegrin & Fage, 1907)

Two specimens of *O. balearica* were found within one quadrant (Figure 3.11). Both specimens were found on the same day (17 August 2001) in the area of Pacug. Specimens were caught in a boulder field under stones covered mainly by the red algae *Jania virgata*, 1846 and *Halopithys incurva* at 2.5 m. In the shadow of stones and rocks, a substratum of coralline belt (*Litophyllum* sp.) was present. Specimens are stored in the ichthyological collection of the MBS: IC-MBP 015 and IC-MBP 029.

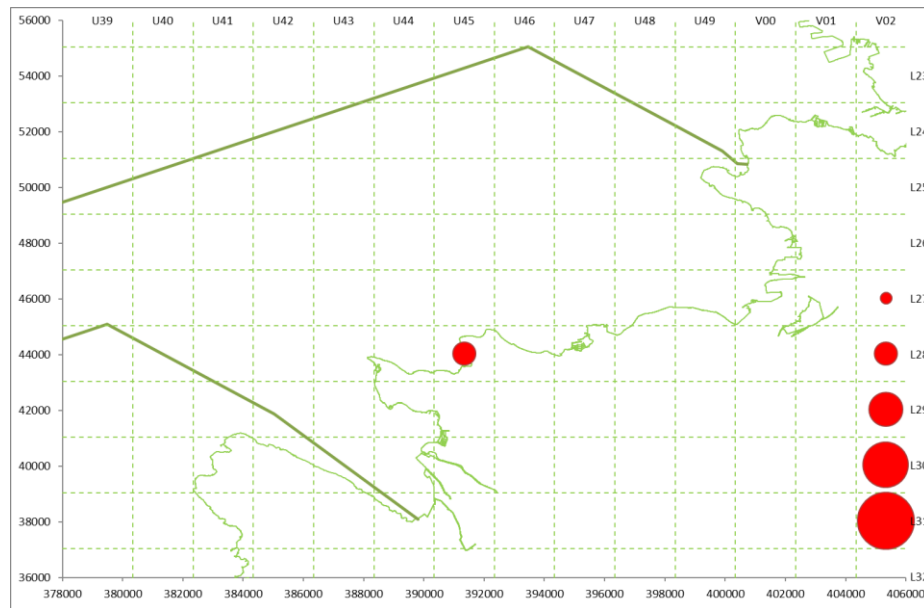


Figure 3.11: Occurrence of *Odondebuenia balearica* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

Thorogobius ephippiatus (Lowe, 1839)

During the survey, only one specimen of *T. ephippiatus* was found. It was observed on 9 August 2005 within the Cape Madonna Nature Monument of Piran (Figure 3.12). The specimen was observed at a depth of 10 m in a cavity beneath sandstone rocks covered with sand (Lipej et al., 2005).

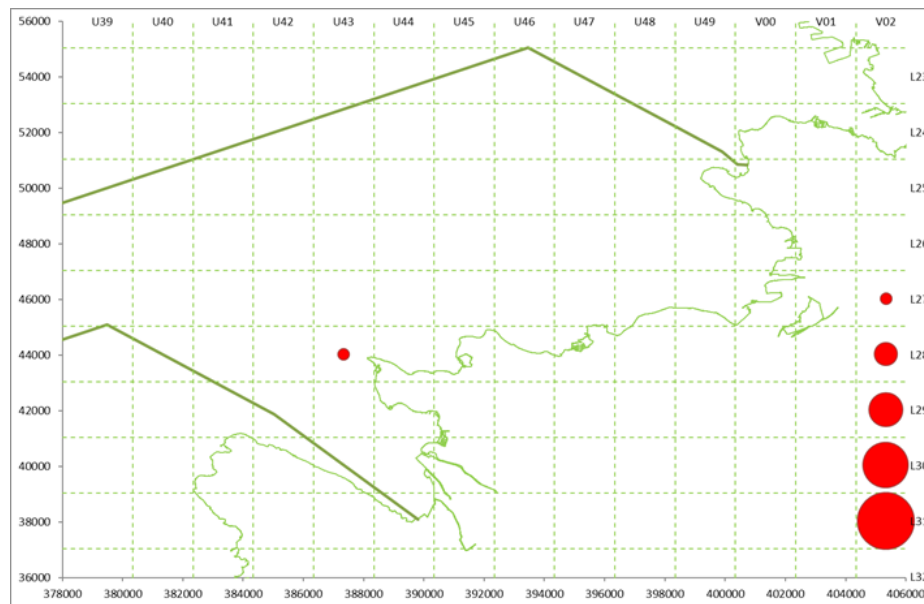


Figure 3.12: Occurrence of *Thorogobius ephippiatus* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

Thorogobius macrolepis (Kolombatović, 1891)

Two specimens of *T. macrolepis* were found in Slovenian coastal waters, both of them in the Cape Madonna Natural Monument of Piran (Figure 3.13). The first specimen was observed and photographed by Borut Mavrič on 22 September 2015, while the second specimen was observed and photographed by a photographer, Borut Furlan, on 18 March 2017. Both specimens were found in a sheltered cavity covered with rough sand at a depth of around 10 m.

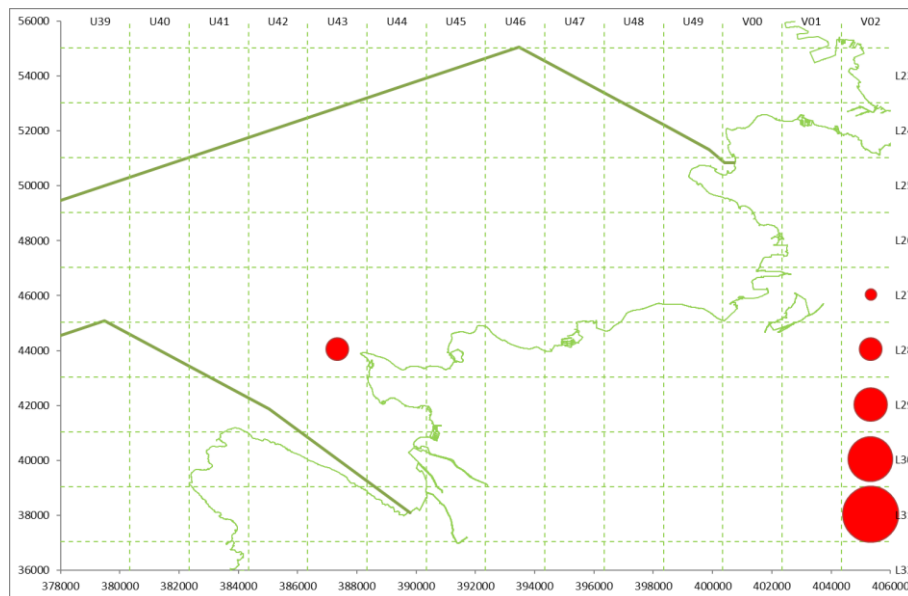


Figure 3.13: Occurrence of *Thorogobius macrolepis* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

Zebrus zebrus (Risso, 1827)

Specimens of *Z. zebrus* were found in 9 quadrants (Figure 3.14). RDA analysis showed that the species is in relation with very shallow waters and stones. Altogether, 42 specimens were recorded in shallow waters, less than 3 m deep (Figure 3.15). Specimens were mostly found in rocky environments under stones and in rocky clefts, while some of them were also recorded in *C. nodosa* and *P. oceanica* seagrass meadows where they were hiding under seashells and single stones. Some specimens were also found under stones in tide pools in lower mediolittoral areas, while two specimens were also found in the bryozoan fouling community of *Amathia verticillata* and *Bugula neritina* on boats. Specimens are stored in the ichthyological collection of the MBS: IC-MBP 063 and IC-MBP 066.

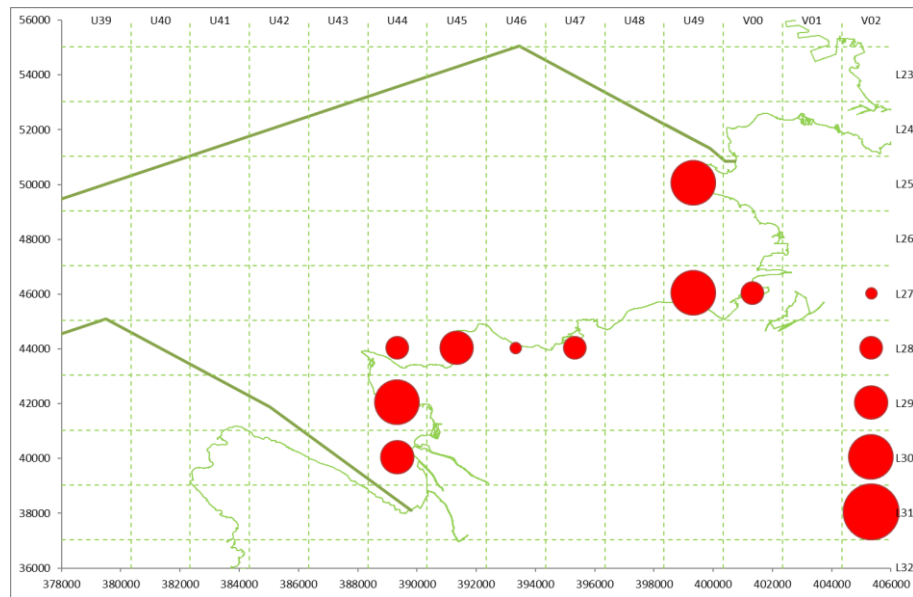


Figure 3.14: Occurrence of *Zebrus zebrus* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

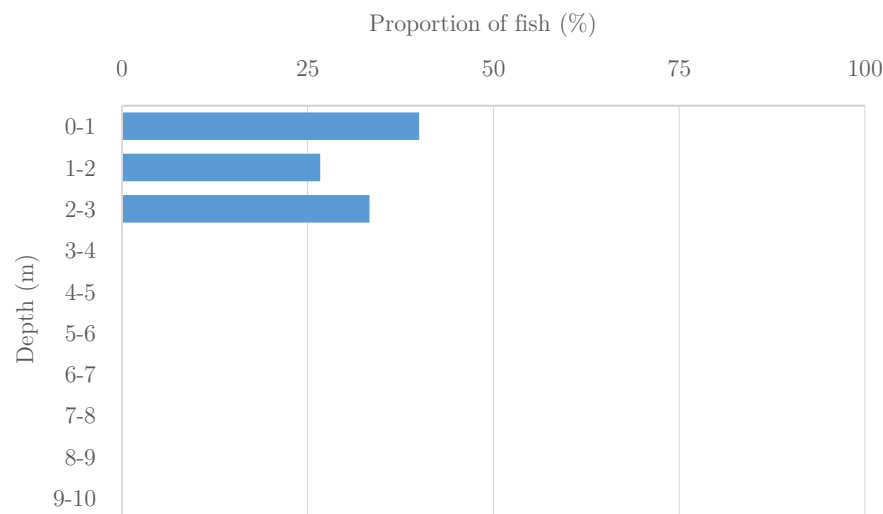


Figure 3.15: Depth distribution of *Zebrus zebrus* (n = 30).

3.2.2 Family Gobiesocidae

Apletodon incognitus Hofrichter & Patzner, 1997

Altogether, 222 specimens of *A. incognitus* were found within 10 quadrants (Figure 3.16). RDA analyses showed that the species is in strong relation with sea shells. Specimens were found in *C. nodosa* and *P. oceanica* meadows where they were hiding in dead oyster shells (*Ostrea edulis* Linnaeus, 1758) attached to noble pen shells (*Pinna nobilis*) and in dead seashells on the sea bottom. More than 97% of the specimens were found at depths between 2 and 6 m, while the majority (50.2%) were present at depths between 4 and 5 m (Figure 3.17). Specimens are stored in the ichthyological collection of the MBS: IC-MBP 252 and IC-MBP 253.

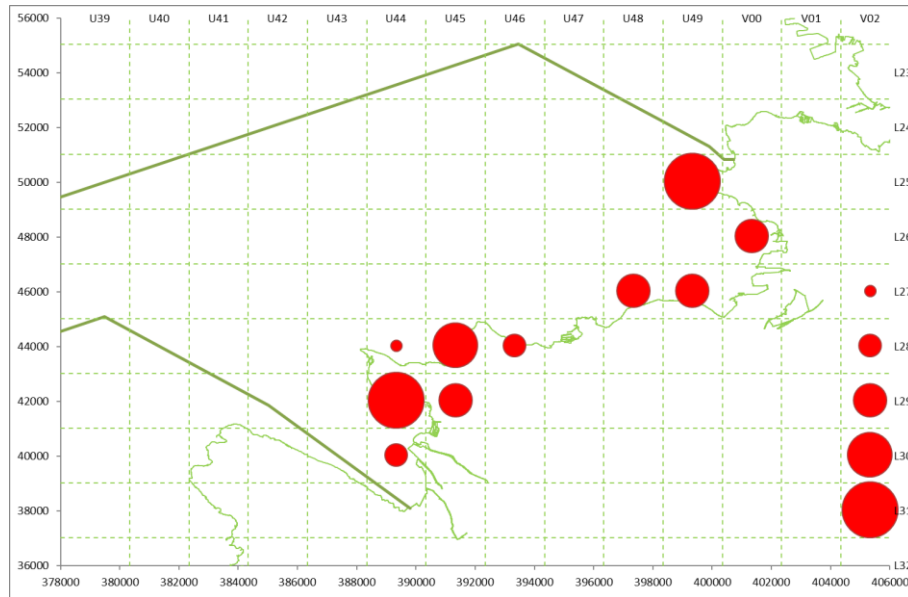


Figure 3.16: Occurrence of *Apletodon incognitus* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

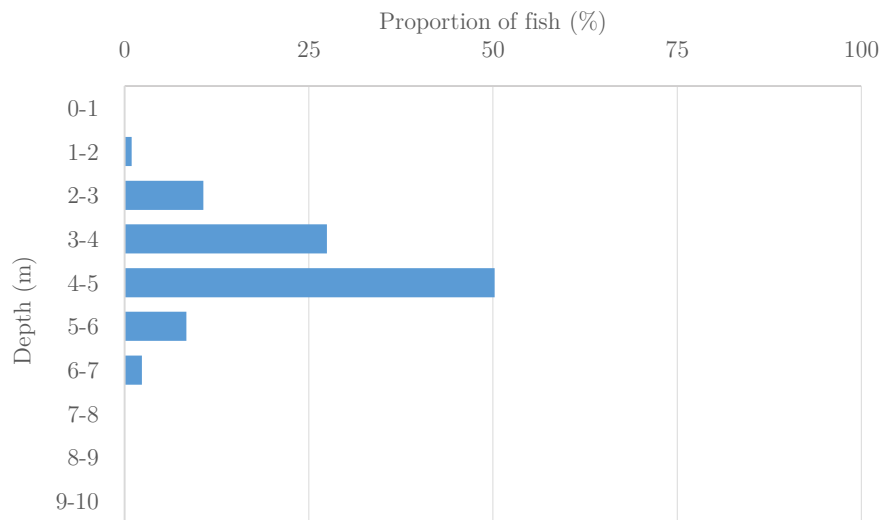


Figure 3.17: Depth distribution of *Apletodon incognitus* (n = 215).

Lepadogaster candolii Risso, 1810

Altogether, 177 specimens of *L. candolii* were found within 7 quadrants (Figure 3.18). RDA analysis showed that the species is in relation with shallow waters and stones. Specimens were mostly found under stones in a rocky environment, while a small proportion of them were also found in *C. nodosa* and *P. oceanica* seagrass meadows where they were hiding under stones and on or under seashells (e.g. *P. nobilis*, *O. edulis*). Most of the specimens (86.4%) were present in the depth range from 1 to 4 m (Figure 3.19). The specimens are stored in the ichthyological collection of the MBS: IC-MBP 031 and IC-MBP 038.

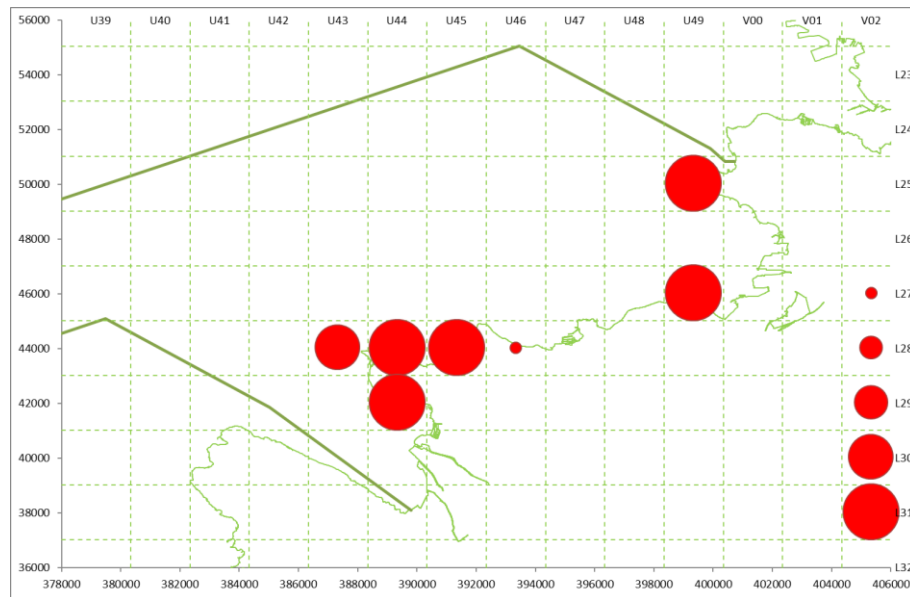


Figure 3.18: Occurrence of *Lepadogaster candolii* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

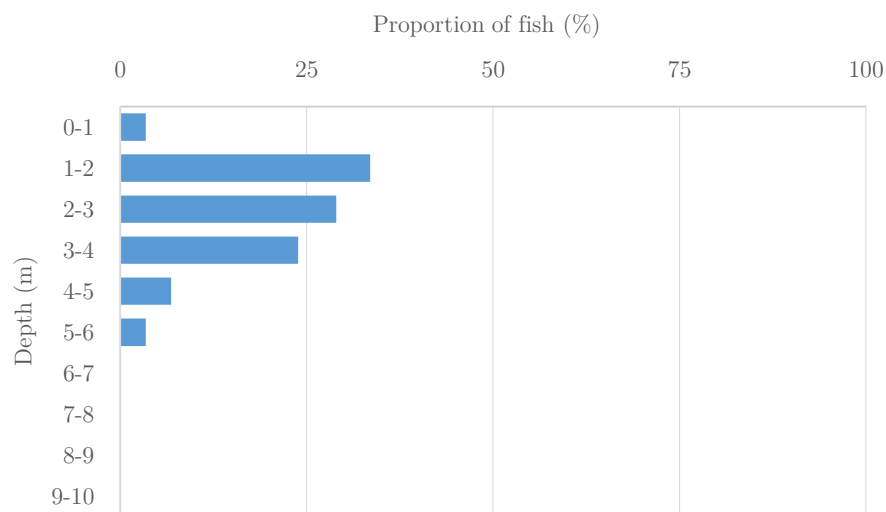


Figure 3.19: Depth distribution of *Lepadogaster candolii* (n = 176).

Lepadogaster lepadogaster (Bonnaterre, 1788)

Specimens of *L. lepadogaster* were found in 8 quadrants (Figure 3.20). RDA analysis showed that the species is in strong relation with very shallow waters and stones. Altogether, 242 specimens were observed and all of them were found under stones in a rocky environment such as boulder fields. The specimens were observed in shallow waters, mostly less than 2 m deep (98.4%), with the majority (66.0%) above 1 m (Figure 3.21). The specimen is stored in the ichthyological collection of the MBS: IC-MBP 281.

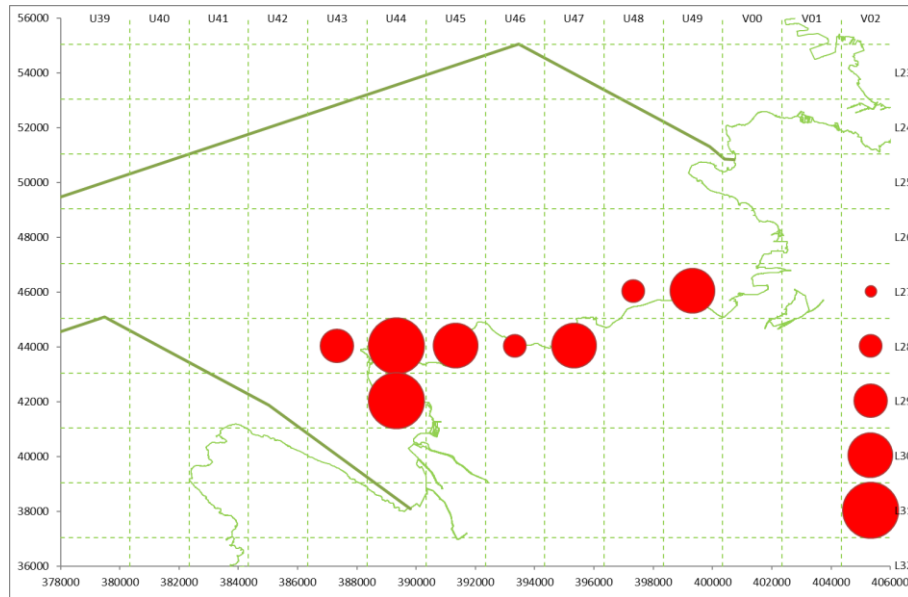


Figure 3.20: Occurrence of *Lepadogaster lepadogaster* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

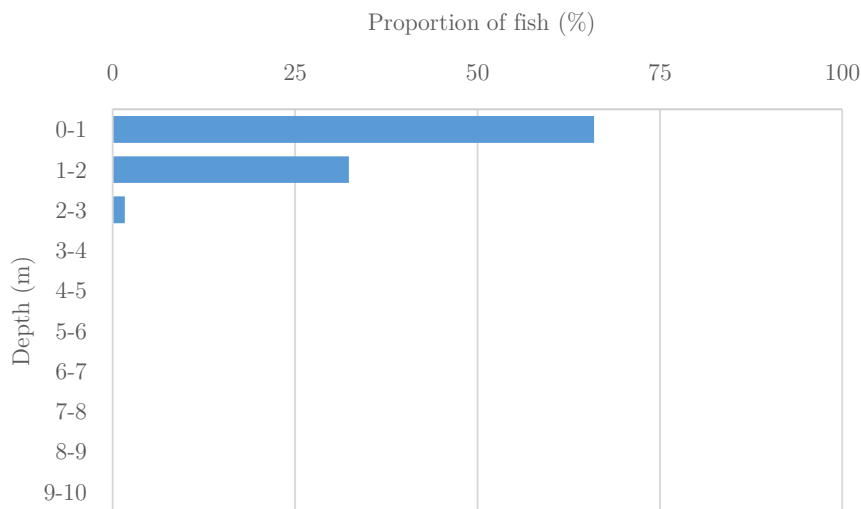


Figure 3.21: Depth distribution of *Lepadogaster lepadogaster* (n = 242).

3.2.3 Family Blenniidae

Microlipophrys nigriceps (Vinciguerra, 1883)

Altogether, 48 specimens of *M. nigriceps* were observed in four quadrants (Figure 3.22). RDA analysis showed that the species is in strong relation with deep-water, vertical walls and sandstone terraces. The majority of specimens (89.6%) were observed in the depth range from 3 to 9 m (Figure 3.23). The specimens were found in the rocky littoral, mostly in dimly lit habitats such as the underside of boulder terraces. They were hiding in precoralligenous formations, where they occupied empty burrows excavated by the date mussel (*L. lithophaga*). One specimen is stored in the ichthyological collection of the MBS: IC-MBP 124.

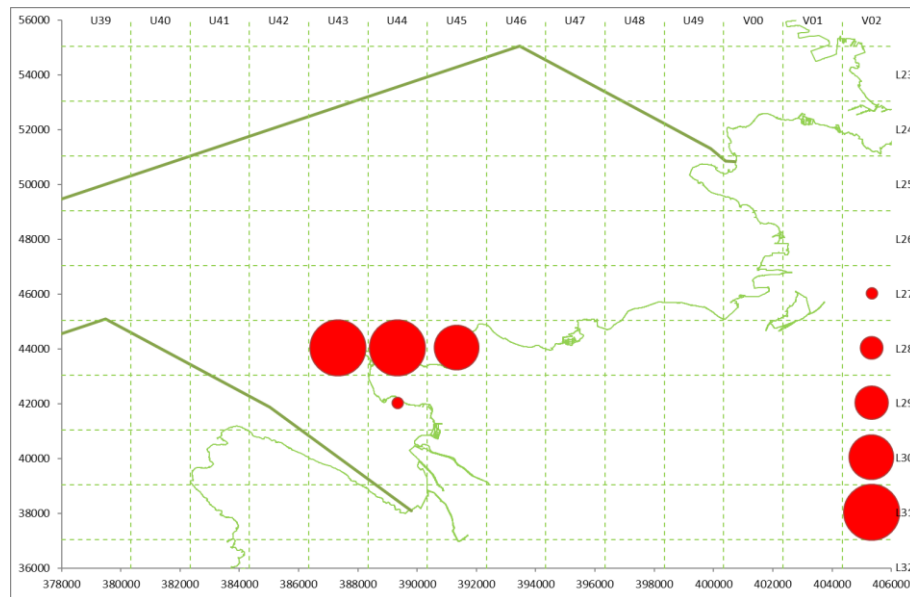


Figure 3.22: Occurrence of *Microlipophrys nigriceps* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

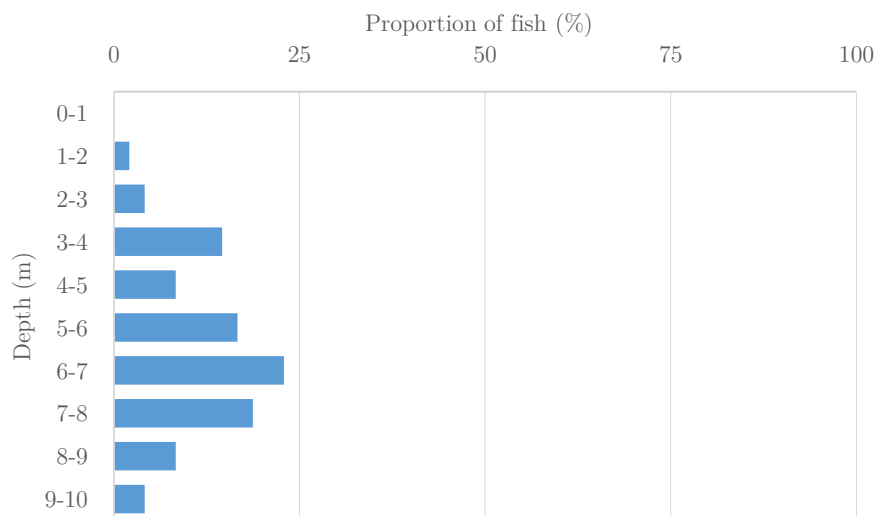


Figure 3.23: Depth distribution of *Microlipophrys nigriceps* (n = 48).

Parablennius zvonimiri (Kolombatović, 1892)

Altogether, 33 specimens of *P. zvonimiri* were found in 5 quadrants (Figure 3.24). RDA analysis showed that species is in relation with deep waters, vertical and artificial walls and a sandstone terrace. Specimens were observed on the shady side of boulders and in precoralligenous formations in a rocky environment. They were recorded in dimly lit habitats such as vertical and artificial walls and overhanging rocks where they were dwelling in empty date mussel burrows. Specimens were found at depths from 1 to 10 m (Figure 3.25). One specimen is stored in the ichthyological collection of the MBS: IC-MBP 145.

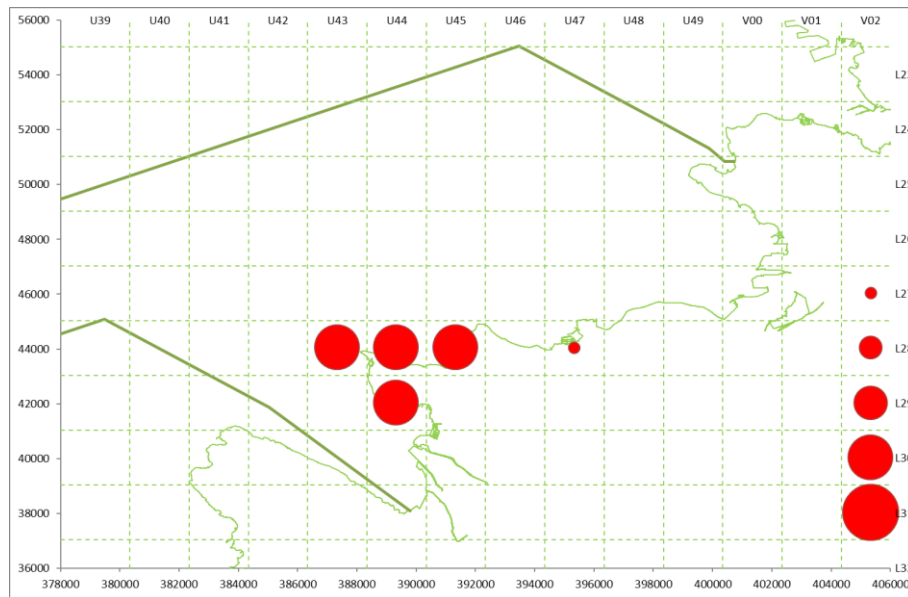


Figure 3.24: Occurrence of *Parablennius zvonimiri* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

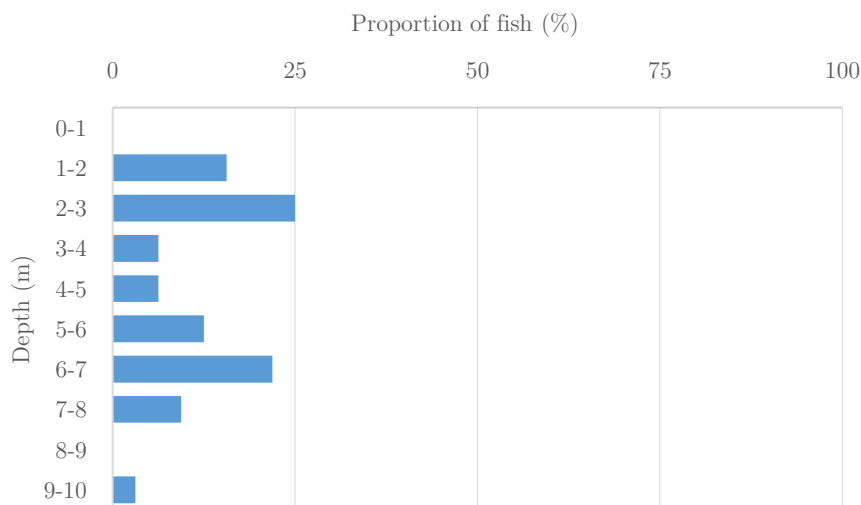


Figure 3.25: Depth distribution of *Parablennius zvonimiri* ($n = 32$).

3.2.4 Family Clinidae

Clinitrachus argentatus (Risso, 1810)

Altogether, 11 specimens of *C. argentatus* were recorded in 8 different quadrants (Figure 3.26). RDA analysis showed that this species is related to shallow waters and macroalgae. All specimens of this species were observed in shallow waters less than 1 m deep (Figure 3.27). With the exception of one which was found among stones, the specimens were found inside the algal cover, such as *Cystoseira* spp., *Halopithys incurva* and *Dictyota dichotoma*. One specimen is stored in the ichthyological collection of the MBS: IC-MBP 146.

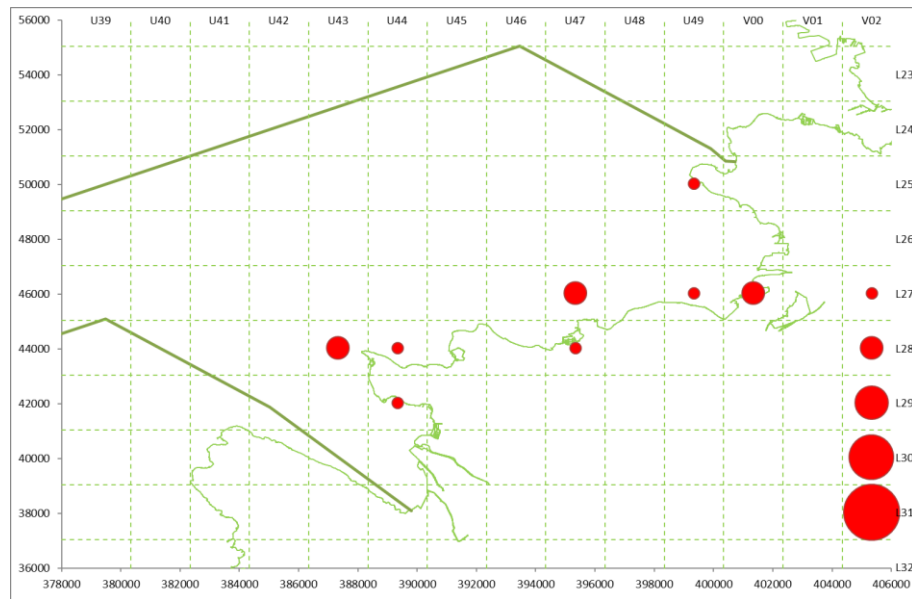


Figure 3.26: Occurrence of *Clinitrachus argentatus* in the Slovenian sea. The size of the dots is shown on the right side of the map and ranks from the smallest to the biggest based on the number of specimens found within the quadrant: 1 specimen, 2-3 specimens, 4-5 specimens, 6-15 specimens, > 15 specimens.

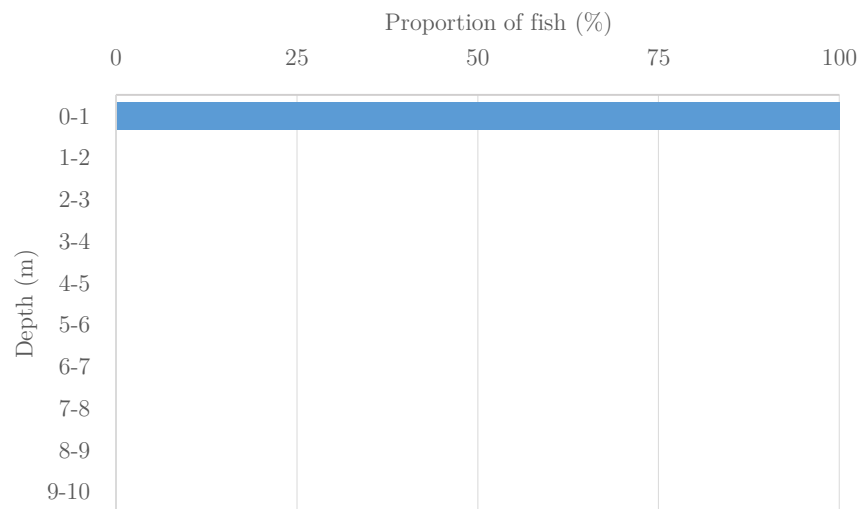


Figure 3.27: Depth distribution of *Clinitrachus argentatus* (n = 11).

3.3 Biometry and Meristic of Clingfishes

Altogether, 242 specimens of *L. lepadogaster* were found, while 194 of them were measured. The size of specimens ranged from 28.06 mm to 78.02 mm (57.60 ± 8.54 mm), while their weight ranged from 0.23 g to 5.85 g (2.17 ± 0.96 g). The majority of specimens belonged to age class 3+ (57.7%), followed by the age classes 4+ (22.2%) and 2+ (18.6%; Table 3.2).

Table 3.2: Proportion (%) of fish specimens per different age classes for three clingfish species.

Age (years)	<i>L. candolii</i>	<i>L. lepadogaster</i>	<i>A. incognitus</i>
0+	17.9	-	6.8
1+	32.5	1.5	25.2
2+	22.2	18.6	31.1
3+	24.8	57.7	36.9
4+	2.6	22.2	-

The sex ratio of 182 specimens of *L. lepadogaster*, whose sex was determined (other were immature), did not statistically differ ($\chi^2 = 2.6593$; $p < 0.1029$) and was 1:1.3 in favour of males. On average, males measure 59.8 mm and they reached maximum 78.02 mm in total length, while females measure on average 55.7 mm and they reached a maximum size of 72.12 mm in total length. Average length of urogenital papilla at males was 1166.6 ± 205.5 μm , while at females 638.2 ± 99.5 μm (Figure 3.28). Our data confirmed that the length of urogenital papilla is in linear relation to the size of the fish (ANCOVA; $p = 3.48\text{e}^{-12}$, $\alpha = 0.05$). Moreover, there are significant differences in fish sex regarding this relation ($p = 1.39\text{e}^{-07}$, $\alpha = 0.05$). Altogether, the length of nasal outgrowths was measured on 74 specimens. Nasal outgrowths of males measure on average 3914.3 ± 1112.6 μm , while in females they measure 2589.1 ± 427.1 μm (Figure 3.29). Our data confirmed that length of nasal outgrowths is in linear relation to the size of the fish (ANCOVA; $p = 1.22\text{e}^{-13}$, $\alpha = 0.05$). Moreover, there are significant differences in fish sex regarding this relation ($p = 0.00147$, $\alpha = 0.05$). On 91 specimens, the number of papillae rows on region A and B at the sucking disc was verified. All specimens had three to four rows of papillae on region A and B at the sucking disc, except two specimens which had four to five rows.

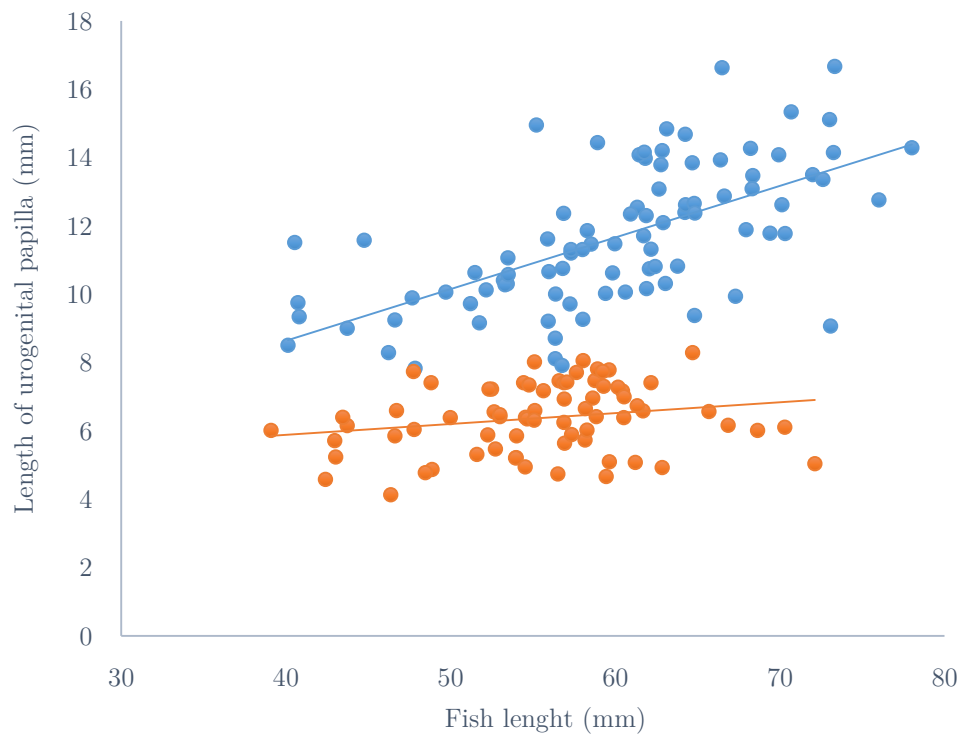


Figure 3.28: Length of urogenital papilla depending on the size of males (blue circles) and females (orange circles) of *L. lepadogaster*. The blue line represents the trend line for males and the orange one for females.

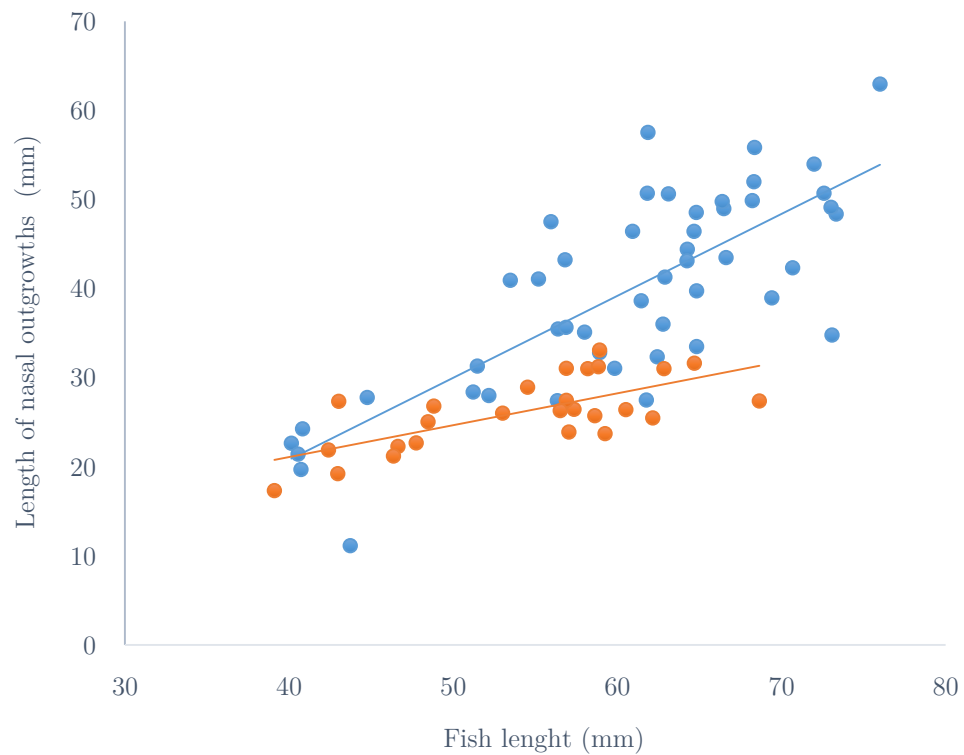


Figure 3.29: Length of nasal outgrowths depending on the size of males (blue circles) and females (orange circles) of *L. lepadogaster*. The blue line represents the trend line for males and the orange one for females.

During the surveys, 177 specimens of *L. candolii* were found, of which 117 specimens were measured and weighed (Table 3.3). The size of specimens ranged from 13.97 mm to 86.94 mm (41.48 ± 16.00 mm) in total length, while the weight of specimens ranged from 0.03 g to 6.05 g (1.08 ± 1.10 g). The most numerous were specimens in the age class 1+ (32.5%), followed by age classes 3+ (24.8%), 2+ (22.2%), and 0+ (17.9%). The sex ratio calculated from 56 specimens, whose sex was determined (other were immature), statistically differed from 1:1 ($\chi^2 = 12.0714$; $p < 0.0005$) and was 1:2.7 in favour of females. Average males measure 64.2 mm and they reached max 86.94 mm in total length, while females measure on average 52.4 mm and they reached maximum 66.08 mm in total length. The average length of urogenital papilla in males was 1471.9 ± 265.3 μm , while in females it was 592.3 ± 104.2 μm (Figure 3.30). Our data confirmed that the length of urogenital papilla is in linear relation to the size of the fish (ANCOVA; $p = 7.23e^{-07}$, $\alpha = 0.05$). Moreover, there are significant differences in fish sex regarding this relation ($p = 5.47e^{-05}$, $\alpha = 0.05$).

Table 3.3: Biometric data of three species of clingfishes.

	<i>L. lepadogaster</i>	<i>L. candolii</i>	<i>A. incognitus</i>
Number of specimens for biometry	194	117	108
Average size (mm)	57.60	41.48	29.30
Average weight (g)	2.17	1.08	0.48
Number of sexed specimens	182	56	66
Sex ratio (females/ males)	1 : 1.3	2.7 : 1	1 : 2.1
Average male papilla (μm)	1166.6	1471.9	630.1
Average female papilla (μm)	638.2	592.3	250.7

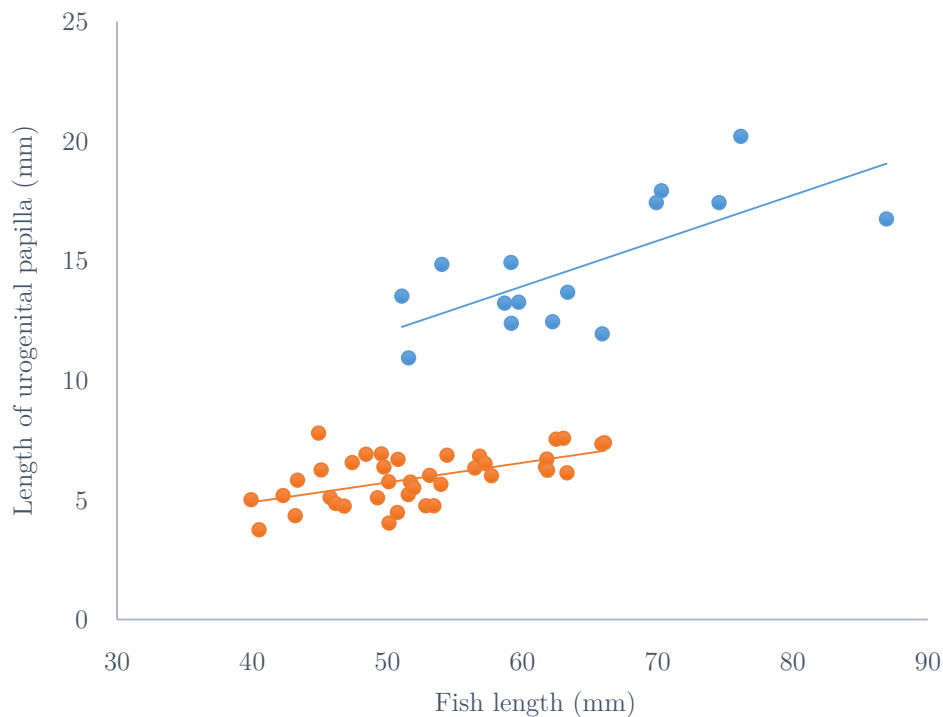


Figure 3.30: Length of urogenital papilla depending on the size of males (blue circles) and females of *L. candolii* (orange circles). The blue line represents the trend line for males and the orange one for females.

During surveys, 222 specimens of *A. incognitus* were found and 108 of them were measured. The size of specimens ranged from 12.24 mm to maximum 50.69 mm (31.16 ± 9.40 mm) in total length. Most of the specimens belong to the age class 2+ (31.1%), followed by age class 3+ (26.2%) and 1+ (25.2%). Most of the studied fish were from MBS collection and preserved in 4% formaldehyde, so weighing was done only on 31 freshly caught specimens. The weight of the specimens ranges from 0.03 g to 1.29 g (0.48 ± 0.37 g). The sex ratio calculated from 66 specimens, whose sex was determined, statistically differed from 1:1 ($\chi^2 = 8.7273$; $p < 0.0031$) and was 1:2.1 in favour of males. Males on average measure 36.00 mm and they reached maximum 50.69 mm in total length, while females measure on average 22.00 mm and they reached maximum 29.35 mm in total length. Average length of urogenital papilla in males was 630.1 ± 202.8 μm , and in females 250.7 ± 81.9 μm (Figure 3.31). Our data confirmed that the length of urogenital papilla is in linear relation to the size of the fish (ANCOVA; $p = 7.53e^{-10}$, $\alpha = 0.05$). Moreover, there are significant differences in fish sex regarding this relation ($p = 0.00811$, $\alpha = 0.05$). However, it should be noted that the sample of females was relatively small. Meristic was done on 61 specimens to verify if all specimens belong to the species *A. incognitus* (Table 3.4).

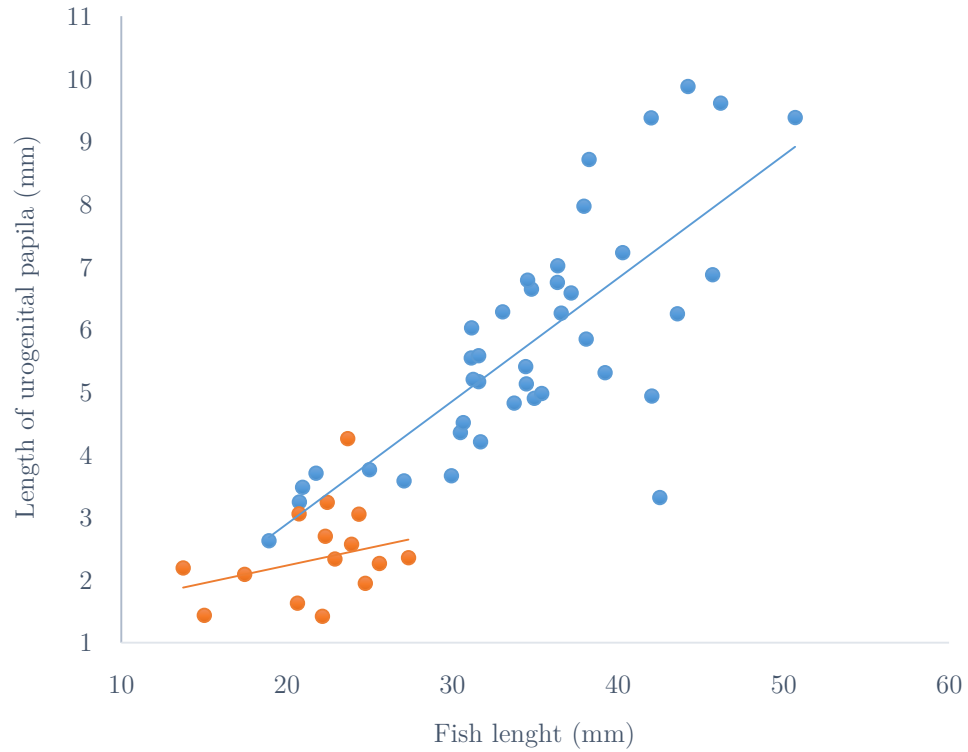


Figure 3.31: Length of urogenital papilla depending on the size of males (blue circles) and females of *A. incognitus* (orange circles). The blue line represents the trend line for males and the orange one for females.

Table 3.4: Meristic counts done on 61 specimens of *A. incognitus*. Average, minimum and maximum numbers of fin rays per fin are presented: D – dorsal, A – anal, P – pectoral, V – ventral and C – caudal fin.

Fin	Number of fin rays		
	Average	min	max
D	5	4	7
A	5	5	6
P	20	17	22
V	4	4	4
C	15	13	17

3.4 Body Colouration of Clingfishes

The general body colouration of *L. lepadogaster* was yellowish brown to greenish grey, with dark spots, which could be immediately changed into dark green-brown with light brown spots. On the top of the head, two blue eye spots were present, surrounded with a pattern of blue lines. Blue eye spots varied in the size and shape between different specimens. Dorsal, anal and caudal fins of adults were coloured reddish, especially intense in males throughout the spawning season, while pectoral fins were not pigmented. Nasal outgrowths were dark purple to red-coloured. The fish were capable of fast body colour changing from dark to light and *vice versa*, especially when they were disturbed (Figure 3.32).

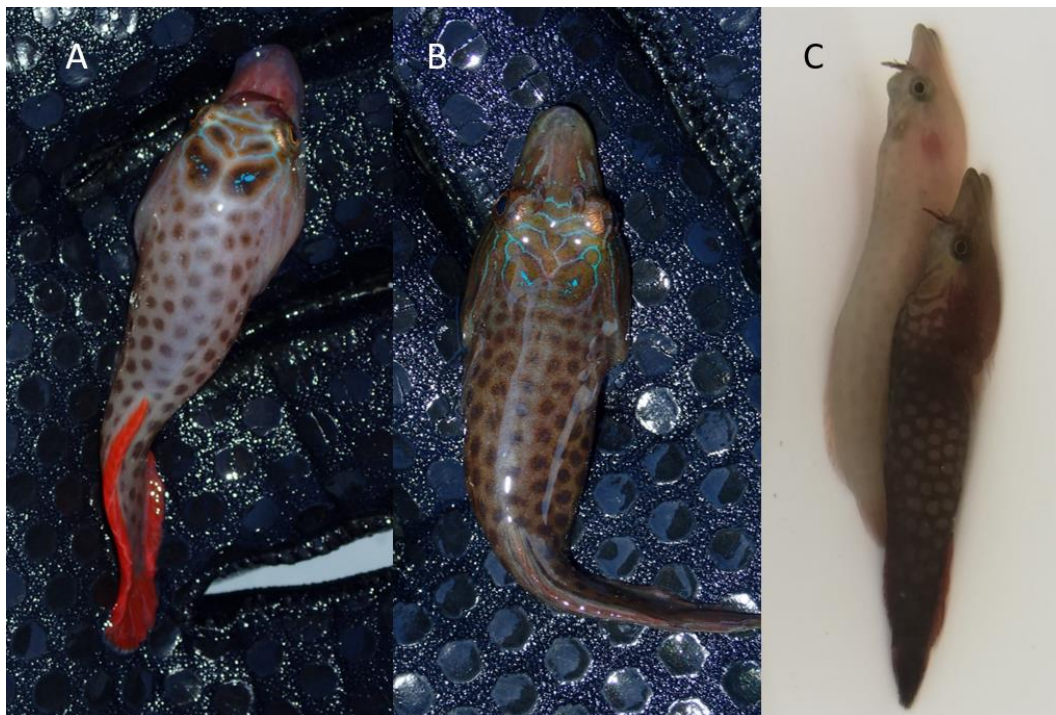


Figure 3.32: Specimens of *L. lepadogaster*: A – male, B – female, C – two males changed body colour because they were disturbed (Photos: D. Trkov).

L. candolii body colour was mostly greenish-brown with variable darker and lighter pattern across the body, which were more pronounced in males, while females were normally more uniformly light green (Figure 3.33). During the spawning season, males

became darker with three to four oblique bright red stripes on the gill cover and from three to five oblique bright red spots extending from the middle of the back to the middle of the basal part of the dorsal fin. Sometimes, red spots on the back were absent and only three spots were present on the basal part of the fin. The red pattern in males was also present outside the mating season, but it was less intense. Juveniles and subadult specimens were dark purple-brown, with white band between eyes, which is less pronounced in adult specimens. White band between eyes was observed in all of 99 examined specimens. On eyes of juveniles, subadults and adults, a pattern of radiating dark and light dashes was present.



Figure 3.33: Specimens of *L. candolii*: A – juvenile specimen; B – female; C – mating male; D and E – male outside the mating season (Photos: D. Trkov).

A. incognitus body colour of adults was yellow-brown to reddish, sometimes with a lighter and darker pattern across the body (Figure 3.34). During the spawning season, the male's head became intensively red-coloured, while dorsal and anal fins became black-coloured. Pectoral fins were never pigmented, while the caudal fin was coloured with the same colour pattern as the body. The body colour of juveniles and subadults is very variable and depends on the substrate. Specimens were mostly green-brown or even reddish with irregular darker and lighter pattern of blotches and mottling. On the body of small specimens, very small blue spots across the body and a white band between eyes were usually present. On juveniles, only the anterior part of the dorsal fin was dark-coloured, while the rear part was transparent. In juveniles, a pale patch was often present under each eye. In the eyes of juveniles and subadults, a pattern of radiating dark and light dashes was present, which was not so pronounced in adult specimens.

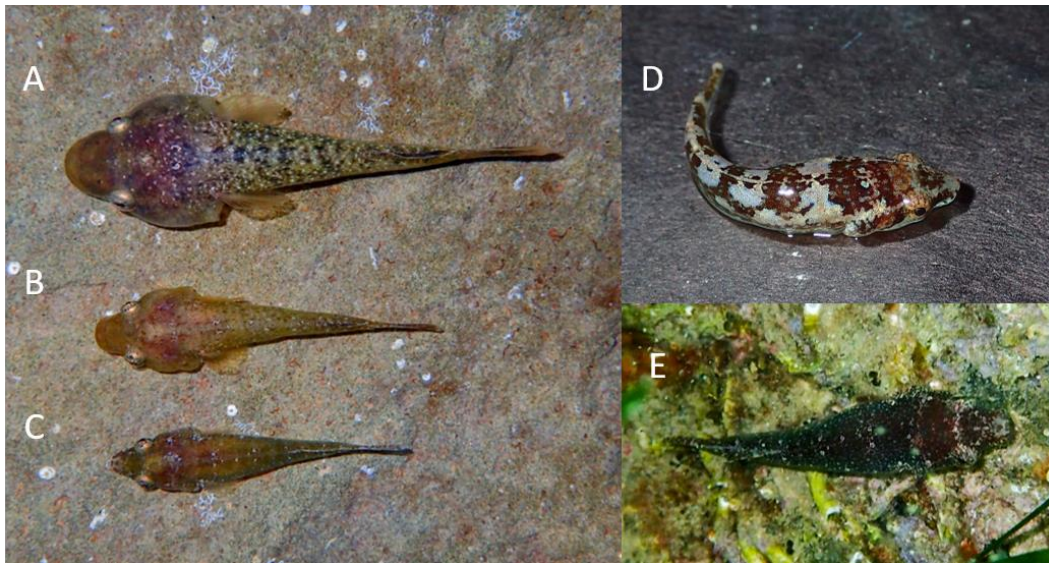


Figure 3.34: Specimens of *A. incognitus*: A and B – males; C – female; D and E – juvenile (Photos: D. Trkov).

3.5 Occurrence, Depth Distribution and Habitat Preferences of Clingfishes

During the survey, altogether 641 specimens of three clingfish species were observed in the field. The locations of the finds are presented in Appendix B.

3.5.1 *Lepadogaster lepadogaster*

All of 242 specimens of *L. lepadogaster* were found in the rocky coastal area. Specimens occurred between 0.4 and 2.2 m of depth (Figure 3.35). However, 75% of specimens were found between 0.7 and 1.2 m. Results showed a significant linear relation (linear regression t-test, $p < \alpha$, $\alpha = 0.05$) between size of the *L. lepadogaster* and depth occurrence, as large specimens occur somehow deeper (Figure 3.36). The majority of the specimens was found in boulder fields and banks of larger stones, while few specimens were also found in banks of pebbles (Figure 3.37). Specimens were mostly attached to the underside of stones which were quite smooth and normally without epibiota cover. In only a few cases they were present under a stone on the substrate. Average stone size, under which specimens were found, measured $26.0 (\pm 11.0) \times 14.3 (\pm 5.7) \times 7.6 (\pm 4.0)$ cm (length x width x height) and it had an average bottom surface 409.8 ± 349 cm², while the average volume of the stone was 4027.7 ± 8269.4 cm³. Bottom surface area positively influenced the clingfish size, as large specimens prefer stones with larger surface area (linear regression t-test, $p < \alpha$, $\alpha = 0.05$). Furthermore, our data showed significant negative linear relation (linear regression t-test, $p < \alpha$, $\alpha = 0.05$) between stone flatness (SA:V) and depth of occurrence, showing that occupied stones were more flat with decreasing depth. Stones were mostly overgrown with turf vegetation (58.4%) or were bare (36.6%). Under stones, where clingfishes were found, the most common substrate were pebbles (63.8%), which were sometimes mixed with sand (20.4%) or rarely occurred together with a sandstone terrace. Pebbles under the stone were of different sizes and rectangular in shape mostly in one layer. Only rarely was the substrate made of sandstone terrace (7.2%). In seven cases, more than one specimen was found under the same stone.

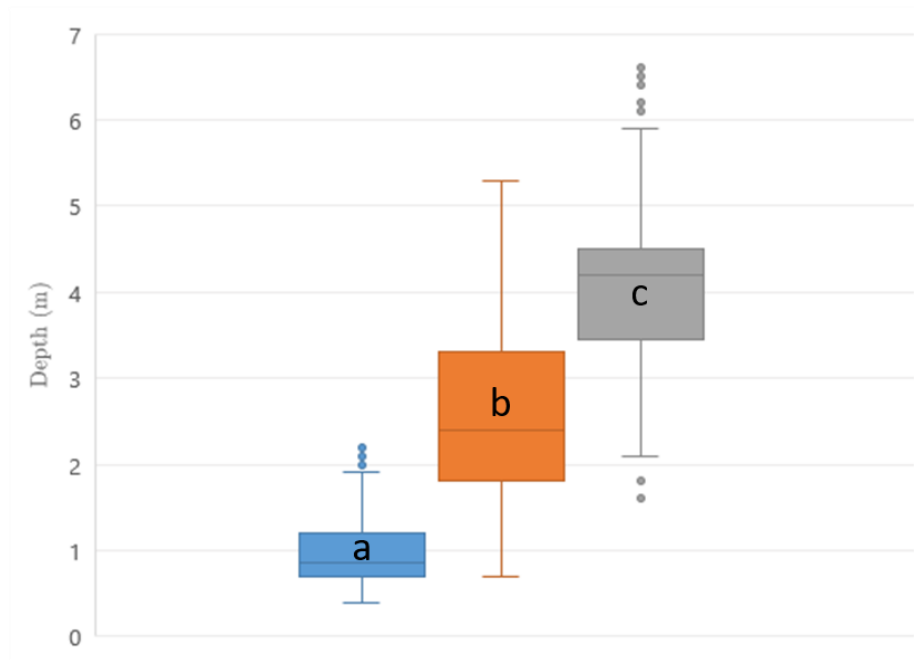


Figure 3.35: Depth distribution of three clingfish species. There is statistically significant difference between species based on depth of occurrence (Tukey's post-hoc test). Legend: blue rectangle – *L. lepadogaster*, orange rectangle – *L. candolii*; grey rectangle – *A. incognitus*.

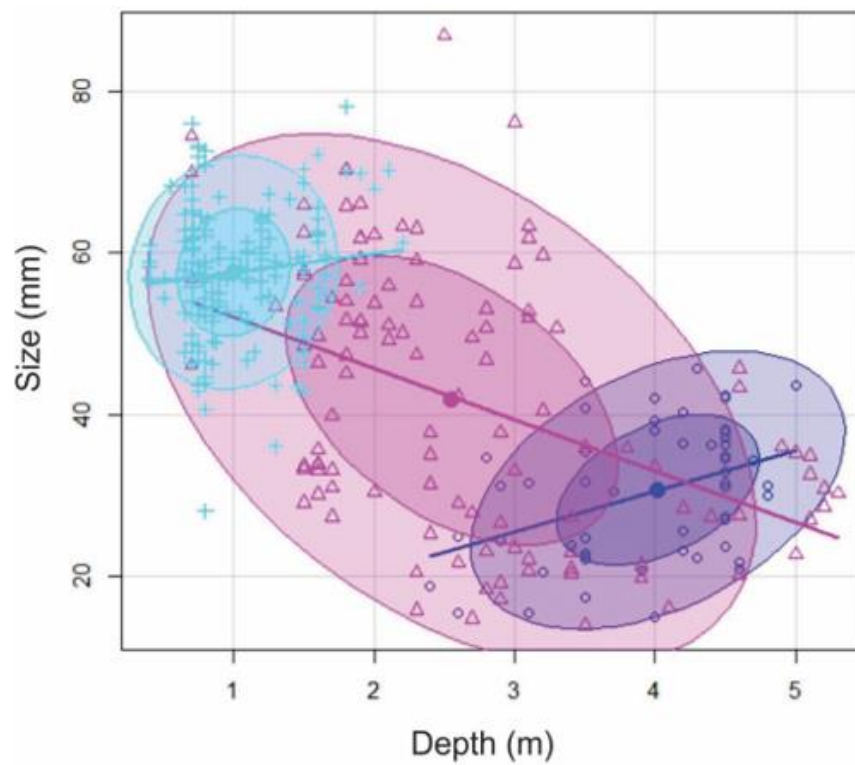


Figure 3.36: Depth distribution of three clingfish species based on their size. Legend: cyan crosses – *Lepadogaster lepadogaster*, red triangles – *Lepadogaster candolii* and blue circles – *Apletodon incognitus*.

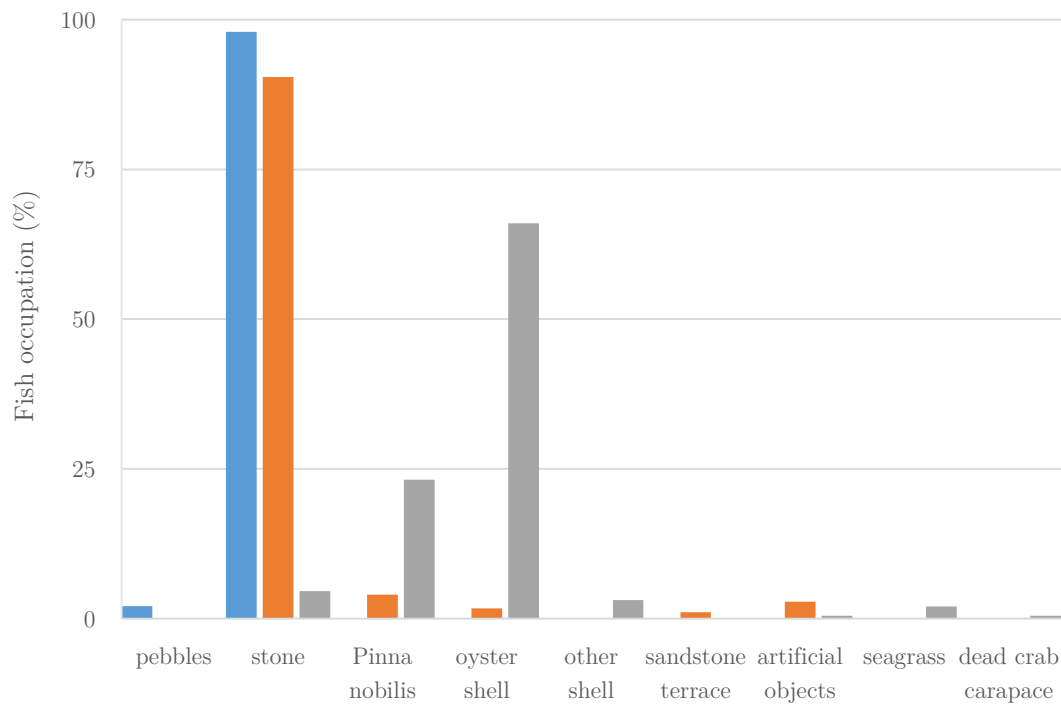


Figure 3.37: Shelters occupied by three species of clingfish. Legend: blue bars – *L. lepadogaster*, orange bars – *L. candolii*, grey bars – *A. incognitus*.

3.5.2 *Lepadogaster candolii*

During the survey, 177 specimens of *L. candolii* were observed in the field. Specimens were found from 0.7 to 5.3 m depth, while most of specimens (75%) were found from 1.8 to 3.3 m (Figure 3.35). Four specimens were found also in tide pools in lower mediolittoral (absolute depth 0.7 m). Results showed a significant negative linear relation (linear regression t-test, $p < \alpha$, $\alpha = 0.05$) between size of the *L. candolii* and depth occurrence, as large specimens occurred in shallower waters and small specimens occurred somehow deeper. Specimens were mostly found on the rocky coastal area (84.2%) in boulder fields and under solitary rocks on sand patches, while 15.8% of them were found in a seagrass meadow of *C. nodosa* and *P. oceanica*. Specimens were mostly attached on the underside of objects (such as stones and seashells) or they were sometimes present on the substrate under objects. Specimens were mostly found under stones (90.4%), and in some cases under seashells (*e.g.* *P. nobilis*, *O. edulis*) and artificial objects (*e.g.* plastic and iron plates) which lay on the sea bottom. In four cases, juvenile specimens were found also on shells of alive *P. nobilis*. Average stone size, under which specimens were found, measured $26.9 (\pm 12.7) \times 17.1 (\pm 7.5) \times 6.7 (\pm 4.0)$ cm (length x width x height) and it had an average bottom surface 530.9 ± 533.1 cm², while the average volume of the stone was 4487.6 ± 9655.5 cm³. The obtained data showed that fish size increases along the bottom surface area gradient, as larger specimens preferred stones with larger surface area (Figure 3.38). Our data showed positive correlation between the depth and the stone flatness (SA:V), which indicated that stones were more flat with increasing depth (Figure 3.39). Furthermore, our data showed significant linear relation (linear regression t-test, $p < \alpha$, $\alpha = 0.05$) between stone flatness (SA:V) and depth of occurrence.

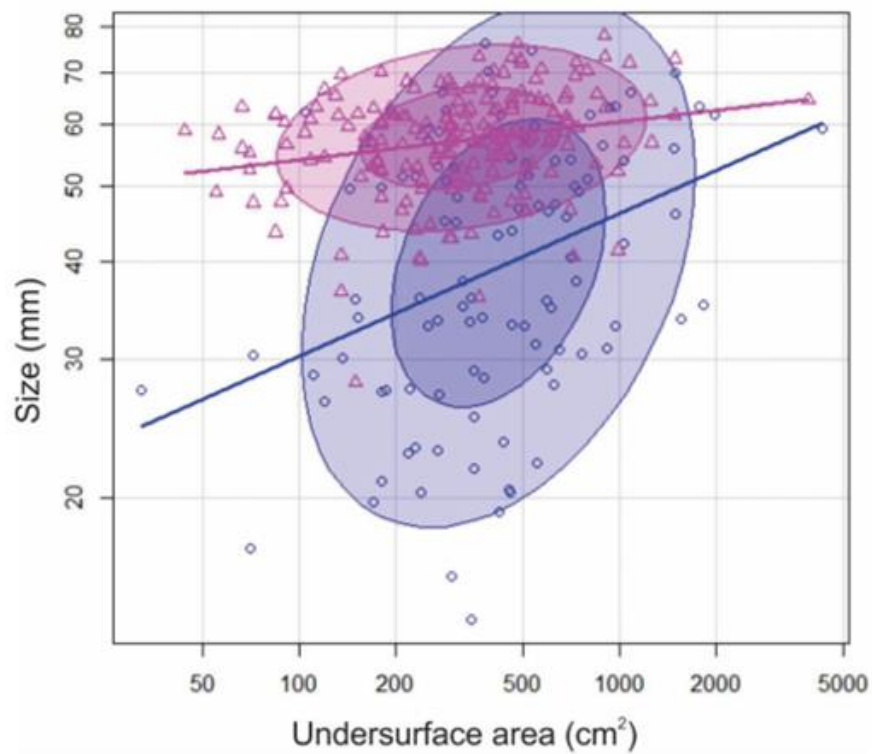


Figure 3.38: Ratio between the undersurface area of inhabited stone and the size of *L. lepadogaster* (red triangles) and *L. candolii* (blue circles). Both axes are log-transformed.

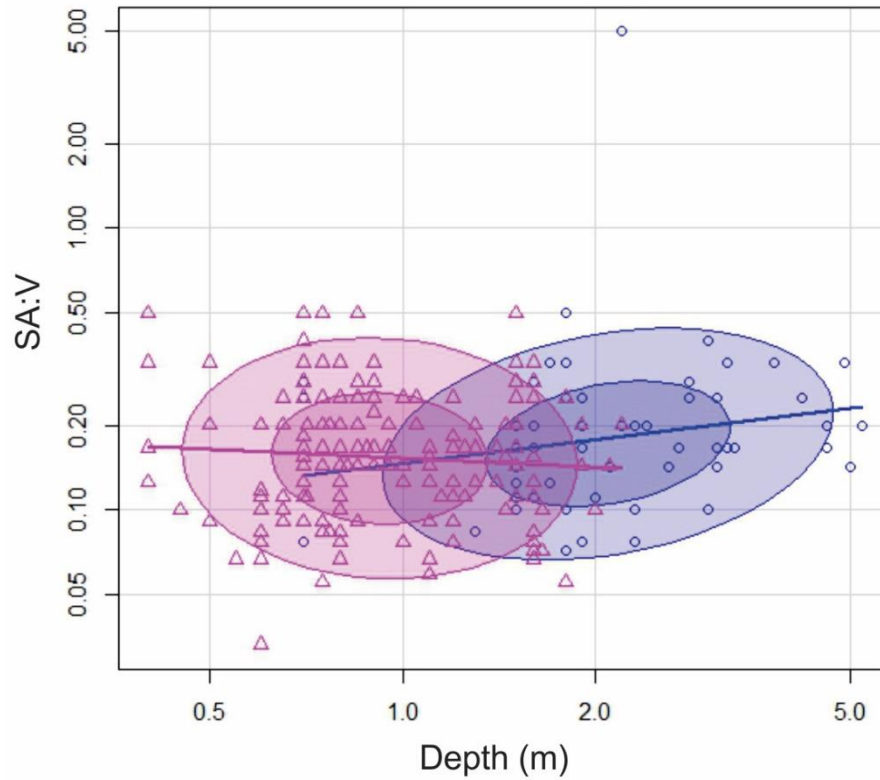


Figure 3.39: Ratio between the depth and the SA:V (surface area to volume ratio) of the stone for *L. lepadogaster* (red triangles) and *L. candolii* (blue circles). Both axes are log-transformed.

The underside of the stones’ surface, occupied by *L. candolii*, was rougher than that of *L. lepadogaster*, due to small holes made by different biota and also its cover such as tubes of serpulid polychaetes. All stones were overgrown with algae (Figure 3.40), 25.3% of them with algal turf, while 62.4% of them with macroalgae (*e.g. Padina pavonica, Dictyota* sp., *Cystoseira* sp.). Less often (12.4%), the stones were overgrown with sponges (*e.g. Aplysina aerophoba*). Different kinds of small sponges were usually present also on the underside of the stone surface, but their presence was not included in the analyses. The most common substrate under the stones were pebbles which were most often mixed with sand (54.2%; Figure 3.41).

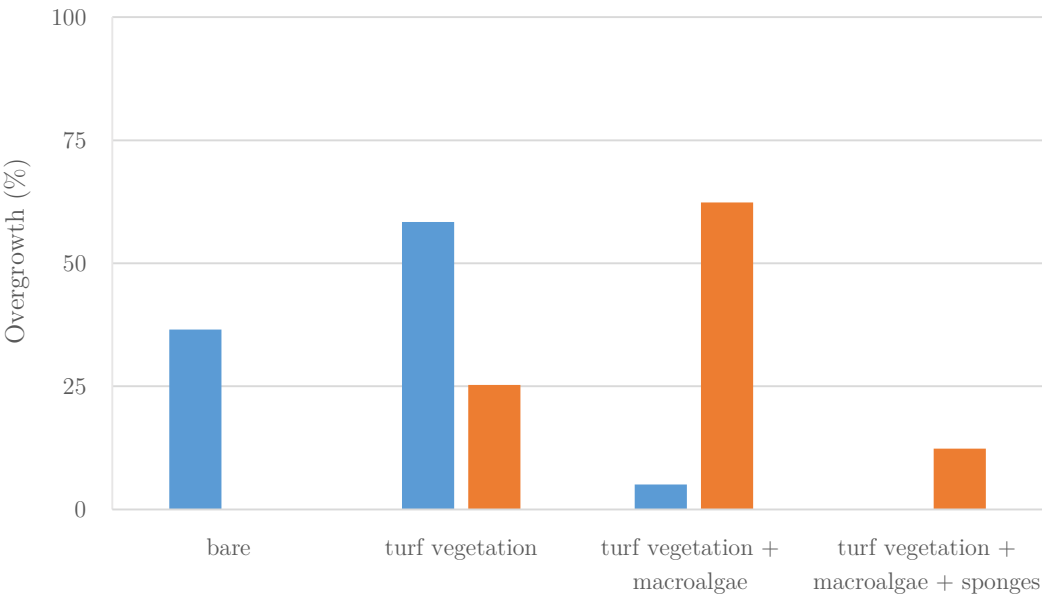


Figure 3.40: Overgrowth on the stone occupied by *L. lepadogaster* (blue bars) and *L. candolii* (orange bars).

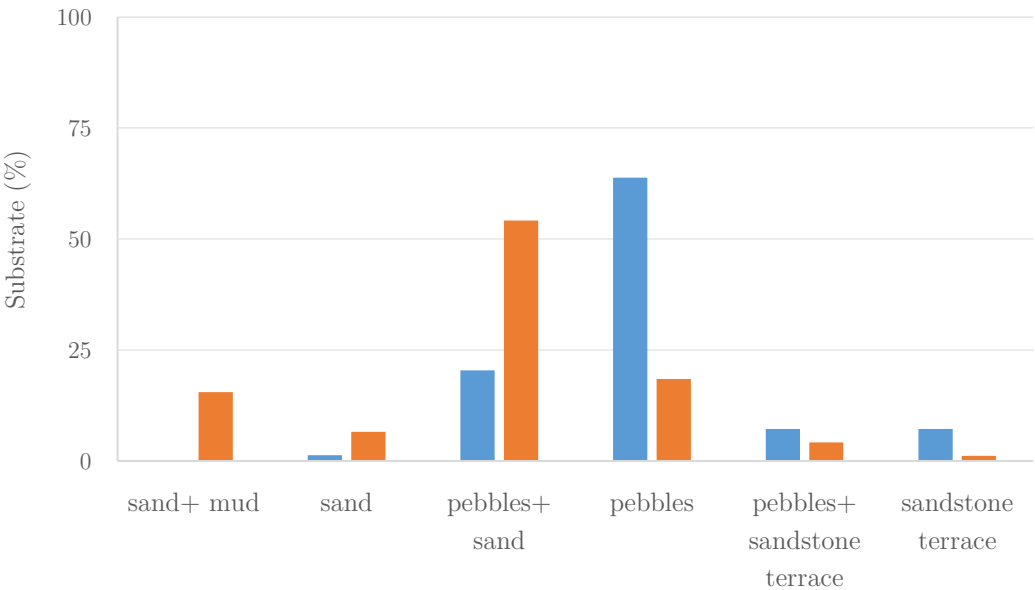


Figure 3.41: Substrate coverage under the stone occupied by *L. lepadogaster* (blue bars) and *L. candolii* (orange bars).

3.5.3 *Apletodon incognitus*

Altogether 222 specimens were observed during the survey. Specimens were found from 1.6 to 6.6 m depth, with the majority of them (75%) occurring between 3.45 and 4.5 m. Results showed a significant linear relation (linear regression t-test, $p < \alpha$, $\alpha = 0.05$) between size of the *A. incognitus* and depth occurrence, as large specimens occur somehow deeper (Figure 3.36). Most of specimens (98.2%) were found in a seagrass meadow of *C. nodosa* and only few in the seagrass meadow of *P. oceanica*. Specimens occupied mostly dead oyster shells (66.0%), especially oysters attached to *P. nobilis* (82.8% of all specimens were found in an oyster shell) and less often in clusters of oyster shells on the sea bottom (9.4% of all specimens found in an oyster shell). Many specimens were found also on a living adult *P. nobilis* (22.2%), which were overgrown with an epibiota cover (*e.g.* oysters, bryozoans, serpulid polychaetes, algae). Only 4.6% specimens were found on isolated stones which were present in the seagrass meadow.

3.6 Reproductive Ecology

Nests of *L. lepadogaster* were found from the end of March to the end of May in the location next to Fiesa. Altogether 88 nests were found during the survey. Nests were observed from 0.4 to 1.4 m depth, while 75% of them were found between 0.6 and 0.75 m. The average stone size on which nests were present measured $27.0 (\pm 9.5) \times 13.9 (\pm 4.0) \times 7.5 (\pm 2.9)$ cm (length x width x height) and it had an average bottom surface 396.3 ± 239.1 cm², while the average volume of the stone was 3260.8 ± 2973.4 cm³. Stones were mostly bare (80.7%) or overgrown with turf vegetation (19.3%). Under stones, where nests were found, the most common substrate were pebbles (34.1%), followed by a sandstone terrace (26.1%) and big stones (diameter > 20 cm; 25.0%). In four cases, more than one nest was found attached to the lower surface of the same stone, and in one case, six separated nests were present on one stone. Only in 27 cases, fish were observed under a stone, and in 16 cases, fish were also caught (15 males and one female).

Altogether 52 nests with total 32.132 eggs were analyzed. Nests normally consisted of one to maximum five (average 1.8) clutches of eggs in different phases of development (Table 3.5). Eggs in the middle were normally the most developed and sometimes already started to hatch. Nests contained 618 eggs on average. One clutch of eggs contained 335 eggs on average. Nests contained from 48 to maximum 7384 eggs. The median value of eggs per clutch, which gives us the average number of eggs laid by a female in one spawning, was 165.5.

Table 3.5: Number of *L. lepadogaster* nests which contained from one to maximum five clutches of eggs.

Number of clutches of eggs per nest	Number of nests
1	28
2	12
3	7
4	4
5	1

The nest of *L. candolii* was observed only once. The nest was found on 1 May 2018 at 3 m depth. It was found under a stone (20 x 19 x 2.5 cm) where a sandstone terrace and

sand were present as the substrate. In the nest, one guarding male was observed. Most of the eggs were already hatched, while in others, well-developed larvae were observed.

Nests of *A. incognitus* were observed from mid-May to mid-July in seagrass meadows. Altogether 22 nests were found in dead oyster shells, which were mostly attached to the living *P. nobilis*. Only one nest was found in a dead oyster shell which was lying on the sea bottom. The nests were mostly extended over both inner parts of the shell and were located near the hinge of the shell. The inner side of the shell was normally overgrown with different organisms, such as bryozoans, serpulid polychaetes and biofilm of microalgae (the shell was coloured green). In each nest, a guarding male was present. Nests were found from 2.8 to 6.6 m, while 75% of nests were distributed in a depth range from 3.2 to 4.4 m.

Altogether, 15 nests of *A. incognitus* with total 8834 eggs were analysed. Nests consisted of one to three clutches of eggs (Table 3.6), and contained 589 eggs on average. On average, nests were composed of two clutches of eggs and each part contained 295 eggs on average. Nests contained from 60 to maximum 1208 eggs. The median of eggs per clutch, which gives us the average number of eggs laid by a female in one spawning, was 189.5.

Table 3.6: Number of *A. incognitus* nests which contained from one to maximum three clutches of eggs.

Number of clutches of eggs per nest	Number of nests
1	6
2	3
3	6

3.7 Diet Studies

3.7.1 Pre-experiments

Specimens of *L. lepadogaster* studied in buckets needed from 6 to 24 hours to produce faecal pellets. During defecation, water temperature ranged from 22 to 27° C. After the defecation, specimens may produce “false” pellets, which were made only of peritrophic membrane but with no content. “False” pellets were smaller than faecal pellets and were white-coloured (Figure 3.42).

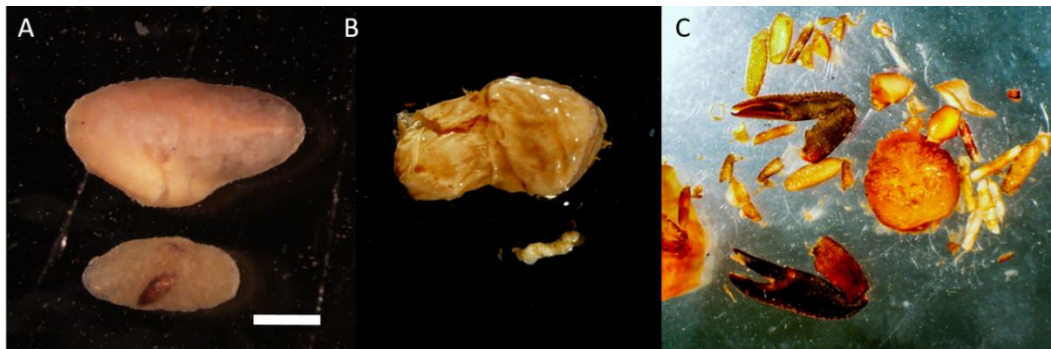


Figure 3.42: A – two faecal pellets of *L. lepadogaster*, the white bar represents 2 mm; B – faecal pellet (above) and “false” faecal pellet (below); C – prey remains from the faecal pellet of *L. lepadogaster* (Photos: D. Trkov).

Experiment on 16 specimens of *A. incognitus*, which had food available all the time, showed that within 24 hours, they produced 53 faecal pellets altogether. Specimens produce from two to maximum six (average 3.3 ± 1.4) faecal pellets per fish per 24 hours. Water temperature during the experiment ranged from 22 to 24° C.

The results of the effectiveness of the method, verified on *L. candolii*, showed that all 29 ingested prey items of four taxa (*Pisidia* sp., *Athanas nitescens*, *Eualus* sp. and Gammaridea) were also found and identified in faecal pellets (Table 3.7).

Table 3.7: Offered and ingested prey items and prey items recognized in faecal pellets of six specimens of *Lepadogaster candolii* used to test the proposed nonlethal method. All the ingested prey was also found and identified in the faecal pellets.

	Offered prey	Ingested prey	Prey recognized in faecal pellets
Specimen 1	2x <i>Pisidia</i> sp.	2x <i>Pisidia</i> sp.	2x <i>Pisidia</i> sp.
	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>
	2x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.
	2x Gammaridea	1x Gammaridea	1x Gammaridea
Specimen 2	2x <i>Pisidia</i> sp.	1x <i>Pisidia</i> sp.	1x <i>Pisidia</i> sp.
	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>
	2x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.
	2x Gammaridea	1x Gammaridea	1x Gammaridea
Specimen 3	2x <i>Pisidia</i> sp.	2x <i>Pisidia</i> sp.	2x <i>Pisidia</i> sp.
	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>
	2x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.
	2x Gammaridea	1x Gammaridea	1x Gammaridea
Specimen 4	2x <i>Pisidia</i> sp.	-	-
	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>
	2x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.
	2x Gammaridea	1x Gammaridea	1x Gammaridea
Specimen 5	2x <i>Pisidia</i> sp.	-	-
	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>
	2x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.
	2x Gammaridea	1x Gammaridea	1x Gammaridea
Specimen 6	2x <i>Pisidia</i> sp.	-	-
	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>	2x <i>Athanas nitescens</i>
	2x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.	1x <i>Eualus</i> sp.
	2x Gammaridea	1x Gammaridea	1x Gammaridea

3.7.2 Test of the non-destructive method on *L. lepadogaster*

To test the effectiveness of the non-destructive method, based on the analysis of faecal pellets, 116 specimens of *L. lepadogaster* were included altogether. Specimens were collected from January to November 2017. Total length of the specimens ranged from 28.06 mm to 78.02 mm (57.19 ± 7.92 mm), while their weight ranged from 0.23 g to 5.85 g (2.08 ± 0.87 g). The sex ratio was 1:1 ($\chi^2 = 0.1923$; $p < 0.66$). All specimens survived the non-destructive method, at least until the release back into nature. Altogether, 177 faecal pellets were produced and 836 prey items were found in the pellets. Almost all clingfishes (95.7%) produced from one to a maximum of three faecal pellets; on average, 1.6 pellets per

specimen. In total, 47 taxa were recognized as prey items, which were grouped in 17 higher taxonomic groups (Table 3.8). Copepods (N% = 33.0), represented mostly by harpacticoids, were by far the most numerous prey group followed by amphipods (N% = 18.9) and decapods (N% = 15.7). Amphipods and decapods were also the most frequent prey group in the samples in terms of frequency of occurrence (both with F% = 69.4). In the diet of more than 30% of fish specimens, copepods (F% = 38.7), gastropods (F% = 34.2) and isopods (F% = 36.9) were also present.

Table 3.8: Major groups of prey items found in the faecal pellets of *Lepadogaster lepadogaster* from the studied area. F (%): relative frequency of occurrence; N (%): relative numerical abundance.

Higher taxon	Lower taxon	F (%)	N (%)
Rhizaria	Foraminifera	1.8	0.2
	Polyplacophora	0.9	0.1
Mollusca	Gastropoda	34.2	7.9
	Bivalvia	13.5	2.8
Crustacea	Copepoda	38.7	33.0
	Amphipoda	69.4	18.9
	Decapoda	69.4	15.7
	Mysida	1.8	0.2
	Anisopoda	2.7	0.5
	Isopoda	36.9	6.5
	Ostracoda	14.4	2.9
	Cirripedia	4.5	0.7
	Crustacea	3.6	0.5
	indet.		
Arachnida	Acari	19.8	5.6
Pantopoda	Pycnogonida	0.9	0.1
Annelida	Polychaeta	8.1	1.6
Echinodermata	Ophiuroidea	17.1	2.6
Indeterminata	Indeterminata	1.8	0.2

3.7.3 Diet study of three clingfish species

Altogether 363 specimens of *L. lepadogaster*, *L. candolii* and *A. incognitus* were included in the study of their diet. Specimens, on which the non-destructive method was used, were collected from January 2017 to July 2019. On 356 fish specimens, the non-destructive method was performed, which all specimens survived up to the point of their release into the nature. Faecal pellets were obtained from 96.9% of specimens, on which the method was performed. Due to the small sample size in *A. incognitus*, we also included eight specimens from the MBS collection whose intestine contents were analyzed.

3.7.3.1 *Lepadogaster lepadogaster*

For the purpose of studying feeding habits, altogether 194 specimens of *L. lepadogaster* were collected. The total length of the specimens ranged from 28.06 mm to 78.02 mm (57.60

± 8.54 mm), while their weight ranged from 0.23 g to 5.85 g (2.17 ± 0.96 g). Out of 183 sexually identifiable specimens 103 were males and 80 females.

Faecal pellets were obtained from 96.9% of the collected fish specimens which produced 294 faecal pellets. Specimens produced from one to maximum three faecal pellets per specimen; on average 1.6 pellets per fish (Table 3.9; Table 3.10). In faecal pellets, 1334 prey specimens were found and recognized, from one to maximum 39 prey specimens per pellet, on average 4.5 prey specimens per pellet. When analysing the number of prey specimens per fish specimen, one to maximum 39 prey specimens were obtained, on average 7.1 prey specimens per fish. Measurements were done on 80 faecal pellets. The average length of a faecal pellet was 4.64 mm, the average width was 2.63 mm and the average weight was 16.64 mg.

Table 3.9: The proportion of *L. lepadogaster* specimens ($n = 188$) which produce from one to maximum three faecal pellets.

Number of faecal pellets per fish	Proportion of fish (%)
1	48.4
2	46.8
3	4.8

Table 3.10: Diet attributes of *L. lepadogaster*.

Proportion of defecated fish (%)	96.9
Number of fish specimens	188
Number of faecal pellets	294
Average number of faecal pellets per fish	1.6
Number of prey	1334
Average number of prey per fish	7.1 ± 7.1
Maximum number of prey per fish	39
Minimum number of prey per fish	1
Average number of prey per faecal pellet	4.5 ± 5.0
Maximum number of prey per faecal pellets	39
Minimum number of prey per faecal pellets	1

In total, 47 taxa were recognized as prey items, which were grouped in 18 wider taxonomic groups (Table 3.11). In faecal pellets, besides prey, different sediment particles were also often present, such as sand grains, old shell fragments of invertebrates and algal particles. Sediment particles could represent up to 16.5% of faecal pellet weight. Copepods ($N\% = 30.1$), represented mostly by harpacticoids, were by far the most numerous prey group followed by amphipods ($N\% = 20.3$) and decapods ($N\% = 12.6$). The latter two prey categories were also the most frequent preyed groups in the samples in terms of frequency of occurrence. Amphipods were present in the diet of 67.6% of specimens, while decapods were present in the diet of 56.4% of fish. The most important prey groups in terms of

biomass were decapods ($B\% = 60.9$) and amphipods ($B\% = 18.3$), which together represent the great majority (79.2%) of biomass. Decapods were the most important prey group in terms of relative importance ($IRI\% = 43.6$), and were mainly represented by the crab *Pisidia* sp. (they represented 82.7% of decapod numerical abundance). Based on determination features, which have been preserved during the digestion, crabs from genus *Pisidia* belong mainly to *P. bluteli* (Risso, 1816) and less often to *P. longimana* (Risso, 1816). Results confirmed that size of *Pisidia* sp. is not in linear relation to the size of the fish (Linear regression t-test; $p = 0.3216$, $\alpha = 0.05$; Figure 3.43). Alternative prey groups were amphipods ($IRI\% = 27.5$), such as species of the family Gammaridae (97.4% of amphipods), and copepods ($IRI\% = 13.0$). Gastropods, which were represented mostly by juveniles of *Bittium reticulatum*, *Alvania* sp. and *Rissoa* sp., constituted 8.3% of IRI. Based on the diet composition we calculated the TROPHs index, which was 3.18 ± 0.44 , and the ITD index, which was 0.83.

The sampling of prey groups under stones inhabited by *L. lepadogaster* showed that amphipods were one of the most numerous and also frequent prey groups in the environment, followed by decapods (Table 3.12). Altogether, 1364 specimens of different prey groups were found within 20 sampling quadrants (25 x 25 cm²) in the lower mediolittoral and the upper infralittoral.

Table 3.11: Major groups of food items found in the faecal pellets of *Lepadogaster lepadogaster*. N (%): relative numerical abundance; F (%): relative frequency of occurrence; B (%): relative gravimetric composition; IRI (%): index of relative importance.

Higher taxon	Lower taxon	N(%)	F(%)	B(%)	IRI(%)
Rhizaria	Foraminifera	0.1	1.1	<0.1	<0.1
	Polyplacophora	0.1	0.5	<0.1	<0.1
Mollusca	Gastropoda	12.1	38.3	8.5	8.3
	Bivalvia	4.2	21.8	0.3	1.0
	Copepoda	30.1	41.0	0.2	13.0
	Amphipoda	20.3	67.6	18.3	27.5
	Decapoda	12.6	56.4	60.9	43.6
Crustacea	Mysidae	0.8	3.7	1.0	0.1
	Anisopoda	0.4	2.7	<0.1	<0.1
	Isopoda	6.6	35.6	4.9	4.3
	Ostracoda	3.4	16.5	<0.1	0.6
	Crustacea indet.	0.5	3.7	4.1	0.2
Arachnida	Acari	4.3	16.0	<0.1	0.7
Pantopoda	Pycnogonida	0.1	1.1	<0.1	<0.1
Annelida	Polychaeta	1.4	8.0	0.4	0.2
Echinodermata	Ophiuroidea	2.1	12.8	0.7	0.4
Actinopterygii	Fish eggs	0.2	1.1	<0.1	<0.1
Indeterminata	Indeterminata	0.4	3.2	0.6	<0.1

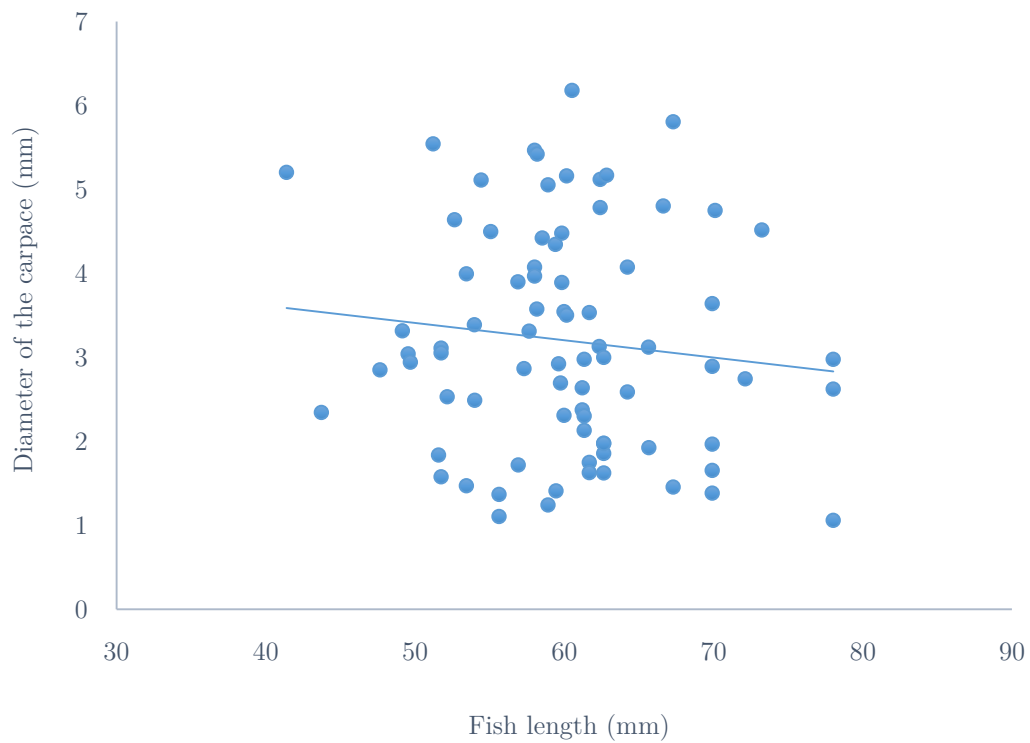


Figure 3.43: Diameter of the *Pisidia* sp. carapace of specimens preyed by *L. lepadogaster* depending on the size of the predator. The blue line represents the trend line.

Table 3.12: Occurrence of different prey groups under stones in the habitat of *L. lepadogaster*.

Higher taxon	Lower taxon	N(%)	F(%)
Mollusca	Gastropoda	3.7	55.0
	Bivalvia	1.4	30.0
	Decapoda	11.5	75.0
Crustacea	Amphipoda	63.1	100.0
	Isopoda	3.8	50.0
	Mysidae	2.6	65.0
Pantopoda	Pycnogonida	0.1	5.0
Annelida	Polychaeta	10.9	90.0
Echinodermata	Ophiuroidea	2.9	70.0

Differences in the diet of *L. lepadogaster* between sexes were not statistically significant (PERMANOVA; $p = 0.648$, $\alpha = 0.05$). However, prey abundance showed that copepods were the most numerous prey group in the diet of both sexes, while the frequency of occurrence showed that amphipods were the most frequent prey group in the diet of both sexes (Figure 3.44, Figure 3.45). Due to the small sample in some age groups, they were grouped in three bigger age groups: < 3 , $3+$ and $4+$ years. There was no significant difference in the diet between different age classes (PERMANOVA; $p = 0.135$, $\alpha = 0.05$). However, copepods dominate in the diet of the age group < 3 years and their abundance and frequency of occurrence were decreasing with the age of the group. This trend was observed also for Acari and ostracods. On the contrary, the abundance and frequency of

occurrence of decapods, isopods and gastropods were increasing with the age of the group (Figure 3.46; Figure 3.47). For different age groups, TROPHs and ITD were also calculated. TROPHs index ranged from 3.18 ± 0.36 for the youngest age group and increased to 3.35 ± 0.52 for the oldest age group, while ITD ranged from 0.75 in the age group < 3 to 0.82 for the age group 4+ (Table 3.13).

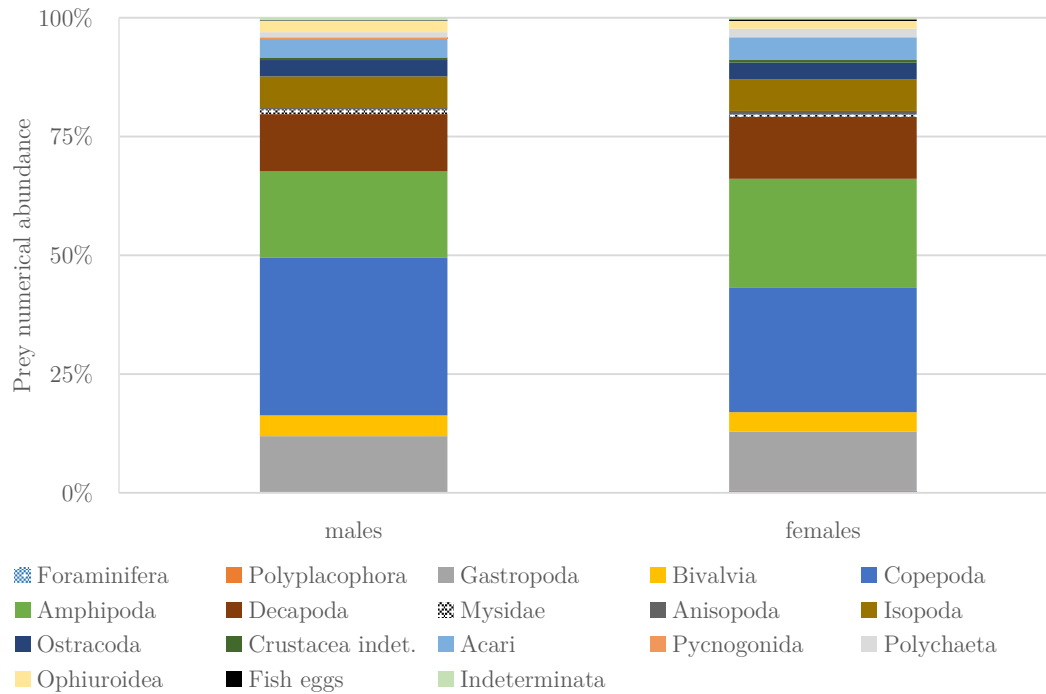


Figure 3.44: The diet of males and females of *L. lepadogaster* in terms of numerical abundance (%).

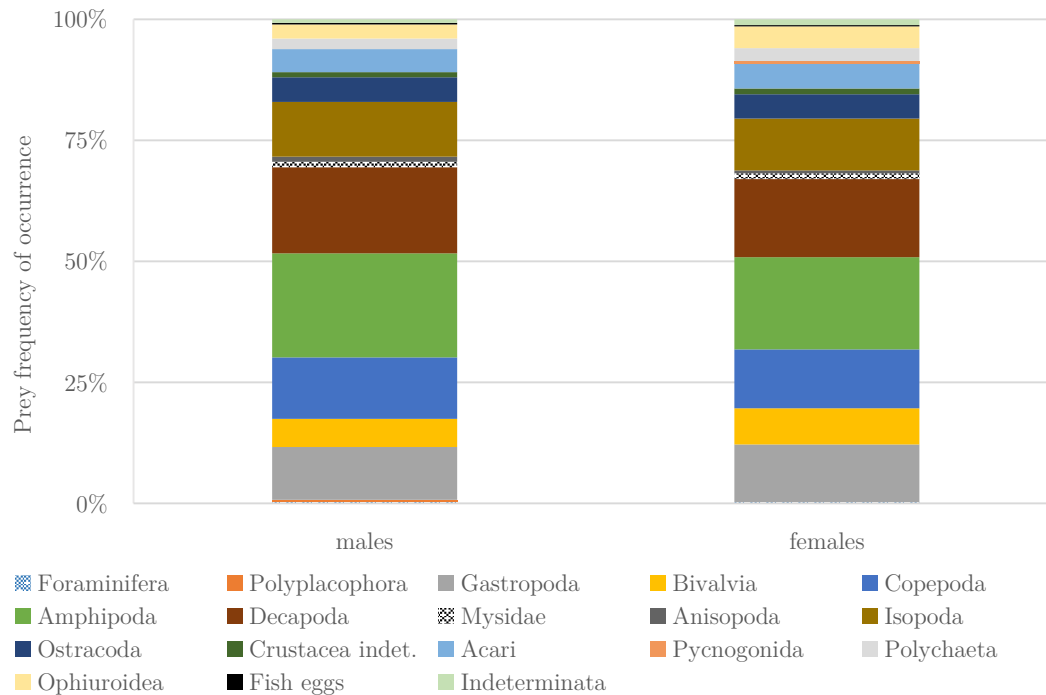


Figure 3.45: The diet of males and females of *L. lepadogaster* in term of the frequency of occurrence (%).

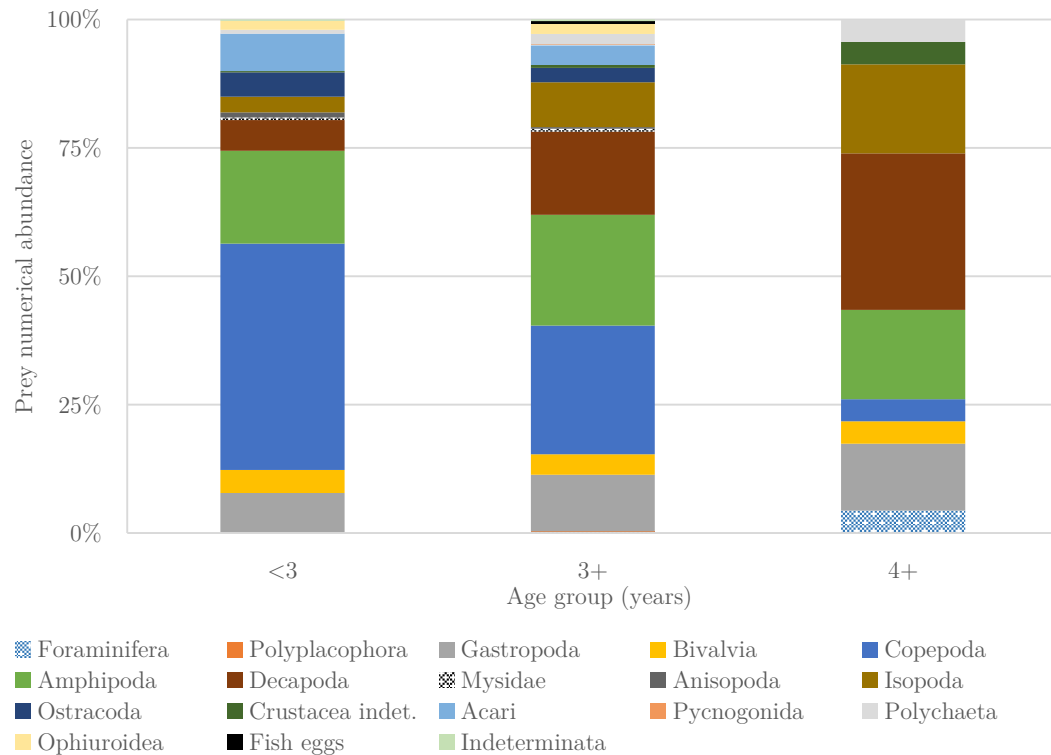


Figure 3.46: The diet of different age groups of *L. lepadogaster* in terms of numerical abundance (%).

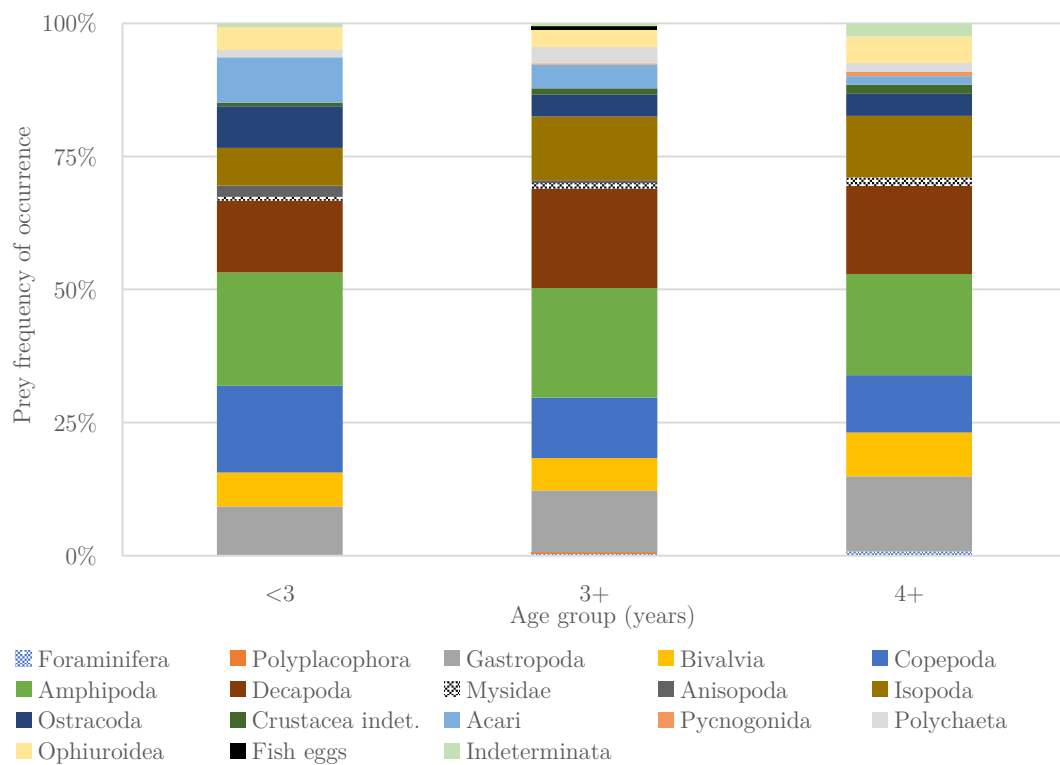


Figure 3.47: The diet of different age groups of *L. lepadogaster* in terms of the frequency of occurrence (%).

Table 3.13: ITD and TROPHs index of different age classes of *L. lepadogaster*. Age classes 1+ and 2+ were grouped together in < 3 years group due to the small sample size.

Age class (years)	Number of specimens	ITD	TROPHs
< 3	39	0.75	3.18
3+	109	0.84	3.21
4+	40	0.82	3.35

Differences in the diet composition of *L. lepadogaster* based on depth segregation were observed and were statistically significant (PERMANOVA; $p = 0.001$, $\alpha = 0.05$). The most significant prey group in shallow water were copepods, while in deeper water it was decapods and gastropods. Furthermore, the SIMPER function showed that decapods were the most important prey group on their maximum depth of occurrence and their importance was decreasing with the decreasing depth (Figure 3.48). The SIMPER function also showed that the importance of copepods was decreasing with the increasing depth. Furthermore, prey numerical abundance showed a higher number and frequency of occurrence of copepods and bivalves in shallower water than in deeper waters. On the contrary, the abundance and frequency of occurrence of decapods, ophiuroids and gastropods was increasing with the increasing depth (Figure 3.49; Figure 3.50).

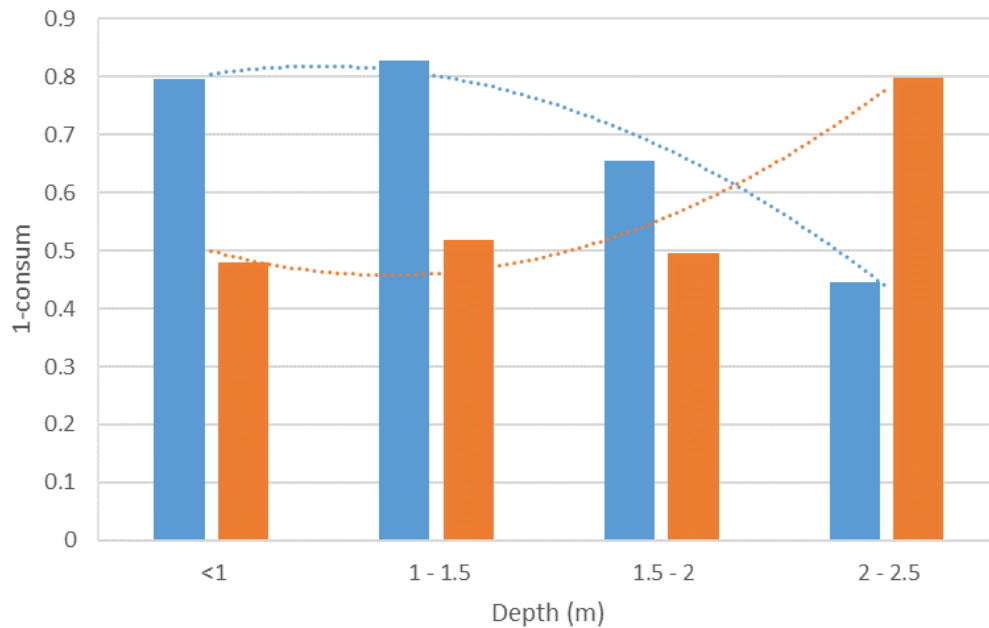


Figure 3.48: SIMPER function showing the importance of copepods (blue bars) and decapods (orange bars) in the diet of *L. lepadogaster* according to the depth distribution. The dotted lines represent trend lines: blue for copepods and orange for decapods.

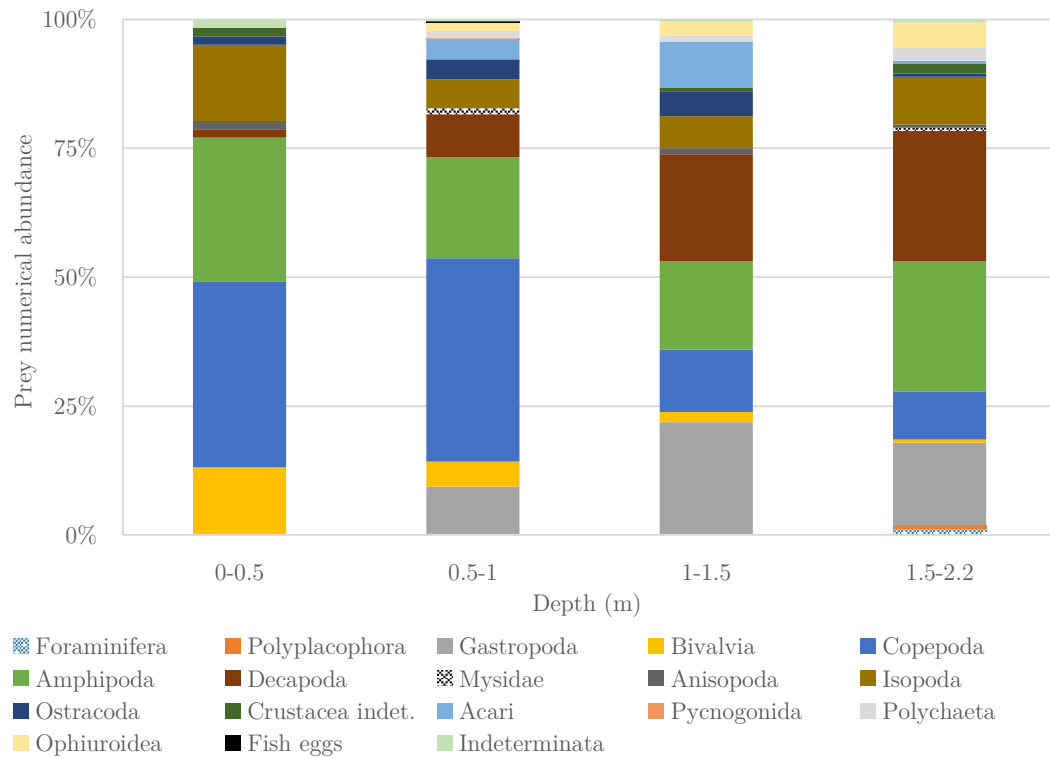


Figure 3.49: Diet of *L. lepadogaster* in terms of numerical abundance (%) at different depth intervals.

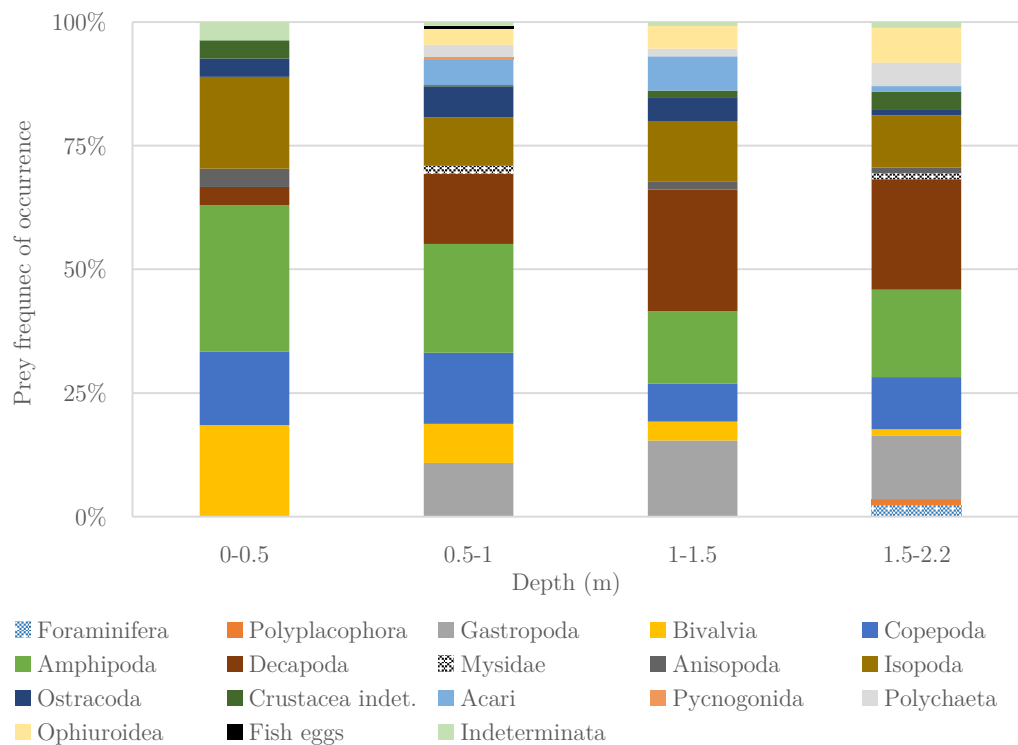


Figure 3.50: Diet of *L. lepadogaster* in terms of the frequency of occurrence (%) at different depth intervals.

Seasonal differences in the diet of *L. lepadogaster* were statistically significant (PERMANOVA; $p = 0.001$, $\alpha = 0.05$). The most significant was the presence of amphipods

in the diet of *L. lepadogaster* in winter, copepods in spring and decapods in summer and autumn. In winter, the most abundant groups were amphipods and gastropods, which were also the most frequent prey groups in the diet (Figure 3.51; Figure 3.52). In spring, copepods were the far most abundant prey group, with more than 52% of prey numerical abundance. In summer, copepods' numerical abundance declined, while the abundance of decapods increased and became the most abundant and frequent prey group in the diet. In summer, the abundance of isopods, acari and amphipods also increased. In autumn, amphipods were the most abundant prey group followed by decapods whose abundance has slightly declined compared to summer. However, decapods' frequency of occurrence in the diet was the highest in autumn (79.3%). Comparing the size of the consumed crabs of *Pisidia* sp. shows that fish consumed the largest specimens of *Pisidia* sp. in winter, and their size was declining through seasons and was the smallest in autumn (Figure 3.53). Seasonal differences in the diet of males of *L. lepadogaster* were also statistically significant (PERMANOVA; $p = 0.001$, $\alpha = 0.05$), and the presence of copepods in the diet was the most significant for spring.

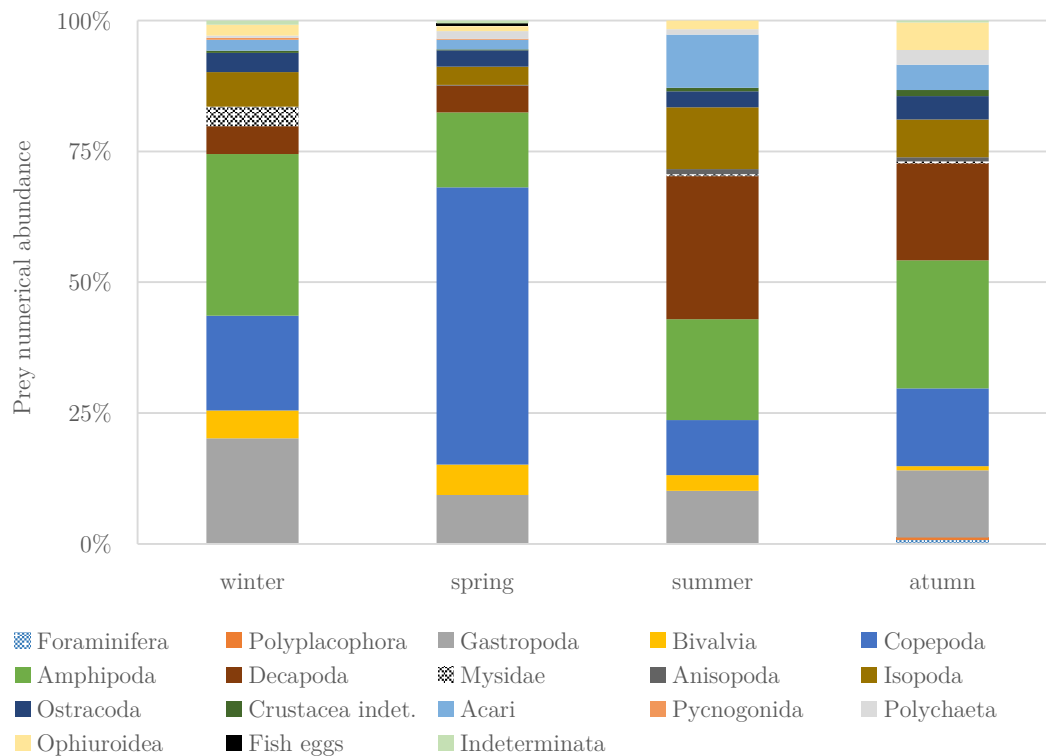


Figure 3.51: Seasonal diet of *L. lepadogaster* in terms of the numerical abundance (%).

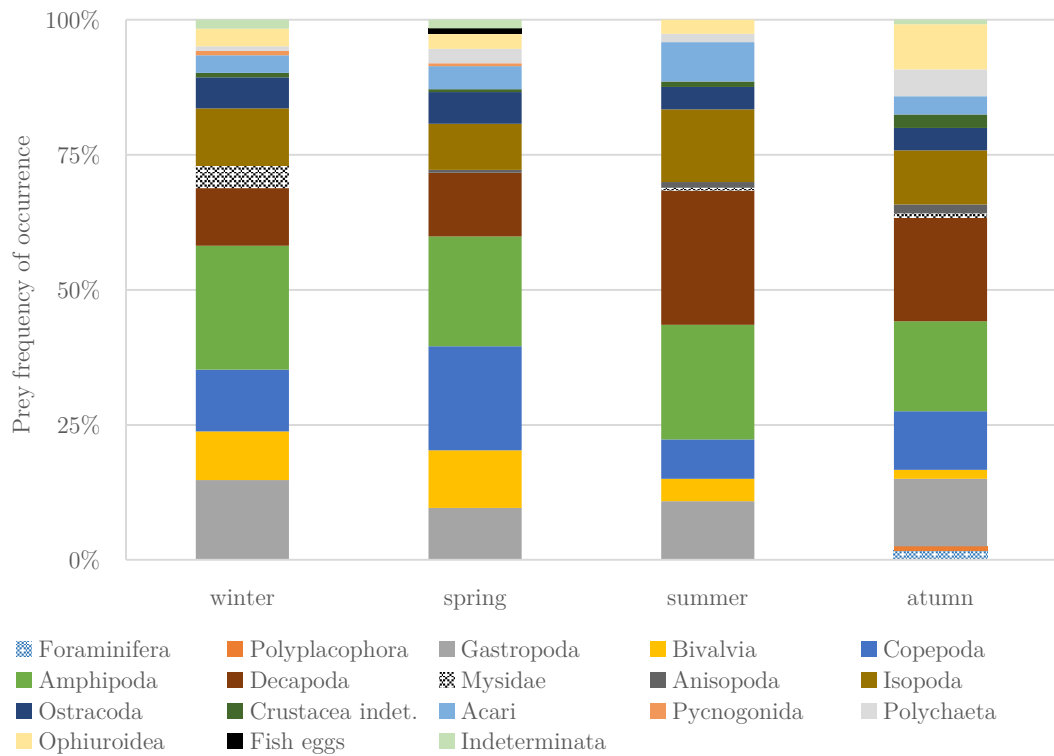


Figure 3.52: Seasonal diet of *L. lepadogaster* in terms of the frequency of occurrence (%).

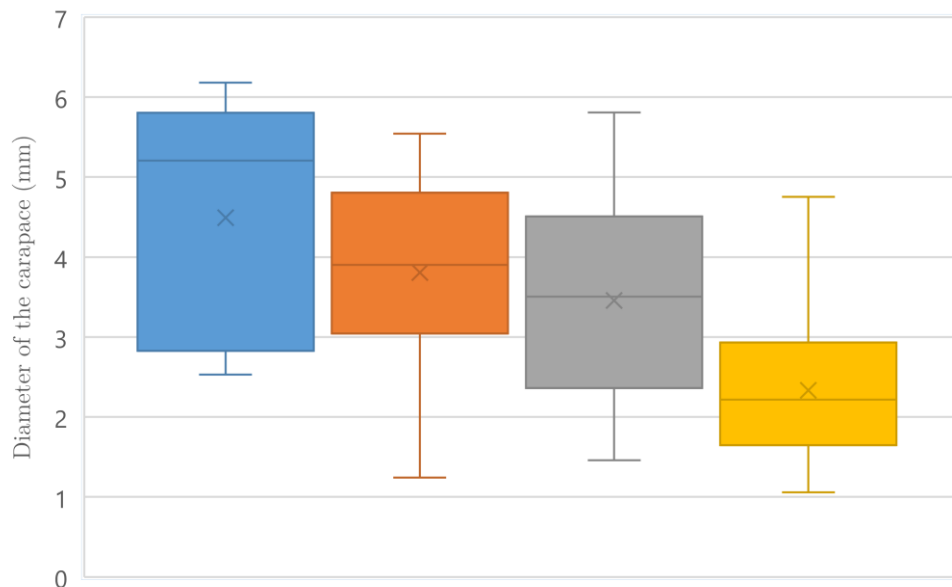


Figure 3.53: The size of the preyed crabs of the genus *Pisidia* consumed in different seasons. Legend: blue rectangle – winter; orange rectangle – spring; grey rectangle – summer; yellow rectangle – autumn.

3.7.3.2 *Lepadogaster candolii*

Altogether, 120 specimens of *L. candolii* were included in the investigation of its diet. 117 specimens were measured. The total length of the specimens ranged from 13.97 mm to 86.94 mm (41.48 ± 16.00 mm), while their weight ranged from 0.03 g to 6.05 g (1.08 ± 1.10 g). Out of 68 sexually identifiable specimens 16 were males and 52 females.

Most of the fish specimens defecated (96.8%) and produced from one to maximum three faecal pellets (Table 3.14), on average 1.3 faecal pellets per fish. Altogether, 161 faecal pellets were obtained, in which 1779 prey specimens were counted and identified (Table 3.15). In faecal pellets, as also per fish, from one to maximum 143 prey specimens were found. On average, 11.0 prey specimens were present in faecal pellets, while 14.8 prey specimens were found per fish specimen. Altogether, 119 faecal pellets were measured. The average weight of the faecal pellet was 10.42 mg and measured on average 3.53 mm in length and on average 2.10 mm in width.

Table 3.14: The proportion of *L. candolii* specimens (n = 120) which produce from one to maximum three faecal pellets.

Number of faecal pellets per fish	Proportion of fish (%)
1	66.7
2	32.5
3	0.8

Table 3.15: Diet attributes of *L. candolii*.

Proportion of defecated fish (%)	96.8
Number of fish specimens	120
Number of faecal pellets	161
Average number of faecal pellets per fish	1.3
Number of prey	1779
Average number of prey per fish	14.8 ± 16.9
Maximum number of prey per fish	143
Minimum number of prey per fish	1
Average number of prey per faecal pellet	11.0 ± 13.7
Maximum number of prey per faecal pellets	143
Minimum number of prey per faecal pellets	1

In total, 42 different prey taxa were found and recognized in the diet of *L. candolii*. Prey items were grouped in 18 wider taxonomic groups (Table 3.16). In faecal pellets, besides prey remains, different sediment particles were sometimes also present, such as sand grains, old shell fragments of invertebrates and algal particles, which could represent up to 64.1% of faecal pellet weight. The most frequent prey groups were copepods (F% = 74.2), followed by decapods (F% = 60.0), isopods (F% = 59.7) and amphipods (F% = 56.7). Copepods were by far the most numerous prey items (N% = 47.2), followed by isopods (N% = 11.3). In terms of biomass, decapods were the most important (B% = 46.3), followed by isopods (B% = 25.4) and amphipods (B% = 20.5). Copepods (IRI% = 30.9) and decapods (IRI% = 28.5) were the most important prey in terms of IRI. Decapods were represented mainly by the crab of the genus *Pisidia* (55.0% of all decapods) and *Athanas nitescens* (38.3% of all decapods), while copepods were represented mostly by harpacticoids. Crabs from the genus *Pisidia* belong mainly to *P. bluteli* and less often to

P. longimana. Alternative prey group were isopods (IRI% = 18.9) and amphipods (IRI% = 13.8). TROPHs index for *L. candolii* was 3.12 ± 0.36 and ITD was 0.73.

Sampling of prey groups under stones inhabited by *L. candolii* showed that amphipods, decapods, polychaetes and ophiuroids were the most numerous and frequent prey groups in the environment. Altogether 913 specimens of different prey groups were found within 10 sampling quadrants (25 x 25 cm²) in the depth range from 2-3 m (Table 3.17).

Table 3.16: Major groups of food items found in the faecal pellets of *Lepadogaster candolii*. N (%): relative numerical abundance; F (%): relative frequency of occurrence; B (%): relative gravimetric composition; IRI (%): index of relative importance.

Higher taxon	Lower taxon	N(%)	F(%)	B(%)	IRI(%)
Rhizaria	Foraminifera	0.2	3.3	<0.1	<0.1
Mollusca	Gastropoda	2.4	30.8	1.8	1.1
	Bivalvia	2.1	23.3	0.7	0.6
Crustacea	Copepoda	47.2	74.2	0.7	30.9
	Amphipoda	7.6	56.7	20.5	13.8
	Decapoda	8.4	60.0	46.3	28.5
	Mysidae	0.2	3.3	0.9	<0.1
	Cumacea	0.3	4.2	<0.1	<0.1
	Anisopoda	1.2	13.3	0.3	0.2
	Isopoda	11.3	59.2	25.4	18.9
	Ostracoda	5.1	35.8	0.1	1.6
	Crustacea indet.	0.7	10.0	0.9	0.1
Arachnida	Acari	0.2	3.3	<0.1	<0.1
Annelida	Polychaeta	11.7	34.2	2.1	4.1
Echinodermata	Ophiuroidea	0.1	0.8	0.1	<0.1
Insecta	Insect larva	0.1	0.8	<0.1	<0.1
Actinopterygii	Fish eggs	0.1	1.7	<0.1	<0.1
Indeterminata	Indeterminata	1.2	2.5	0.1	<0.1

Table 3.17: Abundance (%) and frequency of occurrence (%) of different prey groups under stones in the habitat of *Lepadogaster candolii*.

Higher taxon	Lower taxon	N(%)	F(%)
Mollusca	Gastropoda	0.4	30
	Bivalvia	2.0	70
Crustacea	Decapoda	16.5	100
	Amphipoda	9.6	100
	Anisopoda	0.2	20
	Isopoda	1.3	60
	Mysidae	0.2	10
Pantopoda	Pycnogonida	0.1	10
Annelida	Polychaeta	56.5	100
Echinodermata	Ophiuroidea	13.0	100

Comparison of diet composition between males and females of *L. candolii* showed that there was no statistical difference in the diet between sexes (PERMANOVA; $p = 0.308$, $\alpha = 0.05$). Copepods were the most significant and abundant prey group for both sexes. However, copepods, gastropods and bivalves were more abundant in the diet of males, while decapods, isopods and polychaetes were more abundant in the diet of females (Figure 3.54). Decapods (82.7%) were the most frequent prey group in the female diet followed by copepods (71.2%), while in the male diet, copepods and amphipods (both 62.5%) were the most frequent prey groups, followed by decapods (56.3%; Figure 3.55). Due to a small sample size, age classes 3+ and 4+ were grouped in the 3+ group. The difference in diet composition between specimens from different age groups was statistically significant (PERMANOVA; $p = 0.002$, $\alpha = 0.05$). The most significant prey groups for juvenile specimens were copepods and polychaetes, while for adults it was decapods. Copepods were the most abundant and frequent in the diet of younger age groups and they were decreasing in proportions as the age of the group increases, while the abundance and frequency of occurrence of decapods and isopods were increasing with the increasing age of the group (Figure 3.56; Figure 3.57). For each age group, ITD and TROPHs indexes were calculated (Table 3.18). ITD index ranged from 0.63 in age group 0+ to maximum 0.81 in age group 3+. TROPHs index ranged from 3.03 ± 0.27 at age group 0+ to maximum 3.22 ± 0.40 in the oldest age group 3+.

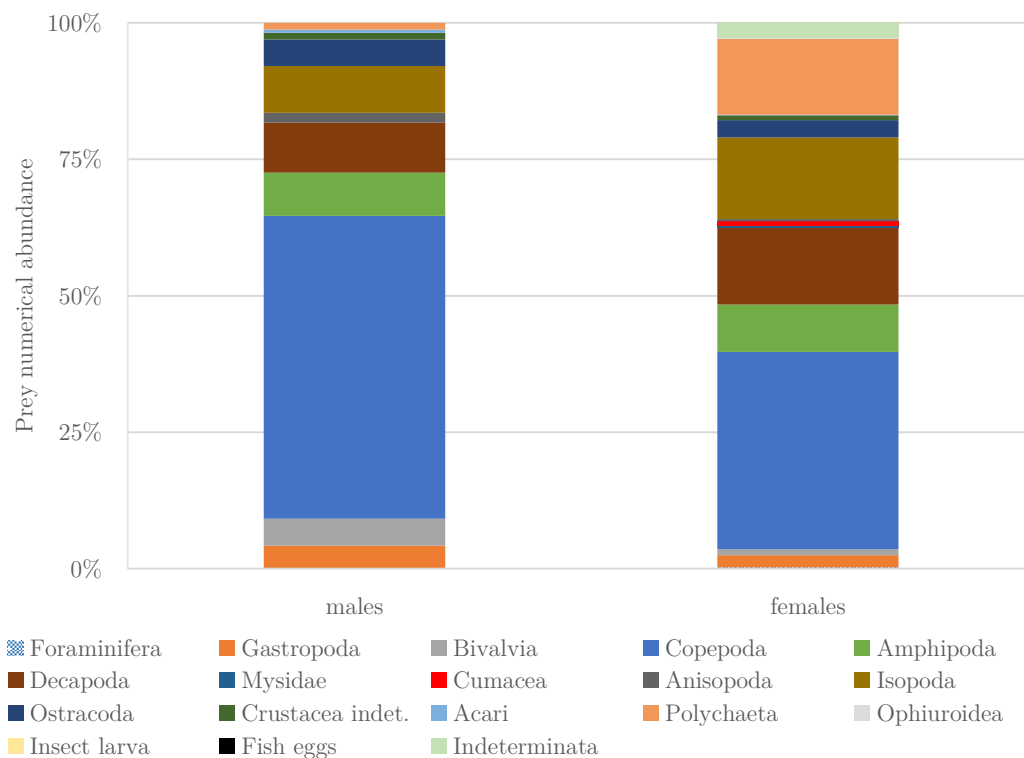


Figure 3.54: The diet of males and females of *L. candolii* in terms of numerical abundance (%).

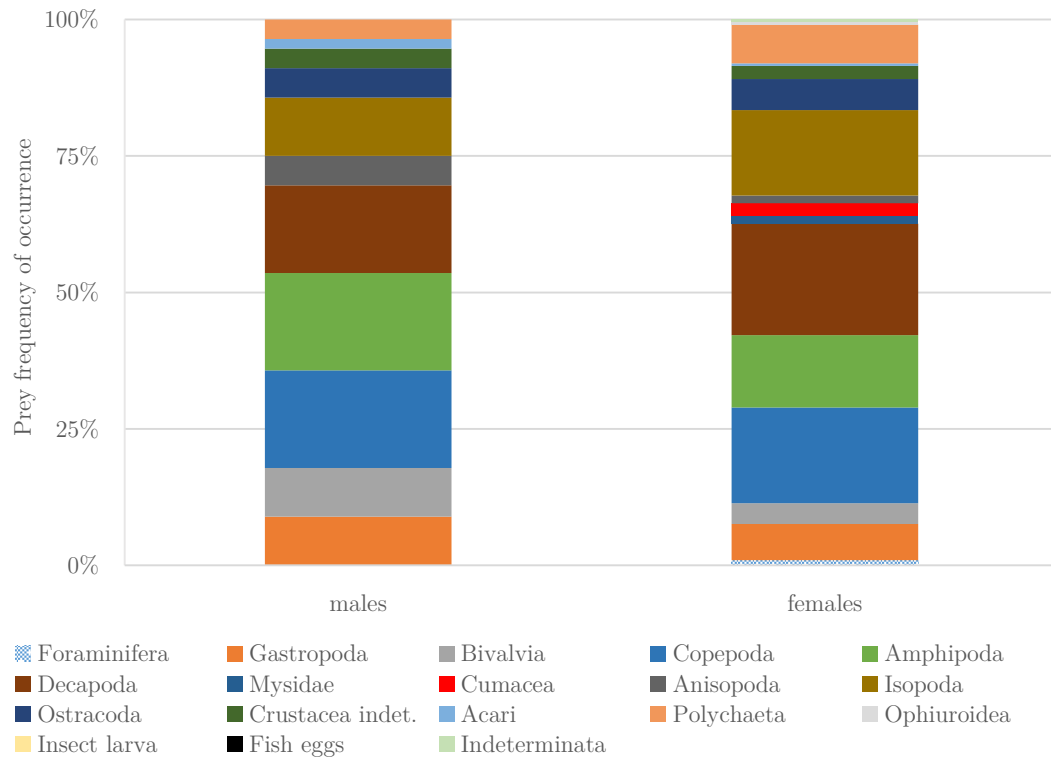


Figure 3.55: The diet of males and females of *L. candolii* in terms of the frequency of occurrence (%).

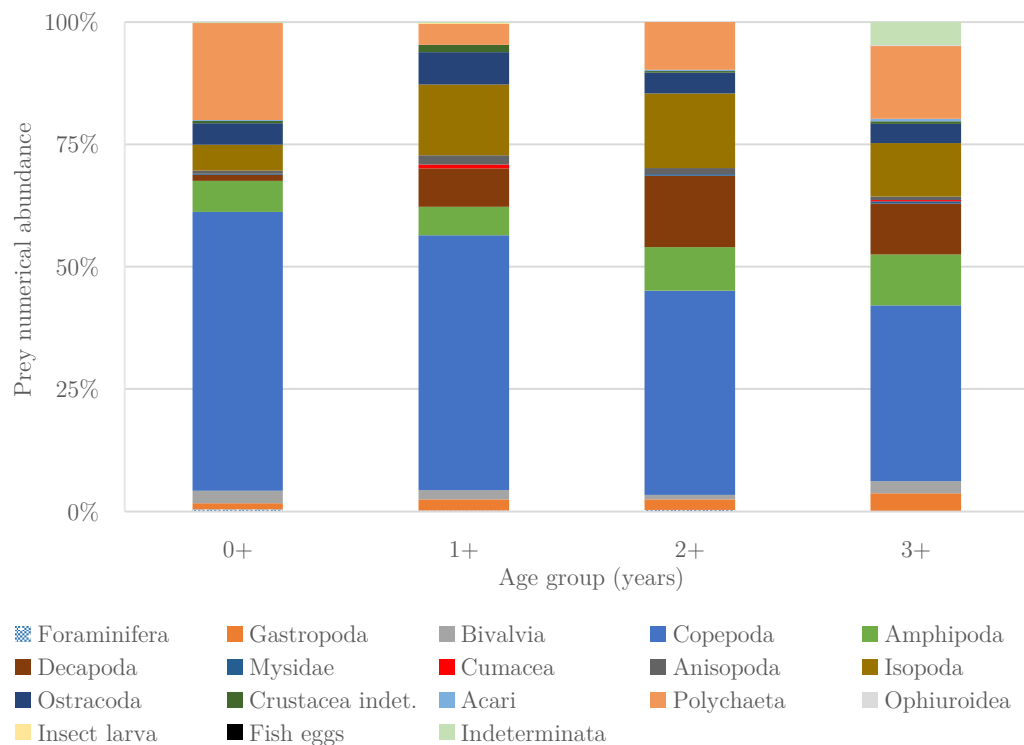


Figure 3.56: The diet of different age groups of *L. candolii* in terms of numerical abundance (%).

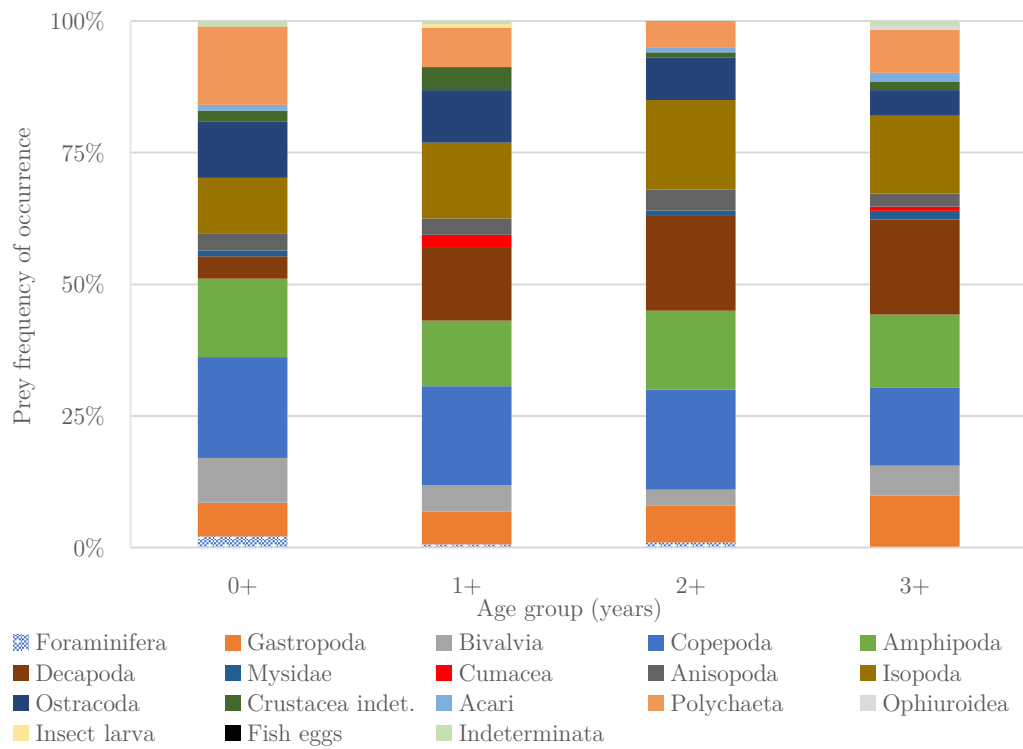


Figure 3.57: The diet of different age groups of *L. candolii* in terms of the frequency of occurrence (%).

Table 3.18: ITD and TROPHs index of different age classes of *L. candolii*. Age classes 3+ and 4+ were grouped together in age group 3+ due to the small sample size.

Age class (years)	Number of specimens	ITD	TROPHs
0+	21	0.63	3.03
1+	37	0.69	3.14
2+	24	0.76	3.19
3+	31	0.81	3.22

Differences in depth segregation between specimens were statistically significant (PERMANOVA; $p = 0.001$, $\alpha = 0.05$). Numerical abundance and frequency of occurrence of copepods and bivalves in the diet of *L. candolii* were increasing with the increasing depth, while gastropod and decapod numbers decreased with the increasing depth (Figure 3.58; Figure 3.59). Furthermore, the SIMPER function showed that decapods were the most important prey group at depth 2-3 m and their importance was decreasing with the increasing depth (Figure 3.60). The importance of copepods did not change with the increasing depth as shown with the SIMPER function. Due to uneven sample distribution across all four seasons, seasonal differences in the diet were not analyzed.

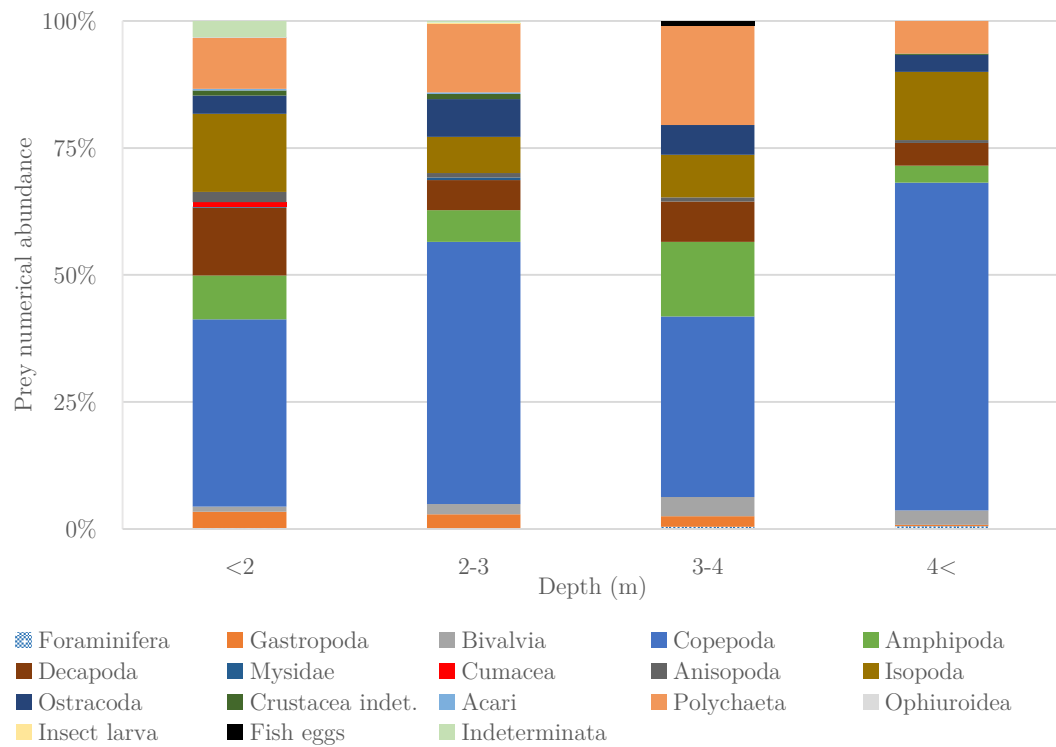


Figure 3.58: The diet of *L. candolii* in terms of numerical abundance (%) at different depth intervals.

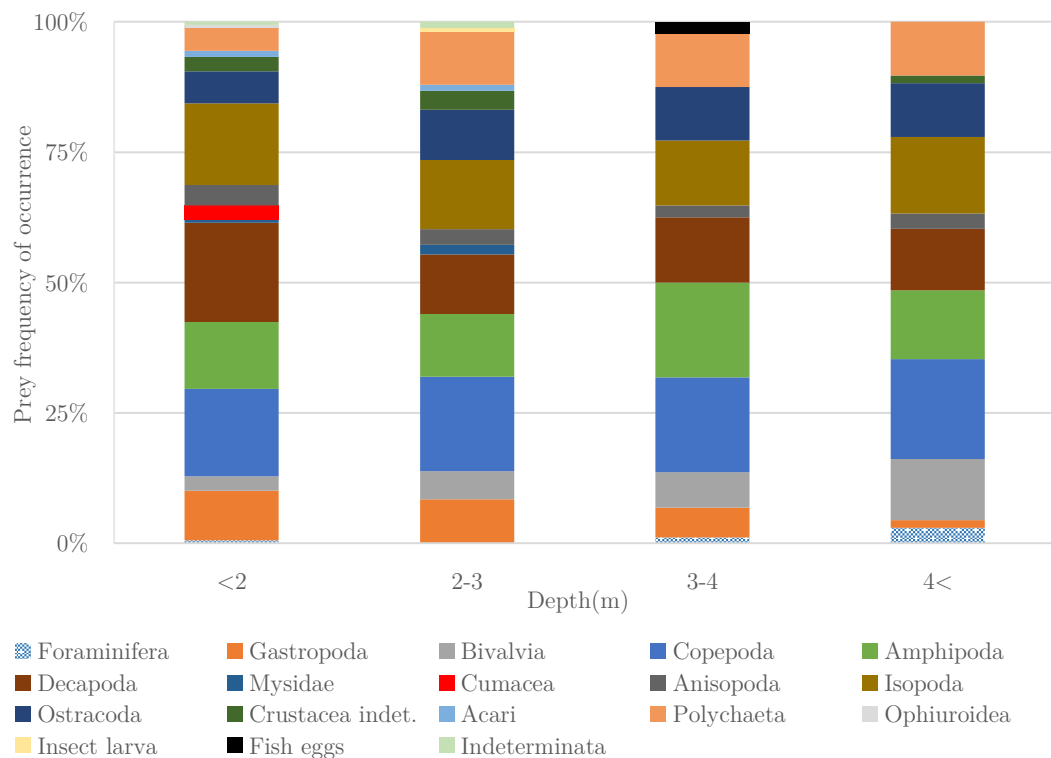


Figure 3.59: The diet of *L. candolii* in terms of the frequency of occurrence (%) at different depth intervals.

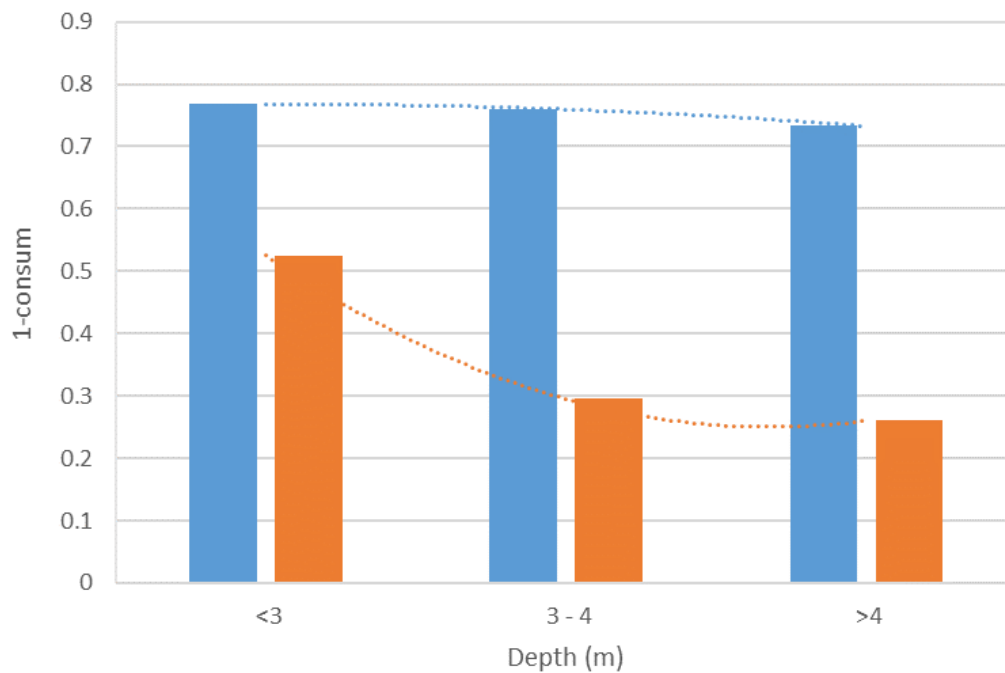


Figure 3.60: SIMPER function showing the importance of copepods (blue bars) and decapods (orange bars) in the diet of *L. candolii* according to the depth distribution. The dotted lines represent trend lines: blue for copepods and orange for decapods.

3.7.3.3 *Apletodon incognitus*

Altogether 45 specimens of *A. incognitus* were included in the diet analysis. Total length of specimens ranged from 15.06 mm to 45.21 mm (32.38 ± 9.27 mm), while their weight varied from 0.03 g to 1.29 g (0.48 ± 0.37 g). Out of 29 sexually identifiable specimens 23 were males and 6 females.

The non-destructive method was used on 37 specimens, and the great majority (97.4%) of them produced from one to maximum five pellets; on average 1.5 faecal pellets per specimen (Table 3.19; Table 3.20). In faecal pellets, 749 prey specimens were found, counted and identified, from one to maximum 70 prey specimens per faecal pellet; on average 12.7 prey specimens per pellet. The highest number of prey specimens per fish was 131, while on average, 16.6 prey specimens per fish were found. In total, 41 faecal pellets were measured. Pellets weighed on average 1.95 mg and measured 2.34 mm in length and 1.58 mm in width.

Table 3.19: The proportion of *A. incognitus* specimens ($n = 37$) which produce from one to maximum five faecal pellets.

Number of faecal pellets per fish	Proportion of fish (%)
1	62.2
2	29.7
3	5.4
5	2.7

Table 3.20: Diet attributes of *A. incognitus*.

Proportion of defecated fish (%)	97.4
Number of fish specimens	37
Number of faecal pellets	56
Average number of faecal pellets per fish	1.5
Number of prey	749
Average number of prey per fish	16.6 ± 26.0
Maximum number of prey per fish	131
Minimum number of prey per fish	1
Average number of prey per faecal pellet	12.7 ± 15.3
Maximum number of prey per faecal pellets	70
Minimum number of prey per faecal pellets	1

In the diet of *A. incognitus*, 23 different taxa were recognized, which were grouped in 18 wider taxonomic groups (Table 3.21). In some cases, besides prey remains, different sediment particles, such as sand grains, shell fragments of invertebrates and algal particles, were also present in faecal pellets. Such particles could represent up to 35.1% of faecal pellet weight. The great majority of prey items in the diet of *A. incognitus* were copepods (N% = 69.0), which were also the most frequent prey items (F% = 77.8), followed by ostracods (F% = 55.6) and amphipods (F% = 46.7). The most important prey group in terms of biomass were decapods (B% = 22.3), amphipods (B% = 17.5) and copepods (B% = 14.6). Decapods were represented mostly by different species of shrimps (*e.g.* *A. nitescens*) and crabs of the genus *Pisidia*. By far the most important prey items based on IRI were copepods (IRI% = 69.4), represented mostly by harpacticoids, followed by amphipods (IRI% = 10.4), which were represented mostly by Gammaridea (88.0% of amphipods) and less by species of the family Caprellidae. Alternative prey groups were ostracods (IRI% = 6.2) and decapods (IRI% = 5.1). Based on the diet composition, the calculated TROPHs index was 3.13 ± 0.26 and ITD was 0.51.

Table 3.21: Major groups of food items found in the faecal pellets of *A. incognitus*. N (%): relative numerical abundance; F (%): relative frequency of occurrence; B (%): relative gravimetric composition; IRI (%): index of relative importance.

Higher taxon	Lower taxon	N(%)	F(%)	B(%)	IRI(%)
Rhizaria	Foraminifera	0.1	2.2	0.6	<0.1
Mollusca	Gastropoda	1.2	13.3	5.7	1.0
	Bivalvia	2.0	22.2	0.7	0.7
Crustacea	Copepoda	69.0	77.8	14.6	69.4
	Amphipoda	3.3	46.7	17.5	10.4
	Decapoda	1.5	20.0	22.3	5.1
	Mysidae	0.4	4.4	4.4	0.2
	Cumacea	0.8	6.7	0.5	0.1
	Anisopoda	1.9	15.6	1.4	0.5
	Isopoda	1.5	13.3	6.4	1.1
	Ostracoda	7.7	55.6	2.8	6.2
	Crustacea indet.	0.8	13.3	4.3	0.7
Arachnida	Acari	0.8	11.1	0.2	0.1
Annelida	Polychaeta	2.5	8.9	9.8	1.2
Actinopterygii	Fish eggs	6.1	24.4	5.7	3.1
Indeterminata	Indeterminata	0.3	4.4	3.1	0.2

The comparison of the diet composition between males and females showed a statistical difference (PERMANOVA; $p = 0.003$, $\alpha = 0.05$). However, it should be noted that the diet sample of females was very small. For the male diet, copepods, fish eggs and ostracods were the most significant. Fish eggs were found only in the diet of males. However, in both species, copepods prevailed in numerical abundance and frequency of occurrence (Figure 3.61; Figure 3.62). In the female diet, ostracods, copepods and decapods were the most frequent prey groups.

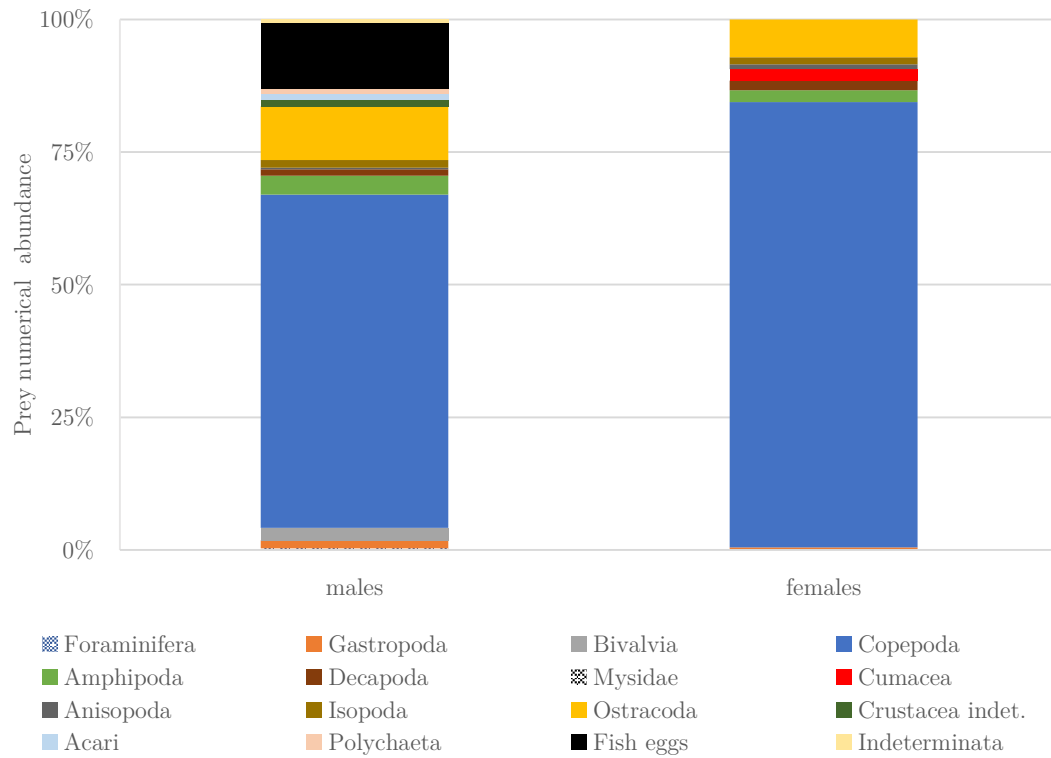


Figure 3.61: The diet of males and females of *A. incognitus* in terms of numerical abundance (%).

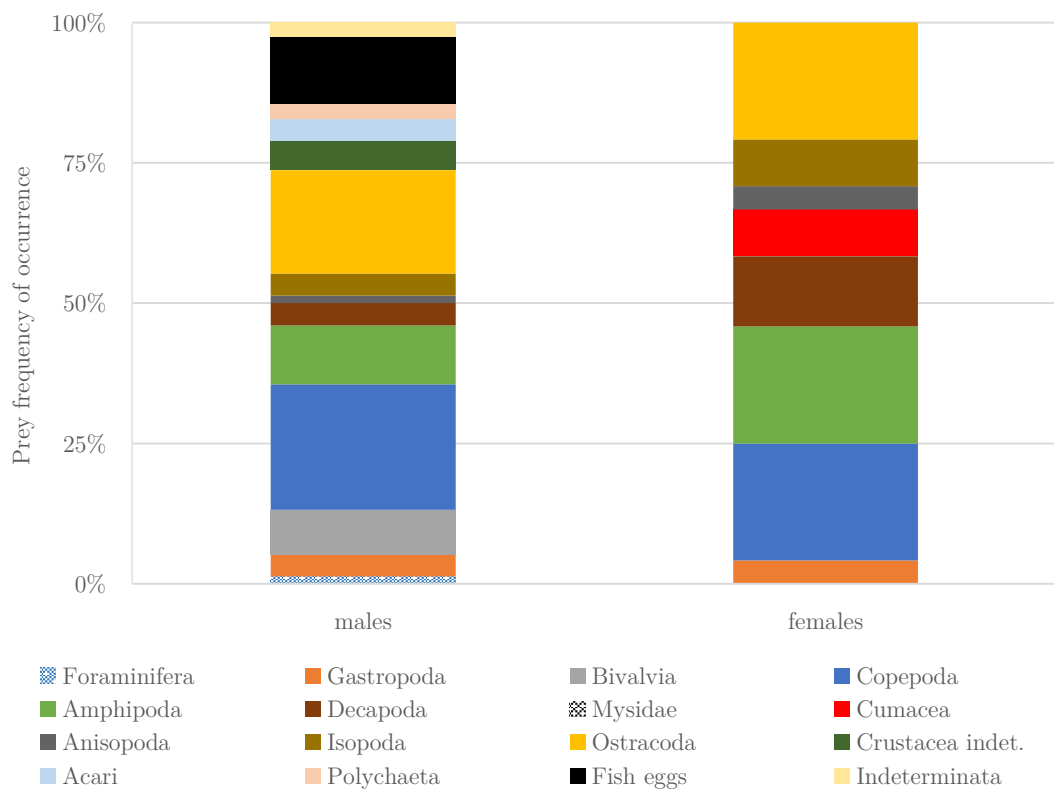


Figure 3.62: The diet of males and females of *A. incognitus* in terms of the frequency of occurrence (%).

Due to the small sample size, age classes were grouped. Specimens from age classes 0+ and 1+ were grouped in < 2 group, while age classes 3+ and 4+ were united in the age group 3+. The difference in the diet between different age groups was statistically significant (PERMANOVA; $p = 0.009$, $\alpha = 0.05$). In all three age groups, copepods represented the most significant and numerous prey group. The youngest age group had the most diverse diet, with higher abundance and frequency of occurrence of amphipods, decapods, anisopods and isopods in comparison to the older two groups (Figure 3.63; Figure 3.64). Only in the diet of the oldest group, which was represented only by males, fish eggs were observed. For each age group, ITD and TROPHs indexes were also calculated (Table 3.22). TROPHs index ranged from minimum 2.98 ± 0.36 in the oldest age group 3+ to maximum 3.26 ± 0.44 in the age group < 2. ITD index was the lowest in the age group 2+ (0.31), while the highest was in the age group < 2 (0.87). Diet composition between nesting and non-nesting males showed that there was a significant difference in their diet (PERMANOVA; $p = 0.022$, $\alpha = 0.05$), however, fish eggs were the most significant prey items of nesting males.

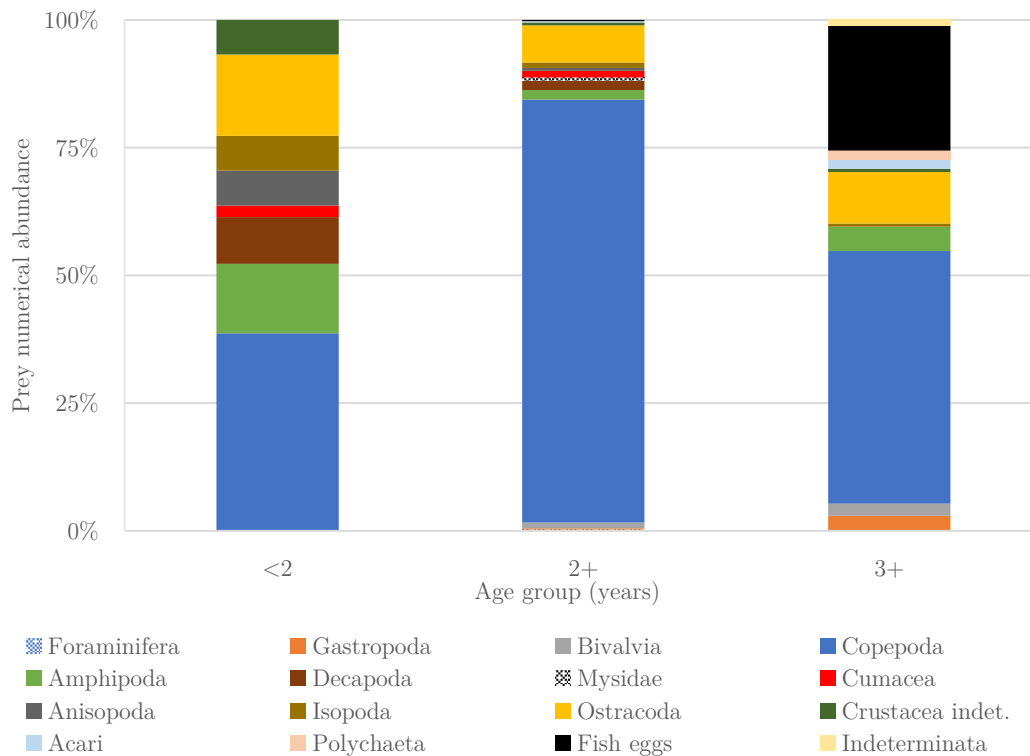


Figure 3.63: The diet of different age groups of *A. incognitus* in terms of the numerical abundance (%). Age classes 0+ and 1+ were grouped in < 2 years group, while age classes 3+ and 4+ were united in the age group 3+.

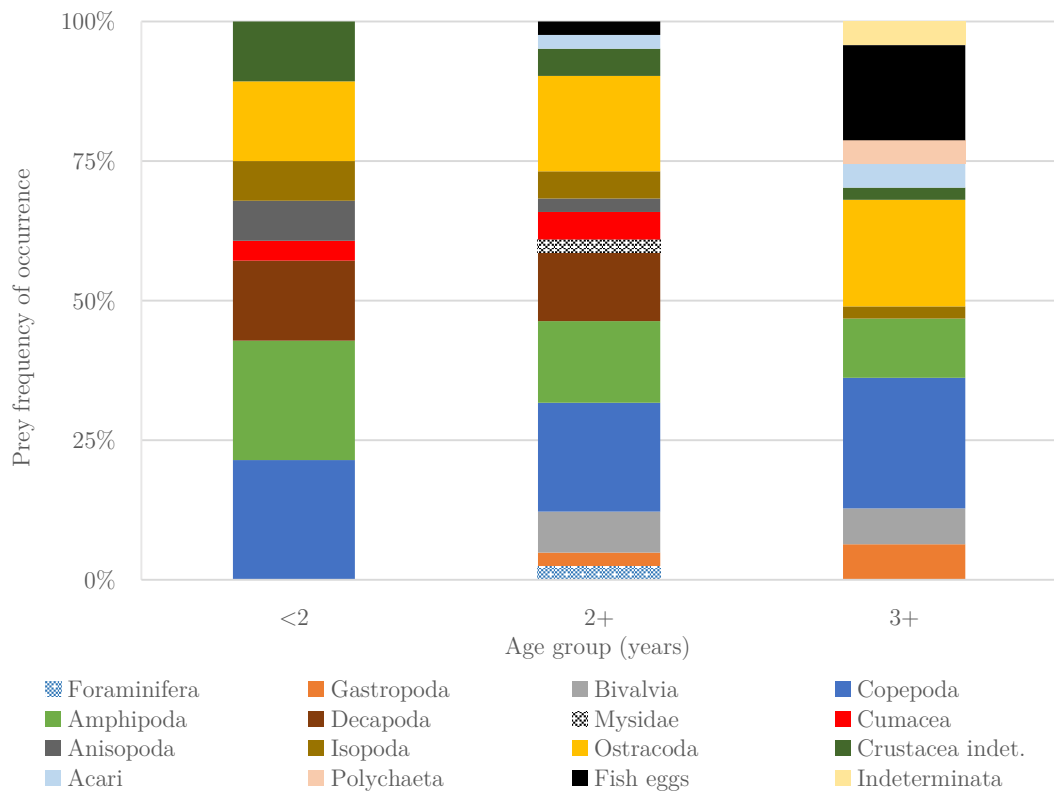


Figure 3.64: The diet of different age groups of *A. incognitus* in terms of the frequency of occurrence (%). Age classes 0+ and 1+ were grouped in < 2 years group, while age classes 3+ and 4+ were united in the age group 3+.

Table 3.22: ITD and TROPHs indexes of different age classes of *A. incognitus*. Some classes were grouped together due to the small sample size.

Age class (years)	Number of specimens	ITD	TROPHs
< 2	9*	0.78*	3.26*
2+	12	0.31	3.05
3+	14**	0.68**	2.98**

* the group also contains specimens of a younger class

** the group also contains specimens of an older class

Based on depth segregation, specimens were grouped in three depth groups (2-3 m, 3-4 m, > 4 m), however, there was no statistical significance in the diet between specimens from different groups (PERMANOVA; $p = 0.247$, $\alpha = 0.05$). In all three depth classes, copepods represented the most abundant and frequent prey group (Figure 3.65; Figure 3.66). Due to uneven sample distribution across all four seasons, seasonal differences in the diet were not analyzed.

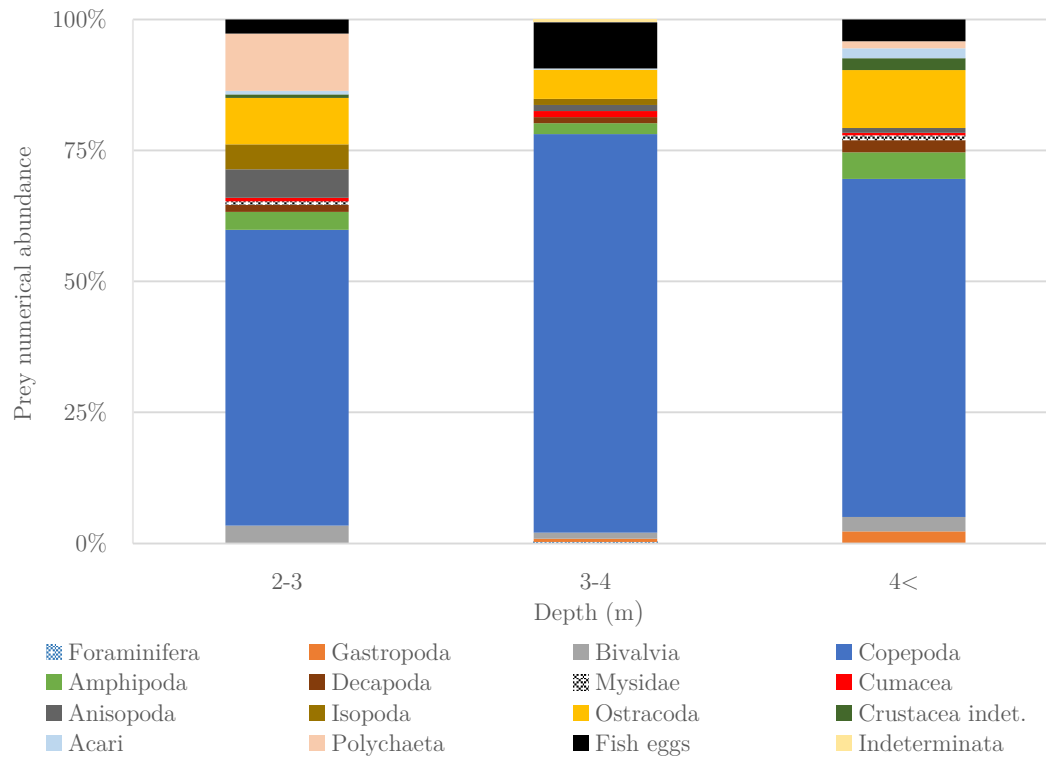


Figure 3.65: The diet of *A. incognitus* in terms of numerical abundance (%) at different depth intervals.

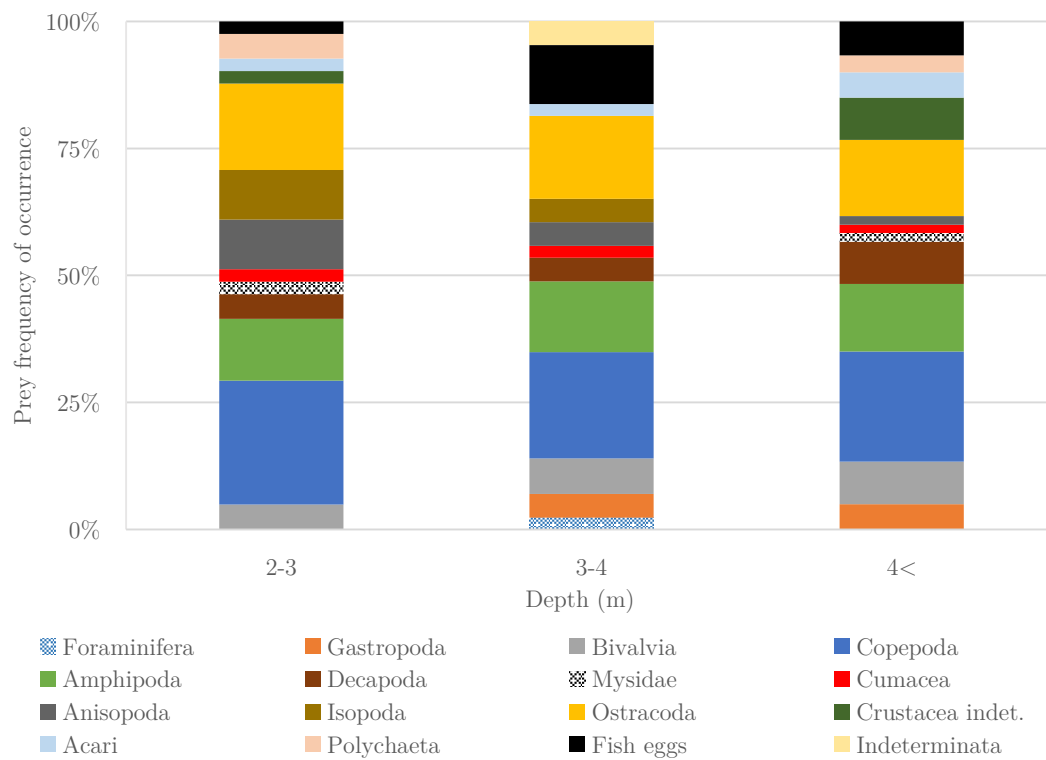


Figure 3.66: The diet of *A. incognitus* in terms of the frequency of occurrence (%) at different depth intervals.

3.8 Fish Observations in Aquarium

L. lepadogaster movements were restricted mostly to the underside surface of the stone. Most of the time they wait in ambush for the prey to come close enough to grab it or they move closer to the prey with small jumps if needed, but they were never observed to leave the shelter.

L. candolii was observed to be much more mobile compared to *L. lepadogaster*. Specimens were mostly hiding, attached to the underside of a stone, but they were also observed moving around the shelter. *L. candolii* did not only wait in ambush for the prey, but they were searching actively for it everywhere in the aquarium. Small specimens were observed to be active especially during the night, when they were picking up copepods from the aquarium walls, while adults were very active also during the day.

Specimens of *A. incognitus* in aquarium behaved similarly to *L. candolii*. Most of the time they were hiding in oyster shells, however they were also observed moving around. Specimens of *A. incognitus* were waiting in the ambush for the prey. In case they observed a potential prey, they moved closer with small jumps and then grabbed it, similar as *L. candolii*. It was also observed that if the prey was not moving, the clingfish did not attack it. This behaviour was observed when they were feeding with specimens of shrimp *Hippolyte* sp.

3.9 Comparison Between Three Clingfish Species

3.9.1 General description

Of all three clingfish species, *L. candolii* reach the largest body size. However, average size of *L. lepadogaster* was the largest, while *A. incognitus* was the smallest clingfish of all three species. In *L. candolii* and *A. incognitus*, specimens in age classes from 1+ to 3+ prevailed, while in *L. lepadogaster* specimens from age classes 2+ to 4+ prevailed. In *L. lepadogaster* and *A. incognitus*, males prevailed in the population, while in *L. candolii*, females were more abundant. In all three species, males were on average larger than females and they also reached a larger maximum body size. Urogenital papillae of males were in all three species larger in males than in females, and reached the maximum average length in *L. candolii*, while the smallest average length was calculated in *A. incognitus*. *L. lepadogaster*, unlike the other two species, has relatively large nasal outgrowths.

All three species showed variable body colouration, which was the most diverse in juvenile and subadult specimens of *A. incognitus*, while adults were more uniform yellow-brown-coloured. In *L. candolii*, juveniles were mostly dark purple-brown, while adults were mostly greenish-brown. In juveniles of *A. incognitus*, a white band between eyes was often present, and it was also observed in specimens of *L. candolii*. Of all three species, *L. lepadogaster* had the most characteristic body colouration. On the body, which was yellowish brown to greenish grey, dark (or light) spots were present, while on the top of the head, two blue eye spots surrounded with a pattern of blue lines were present. In males of all three species, red body parts became more intensively coloured during the mating season. In *L. lepadogaster*, dorsal, anal and caudal fins became intensively red, while in *A. incognitus*, the head became intensively red-coloured during the mating season. The body colour of *L. candolii* during the mating season became darker, while oblique red stripes on the gillcover and red spots extending from the middle of the back to the middle of the basal part of the dorsal fin became intensively red.

3.9.2 Depth distribution

All three species were clearly separated based on depth segregation. Our data confirmed that there is statistically significant difference between species based on depth of occurrence (ANOVA; $p = 2e^{-16}$, $\alpha = 0.05$). Furthermore this results are supported also by results of Tukey's post-hoc test comparison (Figure 3.35). *L. lepadogaster* occurred in the shallowest water from lower mediolittoral to upper infralittoral. The deep distribution of *L. lepadogaster* in the upper infralittoral (about 2 m) overlapped with the upper limit of occurrence of *L. candolii*. Furthermore, *L. candolii* had the overlap in its lower limit of depth distribution with *A. incognitus*, which occurred the deepest out of these three species. However, *L. candolii* occurred mostly below the depth distribution of *L. lepadogaster* and above the depth distribution of *A. incognitus*. There was almost no overlap in depth distribution between *L. lepadogaster* and *A. incognitus*.

In all three species, intraspecific depth segregation based on the size of the specimens was observed. Inside their limit of depth distribution, large specimens of *L. lepadogaster* and *A. incognitus* were present deeper, while juveniles were more abundant in the shallow water. Conversely, large specimens of *L. candolii* were more abundant in shallow water, while juveniles were more abundant deeper. Our data confirmed significant linear relation (linear regression t-test, $p < \alpha$, $\alpha = 0.05$) between size of the fish and depth occurrence. However, the largest specimens of *L. lepadogaster* and *L. candolii* were the most abundant in waters between 1.5 and 2 m, while smaller specimens of *L. lepadogaster* were more abundant in shallower waters and juveniles of *L. candolii* were more abundant in deeper waters.

3.9.3 Habitat selection

L. lepadogaster and *L. candolii* occur mostly in the rocky bottom under stones, while *A. incognitus* occurred mostly inside seagrass meadows, where it occupied dead sea shells (Figure 3.67). However, *L. candolii* was sometimes found in the same depth range as *L. lepadogaster* in the upper infralittoral (around 2 m depth) in a rocky area, as also on the same locations with *A. incognitus* in a seagrass meadow. *L. lepadogaster* and *L. candolii* showed similar microhabitat preferences, as both species prefer stones of a similar size, and a similar substrate under the stone. However, undersurface area of stones inhabited by *L. candolii* was on average somehow larger (1.3 times larger), while in the substrate under the stone, sand was present more often beside pebbles (Figure 3.68). Our data confirmed, that there is a statistical significant difference in the substrate under the occupied stone between *L. lepadogaster* and *L. candolii* ($\chi^2 = 130.59$; $p < 2.2e^{-16}$). Pebbles as substrate under stone were the most significant for *L. lepadogaster*, while pebbles with sand were the most significant for *L. candolii*. Our data also confirmed, that there is a statistical significant difference in the stone overgrown between *L. lepadogaster* and *L. candolii* ($\chi^2 = 228.53$; $p < 2.2e^{-16}$). Bare stones and those with turf vegetation were significant for *L. lepadogaster*, while those with macroalgae were most significant for *L. candolii*. In both species it was observed that the undersurface area is in positive correlation with the size of the fish, as larger specimens occupied stones with a larger undersurface area. Furthermore, our data showed a significant linear relation (linear regression t-test, $p < \alpha$, $\alpha = 0.05$) between undersurface area and size of the fish. The stones' surface area was rougher in *L. candolii* than in *L. lepadogaster* due to small holes made by different excavators and their cover was overgrown with tubes of serpulid polychaetes. In seagrass meadows, specimens of *L. candolii* were mostly found under seashells, where smaller specimens were found under oyster shells, while bigger specimens were found under shells of dead *P. nobilis*. Specimens of *A. incognitus* were exclusively found in seagrass meadows

or nearby, but unlike *L. candolii*, which occurred under the shell, they were mostly found inside the shell of dead oysters. However, juvenile specimens of both species were found also outside the shelter on shells of living *P. nobilis*. *L. lepadogaster* specimens were, unlike the other two species, never observed outside the shelter.

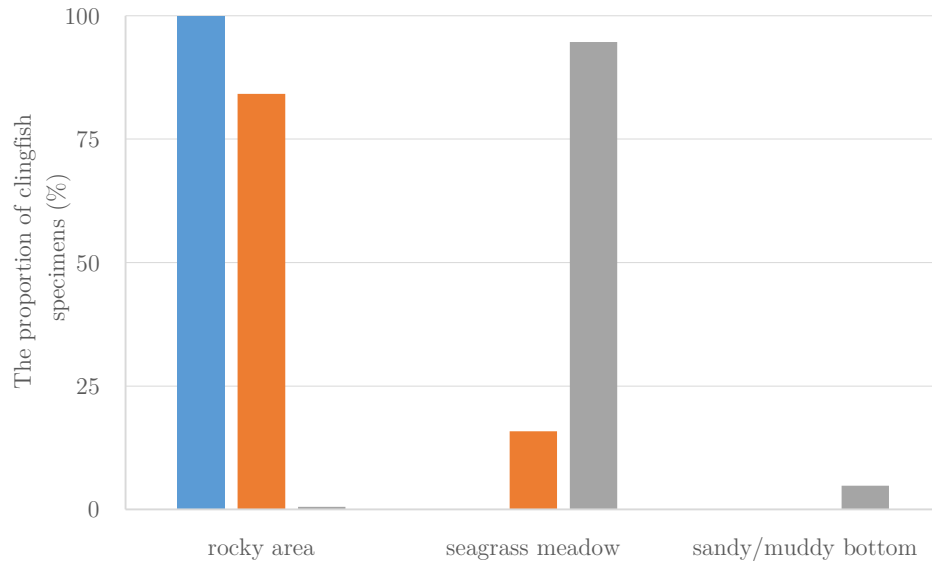


Figure 3.67: Macrohabitat preference of three clingfish species. Legend: blue bars – *L. lepadogaster*, orange bars – *L. candolii*, grey bars – *A. incognitus*.

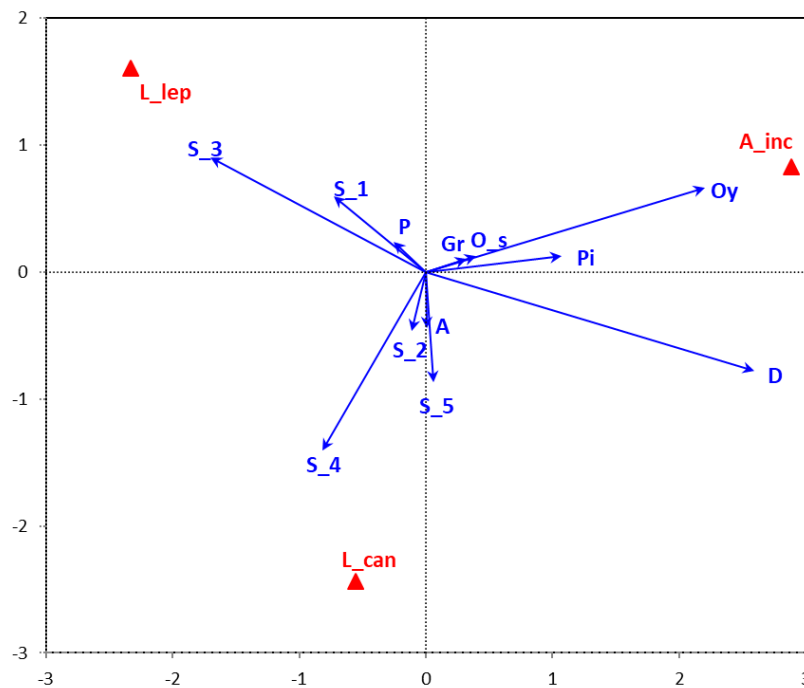


Figure 3.68: Microhabitat choice of three clingfish species (red triangles), presented with the RDA ordinate system. Legend: L_lep – *Lepadogaster lepadogaster*, L_can – *Lepadogaster candolii*, A_inc – *Apletodon incognitus*; P – pebbles; S_1 – stones with sandstone terrace as substrate; S_2 – stones with sandstone terrace and pebbles as substrate; S_3 – stones with pebbles as substrate; S_4 – stones with pebbles and sand as substrate; S_5 – stones with sand as substrate; Te – under sandstone terrace; Pi – on *P. nobilis*; Gr – seagrass; O_s – other seashells; Oy – oyster shells; A – artificial objects.

3.9.4 Reproduction

Among three clingfish species, *L. lepadogaster* is the first to reproduce in early spring. *A. incognitus* reproduction period is from mid-May to the end of July, while the nest of *L. candolii* was found only once in the beginning of May, so it was not possible to do any further analyses regarding its reproduction. *L. lepadogaster* and *L. candolii* spawned under stones, while *A. incognitus* spawned in dead oyster shells which were mostly attached to the *P. nobilis*. In the nest, a guarding male was normally present, however this was not always the case in *L. lepadogaster* where we often failed to notice the fish. *L. lepadogaster* and *A. incognitus* had both around 600 eggs per nest. However, the nest of *L. lepadogaster* could be much bigger as they could contain up to 7384 eggs, while *A. incognitus* nests contained a maximum of 1208 eggs. Females of *L. lepadogaster* on average laid 165.5 eggs per spawn, while females of *A. incognitus* laid 189.5 eggs per spawn.

3.9.5 Diet

Diet study showed that most of the specimens of all three species had defecated and produced mostly one to two faecal pellets. The average lowest number of faecal pellets was produced by *L. candolii* (1.3 per specimen), while *L. lepadogaster* produced the highest average number of faecal pellets (1.6 per specimen). The average number of prey items per faecal pellet, as also per fish, was the highest in *A. incognitus*, followed by *L. candolii*, while *L. lepadogaster* had the lowest average number of prey items per faecal pellet (Table 3.23). However, the highest number of prey items per pellet was found for *L. candolii* (143 prey specimens).

Table 3.23: Diet attributes of three clingfish species.

	<i>L. lepadogaster</i>	<i>L. candolii</i>	<i>A. incognitus</i>
Proportion of defecated fish (%)	96.9	96.8	97.4
Number of specimens	188	120	37
Number of faecal pellets	294	161	56
Number of faecal pellets per fish	1.6	1.3	1.5
Number of prey	1334	1779	749
Average number of prey per fish	7.1	14.8	16.6
Maximum number of prey per fish	39	143	131
Minimum number of prey per fish	1	1	1
Average number of prey per faecal pellet	4.5	11.0	12.7
Maximum number of prey per faecal pellets	39	143	70
Minimum number of prey per faecal pellets	1	1	1

Crustaceans were the most important prey group for all three clingfish species. However, there were significant differences in the diet composition (PERMANOVA; $p = 0.001$, $\alpha = 0.05$). Copepods were the most significant prey group in the diet of *A. incognitus* and somehow lesser for *L. candolii*. For the latter species, the most significant prey groups were also amphipods, decapods and isopods. These results were supported also by IRI (%), which showed high importance of copepods, amphipods, decapods and isopods for *L. candolii*, as

also the highest importance of copepods for *A. incognitus*. Amphipods and decapods were the most significant prey groups for *L. lepadogaster*, which is furthermore supported by IRI (%), which is the highest for these two prey groups. Bray–Curtis dissimilarity index showed that the highest overlap in the diet is between *L. candolii* and *L. lepadogaster* and the lowest between *A. incognitus* and *L. lepadogaster* (Table 3.24), which is supported by IRI (%) of different prey groups for all three species (Figure 3.69).

Table 3.24: Bray–Curtis dissimilarity index for three clingfish species.

	<i>L. candolii</i>	<i>A. incognitus</i>
<i>A. incognitus</i>	0.445	-
<i>L. lepadogaster</i>	0.391	0.458

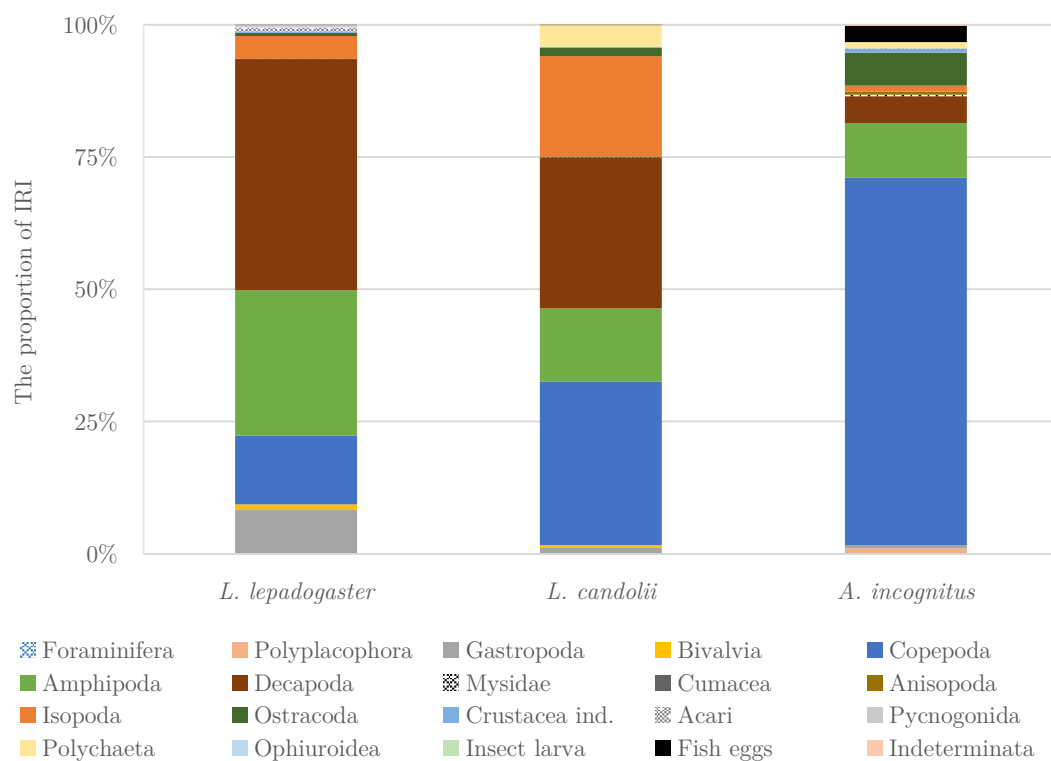


Figure 3.69: Index of relative importance (%) of different prey groups for three clingfish species.

Diet composition showed that there were no evident differences in the diet between sexes in *L. lepadogaster* and *L. candolii*. In *A. incognitus* female sample was quite small to make strong conclusions, however fish eggs were the most significant prey for males. Based on the size of the fish *L. lepadogaster* and *L. candolii*, juvenile specimens fed with smaller prey, such as copepods, while large adults preyed more larger prey groups, such as decapods (Figure 3.46; Figure 3.56). These results were supported also by the ITD and TROPHs indexes, the values of which were increasing with the increasing size of the age group. Conversely, in *A. incognitus* it was observed that smaller specimens had a more diverse diet, with higher proportion of decapods and lower proportion of copepods in the diet than larger specimens. This was reflected also in decreasing ITD and TROPHs indexes with the increasing size of the specimens (Table 3.25).

Table 3.25: ITD and TROPHs indexes for three clingfish species. Some classes were grouped together due to the small sample size.

Age class (years)	ITD			TROPHs		
	<i>L.</i> <i>lepadogaster</i>	<i>L.</i> <i>candolii</i>	<i>A.</i> <i>incognitus</i>	<i>L.</i> <i>lepadogaster</i>	<i>L.</i> <i>candolii</i>	<i>A.</i> <i>incognitus</i>
0+	-	0.63	-	-	3.03	-
1+	-	0.69	0.78*	-	3.14	3.26*
2+	0.75*	0.76	0.31	3.18*	3.19	3.05
3+	0.84	0.81**	0.68**	3.21	3.22**	2.98**
4+	0.82	-	-	3.35	-	-

* the group also contains specimens of a younger class, ** the group also contains specimens of an older class

In *L. lepadogaster* and *L. candolii*, the diet composition is correlated with the size-depth segregation. Large specimens of both species were very abundant in the depth range between 1 and 2 m, where decapods were most abundant in their diet. Smaller specimens of *L. lepadogaster* were more abundant in shallower waters and consequently, copepods were more abundant in diet of fish from that area. This is also supported by SIMPER function (Figure 3.48), which showed that importance of copepods is increasing with decreasing depth. However, importance of copepods based on SIMPER function (Figure 3.60) in the diet of *L. candolii* did not increase with increasing depth, although smaller specimens predominated here. Diet composition of *A. incognitus* did not depend on the depth segregation, although larger specimens were more abundant in deeper waters. Seasonal differences in the diet composition could not be compared due to the uneven sample distribution across all four seasons in *L. candolii* and *A. incognitus*.

3.10 Parasites of Clingfishes

A total of 344 clingfish specimens were examined for external parasites: 33 specimens of *A. incognitus*, 117 of *L. candolii* and 194 specimens of *L. lepadogaster*. External parasites were found in 1.0% of *L. lepadogaster* specimens and in 20.5% of *L. candolii* specimens. In *L. lepadogaster*, parasitic larvae of Gnathiidae were observed (Figure 3.70). In specimens of *L. candolii*, besides larvae of the family Gnathiidae (12.5% of infested specimens), parasitic copepods of the family Caligidae (33.3% of infested specimens) and the family Lernaepodidae (54.2% of infested specimens) were also observed.

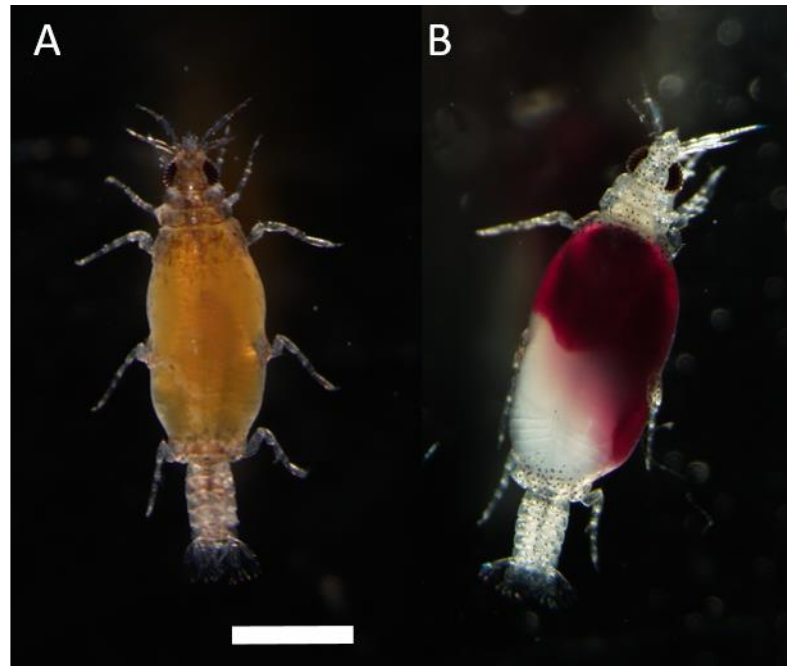


Figure 3.70: Parasitic larvae of the family Gnathiidae: A – from *L. lepadogaster* (white bar represents 1 mm); B – from *L. candolii* which is full of host's blood (Photos: D. Trkov).

Larvae of the families Gnathiidae and Caligidae were observed on the body and fins of the host, while Lernaeopodidae were found on gills, which was visible as a yellowish bulge under the gill cover (Figure 3.71). Lernaeopodidae mostly parasitized juvenile fish specimens, while larvae of families Gnathiidae and Caligidae were found only on adult fish specimens (Table 3.26). Only one specimen of parasitic larvae of the family Gnathiidae was always observed per infected fish. In 23.7% of infection with Lernaeopodidae, more than one specimen was present on the host (one parasite under each gill cover). In the case of parasitic copepods of the family Caligidae, normally more than one parasite per host was present. In one case, altogether 27 specimens of copepods were found on one fish which was physically very weak. The clingfish was placed in the aquarium, but it lived only for a few days. Specimens of Caligidae were mostly attached on the dorsal part of the host body behind the eyes (Figure 3.72), while parasites were also moving around the body of the host. On the fish body, patches of damaged tissue where parasites were attached most of the time were observed. Lernaeopodidae were observed to parasitize on fish from June to October, larvae of the family Gnathiidae from April to October and Caligidae from July to August.



Figure 3.71: *L. candolii* with a yellowish bulge on the gill cover which indicates an infection with Lernaepodidae; B – specimen of Lernaepodidae dorsal side (white bar represents 0.5 mm); C – specimen of Lernaepodidae ventral side (white bar represents 0.5 mm; Photos: D. Trkov).

Table 3.26: Parasite groups observed to parasitize *L. candolii* (n = 22). One fish had parasites from two different groups.

	male	female	juvenile
Lernaepodidae	-	2	9
Caligidae	3	5	1
larvae of Gnathiidae	-	3	-

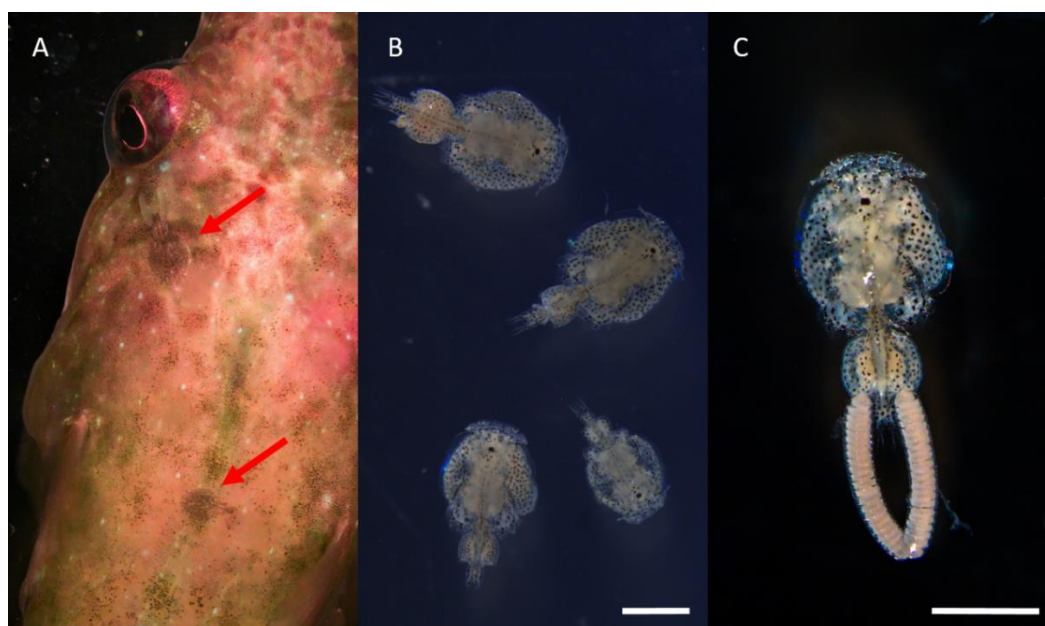


Figure 3.72: A – two specimens of Caligidae (red arrows) parasitize on *L. candolii*; B – specimens of Caligidae found on *L. candolii* (white bar represents 1 mm); C – specimen of Caligidae with clusters of eggs (white bar represents 1 mm; Photos: D. Trkov).

In specimens of *A. incognitus*, no external parasites were observed. However, 20 randomly selected specimens (males) of *A. incognitus* were checked for internal parasites, and 50.0% of them were infested with trematodes. Furthermore, two specimens were found in very bad condition (visible bones due to malnutrition; Figure 3.73) and were also examined for internal parasites. Both specimens were infested with trematodes and in one case also with cestodes which were present in their intestine. Eggs and larval stages of trematodes were observed also in liver and muscles of the fishes. In the specimen that was infested with a cestode, bumps (eggs of cestode) in the fins were also observed.

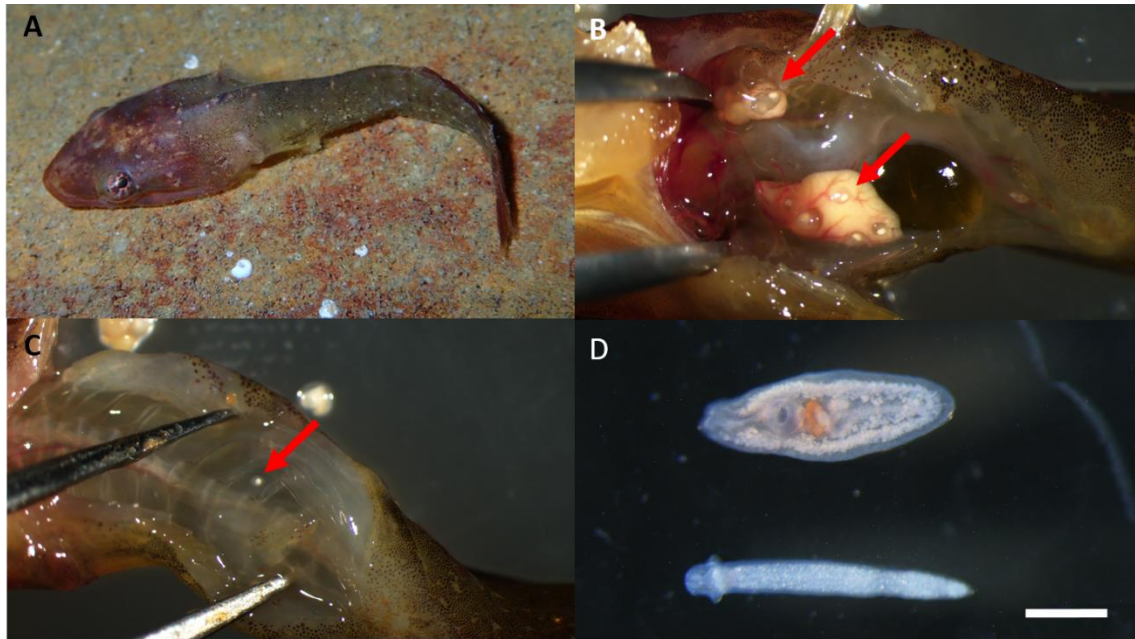


Figure 3.73: A – specimen of *A. incognitus* with visible bones due to strong infestation with internal parasites; B – eggs of trematodes in liver; C – egg of trematode in muscles; D – adult specimen of trematode (above) and cestode (below) from the intestine of *A. incognitus* (white bar represents 0.5 mm; Photos: D. Trkov).

Chapter 4

Discussion

4.1 Advantages and Disadvantages of the Selected Methods

Cryptobenthic fish species are often overlooked due to their cryptic life style (Henriques et al., 2002). Because they live in a heterogeneous environment, such as caves, cavities, holes, under stones and clefts, sampling is very demanding. Therefore, anaesthetics (*e.g.* quinaldine, tricanin, clove oil) are very helpful to facilitate the fish catch (Gibson, 1967; Munday & Wilson, 1997). Sampling of cryptobenthic fishes is usually not even possible without the anaesthetic and hardly leads to success (Hofrichter, 1993). However, the use of anaesthetics and ichthyocides is considered as a destructive technique (Ackerman & Bellwood, 2000; Kovačić et al., 2012). Destructive techniques are considered those that remove a fish from a particular area and have a harmful effect on the investigated fish (Ackerman & Bellwood, 2000). The anaesthetic quinaldine was used in this study because it is the most effective compared to other anaesthetics such as tricanin (MS 222), clove oil, benzocaine and 2-phenoxyethanol (Munday & Wilson, 1997). Quinaldine has a high space and time efficiency as an anaesthetic and consequently it is the most appropriate anaesthetic to use for the cryptobenthic fish to survive (Glavičić et al., 2016). Furthermore, quinaldine is considered as a nontoxic anaesthetic, the exposure to low concentration is not lethal and the recovery of fish is fairly rapid (Gibson, 1967). However, it should be handled with caution since its effect on the human by prolonged contact and repeated exposure is not known, while in contact with eyes and mouth it can produce painful irritation (Gibson, 1967).

Although quinaldine is a specific anaesthetic for vertebrates (Gibson, 1967) and should not affect other organisms in the environment, we observed that at high concentrations, it is lethal to different crustaceans (*e.g.* *Pisidia* sp., *Alpheus* sp.) and sometimes also to small fish specimens in hiding places where it was used. The anaesthetic impact of quinaldine on invertebrates was observed also by other researchers (Hofrichter, 1993; Gibson, 1999). Furthermore, invertebrates, which tried to escape away from the quinaldine spray, often became a prey of other predators, usually of epibenthic fishes (*e.g.* *Salaria pavo* (Risso, 1810), *Parablennius tentacularis* (Brünnich, 1768), *Gobius* spp.)), and nectobenthic fishes (*e.g.* *Serranus scriba* (Linnaeus, 1758), *S. hepatus* (Linnaeus, 1758))(Figure 4.1). Due to its negative effect, such as death of fish specimens and other invertebrates, which were exposed to excessive concentration of quinaldine for too long, we tried to use it as little as possible. Therefore, the specimens of *L. lepadogaster* and *A. incognitus* were usually caught only with a hand net or with a plastic bag without the use of quinaldine. Quinaldine was used only on small areas (normally less than 50 x 50 cm²) where individual fish hiding places were squirted with the anaesthetic. Consequently, quinaldine was quickly diluted with the seawater, which prevented deaths of fish and other invertebrates due to overdose. In order to prevent unnecessary death of marine organisms that remain in a hiding place, the concentration of used quinaldine should be reduced. This was achieved by creating a

water current with a hand directed to the hiding place where quinaldine was squirted. Consequently, it was diluted and blown away. The use of quinaldine in tide pools is not advised due to the small volume of seawater, which can lead to a high concentration of quinaldine and consequently to the death of marine organisms in these specific habitats. The use of quinaldine for sampling in isolated sea pools and in MPAs is therefore unethical and controversial.



Figure 4.1: *Alpheus* sp., which escaped from the area where quinaldine was sprayed, was caught by *Serranus hepatus* (Photo: D. Trkov).

Studies of cryptobenthic fish abundance require destructive techniques (Glavičič et al., 2016) where the anaesthetic is used on larger areas (usually the sample area is one-square-metre large), so we did not perform quantitative samplings. A disadvantage of such sampling is also that most anaesthetized fish float out of their hiding places due to water current movements and the exact origin and position of them at the bottom cannot be known (Glavičič et al., 2016). Furthermore, such use of anaesthetic in the mediolittoral and the upper infralittoral would be less efficient or inefficient due to strong water movements which could blow away the anaesthetic immediately. The problem of the water flow which blew away the anaesthetic and consequently disabled the sampling was exposed also by Gibson (1967).

However, some quantitative samplings were performed in the mediolittoral and upper infralittoral belts, where all hiding places (stones) within 1 x 1 m² quadrant were checked for clingfishes (without using quinaldine). Samplings were then no longer performed, as it has shown to be a very time-consuming method, especially in a heterogenic environment with a lot of hiding places.

Samplings in seagrass meadows were done with the use of sliding dredge, but it proved to be unsuccessful (only one specimen *A. incognitus* was caught) and also unsuitable for implementation due to the large number of *P. nobilis* present in the environment. Inefficiency and unsuitability of dredge for quantitative clingfish sampling were also highlighted by Hofrichter (1993, 1995). Furthermore, such sampling does not provide any data about the hiding place of clingfishes (Hofrichter, 1993, 1995). The cube method for collecting fish, which was used on seagrass meadows, was also ineffective, which is probably due to the use of a hand net instead of a suction-sampler for catching the anaesthetised fish as described by Hofrichter (1995). The suction-sampler was not used as it requires a lot of effort and at least two to three divers (Hofrichter, 1995).

The study was conducted in non destructive way and fish were therefore not sacrificed for the purpose of obtaining otoliths in order to determine the age of the fish. Instead, fish age was determined based on fish size and age-size classes as proposed by Hofrichter (1995), as it was done on specimens from Krk island, which is the closest location (compared to other researches) to Slovenian sea. However, as cohorts of fishes usually have overlapping ranges, some fish grow fast, some fish grow slow, and with ageing, the difference accumulates and overlap increases, so results of age structure may deviate from real one and so they are only informative. Furthermore, age determination need to be taken with caution, as age-size classes differ between authors, due to demanding interpretations of the age from otoliths (Hofrichter, 1995; King, 1989; Wilson, 1981). Although we used age classes in this study to facilitate the interpretation of the results, it makes more sense to consider them as size classes.

4.2 Fauna of Cryptobenthic Fish in Slovenian Sea

Among the 12 cryptobenthic species found in Slovenian coastal waters, three species of the family Gobiidae: *O. balearica*, *T. macrolepis* and *C. quadrivittatus*, were observed for the first time in Slovenian territorial waters in recent years. Marčeta (1999) mentioned at least one finding of *C. quadrivittatus* near Trieste and considered it as an expected species for the adjacent Slovenian waters. All three species were recorded in the nearby Kvarner area as well (Ahnelt & Kovačić, 1997; Kovačić, 1997, 2005), while *O. balearica* and *C. quadrivittatus* were recorded also near Rovinj (Kovačić, 2005). *T. macrolepis* was often overlooked in the past, probably not only due to its cryptic lifestyle, but also because it occurs in deeper environments (Bilecenoglu & Yokes, 2016; Miller, 1986), which are more difficult to access and were consequently less explored. These new observations indicate that the species is more widespread than it was previously assumed (Bilecenoglu & Yokes, 2016; Miller, 1986). *C. quadrivittatus* was also often overlooked in the past (Ahnelt, 1990, 1991; Kovačić, 1997, 2005). Specimens are rarely found, and it is presumed that they are not numerous (Ahnelt, 1991). *O. balearica* is the smallest out of these three species (3 –4 cm) and was until recently considered as a rare species (Kovačić et al., 2012). In the Adriatic Sea, it is present in its eastern part, mainly in the southern and central Adriatic, but also in the northern Adriatic: in the Kvarner area and along the Istrian peninsula (Ahnelt, Miller, & Patzner, 1994; Kovačić, 2005; Miller & Tortonese, 1968)). Findings of *O. balearica* and *T. macrolepis* represent northernmost sightings of these species in the Adriatic Sea as well as the Mediterranean Sea, extending the total species distribution to north. *O. balearica* was probably overlooked in the past due to its small body size and cryptic lifestyle. Furthermore, fluid-preserved fish staining proposed by Saruwatari et al. (1997) enabled us an easier and more accurate determination of cryptobenthic fish species, in which important determination characteristics cannot be noticed without such techniques. This was shown also by revising the collection of cryptobenthic fish fauna of the ichthyological collection of MBS, where two *O. balearica* specimens were found, which were wrongly determined as *M. macrocephalus*.

Among cryptobenthic fish fauna in Slovenian waters, gobies are represented with the highest number of species (Table 3.1), which is in accordance with the results obtained for nearby areas, as reported in many studies (Glavičić et al., 2016; Kovačić, 1997; Kovačić et al., 2012). Glavičić et al. (2016) also reported that species of the family Gobiidae can represent more than 90% of the total cryptobenthic fish abundance at deep littoral cliffs in the Mediterranean. However, clingfishes were by far the most numerous cryptobenthic fishes in Slovenian waters. *L. lepadogaster* was the most numerous species (n = 242 specimens), followed by *A. incognitus* (n = 222) and *L. candolii* (n = 177). These three

species represent 76.7% of all observed cryptobenthic fish specimens, but the number does not reflect the actual density of fish species, as clingfishes were sampled with the highest effort.

Different species showed different habitat preferences. *C. quadrivittatus* and *L. lepadogaster* occurred only in the rocky environment where they were hiding under stones. Species such as *T. ephippiatus* and *T. macrolepis* require a specific rocky habitat with caves and cavities of steep rock faces under which sandy patches are present (Bilecenoglu & Yokes, 2016; Kovačić, 1997; Miller, 1986; Patzner, 1999). Probably, as regards Slovenian waters, this type of habitat is only found in the Natural Monument Cape Madonna protected area, where the species were in fact recorded. There is also the deepest part of the Slovenian sea, which explains the occurrence of these two species known to occur in deeper waters (Miller, 1986). Moreover, this restricted area is inhabited by a high number of cryptobenthic fish species per 2 x 2 km quadrant, as well. This is in accordance with Lipej, Orlando-Bonaca & Šiško (2003), who reported on the correlation between the high diversity of a fish assemblage and the diversity of habitat types in this area. Similar dimly lit habitats, such as overhanging rocks, are also important habitats for two syntopical sciaphilic blennies, *M. nigriceps* and *P. zvonimiri*, which generally hide in precoralligenous formations and dwell in empty *L. lithophaga* burrows (Lipej et al., 2016, 2005; Orlando-Bonaca, 2006; Orlando-Bonaca & Lipej, 2008a; Patzner, 1999). Another species, *O. balearica*, also seems to be positively correlated with the coralline algal belt but in circalittoral biocenosis of the coastal detritic bottom (Consoli et al., 2019; Miller & Tortonese, 1968). However, our specimens were found in a boulder field with a coralline algae belt in shallow waters (2.5 m) near the coast. In a similar, mixed hard bottom (cobbles, bedrock and boulders), covered with soft thallus algae, but deeper in the infralittoral, species were observed in relatively high numbers by Kovačić et al. (2012).

The presence of three cryptobenthic species, *L. candolii*, *M. macrocephalus* and *Z. zebrus*, is not related only to the rocky environment, but they were also observed in seagrass meadows where they were hiding under shells and other objects on the sea bottom. Other authors have also reported about generalist habitat preferences of *L. candolii* and *Z. zebrus* (Hofrichter & Patzner, 2000; Patzner, 1999). *C. argentatus* occurs in a rocky environment, but it is the only species that is strongly associated with a dense macroalgae belt (e.g. *Cystoseira* spp., *Halopithys incurva*), which provides shelter for this species (Orlando-Bonaca & Trkov, 2016). Santin (2008) reported that the removal of the canopy forming algae did not affect the structure of cryptic fish assemblages, but we believe this result does not apply to *C. argentatus*.

Regarding depth distribution, three species, *C. argentatus*, *L. lepadogaster* and *C. quadrivittatus*, occurred only in very shallow waters, mostly less than 2 m deep, while *Z. zebrus* also occurs in slightly deeper areas (down to 3 m). As tidal amplitude is around ± 90 cm in this part of the Adriatic (Cosoli, Ličer, Vodopivec, & Malačič, 2013; Janeković & Kuzmić, 2005), tidal dynamics have a big impact on the distribution of these species. With the exception of *C. argentatus*, which probably withdraws to deeper water at the low tide, the other three species (*C. quadrivittatus*, *L. lepadogaster*, *Z. zebrus*) were found in lower mediolittoral tide pools. In few cases, four specimens of *L. candolii* were also found in tide pools in the lower mediolittoral (0.5 – 1 m). These were also the only findings of *L. candolii* in the mediolittoral stage. The occurrence of *Z. zebrus* and *C. quadrivittatus* in tide pools has been observed also by other authors (e.g. Ahnelt, 1990, 1991; Nieto & Alberto, 1992; Patzner, 1999). However, Patzner (1999) reported that only juvenile specimens of *Z. zebrus* use a wide variety of living spaces, while adults are confined to tidal pools (Miller, 1977; Nieto & Alberto, 1992). This is in accordance with our observations, as the largest

specimens (total length of the largest specimen was 47.29 mm) were found only in tide pools in the lower mediolittoral.

Other species, such as *M. macrocephalus*, *L. candolii* and *A. incognitus*, were mostly present in deeper waters in the infralittoral belt (depth range from 1 to 7 m). The presence of *M. nigriceps* and *P. zvonimiri* is mainly related to the availability of suitable hiding habitats (dimly lit habitats such as the underside of boulder terraces with empty burrows of *L. lithophaga*), which mostly occur deeper on the sea bottom (depth range from 1 to 10 m) which is in accordance with Patzner (1999). Overall, *T. ephippiatus* and *T. macrolepis* were observed in the deepest areas, which is in accordance with Miller (1986), who stated that other species from this genus also prefer habitats somewhat deeper than other Gobiidae species. However, based on different research, *T. macrolepis* occurs in the Adriatic Sea in shallower waters (most frequent from 11 to 25 m: Ahnelt & Kovačić, 1997; Kovačić, 1994) than in other parts of the Mediterranean (around 40 m; Patzner, 1999). Also, *O. balearica* was found in very shallow waters (2.5 m), compared to other studies, where specimens were found in deeper infralittoral (15 m) down to 60 m depth (Ahnelt & Dorda, 2003; Ahnelt et al., 1994; Consoli et al., 2019; Kovačić et al., 2012; Miller & Tortonese, 1968; Parenzan, 1973). This may be due to the shallowness of the Slovenian sea and consequent occurrence of specific suitable habitats at shallower depths.

In comparison with the rocky environment, the sedimentary environment is much poorer in cryptobenthic fish fauna. In the shallowest part of the sedimentary bottom, no cryptobenthic fish species were observed, probably due to the lack of hiding places, which is related to the low spatial heterogeneity of this habitat. Macpherson (1994) came to similar observations on blennies, as their presence is negatively correlated with the sandy bottom. Deeper on the sedimentary bottom, where seagrass meadows appear, four cryptobenthic fish species were recorded. Unlike *L. candolii*, *M. macrocephalus* and *Z. zebrus*, *A. incognitus* showed a strong preference only for seagrass meadows, where it occupies seashells, which is in accordance with the conclusions of Hofrichter (1995), Hofrichter & Patzner (2000) and Patzner (1999).

Cryptobenthic fishes were only found down to a depth of 10 m, since in deeper waters, the bottom is mainly sedimentary and there is a lack of cryptic habitats. This is also the reason why cryptobenthic species were only found in the quadrants located near the coast. Shallow waters and the lack of cryptic habitats on sedimentary bottom are probably the main reasons for lower species richness and absence of some species compared to the results of other authors (e.g. Glavičić et al., 2016; Kovačić, 1997; Kovačić et al., 2012; Patzner, 1999). However, further sampling on the sedimentary bottom is needed, especially on biogenic formations, coastal detritic bottom and secondary hard bottoms, which offer potential hiding places for cryptobenthic fish fauna.

In our research, stones (6 species; Table 4.1) were the most common hiding place in the rocky environment, followed by overhanging rocks (4 species), which is in accordance with the results published by Kovačić (1997). Within seagrass meadows, seashells seem to be an important microhabitat, since three cryptobenthic fish species were observed using them as a hiding place.

Clearly, the results show that cryptobenthic species richness is much higher on the rocky coast than on sedimentary bottoms, due to the higher spatial heterogeneity of the first environment. Santin et al. (2005) reported that rugosity, as a qualitative measure of habitat complexity, is important for explaining the variability in cryptic assemblages, underlining the importance of hiding places for the occurrence of cryptobenthic fishes. Certain epibenthic fishes, such as gobies *Gobius cruentatus* (Gmelin, 1789), *G. cobitis* (Pallas, 1814), *G. paganellus* (Linnaeus, 1758) and blennies *Microlipophrys canevae* (Vinciguerra, 1880), *Parablennius incognitus* (Bath, 1968) were found in similar hiding places. Kovačić (1997) also reported on similar observations of hiding non-cryptobenthic

fish species in cryptic habitats. This could lead to interspecific competition for space, which is well-known for blennies (Goldschmid & Kotrschal, 1981; Koppel, 1988; Orlando-Bonaca, 2006; Trkov, 2014).

Table 4.1: Hiding places occupied by cryptobenthic fish species.

	Stones and rocks	Precoralligen with endolithic burrows*	Overhanging rocks*	Seagrass meadows**	Dense algal belt
<i>Lepadogaster lepadogaster</i>	+	-	-	-	-
<i>Lepadogaster candolii</i>	+	-	-	+	-
<i>Apletodon incognitus</i>	-	-	-	+	-
<i>Cliniceps argentatus</i>	-	-	-	-	+
<i>Chromogobius quadrivittatus</i>	+	-	-	-	-
<i>Millerigobius macrocephalus</i>	+	-	-	-	-
<i>Odondebuenia balearica</i>	+	-	-	-	-
<i>Zebrus zebrus</i>	+	-	-	+	-
<i>Thorogobius ephippiatus</i>	-	-	+	-	-
<i>Thorogobius macrolepis</i>	-	-	+	-	-
<i>Microlipophrys nigriceps</i>	-	+	-	-	-
<i>Parablennius zvonimiri</i>	-	+	-	-	-

Exclusive shelters for fish in the marked habitats listed in the table: *cavity beneath boulders, **seashells

Up to now, 243 fish species have been confirmed in the Slovenian sea (Lipej & Orlando-Bonaca, 2020). Based on that, cryptobenthic fish species represent 4.9% of fish fauna in the Slovenian sea and 5.8% in terms of bony fish species. A comparison between cryptobenthic fish fauna lists produced by other northern Adriatic authors (Kovačić, 1997, 8 species; Kovačić et al., 2012, 6 species; Santin et al., 2005, 17 species) can be misleading as some species of epibenthic fishes were considered as cryptobenthic by certain authors (Santin et al., 2005), while for some fish species, such as *P. zvonimiri*, classification differs between authors (e.g. Illich & Kotrschal, 1990; Kovačić et al., 2012). In this research *P. zvonimiri* and *M. nigriceps* were considered as cryptobenthic species, as they occupy empty *L. lithophaga* burrows and inhabit dimly lit habitats. However, based on the published literature (Brandl et al., 2011; Hofrichter & Patzner, 2000; Kovačić, 1997; Kovačić et al., 2012) dealing with the occurrence of cryptobenthic fish species in the northern Adriatic, and new sampling techniques, other species are expected to be found in Slovenian waters in the future. This could be the case for two clingfish species, such as *Opeatogenys gracilis* (Canestrini, 1864) and *Gouania wildenowi* (Risso, 1810), which were previously confirmed in the northern Adriatic Sea (Hofrichter & Patzner, 2000). *O. gracilis* is expected to be

found in seagrass meadows, where we tried to catch it with a bottom dredge and a cube method but without success. *G. wildenowi* occurs in a rocky environment on limestone gravel beaches (Patzner, 1999; Wagner et al., 2019). Natural limestone beaches, as the most suitable habitat, are present on the Slovenian coast only at two locations in Izola, but there are also some artificial beaches with gravel along the Slovenian coast, and natural sandstone gravel beaches, which were checked. We checked altogether eleven of the most suitable locations, at different tide levels, and more than 100 samples of gravel were checked as proposed by Wagner et al. (2019), but the species could not be found.

As a result of this research, studies of occurrence of cryptobenthic fish species in the Slovenian sea were published in scientific journals (Trkov & Lipej, 2020; Trkov, Mavrič, Orlando-Bonaca, & Lipej, 2019).

4.3 Three Clingfish Species from Slovenian Sea: *L. lepadogaster*, *L. candolii* and *A. incognitus*

4.3.1 Biometry and meristic of clingfishes

L. lepadogaster, *L. candolii* and *A. incognitus* show adaptation to the environment in which they can be found. All three species are smaller than 10 cm, which enables them an efficient exploitation of cryptic habitats and better food availability (Miller, 1979). Although *L. candolii* reach the largest maximum sizes of all three species, specimens were on average smaller than those of *L. lepadogaster*, while *A. incognitus* was the smallest of all three species. The size of the fish species is correlated with their preferred microhabitat choice. The small size of specimens of *A. incognitus* enables them to effectively explore oyster shells, which are the smallest shelters compared to the other two species. Other two species hide mostly under stones, where space is not so restricted and consequently the species can reach larger sizes. All three species are dorsoventrally flattened, which is another adaptation of exploiting their preferred cryptic habitats where space is a limiting factor (Abel, 1962; Gibson, 1969).

All three clingfishes are short-living species and based on age-size correlations (Hofrichter, 1995) they reached approximately four years. The oldest specimens on average were those of *L. lepadogaster* (3+ years), followed by specimens of *A. incognitus* (2+ years), while the youngest specimens on average were *L. candolii* (1+ year). King (1989) and Hofrichter (1995) came to a similar conclusion in *L. lepadogaster*, as only few specimens of year class 0+ were found. Using any fishery or non-fishery collecting techniques we miss the youngest/smallest fish, so the length or age frequency curve is usually roughly bell-shaped, while the real population, due to mortality, follows exponential decay function along size or age increase. Fish growth is the fastest in the first two years and then slows down (Hofrichter, 1995; King, 1989), which is characteristic for many benthic fish species (Gibson, 1969). Observations of King (1989) indicates that specimens of *L. lepadogaster* become sexually mature when they reach at least 39 mm in total length, which would mean that specimens from the Slovenian sea become mature at 2+ age class.

The sex ratio in *L. lepadogaster* and *A. incognitus* was in favour of males, while in *L. candolii* it was in favour of females. However, sex ratio of *L. lepadogaster* was not significantly different from 1:1, which is in accordance with other authors (Hofrichter, 1993; King, 1989). The higher number of collected males than females of *A. incognitus* could be related to their habitat choice; males are bigger and were hiding mostly in seashells, while females are smaller and could hide in seagrass meadows and other cryptic habitats where they were much harder to be observed. Sex determination of *L. candolii* and *L. lepadogaster* was done based on the shape and length of urogenital papilla (Figure 4.2), which were the

most useful characters, rather than studying the gonads where specimens need to be killed and dissected. The problem is that specimens need to be sexually mature, otherwise urogenital papilla is not developed enough and sex could not be determined based on its shape and size. In *L. lepadogaster*, specimens need to be longer than 38 mm to be mature (King, 1989; Hofrichter, 1995). However, there was no available data indicating at which length *L. candolii* become sexual mature, so we presumed that sexual maturity is probably similar to that of *L. lepadogaster*. To this end, specimens of *L. candolii*, which are at least longer than 38 mm, were treated as sexually mature.

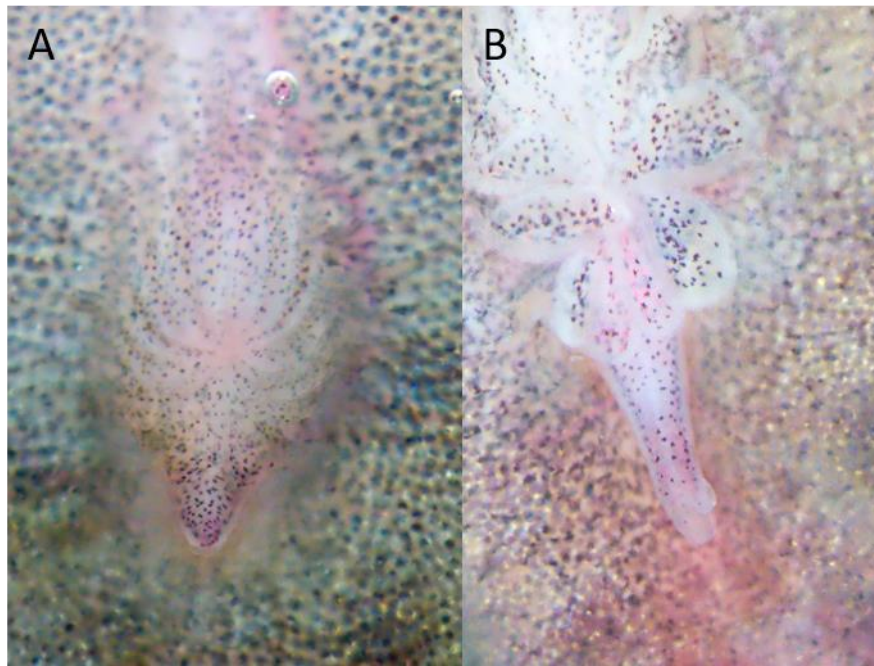


Figure 4.2: Urogenital papilla of *L. lepadogaster*: A – female; B – male (Photos: D. Trkov).

In *A. incognitus*, the sex was determined based on specimens of gonads from MBS collection, length and shape of urogenital papilla and the size of caniniform teeth (Hofrichter, 1995; Figure 4.3). The latter method was the most useful, as the shape of caniniform teeth could be observed already in juvenile specimens, when gonads were not developed enough to determine the sex of the specimens. Furthermore, based on the size of caniniform teeth, the sex of the fish can be determined in live specimens. Unfortunately, caniniform teeth are present only in species of the genus *Apletodon* (Briggs, 1955), so this character could not be used for sex determination in other clingfish species. The length of urogenital papilla was not informative as a sexual character to distinguish sex in *A. incognitus*. The problem was that male and female urogenital papillae have a quite similar shape and size, especially in smaller specimens, which was sometimes observed also in small barely sexually mature specimens of *L. lepadogaster* and *L. candolii*. Furthermore, urogenital papilla in all three species can be slightly retracted or covered with anal papillae and consequently, sex was much more difficult to be determined. There are also some other external sexual characters, which can help us in sex determination. The colour pattern could also be used as a sexual character and it can be useful mostly in large-sized mature specimens during reproductive season (discussed in Chapter 4.3.2). However, outside the breeding season and in barely sexually mature specimens, colour patterns were not so useful, as male and female patterns were too similar. Another sexual character in *L.*

lepadogaster is the length of nasal outgrowths, which was in males on average 1.5x longer than in females. In *A. incognitus*, the sexes can be distinguished also by the width of the head. It is wider in males, which is characteristic of the genus (Hofrichter, 1995). Different head shape and size between males and females are associated with a different type of food (Sakashita, 1992), but this was not confirmed in this research, as males with bigger heads feed with smaller prey than females. However, in all three species, males reached larger maximum size and were on average larger than females. Larger body size could be correlated with territoriality and nest guarding, as bigger males could protect their nests more easily. This dominance rank, positively correlated with the size of the fish, was observed in blennies, in which male territoriality and nest guarding is also known (Lengkeek, 2006; Orlando-Bonaca & Lipej, 2008b; Trkov, 2014).

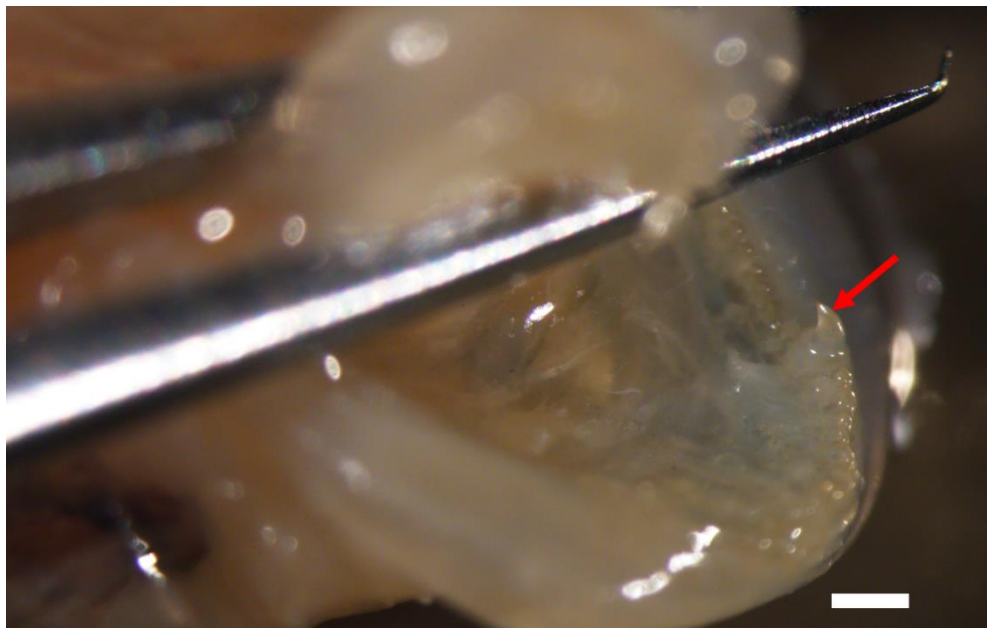


Figure 4.3: Caniniform tooth at *A. incognitus* (white bar represents 0.5 mm; Photo: D. Trkov).

L. lepadogaster is very similar to the related species *L. purpurea*, which was until recently considered as its subspecies *L. lepadogaster purpurea* (Henriques et al., 2002). Knowledge on the distribution of *L. purpurea* is still very poor and there is a possibility that this species could also be present in the Slovenian part of the Adriatic Sea. In order to ascertain the presence of this species in the studied area, distinctive characters, such as the size and shape of two blue eye spots surrounded with blue line patterns on the head and the number of rows of papillae on the anterior and posterior region of the sucking disc (Henriques et al., 2002; Wagner et al., 2017), were checked in 91 specimens. The size and shape of two blue eye spots were very variable and they consequently could not be considered as a reliable character (Figure 4.4). Based on the number of rows of papillae on the anterior and posterior region of the sucking disc, all specimens belonged to species *L. lepadogaster*.

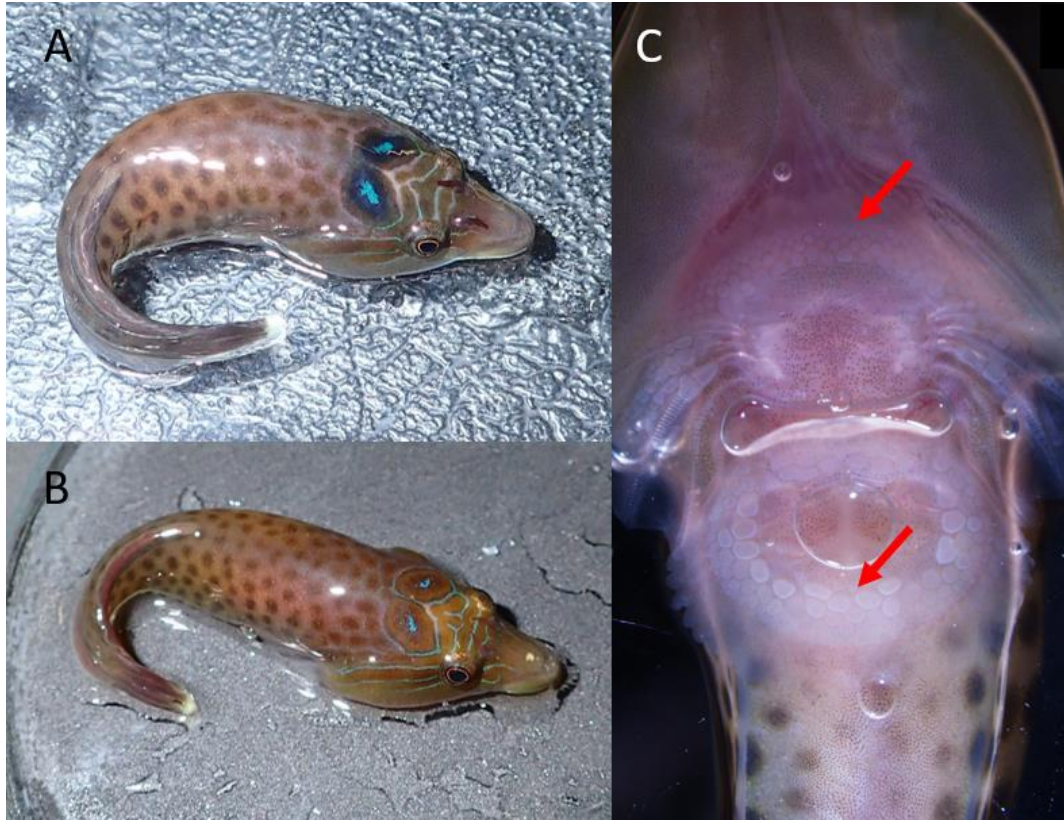


Figure 4.4: A and B – different size of two blue eye spots at *L. lepadogaster*; C – papillae on the anterior and the posterior region of the sucking disc at *L. lepadogaster* (Photos: D. Trkov).

The species *A. incognitus* was described only recently and was in the past often misidentified with the related species *Diplecogaster bimaculata* and *A. dentatus* (Hofrichter, 1995; Hofrichter & Patzner, 1997). Therefore, in addition to checking determination characters, we also did meristic counts on fin rays in 61 specimens, which further confirmed the correct determination (Table 4.2). There was a deviation in the number of fin rays with the results of Hofrichter (1995), but due to other determination characters, we assumed that it is just a morphological variance.

Table 4.2: Meristic counts done on 61 specimens of *A. incognitus* compared with results of Hofrichter (1995). Average, minimum and maximum numbers of fin rays per fin are presented: D – dorsal; A – anal; P – pectoral; V – ventral; C – caudal fin.

Fin	Number of fin rays					
	This study			Hofrichter (1995)		
	Average	min	max	Average	min	max
D	5	4	7	5	4	6
A	5	5	6	5	4	6
P	20	17	22	19	17	22
V	4	4	4	-	-	-
C	15	13	17	14	13	16

4.3.2 Body colouration of clingfishes

All three species have a different body colour pattern, which differs also between juveniles and adult specimens of the same species. The body colour of *L. lepadogaster* varies only little among specimens. The smallest caught specimen of *L. lepadogaster* measured 28.06 mm and was already coloured as an adult, so we had no opportunity to observe the colouration of juvenile specimens. Specimens of *L. lepadogaster* can change the body colouration from a bright general colour with dark spots to darker general colouration with brighter spots. Such colour changing was usually observed when the fish was disturbed. In all three clingfish species it was observed that they are able to quickly change colour from dark to bright and *vice versa*, which usually happened when they were transferred to a darker or paler substrate. Mood-related colour change in clingfishes was observed also by Hutchins (1992), while daily colour changing from paler at night to darker at day, which is known for several benthic fish species (Hofrichter & Patzner, 2000), was observed in *L. lepadogaster* and *L. candolii* by Ermin (1948). A quick change of body colour, intensity and/or area of black, grey and brown colours in fish is achieved by melanin granule dispersion in the melanocytes through melanocyte-stimulating hormone (Price, Weadick, Shim, & Rodd, 2008). However, behaviour of pigment cells is under the control of the endocrine and neural system, where rapid change of colour is typical for the latter one (Fujii, 2000). Such body colour change can signal static properties of the individual (*e.g.* sex, identity, development stage) and individual current motivation and quality state (Price et al., 2008). As clingfish are able to change their coloration quickly upon disturbance, it is quite difficult to photograph specimens in their “normal” colouration. This applies especially to the mating males, which become much less intensely coloured, if they were disturbed.

The colour pattern was the most diverse in juveniles and subadults of *A. incognitus* where the colour highly depended on the substrate. Juvenile specimens are able to change their general body colour pattern at least within a few months, which was observed in a specimen kept in an aquarium. The specimen changed its body colour from a generally dark green pattern to light brown. Such colour changing in *A. incognitus* was also previously observed by Hofrichter & Patzner (2000). Colour changing enables them to adapt to a given environment, which is very important as they can also be found outside the shelter, often on the shell of *P. nobilis*. Cryptic colouration enables them to stay undetected by predators, so they can move around outside of the shelter. This is in agreement with Gibson (1969) who stated two factors which influence the colouration changes: substrate colour and fish disposition. Furthermore, benthic fish species have a stronger tendency towards variability of the body colouration and changing colouration than pelagic species (Gibson, 1969).

The body colour of *L. candolii* juveniles is not so diverse in colour patterns, but rather uniformly dark purple-brown. This is in accordance with their hiding behaviour, as they were found mostly in shelters, where darker colouration seems to be more suitable. However, juvenile specimens of *A. incognitus* and all specimens of *L. candolii* have a white band between the eyes, previously observed also by Hofrichter (1995) and Hofrichter & Patzner (1997). A white band between the eyes was also observed in juveniles of *M. macrocephalus* and *Z. zebrus* (*personal observations*). This white stripe could have the purpose of obscuring the fish silhouette, as colour patterns with stripes are known to have the role of silhouette concealment in fish (Castro & Huber, 2005). Furthermore, observations indicate that distinctive the mottled body pattern possessed by fish outside the breeding season is actually cryptic in their natural habitat (Erisman & Allen, 2005). Variable body colouration among clingfishes and their high tendency for adaptation to habitat was already reported by Hofrichter & Patzner (2000).

In the spawning season, adult specimens of clingfishes get a mating colouration. In *L. lepadogaster* both sexes get an intensively red colouration of dorsal, anal and caudal fins, which is more intense in males. Furthermore, the body becomes more dark coloured. Similar findings were observed in *A. incognitus*, where males' dorsal, anal and caudal fins become dark-pigmented, while the head becomes reddish. Males of *L. candolii* also become dark-coloured during the spawning season, while the red colour occurs as stripes on the gill cover and as spots on the back and the dorsal fin. Dark body colour is known to be correlated with aggressive behaviour (Horth, 2003) and occurs in many fish species during the mating season (e.g. *Coryphoblennius galerita* (Linnaeus, 1758), *Parablennius sanguinolentus* (Pallas, 1814); Lipej et al., 2008). The red and dark body colouration thus have an important role in mating of the three species of clingfishes. The red and black nuptial colouration in males is also well known in the coastal blenniid, the red triplefin *Tripterygion tripteronotum* (Risso, 1810) (Lipej et al., 2008), which is considered by some authors (e.g. Kesici & Dalyan, 2018) as a cryptobenthic fish species. Red mating colouration is known also in many other marine as well as freshwater fish species which defend available resources (Price et al., 2008). In some fish species, it is known that only sexually mature individuals or only males who successfully establish a breeding territory develop mating colouration (Erisman & Allen, 2005; Sakashita, 1992).

Pigments or their precursors, especially carotenoids (red, orange, yellow colour), cannot be synthesized, so they must be obtained from the diet, which results in the colour expression being dependent on the individual foraging success and physiological efficiency (Erisman & Allen, 2005). Carotenoid-based colouration is associated also with parasite load (specimens infested with a parasite have duller colours; Price et al., 2008) and involved in the immune system (Grether, Kasahara, Kolluru, & Cooper, 2004; Lozano, 1994). The colour intensity of the individual thus shows its quality as a mate partner and a competitor as well as their escape potential for predators (Price et al., 2008). Male colouration consequently has a great influence on the female choice of a mating partner (Brownell, Shapiro, & Beltran, 2014; Grether, 2000). As carotenoids are limited in their availability and one of their most important sources are crustaceans (e.g. crabs; García-Chavarría & Lara-Flores, 2013), this could indicate that red colouration in male clingfishes is associated with their foraging success, which depends on their ability to defend a territory (hiding place) that provides a sufficient amount of crustaceans as prey. This is another indicator of how important is the role of crustaceans as a prey group for these three clingfish species.

4.3.3 Habitat selection patterns in three clingfish species

All three species of clingfishes were found in the coastal ecosystem in shallow waters where they were distinctly in conjunction with the substrate, which is typical for many clingfish species in general (sensu Hofrichter, 1993). Of all the three species, *L. lepadogaster* specimens were most often found, which is in accordance with many authors (Briggs, 1986, 1990; Dunne, 1983; Hofrichter, 1993; Illich & Kotrschal, 1990; P. A. King, 1989; Wilson, 1981), who stated that this species is locally common and is also considered as one of the most common clingfish species in the Mediterranean Sea.

4.3.3.1 Depth

Many coastal fish are syntopic, which means that they live in the same habitat. Separation into different ecological niches enables them to coexist in the ecosystem (Zander & Berg, 1984). Separation of ecological niches can also be clearly seen in three clingfish species, which can be found in Slovenian coastal waters. The study showed that all three clingfish species, *L. lepadogaster*, *L. candolii* and *A. incognitus*, differ in terms of depth segregation and there is just a little overlap between species. *L. lepadogaster* has the narrowest depth

range and it was the most abundant near the tide line in the lower mediolittoral and the upper infralittoral, which agree with observations of Hofrichter & Patzner (2000) who reported the species to be the most abundant at the depth of 0.8 m. Gibson (1972) and Hofrichter (1993; 12 m depth) reported that specimens were also found much deeper in the infralittoral. Hofrichter (1993) observed that in *L. lepadogaster*, there is no seasonal shift to deeper waters due to low winter temperatures. However, he observed that fish moved in deeper water in bad weather conditions (*e.g.* storms, extreme surf).

L. candolii occurred deeper in the infralittoral and were most abundant below the limit of the distribution of *L. lepadogaster*. Although there is a slight overlap in the depth range with *L. lepadogaster*, it is necessary to take into account the preference to habitat exposure. In sheltered locations, where *L. candolii* occurred in lower mediolittoral, *L. lepadogaster* was absent, so there was no habitat overlap between species. However, in the same location, *L. candolii* was mostly found below the lower limit of distribution of *L. lepadogaster*, which is in agreement with Brandstätter & Körbek (1989). Hofrichter & Patzner (2000) reported that the species is most abundant at the depth around 2 m, where it reaches maximum densities of 10 ind./m². From all three species, *A. incognitus* occurred in deeper layers, which is related to the availability of seagrass meadows that are present in the Slovenian sea from 1 to 11 m depth (Lipej et al., 2018).

In all three species, size-depth segregation was observed. In general, large specimens of *L. lepadogaster* and *A. incognitus* were more abundant near the lower limit of its depth distribution, while smaller specimens were more abundant near the upper limit of its depth distribution. Conversely, large specimens of *L. candolii* were more abundant near the species' upper limit of depth distribution, while smaller specimens were more numerous at the species' lower limit of depth distribution. Small specimens of both species in that way could avoid mutual competition, as well as intraspecific and interspecific predation of large specimens. *L. lepadogaster* predation on smaller *G. wildenowi* was observed by Hofrichter (1993), while we never observed interspecific predation among clingfishes. Depth segregation of different sized fish of the same species is known in many fish species and it is the result of intense intraspecific predation or competition (Freeman & Stouder, 1989; Guttridge et al., 2012). Intraspecific separation of depth distribution could be the avoidance of competition for food and shelters between specimens of different size, as also avoidance of cannibalism, since large specimens could prey on smaller specimens. Predation of large specimens of *L. candolii* on smaller ones was observed when they were kept together in a 10 l bucket. Cannibalism is also known in other clingfishes (Johnson, 1970; Pires & Gibran, 2011). However, diet analysis had never confirmed predation on smaller specimens. Another very likely reason is that larger specimens of *L. lepadogaster* occurred somehow deeper due to the presence of decapods (*Pisidia* sp. in particular), which are important food items and occur mainly in the upper infralittoral (Holthuis, 1961; Melzer et al., 2019). This is supported also by the occurrence of largest specimens of *L. candolii*, which were abundant especially in the upper infralittoral belt, where decapods represent important prey items for them. Occurring of large specimens of *L. lepadogaster* in the upper infralittoral could also be linked to strong territorial behaviour in males (Gonçalves et al., 1996), which hide under the same stone for several months (Hofrichter, 1993). Specimens of *L. lepadogaster* were found under a stone right under the water line at low tide, but never outside the water. At low tide, specimens from the lower mediolittoral need to move somehow deeper or remain in tide pools, while specimens which occur deeper in the upper infralittoral can stay in their shelter. Furthermore, movement of males into slightly deeper water during the mating season was previously reported by Hofrichter (1993).

4.3.3.2 Macrohabitat

All three species showed preference to different macrohabitats. *L. lepadogaster* and *A. incognitus* are very specific in their habitat choice. *L. lepadogaster* was found only in the rocky environment, while *A. incognitus* is strictly associated with the seagrass meadows. *L. candolii* is the most euryecious of all three species and was found in the rocky environment as well as in seagrass meadows.

L. lepadogaster specimens were mostly found in boulder fields and banks of large stones, where this species achieves densities to maximum 8 ind./m² (Patzner, 1999), while Hofrichter & Patzner (2000) reported as much as maximum 23 ind./m² in such habitat. Species of *L. lepadogaster* occurred in exposed or partially exposed areas, while they were absent in sheltered areas. Adaptations to strongly exposed habitats and avoiding of calm waters are also known in other clingfishes, although adequate shelters and food are provided (Johnson, 1970; Paine & Palmer, 1978). In a sheltered area, such as the bay of St. Jernej (Ankaran), *L. lepadogaster* was absent and this was the only location where *L. candolii* occurred in tide pools in the lower mediolittoral. This shows that this species probably has different preferences for habitat exposition or just take advantage of a free habitat niche. The preference for less turbulent water by *L. candolii* was previously reported by Gonçalves, Gonçalves, Almada, & Almeida (1998). However, *L. candolii* was mostly found in a similar rocky habitat as *L. lepadogaster*, but it occurs in deeper waters. Only in few cases *L. lepadogaster* and *L. candolii* were found together at the border of their occurrence in the upper infralittoral. Boulder fields were recognized as the most suitable habitat for those two species also by Hofrichter (1993, 1995) and Patzner (1999), as well as for other Mediterranean clingfishes (Hofrichter & Patzner, 2000). Patzner (1999) stated that in boulder fields, *L. candolii* can reach maximum densities up to 12 ind./m². *L. candolii* occurred also in seagrass meadows, as observed also by other researchers (Hofrichter, 1995; Hofrichter & Patzner, 2000; Patzner, 1999), where it is syntopical with *A. incognitus*. Although *A. incognitus* mostly occurred deeper than *L. candolii*, both species were also found together in certain locations.

4.3.3.3 Shelter

All three clingfish species exhibit a cryptic life style, which provides them protection against predators (Hofrichter & Patzner, 2000). Preferred shelter of *L. lepadogaster* and *L. candolii* were big stones under which they were hiding. These were mostly stones of sandstone, which were mostly rectangular in shape. Both species occupied stones, which were big enough that wave action could not move or turn them around, but not too big, otherwise stones were too heavy and they sank into the sediment. In *L. candolii*, which occupied stones with average larger undersurface area than *L. lepadogaster*, stones were flatter, which prevents the stone to sink into the sediment. Deeper in the infralittoral where *L. candolii* occurred, a softer sediment (*e.g.* sand) was present, so only flat stones were not sunken into the sediment. This is furthermore supported by observations that stones occupied by *L. candolii* were flatter with the increasing depth.

Big stones form the basis of a more stable community and they are more suitable as shelters. This is in accordance with a high degree of algal overgrowth of stones. In fact, algal vegetation cannot overgrow smaller stones, which are moving and turning around due to wave action. The habit of *L. lepadogaster* to occupy flat stones with an algal cover was previously reported by King (1989). However, due to high water turbulence and exposure to factors associated with a low tide, many stones occupied by *L. lepadogaster* were without vegetation (Figure 4.5). The occurrence of *L. candolii* in the biocoenosis of photophilic algae was also reported previously by other authors (Hofrichter, 1995; Hofrichter &

Patzner, 2000; Kovačić, 1997). The latter one also reported on the choice of stones without vegetation by *L. lepadogaster*.

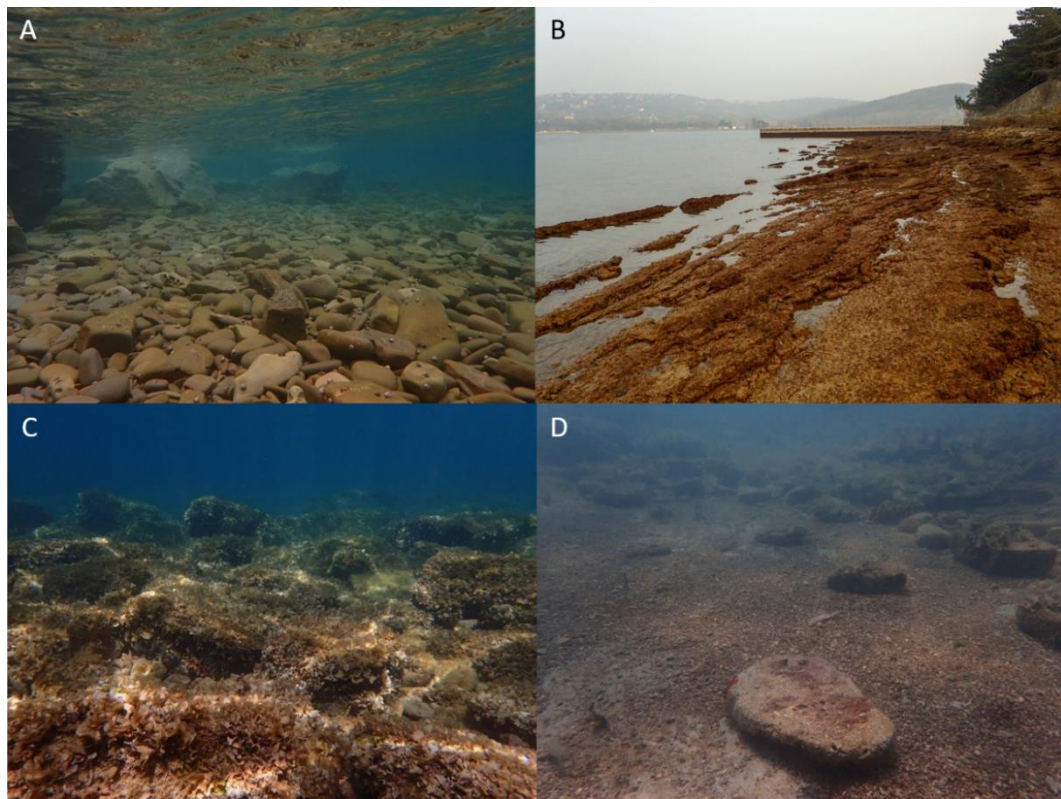


Figure 4.5: A – stones in the exposed mediolittoral occupied by *L. lepadogaster* were mostly bare or covered with turf algae; B – stones in the sheltered mediolittoral were covered with macro algae and at such a location (Debeli rtič), *L. candolii* occurred in the mediolittoral; C and D – stones in the infralittoral occupied by *L. candolii* were mostly overgrown with macro algae (Photos: D. Trkov).

Analyses showed that *L. lepadogaster* occupied stones with an average undersurface area of 409.8 cm², which is in accordance with Henriques et al. (2002), who reported that *L. lepadogaster* occupied stones with a similar bottom surface area (477cm²). However, *L. candolii* occupied stones with on average 1.3 times larger undersurface area (530.9 cm²) than *L. lepadogaster*. As *L. lepadogaster* specimens were on average bigger than specimens of *L. candolii*, the size of the fish species is probably not related to the area. At the same time, it should be noted that the size of stones decreases with the distance from the coast. One of the reasons why *L. candolii* prefer stones with a larger undersurface area could be due to the fact that they inhabit deeper and less turbulent waters, which bring less food for potential prey (detritivore crustaceans). This is reflected in lower prey abundance and consequently the clingfish needs a bigger shelter to obtain enough food. Another reason could be the substrate under stones (*e.g.* sand which is more common in deeper areas) provides less shelter for potential prey groups (especially decapods) and that could also be compensated with the choice of stones with a larger undersurface area. However, *L. candolii* also prey outside the shelter, as observed by Almada, Garcia & Santos (1987), which could be due to an insufficient amount of suitable prey under the stones. Conversely, specimens of *L. lepadogaster* were never observed outside the shelter, which is in accordance with observations of many researchers (*e.g.* Gonçalves, Gonçalves, Almada, & Almeida, 1998; Hofrichter, 1993, 1995). Gonçalves et al. (1998) stated that *L. lepadogaster* does not make

many movements, probably due to the turbulent environment in which it lives. Association between a small movement and turbulent environment was also observed in blennies (Almada & Santos, 1995). Velasco et al. (2010) stated that in the rocky intertidal habitat, the availability of prey is relatively high, which is reflected in higher dietary overlap of intertidal fish. Furthermore, high density of suitable prey could be an important factor, which allows feeding without major movements. However, suitable stones in the mediolittoral are more abundant and usually one next to the other compared to those deeper in the infralittoral, so specimens of *L. lepadogaster* can easily move from stone to stone, without leaving the shelter during feeding. In the aquarium it was observed that if specimens left the inhabited stone, they just switched from one to another nearby shelter under the stone. The similar behaviour was observed in the field when the occupied stone was moved and fish moved just to the nearby stone. These observations indicate that *L. lepadogaster* exhibit the most cryptic behaviour, compared to *L. candolii* and *A. incognitus*. In *L. lepadogaster* and *L. candolii*, the area of the underside of stones is positively correlated with the size of the clingfishes, as bigger specimens preferred a larger surface area. The reason for occupying stones with a larger undersurface area by large specimens could be that it provides more space as well as a larger area for prey searching.

L. lepadogaster and *L. candolii* showed a preference to the similar substrate under occupied stones. In both species, pebbles were the most common substrate. Under stones occupied by *L. candolii*, pebbles were usually mixed with sand. They occur in deeper waters distant from the coast, where finer sediments prevail. Pebbles probably provide just enough space under stones for clingfish specimens to live under them. Furthermore, pebbles are a favourite habitat for decapods (*Pisidia* sp. and *A. nitescens*; Pallas, Garcia-Calvo, Corgos, Bernardez, & Freire, 2006), which are important food items for clingfishes. In case that there was only fine substrate (e.g. sand) under the stone, there was usually not enough space between the stone and the substrate (stones were usually plunged in) for the clingfish, while big stones as substrate cause that the hiding place under the stone was too exposed and offers no protection against predators.

Specimens of *L. candolii* also occupied seashells (mostly *P. nobilis* and *O. edulis*) and artificial structures. *L. candolii* exploited these microhabitats mostly in seagrass meadows, where shells were almost the only hard structures which can be used as shelters and consequently their availability is a limiting factor. Artificial structures, such as metal and plastic plates, were consequently used as an alternative shelter. The exploitation of artificial structures as hiding places by *L. candolii* and related species *D. bimaculata* was reported also by Brandl et al. (2011). Most of the stones on the sedimentary bottom are also the consequence of human activities. However, most of these stones are unsuitable for clingfish occupation as they were sunken into the seabed due to their weight. Under such stones, specimens of *Gobius niger* (Linnaeus, 1758) were mostly observed, which can dig a shelter under a stone (*personal observation*). *G. niger* and big males of *Parablennius tentacularis* (Brünnich, 1768) were the most common fish species observed under shells of dead *P. nobilis* as well. As the shell was normally occupied only by one fish, we could assume an ongoing interspecific competition for such shelters. Strong intra- and interspecific competition for hiding places, which are limiting resources in the environment, was already demonstrated in blennies (Koppel, 1988; Orlando-Bonaca, 2006; Orlando-Bonaca & Lipej, 2008b; Trkov, 2014).

Specimens of *L. candolii* were mostly found when they were hiding under an object on the sea bottom and only in few cases were small specimens found outside the shelter on shells of *P. nobilis*. This confirms that specimens also move around outside the shelter, which was already suspected by Kovačić (1997) and reported by other researchers (Almada et al., 1987; Hofrichter, 1995; Hofrichter & Patzner, 2000). However, specimens were never

observed freely swimming between shelters in the field as other authors reported (Hofrichter & Patzner, 2000; Patzner, 1999), except when they were disturbed from the shelter. In that case, specimens sometimes swim more than 1 m away to the next shelter and not only to the nearest one, such as it was observed in *L. lepadogaster*.

In seagrass meadows, specimens of *A. incognitus* were found, where they showed a great preference for occupying dead shells, especially empty oyster shells (66.0% of all shelters) which were mostly attached to *P. nobilis* (54.6% of all shelters). Flatted body and small body size enable them to effectively occupy dead oyster shells. In the latter, small specimens of *P. tentacularis* were also often observed. The blenny could compete with specimens of *A. incognitus* for available hiding places. In oyster shells, other fish species were also found, such as *Symphodus cireneus* (Bonnaterre, 1788) and *Z. zebrus* (Figure 4.9). However, it seems that *A. incognitus* prefers empty oyster shells which are more closed than those occupied by *P. tentacularis*, especially during the nesting period. Occupation of such shells is possible due to their reduction of body height, which is besides Blenniidae and Gobiidae, the most pronounced in Gobiesocidae (Abel, 1962; Gibson, 1969). Furthermore, adult males of *A. incognitus* have wide heads in the cheek area (Hofrichter, 1995), which make them seem bigger and allow them to expose caniniform teeth, which probably serve for defence, as they are not used for diet. Many other small territorial fish species also have big caniniform teeth, which do not serve for feeding, but rather for predator deterrence and agonistic interactions (Kotrschal & Goldschmid, 1992). Teeth showing (yawning) was observed in the interactions of specimens of all three species kept in the aquariums (Figure 4.6). Such behaviour was also observed in *L. lepadogaster* by Hofrichter (1993).



Figure 4.6: Teeth showing (yawning) at *L. candolii* (Photo: D. Trkov).

Although specimens of *L. candolii* also occurred in the same habitat and as deep as *A. incognitus* and both specimens occupied seashells, there is probably only little competition between these two species for shelters. Adult specimens of *L. candolii* occupied shells of dead *P. nobilis*, which lay on the sea bottom (Figure 4.7). Such pen shells were mostly occupied also by big males of *P. tentacularis* and *G. niger*, but almost never by *A. incognitus*. The latter species mostly hide inside the empty oyster shells, which were too

small for adult *L. candolii*. Furthermore, juvenile specimens of *L. candolii* mostly occupied the undersurface of oyster shells on the sea bottom, and were almost never observed inside the shell unlike specimens of *A. incognitus*. However, *A. incognitus* prefer oyster shells which were attached to the living *P. nobilis*, which furthermore reduce the possibility of competition with *L. candolii* and also with other invertebrates and potential predators (*e.g.* crabs) that are present on the sea bottom. Many researchers (Hofrichter, 1995; Hofrichter & Patzner, 2000) reported that juvenile and subadult specimens were commonly found on leaves of *P. oceanica* and under different species of sea urchins, where they reached maximum abundance of up to 40 ind./m². However, in this study, specimens of *A. incognitus* and also *L. candolii* were never found under sea urchins, despite the fact that these hiding places were checked many times.



Figure 4.7: A – In seagrass meadows, hard objects were a limited resource; B – dead *Pinna nobilis* shells laying on the bottom were the preferred shelter of adult *L. candolii*; C – cluster of oyster shells laying on the sea bottom were occupied by juvenile *L. candolii* and specimens of *A. incognitus*. D – oyster shells attached on *P. nobilis* were the preferred shelter of *A. incognitus* (Photos: D. Trkov).

Of all three species, specimens of *A. incognitus* most often occurred outside the shelter. While adult specimens of *A. incognitus* were mostly found hiding inside the empty oyster shells, juvenile and subadult specimens were also found outside of the shelter on shells of living *P. nobilis* (Figure 4.8). This is associated also with the body colouration of small clingfish specimens. Specimens of *P. nobilis* associated with *A. incognitus* were mostly adult and they did not have spines (a characteristic feature of juveniles Noble pen shell) which would disable fish clinging. Shells of *P. nobilis* were normally overgrown with epibiota, which provided cover for fish. Disturbed fish sometimes move to seagrass leaves, but they were never found hiding on them.

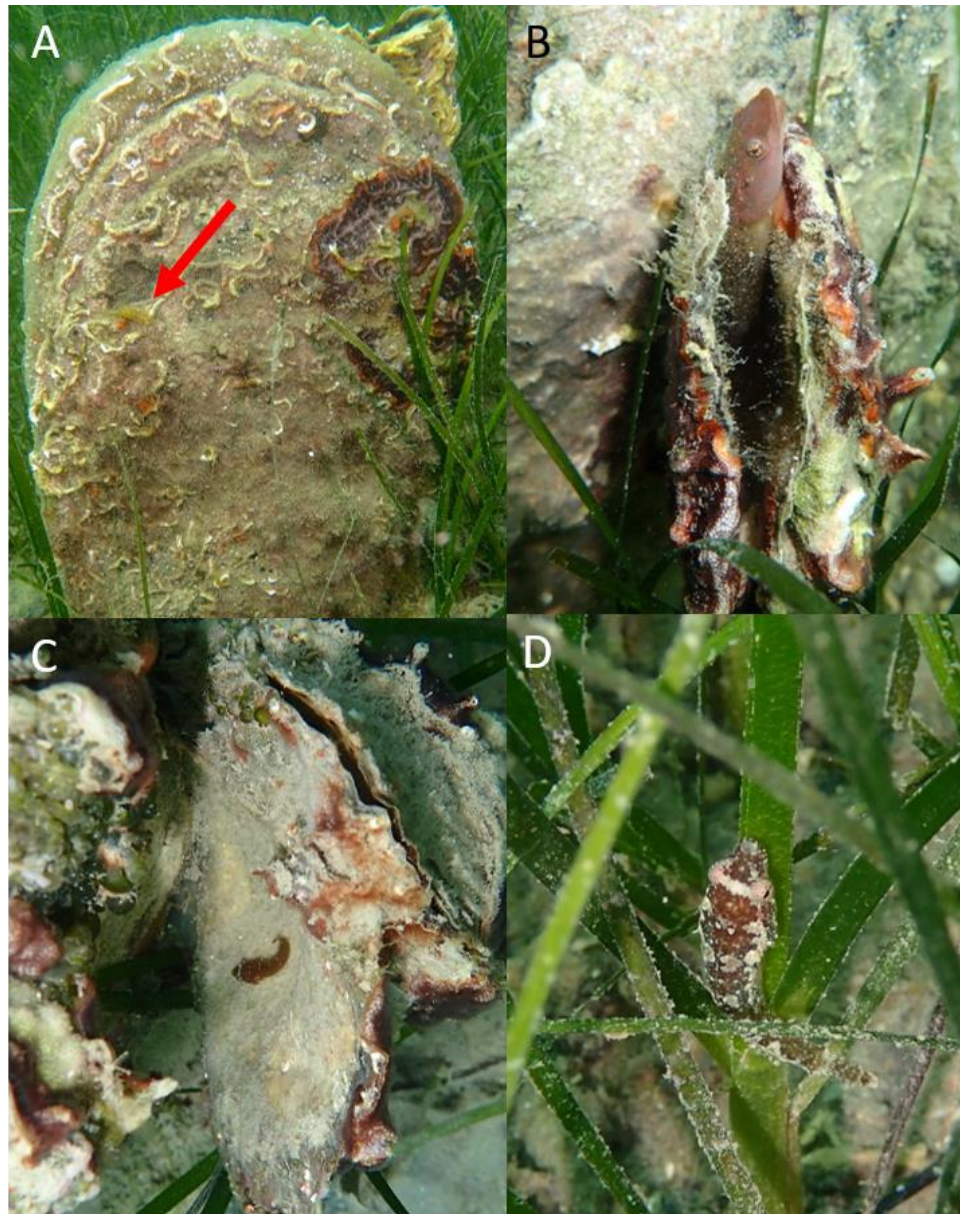


Figure 4.8: Specimens of *A. incognitus* hiding: A – on shell of *Pinna nobilis*; B – nesting male occupying a dead oyster whose shell is slightly open; C – non-nesting specimen occupying a dead oyster whose shell is wide open; D – disturbed specimen moved from shelter to the seagrass leaf (Photos: D. Trkov).

P. nobilis has proven to be a very important bioconstructor, since in the seagrass meadows, it represents almost the only natural hard and sufficiently large base for the settlement of oysters (and also other organisms), whose shells represent important hiding places for many marine organisms (Cabanellas-Reboredo et al., 2019; Giacobbe, 2002; Lipej, Mavrič, & Orlando-Bonaca, 2012; Figure 4.9). By peeling off the top layer of shells of *P. nobilis*, the oysters fall to the sea bottom (*personal observations*), which increases the habitat heterogeneity and significantly influences the appearance of many organisms depending on hiding places.



Figure 4.9: Different coastal fish species occupying dead oyster shells attached to the shell of *Pinna nobilis*: A – *Zebrus zebrus*; B – *Parablennius tentacularis*; C – *Symphodus cireneus*; D – *Gobius niger* often occupied shell of dead *P. nobilis*, which lay on the sea bottom; E – artificial object in a seagrass meadow used as a shelter by *L. candolii* (Photos: D. Trkov).

4.3.4 Reproductive ecology

L. lepadogaster reproduce seasonally, as nests were observed from the end of March till the end of May in the same habitat as adult specimens were found. This is in accordance with other authors who reported that species spawn from March to July (Henriques et al., 2002; Hofrichter, 1993), while in the northern Atlantic, eggs were found only in July and August (King, 1989), which is probably due to lower water temperatures. Nests were found in the lower mediolittoral and the upper infralittoral, in boulder fields, where they were attached on undersurface of stones of a similar size and the substrate as preferred by adult specimens. Similar observations were reported also by Hofrichter (1993). However, in some cases, there were big stones in more than one layer, forming a three dimensional multilayered habitat. Nests in such habitat were sometimes much bigger and extending on more than one stone. Such habitat was very heterogeneous and very demanding for sampling, furthermore quinaldine could not be used, due to the high turbulence and waves. This is the reason why we were not able to catch fish specimens on such a habitat, but rather just found their nests. Due to the high number of nests, which were quite large (the largest contained 7384 eggs), we assumed that also the number of fish specimens was quite high in such

environments. The habitat complexity of the substrate thus enlarged the biotope of *L. lepadogaster* and so its abundance increased (Hofrichter, 1993). However, only in 16 cases, we were able to catch the fish on the nest and a great majority of them were males. This is in accordance with the results of other authors (*e.g.* Hofrichter, 1993, 1995; King, 1989), who stated that males care for nests until the eggs do not hatch. This was observed also in *L. candolii* and *A. incognitus*, and was already known for many clingfishes (Hofrichter, 1995; Pires & Gibran, 2011) and other benthic fish species (Almada & Santos, 1995; Coleman, 1999). A female, which was found in the nest, was there probably due to its spawning. On some stones, multiple nests were present, so it is not clear if all of them belonged to one male or many of them. As nesting males are territorial and protect their stone (Hofrichter, 1993), it is strange that more males would have nests under the same stone, especially because nests were very close to each other (the shortest distance between nests was 1.5 cm; Figure 4.10). However, multiple nests would be much harder to protect by one male.

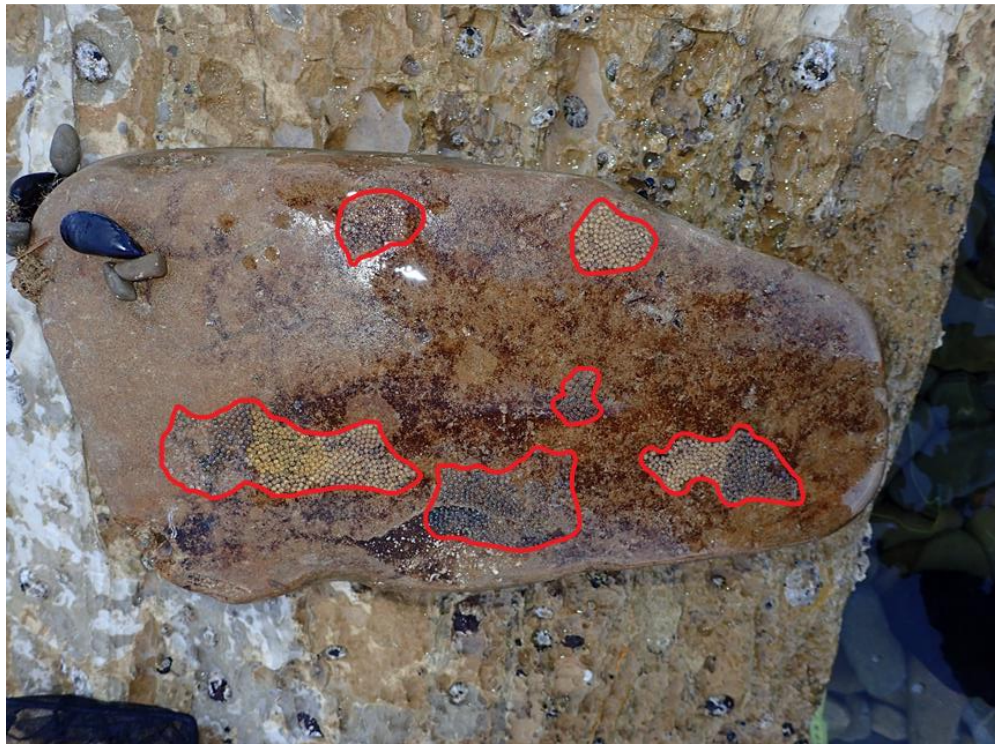


Figure 4.10: Multiple nests of *L. lepadogaster* attached to the undersurface of the stone (Photo: D. Trkov).

Eggs were attached to the stone surface with attachment filaments (Breining & Britz, 2000) in one layer. Nests normally contained more than one egg clutches, which can be observed because of different stages of egg development (Figure 4.11). Eggs in the middle of the nest were normally the most developed, suggesting that the first female laid eggs in the middle of the nest and then other females laid eggs around them. This indicates that the males spawned on multiple occasions, which is in accordance with the observations of Hofrichter (1993), who noticed that females laid eggs in several clutches in a few weeks and not all at once. Females on average deposited 165.5 eggs per spawning, which is in agreement with King (1989; 156 eggs) and Hofrichter (1993; 150-200 eggs). The latter one also described the process of egg deposition in *L. lepadogaster*. Although nests contained eggs in on average 1.8 different development stages, we estimated, based on the number of eggs laid by one female at one spawning, that the male spawns on average 3.7 times per

nest, while at the largest nest (with 7384), the male could spawn around 44 times. The reason why eggs were not in so many different stages is because the male probably spawned with multiple females, one after another in a short period of time, so eggs were in the same development stage. Similar observations regarding the egg abundance were reported also by Hofrichter (1993, 1995). Measurements done by Hofrichter (1995) show that eggs of *L. lepadogaster* are 1.78 to 1.92 mm long, 1.46 to 1.55 mm wide and 1 mm high. In water temperatures from 20 to 22 °C, larvae hatch after 15 to 16 days and measure 5.4 - 5.5 mm (Kalinina, 1976).

These results are one of the first more detailed pieces of information about the reproduction of the recently described species *A. incognitus*. New findings also support the observation of Hofrichter (1995), who reported that one nest was found on 22 July 1995 in an empty oyster shell. In empty mollusc shells, a related species *D. bimaculata* also nests (Hofrichter, 1995). However, it was observed that *A. incognitus* used shells, in which the inner parts were covered with different epibiota and were consequently not white, while Hofrichter (1995) stated that *D. bimaculata* use shells, whose inner side of the shell is without epibiota and consequently smooth and white-coloured. Oyster shells used for nests of *A. incognitus* were mostly attached to the living *P. nobilis*, which probably served as protection from predators, which were present on the sea bottom (*e.g.* crabs *Maja crispata* Risso, 1827 and *Eriphia verrucosa* (Forskål, 1775)). Furthermore, shells used for nests were narrowly opened, which further prevents access to the nest for intruders and predators.

Only one nest of *L. candolii* was found in the beginning of May under a stone, which is in agreement with the authors who stated that the species spawn from March to late August in the infralittoral (Henriques et al., 2002; Hofrichter, 1995).

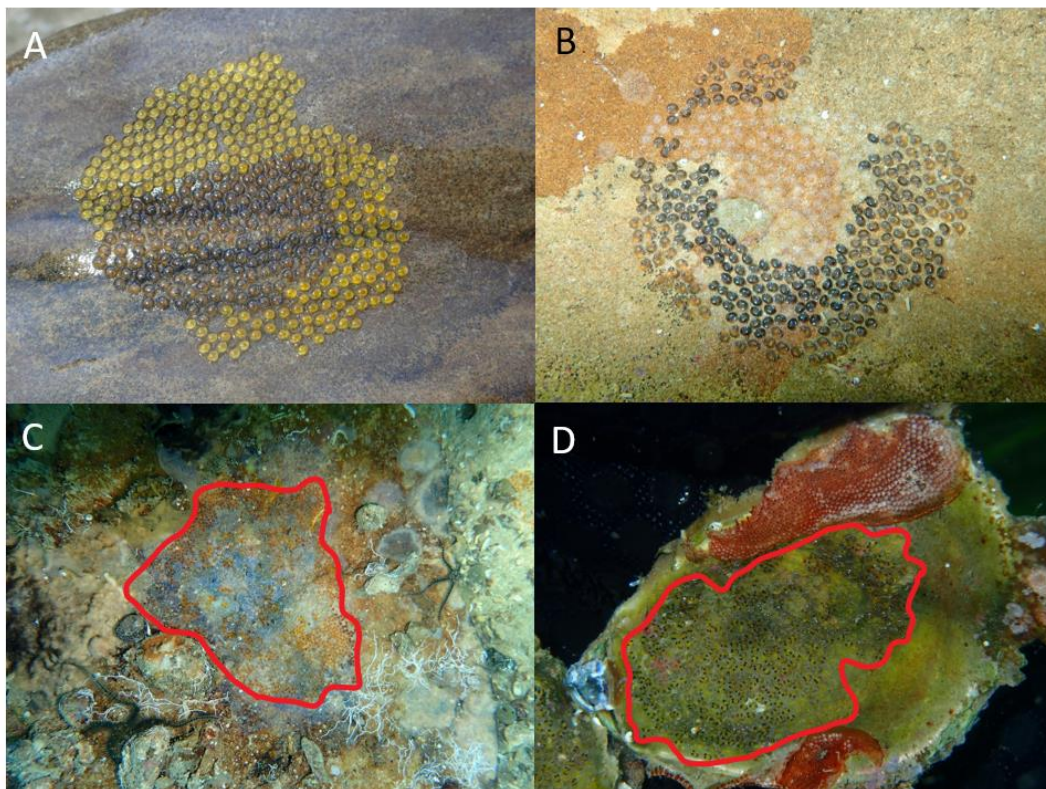


Figure 4.11: A – nest of *L. lepadogaster* composed of two clutches of eggs in different development stages; B – nest of *L. lepadogaster*; eggs in the middle are already hatched, while two clutches of eggs around it are still developing; C – nest of *L. candolii*; D – nest of *A. incognitus* (Photos: D. Trkov).

For all three species we thus confirmed the claims that European clingfishes reproduce in spring and summer and that males guard nests (Hofrichter, 1995). Care for eggs (*e.g.* nest cleaning and protection against predator) is one of the most common forms of parental care of mediolittoral fish species (Coleman, 1999). However, reproduction in spring and summer, due to high availability of food for offspring, is characteristic of most living organisms (Bye, 1984).

4.3.5 Diet study

4.3.5.1 Pre-experiments

Pre-experiments give us important information about the excretion of faecal pellets. Based on our results, 24 hours after feeding is enough time for clingfishes to produce the faecal pellets containing the ingested food. This was supported by the production of “false” pellets (without prey) within this period of time. “False” pellets were smaller compared to the regular ones and white-coloured. The first “false” pellet excreted after the regular one was checked if it contained any prey to be sure that the specimen certainly excreted all prey from its digestive tract.

Results of the pre-experiment with *A. incognitus* were in accordance with our observations that clingfishes have a rapid digestion, as specimens produced on average more than three faecal pellets during 24 hours of the pre-experiment. These results support our observations in aquarium, where it was estimated that fish need between 6 and 24 hours from the time of feeding to the excretion of the faecal pellets.

Results of the pre-experiment with *L. candolii* showed that all ingested prey items were found and identified in faecal pellets, which shows the efficiency of the method. The reason why specimens of *L. lepadogaster*, which were primarily used in the experiment, did not feed, could be explained by the fact that unlike specimens of *L. candolii* and *A. incognitus*, they were never found in nature outside their shelter. Consequently, shelter should be provided in experiments with specimens of *L. lepadogaster*, but in this manner, it would be more difficult or even impossible to control the experiment.

4.3.5.2 Accuracy of new method tested on *L. lepadogaster*

The aim of this part of the study was to test the diet of the clingfish *L. lepadogaster* introducing a new non-destructive method, which does not necessitate the sacrifice of fish specimens. *L. lepadogaster* has a short digestive tract (Hofrichter, 1993; Figure 4.12) and consequently rapid digestion (*personal observation*), which enables us to obtain samples relatively fast. This is important since the method is based on faecal pellets.



Figure 4.12: *L. lepadogaster* has a short digestive tract. Red arrow points to the midgut where faecal pellets form. The midgut represents around one third of the digestive tract, while other two thirds are represented by the esophagus and the hindgut (Photo: D. Trkov).

Short digestive tract and fast digestion have also been observed in other clingfish species such as *Sicyases sanguineus* (Müller and Troschel, 1843) (Cancino & Castilla, 1988). In general, most specimens produced from one to two faecal pellets during 24 hours in captivity. Due to rapid digestion and high numbers of prey items in faecal pellets, we assume that they feed very often. Furthermore, we observed that even after defecating, clingfishes still produce false faecal pellets without prey, which is likely an adaptation to continuous feeding. Prey was normally eaten as a whole (Day, 1884), but in cases where the specimen of *Pisidia* sp. crab was too big and tried to defend itself, the clingfish tore off its claw and ate it (*personal observations*). Rapid digestion was reflected in poorly digested food. A large proportion of digested prey items were consequently whole or almost whole, which facilitated the identification, quantification and measuring of the prey. Cancino & Castilla (1988) found out that in the related clingfish species *S. sanguineus*, some type of prey can even survive the passage through the digestive tract due to its short gut and brief food passage time. The most digested prey items were identified based on the hard body parts (*e.g.* jaws) that did not degrade during digestion. During the reproductive season, eggs assumed to be *L. lepadogaster* eggs were found in prey samples as well. Consequently, we could assume that if other soft body parts of organisms were present in the diet, their remains would also be observed. Furthermore, also King (1989), who studied the diet of *L. lepadogaster* based on the intestine content, recognized softer-bodied animals only by certain distinguishable parts. This indicates that even with obtaining prey from the intestine would not give us more accurate results. However, care must be taken to not overlook the soft-body prey, which rapidly decomposes.

The comparison of our data on the *L. lepadogaster* diet with the data obtained in existing studies outside the Adriatic Sea of King (1989), Mazé (2007), Velasco et al. (2010) and Wilson (1981), who analysed intestine contents, showed similarity and consistency between studies, which proves the usefulness and applicability of the method for studying clingfish feeding ecology. Furthermore, even higher numbers of preyed taxa were identified with our method compared to the methods used by other authors (Table 4.3), thus showing the accuracy of the tested method.

Amphipods were the most preyed invertebrates in the diet of *L. lepadogaster* in terms of numerical abundance or frequency of occurrence in the studies of Compaire, Cabrera, Gómez-Cama & Soriguer (2016), Gibson (1972), Mazé (2007) and Velasco et al. (2010), while copepods were the most numerous prey group in the study of Wilson (1981). These results are in agreement with our observations, as copepods and amphipods were the most abundant and frequent prey groups. The high abundance of decapods found in our study in contrast to other studies is probably due to the substantial availability of *Pisidia* sp. in the clingfish habitat ($N\% = 11.5$, $F\% = 75$; up to 26 specimens per 25 x 25 cm quadrant). The presence of a particular prey in the diet is highly dependent on the local occurrence of prey, as previously observed by Gibson (1972). Besides crustaceans, gastropods were the most abundant prey group, which is consistent with the studies of King (1989), Mazé (2007) and Wilson (1981). By comparing data from different studies, some differences also emerged, which are probably a consequence of the average size of the studied fish (decapods are more numerous in the diet of larger specimens), local availability of prey, and also the size of the clingfish sample (number of studied fish), which can significantly affect the results (Gibson, 1972).

Table 4.3: Comparison of relative prey abundance ($N\%$) data obtained for *L. lepadogaster* by different authors.

Author		Wilson, 1981	King, 1989	Mazé, 2007	Velasco et al., 2009	This study
Number of specimens		42	47	178	6	116
Rhizaria	Foraminifera		1.6			0.2
	Polyplacophora				25.0	0.1
Mollusca	Gastropoda	28.8	13.0	33.9		7.9
	Bivalvia		0.9	1.5		2.8
	Copepoda		43.4	1.4	12.5	33.0
	Amphipoda	21.2	10.9	48.7	37.5	18.9
	Decapoda	23.1	2.5	1.5		15.7
	Mysida					0.2
	Anisopoda					0.5
Crustacea	Isopoda	3.8	3.2	4.5	12.5	6.5
	Ostracoda	11.5	14.1	1.5	12.5	2.9
	Cirripedia					0.7
	Crustacea indet.					0.5
Arachnida	Acari		7.5			5.6
	Pantopoda					0.1
Annelida	Polychaeta		1.4	2.9		1.6
Echinodermata	Ophiuroidea		1.1	1.7		2.6
Insecta	Chironomidae larvae	11.5	0.4			
Indeterminata	Indeterminata			1.4		0.2
Other	Other			1.1		

A method of collecting faecal pellets immediately after defecation has proved to be very useful and effective. We did not obtain prey items from only a small proportion of specimens (4.3%), which is probably due to the fact that they had already defecated before being caught. As the specimens started to excrete empty peritrophic membranes (“false”

pellets) after defecation, it was certain that all prey items in the digestive tract had already been defecated. The pre-experiment with six specimens *L. candolii* showed that all eaten prey was also found and recognized in faecal pellets, which confirms the effectiveness of the proposed method for diet analysis. In addition, checking if all ingested prey items were found in intestines or stomachs when using other nonlethal methods of diet analysis would be a proper test for evaluating effectiveness. Based on the results we decided that the method based on faecal pellets is the most suitable method for future use in our study of clingfish diet. However, as a result of this research, a study of the non-destructive method was also published in a scientific journal (Trkov & Lipej, 2019).

4.3.5.3 Diet of three clingfish species

All fishes survived the faecal collecting method, so it can be considered as non-destructive and appropriate for its use inside MPAs as well as on protected fish species. These results are in accordance with the results of pre-experiments on *L. lepadogaster*. The method based on faecal pellets showed to be very useful and efficient for the use on clingfishes, as faecal pellets were obtained from 96.9% of all investigated specimens, on which the method was performed. There were minor differences in the proportion of defecated specimens between all three species. High proportion of *L. lepadogaster* specimens (93%) that contained prey in the gut was also reported by King (1989). Furthermore, Hofrichter (1995) stated that *L. lepadogaster* feed throughout the whole day. Specimens of all three species often produce more than one faecal pellet. Regarding their very short digestive tract, this indicates a high degree of it fullness with prey, which is in accordance with Depczynski & Bellwood (2003), who state that cryptobenthic fishes feed continuously or with high-energy foods. This is also supported by observations of specimens of *A. incognitus*, which showed that specimens produce an average of 3.3 faecal pellets per day (one faecal pellet per 7 - 8 hours), which means that species feed quite often and that prey items obtained by diet sampling represent only about a third of their daily consumption.

The most abundant prey group in the diet of *L. lepadogaster* were crustaceans, represented by copepods and amphipods, which are also the most numerous prey groups in the diet of many other intertidal fish species (Velasco et al., 2010). Amphipods and copepods are also one of the most frequent prey groups in the diet of *G. paganellus* (Azevedo & Simas, 2000; Dunne, 1978; Gibson, 1972), which was often observed in the same habitat as *L. lepadogaster*. The most important prey groups in terms of IRI for *L. lepadogaster* were represented by decapods and amphipods in particular, which was previously also observed by Wilson (1981). Amphipods and decapods were also the most numerous and frequent prey groups under stones, which shows the opportunistic feeding habits of *L. lepadogaster*. Decapods in the diet were mainly represented by the crab *Pisidia* sp. (82.7% of decapods), while amphipods were dominated by gammarids. Both groups were abundant and frequent under stones in the habitat of *L. lepadogaster*, which is in accordance with their high abundance and frequency of occurrence in the diet, which indicates opportunistic feeding habits of *L. lepadogaster*. Despite the high abundance of copepods, their importance (IRI) is rather small compared to decapods and amphipods, due to their negligible biomass. In fact, the average weight of the decapod in the diet of *L. lepadogaster* was thus 1026 times greater than the average weight of the copepod. This is in accordance with the observations of King (1989), who stated that copepods, despite their high abundance, represent only a small total volume of prey, while decapods *vice versa*, despite their low abundance, represent quite a large total volume of prey. However, prey weights need to be interpreted with caution, since they were calculated from correlation curves (*e.g.* Decapoda), or average weights were used (*e.g.* Copepoda), so their weight can differ from the actual values. Due to the method, the fauna was sampled (net mesh size), information

of abundance and frequency of copepods as well as other meiofauna organisms from the environment were not provided. However, a sample square measuring 625 cm² (25 x 25 cm) was used, as the 400 cm² frame (20 x 20 cm) is considered as the minimum sampling area for Mediterranean infralittoral communities (Montesanto & Panayotidis, 2001).

In the diet of *L. candolii*, copepods were by far the most numerous prey group and represented 47.2% of numerical abundance. Consequently, besides decapods, copepods were the most important prey items in terms of IRI, followed by isopods (18.9%) and amphipods (13.8%) in the diet of *L. candolii*. High abundance of decapods in the diet is thus in accordance with their high abundance and frequency of occurrence under a stone inhabited by *L. candolii*. Despite the high number of polychaetes and ophiuroids under stones, they were less abundant in the diet of *L. candolii*. Polychaetes were hiding inside the sediment or in case of serpulid polychaetes in tubes, so they are less accessible for clingfishes, while ophiuroids do not seem to be a preferential prey, as they were only rarely found in the diet. The importance of copepods and amphipods in the diet of *L. candolii* was observed also by other authors (Dunne, 1983; Gibson, 1968; Velasco et al., 2010). Copepods were almost exclusively represented by harpacticoids, but also seven parasitic copepods (family Caligidae) were found in the diet. Furthermore, among isopods, there were many parasitic larvae of Gnathiidae present in the diet, and in one case also fish scales (which were most likely ingested together with the parasite) were found in the faecal pellet. Feeding with parasites is in accordance with observations of Mazé (2007). This confirms that *L. candolii* is an occasional cleaning fish, whose cleaning activity was firstly observed in the Mediterranean Sea by Weitzmann & Mercader (2012). Among Mediterranean clingfish species, cleaning behaviour was observed also in *D. bimaculata* (Patzner & Debelius, 1984). In few cases, parasitic larvae of Gnathiidae were also found in the diet of *L. lepadogaster*. However, parasitic larvae of Gnathiidae were observed to parasite on specimens of *L. lepadogaster* and *L. candolii*, while on the latter species, parasitic copepods were also observed. Based on that, there is a possibility that intraspecific cleaning behaviour is present in clingfishes. Unlike in the diet of *L. lepadogaster*, where decapods were mainly represented only by *Pisidia* sp., in the diet of *L. candolii*, besides *Pisidia* sp. (55.0% of decapods), a high number of *A. nitescens* (38.3% of decapods) was also present. This is probably correlated with the occurrence of shrimps *A. nitescens*, which are more abundant in somehow deeper waters (infralittoral) than in the mediolittoral (Holthuis, 1961; Melzer et al., 2019; Pallas et al., 2006). That was observed also in prey sampling under stones, where in the lower mediolittoral, *A. nitescens* represent 7.8% of decapods' numerical abundance, while in the infralittoral, they represent 26.5%.

A. incognitus, as the smallest of all three clingfish species (average length 32.38 mm), feed mostly with small prey groups, such as copepods, which represented 69.0% of the numerical abundance and 69.4% of IRI. Alternative prey in the diet of *A. incognitus* were amphipods (10.4%), while decapods represented only a small proportion of IRI. Harpacticoid copepods were recognized as the most important food also for small syntopical clingfish *O. gracilis* (Vizzini, Mazzola, & Scilipoti, 2001). Also many fish species living in phytal habitats and preying on meiofauna (benthic organisms which pass through a 0.5 mm sieve and are retained on a 45 µm sieve) preyed on high numbers of copepods in the diet (Gee, 1989).

Based on a wide range of prey taxa found in the diet of all three clingfishes could be considered as carnivorous opportunists. This is consistent with the studies of many authors (Gibson, 1972; King, 1989; Mazé, 2007; Velasco et al., 2010) and was observed also in other clingfish species (*e.g.* Paine & Palmer, 1978). In fact, the highly opportunistic foraging on a vast array of small prey is a general characteristic of many cryptobenthic fish species (Brandl et al., 2018; Depczynski & Bellwood, 2003). These three species of clingfishes also

share other characteristics with cryptobenthic fishes, such as a small body size, short gut, rapid digestion and others (sensu Brandl et al., 2018). A limited home range of cryptobenthic fish species, which naturally confines access to prey (Depczynski & Bellwood, 2003), may well explain their opportunistic feeding. Species with a smaller home range should thus have a more opportunistic diet than species with a large home range. This is supported by the ITD value which tells us the degree of the opportunistic diet and was the highest in *L. lepadogaster*. This species has the smallest home range and is very territorial. Based on the undersurface of the stone, *L. candolii* has a wider home range and it also feeds outside the shelter (Almada et al., 1987; Gonçalves et al., 1998), which is reflected in a less opportunistic diet (ITD = 0.73). *A. incognitus* was the species most often found outside the shelter, so it is supposed to have the widest home range of all three species, which is reflected in the least opportunistic diet of all three species (ITD = 0.51). Furthermore, these findings are supported by prey diversity in the diet of clingfishes. *L. lepadogaster* had the most diverse diet, which consists of 47 different taxa, followed by *L. candolii* with 42 taxa in the diet, while *A. incognitus* had the lowest number of different taxa (23) in the diet. Comparison of the average size of the fish species shows that larger species have a wider food niche. These results are in accordance with previous findings of other authors (Gee, 1989; Hofrichter, 1993; Zander & Berg, 1984), who highlighted that a larger body size enables the fish to feed with a wider range of prey.

For all three clingfish species, the most important prey group were crustaceans, which is in accordance with many authors (Day, 1884; Dunne, 1983; Gibson, 1968, 1972; Hofrichter, 1993, 1995; Mazé, 2007; Velasco et al., 2010). The importance of crustaceans as the prey of clingfishes is also reflected in the red mating colouration (discussed in Chapter 4.3.2). The importance of crustaceans, where amphipods, copepods and decapods present the most important taxa, in the diet of opportunistic cryptobenthic fishes, was already highlighted by Brandl et al. (2018). In all three clingfish species, harpacticoid copepods were the most numerous prey group, which was observed in many fish species, which feed on meiofaunal organisms (Gee, 1989). A high proportion of relatively small harpacticoid copepods in the diet could be a consequence of the fact that they have a 35% higher calorific value than amphipods, and the proportion of successful prey capture is much higher for harpacticoid copepods than that of amphipods (Volk, Wissmar, Simenstad, & Eggers, 1984). However, the species of copepods and amphipods used in the study should be considered. High caloric value and low cost of capturing are probably the main reasons for the large proportion of copepods in the diet of clingfishes, although their jaw anatomy allows them to eat much larger prey.

Analyses showed that the proportion of small prey, such as copepods in the diet of *L. lepadogaster* and *L. candolii*, decreases with the increasing size (age) of the specimens, while in smaller species *A. incognitus*, copepods were abundant also in the diet of adult specimens. This is in accordance with the assertion of Gee (1989) that small animals, such as copepods, are most likely to be consumed by young stages of fish or those with a small adult size. Conversely, the proportion of large prey, such as decapods, increases in the diet of larger (older) specimens of *L. lepadogaster* and *L. candolii*. It is also reflected in the TROPHs index, which increases with the size (age) of the fish specimens. This indicates an ontogenetic shift in the diet of *L. lepadogaster* and *L. candolii*. In general, we can say that young specimens of both species feed mostly on small prey, such as copepods, and with increasing size, specimens switch to larger prey, such as decapods. This is in accordance with the findings of Stoner & Livingston (1984), who discovered that the differences in the composition and size of prey with the increasing fish size are related to optimizing the energy input for growth. Therefore, larger predators will feed with larger prey, considering that the prey has theoretically equivalent energy values (Gee, 1989). Ontogenetic shift in the diet is present in many fish species. Shift from meiofauna (e.g.

harpacticoid copepods, ostracods) to larger prey is normally very rapid, which suggests that there is a general fish size threshold, above which meiofauna is not important as a prey group anymore (Gee, 1989). Meiofauna (mostly harpacticoid copepods) represents an important prey group mostly for fish up to around 30 to 60 mm, depending on the jaw structure (Gee, 1989). The threshold can be observed in *L. lepadogaster* in the age group 4+ (> 64 mm), where copepods represented only a small proportion of prey abundance compared to a younger (smaller) age group. A fish size threshold for preying copepods at age group 4+ (> 71 mm) was also observed in *L. candolii*, but the sample was too small to be taken into account. In *A. incognitus*, the shift to larger prey was not observed, probably due to the small adult size, which enables the efficient exploitation of small prey.

Diet sample of *A. incognitus* was relatively small to make strong conclusions. However, it was observed that the smallest specimens had a more diverse diet than the larger ones, with the lowest proportion of copepods in the diet. This is reflected also in the TROPHs index which was the highest in smaller specimens and was decreasing with the size of the specimens. High proportion of copepods in larger specimens could be connected with their way of hiding. Larger specimens hide mostly in oyster shells and they depend mainly on the prey that randomly swims in the shelter (*e.g.* copepods and ostracods), while smaller specimens of *A. incognitus* were often found outside their hiding places where they were seeking for prey. Such behaviour was also reflected in a more diverse diet of small specimens. This is supported by the fact that the largest specimens were mostly nesting males. During that time, their movements were restricted to the nest and they were probably dependent on prey that randomly came in the immediate vicinity of their shelter. Such feeding is possible due to extensive diurnal migration of harpacticoid copepods between sediment and grass blades (Hicks, 1986; Walters & Bell, 1986), which in this way get into empty oysters on *P. nobilis* present in seagrass meadows.

In the diet of males of *L. lepadogaster* during their mating season (spring), copepods were also the most significant prey. It is necessary to take into account that infralittoral harpacticoid copepods in the Gulf of Trieste are the most abundant in summer (Vrišer, 1996), however, there is no data about harpacticoid copepods from the mediolittoral. Furthermore, this is supported by a higher proportion of copepods in the diet of males of all three species, although they were larger than females, and it would be expected that they would feed with larger prey, such as decapods. A high proportion of copepods in the male's diet could be to this end linked with their territoriality behaviour. However, this may result in more larger prey remaining for females during the mating season, as they need more energy to produce eggs.

The jaw shape which was investigated by Hofrichter (1995) seems to also be closely related to food choices. Jaw is the most extended in *L. lepadogaster* and the least in *A. incognitus*. As clingfishes are not able to produce suction pressure, such shape of the jaw and the possibility that jaws can be opened to angle 60° (Hofrichter, 1993, 1995) enable *L. lepadogaster* to grab big prey, such as decapods. Furthermore, teeth of clingfishes are not intended for crushing their prey but for retention only (Hofrichter, 1993, 1995). Prey is consequently eaten as a whole and due to fast digestion it is often relatively undigested. This could be the reason that faecal pellets contain a peritrophic membrane which surrounds prey remains and protects the intestinal walls against damage that could be caused by sharp remnants of prey (Stobbs, 1980). As the peritrophic membrane is formed in the midgut (*personal observation*), this indicates that all digestion happens there.

Because of their cryptic way of life, it is almost impossible to observe the feeding behaviour of clingfishes in the field. Hofrichter (1993, 1995) pointed the importance observations in the aquarium for studying clingfish ecology. Aquarium observations gave us important information about feeding habits of three species of clingfishes. *L. lepadogaster* movements were restricted mostly to the underside surface of the stone, while *L. candolii*

and *A. incognitus* were observed to be much more mobile and were also observed moving around the shelter. *L. candolii* and *A. incognitus* did not only wait in ambush for the prey, but they were searching actively for it everywhere in the aquarium. Such feeding behaviour was reflected also in the diet composition, as specimens of *L. candolii* and *A. incognitus* had more prey items in the diet, which are present mostly on the upper side of stones (*e.g.* Cumacea and Caprellidae) than *L. lepadogaster*. Observations shows that *A. incognitus*, as is also true for *L. lepadogaster* and *L. candolii*, rely primarily on the sight when they hunt, which is characteristic for many lurking fish species (Hogue & Carey, 1982; Magnhagen & Wiederholm, 1982).

The diet composition of *L. lepadogaster* and *L. candolii* varies based on the depth. As the depth increased, the proportion of copepods and molluscs in the diet of *L. lepadogaster* decreased, while conversely the proportion of decapods, gastropods and ophiuroids increased. These results are probably a reflection of the occurrence of large specimens of *L. lepadogaster* in the deeper regions (1-2 m), where they feed with larger prey, as also reflected in the occurrence of the certain prey in that depth. Occurrence of big specimens of *L. lepadogaster* in deeper waters could be the result of more suitable breeding habitat (less chance of moving and turning stones due to wave action) as well as the occurrence of the most important prey (*e.g.* decapods) from 1 to around 2 m. The latter is supported also by the occurrence of large specimens of *L. candolii* at that depth, which had a high proportion of decapods in the diet. Based on prey determination features (Koukouras, Mavidis, & Noël, 2002), which have been preserved during the process of digestion, crabs from genus *Pisidia* belong to *P. bluteli* or *P. longimana*. These two species with *A. nitescens* represent the majority of decapods in the diet of *L. lepadogaster* and *L. candolii*. Crabs of the genus *Pisidia* are also known to be important food for many other species (Robinson & Tully, 2000).

P. bluteli and *P. longimana* occur mostly in the upper infralittoral zone near the tide line, where they hide under stones (Holthuis, 1961; Melzer et al., 2019), while *A. nitescens* occurs on a mixed sediment, phytal and hard bottom (Melzer et al., 2019), and is the most abundant on pebbles in the upper infralittoral (Pallas et al., 2006). This is consistent with our results on fauna sampling under stones and with the results showing that *L. candolii* had a higher proportion of *A. nitescens* in the diet than *L. lepadogaster*, which occurs mostly in the mediolittoral. High abundance of decapods in the diet of *L. lepadogaster* and *L. candolii* in the lower mediolittoral and the upper infralittoral thus coincides with the occurrence of decapods in this habitat. The high number of copepods in the diet of *L. candolii* from deeper waters could be correlated with sand, which deeper on the sea bottom replaces pebbles. The proportion of decapods, which as substrate prefer pebbles, consequently decreases in the diet of *L. candolii* with depth, while the proportion of harpacticoid copepods in the diet, which live in/on the sand (Hicks, 1986), increases with depth. The occurrence of decapods on the pebbles is also reflected in their lower abundance in the diet of specimens of *L. lepadogaster* and *L. candolii*, which inhabit stones under which bare sandstone terraces were present. Furthermore, in the diet of those fish, copepods were more abundant, which is probably a compensation due to the small amount of larger prey. These results could show that preferential prey determines the occurrence of the clingfish species and specimens of a particular size. This is not surprising since cryptobenthic fishes, due to their small body size, appear to have higher mass-specific metabolic rates compared to the larger fish species (Brandl et al., 2018). Consequently, in small fish, energy consumption is very limited, with little tolerance for periods of hunger or reduced food intake (Miller, 1996). Small fish thus rely on constant feeding with high-quality food (*e.g.* crustaceans) (Brandl, et al., 2018), exploitation of which is limited by the size of the prey that the fish can catch and ingest by their gape size (Mihalitsis &

Bellwood, 2017). Diet composition is thus not dependent only on the availability of food, but also depends on the fish size and energetic benefits of preying certain prey (Gee, 1989). The size of the fish is one of the most important factors underlying food selection (Gee, 1989). *L. lepadogaster* with the largest average size had the lowest average number of prey per fish (7.1), while *A. incognitus*, whose average size was the lowest of all three species, had the highest number of prey per fish (16.6). This is because *L. lepadogaster* feeds on larger prey (*e.g.* decapods) and consequently it needs to consume less prey specimens than *L. candolii* and even less than *A. incognitus*, which preys mostly on much smaller copepods. Different coexisting fish species thus prey on different types and/or sizes of their respective prey, which is related to the avoidance of competition among non-specialized consumers at the same trophic level (Morton, Platell, & Gladstone, 2008). Separation of ecological niches of different fish species enables them to coexist in the same ecosystem (Zander & Berg, 1984).

Seasonal differences in the occurrence of certain prey items in the diet *L. lepadogaster* were observed. Summer peak of decapods in the diet coincides with their high abundance of larvae in summer in temperate latitudes (Pfister, 1997). For *P. bluteli* and *P. longimana*, no reproduction data were available, while summer larval settlement is known for closely related species *P. longicornis*, which live in somehow deeper waters. Furthermore, in that species, the highest mortality was observed during summer (Robinson & Tully, 2000). The newly settled *Pisidia* are the most numerous at the end of the summer and at the beginning of autumn (Robinson & Tully, 2000), which explains their high abundance and frequency in the clingfish diet. Furthermore, it is supported by results, showing that *Pisidia* sp. from *L. lepadogaster* diet were the smallest in summer and especially in autumn. As *L. lepadogaster* and *Pisidia* sp. are poorly mobile species (Hofrichter, 1993; Robinson & Tully, 2000), this probably results in the fact that when fish eat most of the *Pisidia* sp. in their hiding place, consequently the abundance and frequency of *Pisidia* sp. in the diet decline, which was observed during the winter and spring. The decline of decapods in the diet of *L. lepadogaster* may also be due to the fact that decapods grew to a size where they are much harder or even impossible to be eaten by clingfishes. However, this is less likely as *P. bluteli* and *P. longimana* have a relative small adult size and there was no correlation between the size of the consumed *Pisidia* and the clingfish size. In winter and spring when there is a lack of suitable decapods, *L. lepadogaster* feed more with alternative prey groups (*e.g.* gastropods, copepods). Compaire, Cabrera, Gómez-Cama & Soriguer (2016) reported on observations that clingfishes feed more on decapods and isopods during the warmer season and with molluscs in the colder part of the year. Zander & Hagemann (1989) on the other hand reported on fish feeding with alternative prey during spring when there is a shortage of suitable-sized benthic crustaceans. However, King (1989) has not observed any major seasonal differences in the diet of *L. lepadogaster*. Seasonal differences in the diet could not be observed in *L. candolii* and *A. incognitus*, due to the unevenly distributed sample throughout the seasons.

In the diet of all three clingfish species, fish eggs were found. Predation on fish eggs by *L. candolii* and *L. lepadogaster* was observed also by Almada et al. (1987) and Hofrichter (1995). Eggs were mostly found in the diet of nesting males, especially in *A. incognitus*. Fish eggs in the diet could be the result of predation on other fish nests, cannibalism, or the removal of hatched eggs remains. Cannibalism can occur when specimens prey on other male's nests, or when nesting males prey on their own eggs due to the lack of food during nesting (small amount of potential prey enters the nest). Guarding males thus sometimes eat some of their own eggs or even all of them to keep themselves alive throughout the nesting season (Gross & Sargent, 1985). Eggs predation of own spawn was observed also at guarding males of *Gobius auratus* Risso, 1810 (Zander & Hagemann, 1989).

Other interesting prey items were serpulid polychaetes identified by their operculum. These sedentary polychaetes represent the majority of the polychaete group. They were the most numerous in the diet of *L. candolii*, where they represented 11.7% of numerical abundance. This is in accordance with field observations where a lot of serpulid polychaetes were observed on the undersides of stones occupied by *L. candolii*. Serpulid polychaetes appeared also in the diet of large males of *A. incognitus*. This could indicate that serpulid polychaetes are an alternative prey during the mating season when *A. incognitus* males care for the nest and their movements are restricted to the oyster shell. Fish feeding with polychaetes at a time when there is a lack of more suitable food was observed previously by Zander & Hagemann (1989).

Different sediment particles (*e.g.* sand grains) were often observed in the faecal pellets, which was reported also by Hofrichter (1993). Such particles were probably consumed accidentally, along with the prey.

4.4 Parasites of Clingfishes

Cryptobenthic fish species are important secondary consumers and play an important role in energy transfer to higher level consumers (Brandl et al., 2018). Consequently, they serve as vectors for parasites. Although parasites of commercial fish are quite well known (*e.g.* Mladineo, 2005; Raibaut, Combes, & Benoit, 1998), there is a lack of knowledge on cryptobenthic fish parasites (Hofrichter, 1995).

All external parasites found on clingfishes in this study were crustaceans, which are besides the monogeneans the most numerous ectoparasite group among marine fish (Rohde, 2005). There were three groups of external parasites found on clingfishes: parasitic larvae of Gnathiidae belonging to isopods, and Lernaepodidae and species of the family Caligidae, which belongs to parasitic copepods. Parasitic isopods and copepods are also the main crustacean groups of parasites found on gobies (Alberto & Nieto, 1994; Raibaut et al., 1998; Van Damme, 1997). Isopods parasitizing on fish belong to cymothoids and gnathiids (Ravichandran, Rameshkumar, & Kumaravel, 2009). Cymothoids parasitize on fish as immature and adult, while gnathiids parasitized on fish only at larval stages, while adults are non-feeding (Tinsley & Reilly, 2002). However, Hofrichter (1995) reported on parasitizing cymothoids on clingfishes, which we never observed.

Parasitic copepods and isopods attach themselves to the host body where they feed with the host's tissue, blood or haemolymph (Ravichandran et al., 2009; Smith, Wootten, & Sommerville, 2007). Skin damage, due to parasite penetration, can thus result in local lesions, which can cause secondary infection. The latter can lead to necrosis and epidermal erosion (Smith et al., 2007). Gill parasites can cause a loss of the respiratory surface area due to fusion of secondary lamellae and hypertrophy of the gills. Gill infestation in fish can result as anaemia and haemorrhagia (Toksen, 2007). Furthermore, parasitic copepods have been suggested as the vector for viral and bacterial diseases (Nylund, Wallace, & Hovland, 1993). Fish parasites can thus inhibit reproduction and growth of the fish, while they can also have mortal consequences on the host (Ravichandran et al., 2009), which was observed in the case of *L. candolii*.

In the case of *A. incognitus*, only males were examined. Males dominated also in examined specimens of *L. lepadogaster*, whereas in the *L. candolii*, females and juveniles predominated. The greater presence of parasites in females is thought to be related to their mobility, as males are less mobile (territoriality characteristic especially during the breeding season) and thus have fewer opportunities to come in contact with parasites (Pampoulie et al., 1999). In contrast, an immunosuppressive effect of a testosterone synthesis could explain a large number of parasites in males (Folstad & Karter, 1992).

However, it was observed that Lernaeopodidae were parasites mostly on juvenile specimens of *L. candolii*, while they were rarely found in adult specimens. The reason for that remains unknown. Parasites can approach and infest the hosts by themselves (*e.g.* parasitic copepods and isopods; *personal observations*; Heuch, Øines, Knutsen, & Schram, 2007; Ravichandran et al., 2009) or via food (many internal parasites; Zander, 2003). Snails and crustaceans are preferred intermediate hosts for many parasite groups (Zander, 1998), which results in clingfishes being important vectors and final hosts for parasites, as was observed especially at *A. incognitus*, where 54.5% of specimens were infested with internal parasites.

Due to the relatively frequent occurrence of internal and external parasites in clingfishes, their role in the expression of mating colouration and with that related female choice should also be taken into consideration. As parasites use the host resources, the hosts need to skip some activities in order to save some energy or consume more food (Lindström, 1998), which could lead to reduced reproductive success. Positive correlation between parasite load and loss of fish eggs was observed by Sasal (2006). Eggs loss is a result of filial cannibalism or reduced ability to defend the nest against predators (Sasal, 2006). It is not surprising that the expression of secondary sexual characteristics (colour intensity, fin surface area) are so associated with the presence of parasites (Barber, 2002; Price et al., 2008). Less pronounced secondary sexual characters may reveal that the male is not in a good condition due to parasite load (Barber, 2002).

The results of this study are one of the few data on cryptobenthic fish parasites from the Mediterranean Sea. Cryptobenthic fish species obviously play an important role in the ecology of many parasites, serving them as the final host or as a vector in transmission to the final hosts. From this point of view, more time will be needed to explore cryptobenthic fish parasites in the future in order to better understand their role in the fish ecology.

Chapter 5

Conclusions

5.1 Importance of the Research

Teleost fish are the most diverse vertebrate taxa (approximately 35.500 formally described fish species) and more than a half of the species are present in marine ecosystems (Fricke, Eschmeyer, & Van der Laan, 2020). Marine fish are involved in many critical ecosystem processes and provide a variety of ecosystem goods and services (Mora, 2015). Research of cryptobenthic fish species is thus essential to complete the understanding of the marine ecosystem. Due to the cryptic way of life, their importance was usually overlooked in the past. However, new discoveries about cryptobenthic fish species show that they represent a very essential part of coastal biodiversity, which can importantly affect the ecosystem functions (Ackerman & Bellwood, 2000). Recently, we faced a great decline in the fauna and flora all around the world, which is also reflected in the functioning of the ecosystem we depend on (Loreau et al., 2001). The more species are present in the ecosystem, the more stable is an ecosystem and resistant to different disturbances (Loreau et al., 2001). Degraded environments with low species richness are also more vulnerable for the settlement and spreading of invasive alien species (Didham, Tylianakis, Gemmell, Rand, & Ewers, 2007), which represent one of the major threats to native species and biodiversity (Sala et al., 2000). Invasive alien species have a negative impact not only on the environment but also on humans (Pejchar & Mooney, 2009). Knowing the species ecology is thus essential for the development of effective species management and protection programme. However, there is a big knowledge gap about the ecology of small cryptobenthic gobies and gobiesocids from the Mediterranean Sea (Patzner, 1999), as well as in the Adriatic Sea, which we would like to fill at least slightly.

Habitat loss and fragmentation are major threats to biodiversity (Fahrig, 1997), and also the biggest threat to cryptobenthic fish communities (Brandl et al., 2018). Cryptobenthic fish species with a specific habitat preference are usually the most vulnerable to changes of microhabitats, even if some species show plasticity to habitat use (Taylor & Van Tassell, 2002). Cryptobenthic fishes in general have a small dispersal range (due to the small body size; Brandl et al., 2018; Luiz et al., 2013; Pires & Gibran, 2011), so they can be affected by the fragmentation of a suitable habitat, which makes it difficult to recolonize (Brandl et al., 2018). Habitat degradation is also very problematic in the Slovenian sea, especially in the coastal part, as there is less than 20% of it still in natural the state (Turk, 1999). Decreased biodiversity and increasing of its vulnerability are consequences of such modification of shoreline (Turk, 1999). As the most degraded are riparian zones of the supralittoral, mediolittoral and also the infralittoral (Turk, 1999), this has a big impact on *L. lepadogaster*, which exclusively occur in this coastal zone. Due to specific habitat requirements of *L. lepadogaster* and isolated locally abundant populations, a serious disturbance of the habitat and specimens over the collection might seriously affect

their populations (King, 1989). In order to protect and conserve *L. lepadogaster* in urbanized parts of the coast, it would therefore be necessary to maintain a suitable habitat with sufficiently large stones (used as hiding places), and construction work in the mediolittoral zone should be carried out outside the breeding season in order to prevent the disturbance of the reproductive process and unnecessary destruction of nests.

Problematic are not only costal parts, which are totally destroyed (built-up area), but also parts where natural elements of the coast were modified, such as beaches. The problem of the beaches is mostly the pouring of material which is inappropriate for the cryptobenthic fish species settlement due to its small granulation. Due to the loss of cryptic habitats in such an environment, many cryptobenthic fishes disappear. Such a decline in the *L. lepadogaster* abundance was observed in the new beach constructed in 2017 in front of MBS (Figure 5.1). *L. lepadogaster* was previously present as a rather common species on the location before the construction, and then it almost disappeared after the pouring of river gravel (*personal observations*). The beach construction also caused the disappearance of *Cymodocea* seagrass meadow deeper in the infralittoral, which changed into a bare sediment bottom. As *A. incognitus* is strictly associated with seagrass meadows, this also led to the disappearance of this species. In the past, this location was an important habitat for this species, which is confirmed by many findings ($n = 129$). After the beach construction, in the first year, we were able to find only two specimens, while in the second year, we could not find any specimens on this location. After that, the only location where dense population of *A. incognitus* was found, was on Debeli rtič. It is also the area where the seagrass meadow of *C. nodosa* is still in the best condition in the Slovenian sea (Martina Orlando-Bonaca et al., 2015), which could indicate that the occurrence of *A. incognitus* depends not only on the presence of seagrass meadows, but also on their condition. In recent years, seagrass meadows are disappearing not only along the Slovenian coast (Lipej et al., 2018), but also in other parts of the Mediterranean Sea and the Atlantic Ocean (Pergent et al., 2014; Telesca et al., 2015). Furthermore, we are facing high mortality of *P. nobilis* in the Mediterranean Sea (Cabanellas-Reboredo et al., 2019), whose population is declining and is critically endangered (Kersting et al., 2019). *P. nobilis* is a very important bioconstructor as its big shell serves as a shelter or a solid basis for the settlement of many other organisms. One of them are oysters whose empty shells offer an important hiding and nesting place, as shown in the case of *A. incognitus*. The decline of *P. nobilis* and seagrass meadows can have a significant impact on populations of *A. incognitus* and also other fish species, which are strongly associated to them.

From all three species, it seems that *L. candolii* is the less negatively affected by human impacts. This is due to its distribution in the infralittoral belt, which is less affected by humans, its euryecious habitat and microhabitat preferences, and even to its potential to exploit artificial objects on the sea bottom as hiding places.

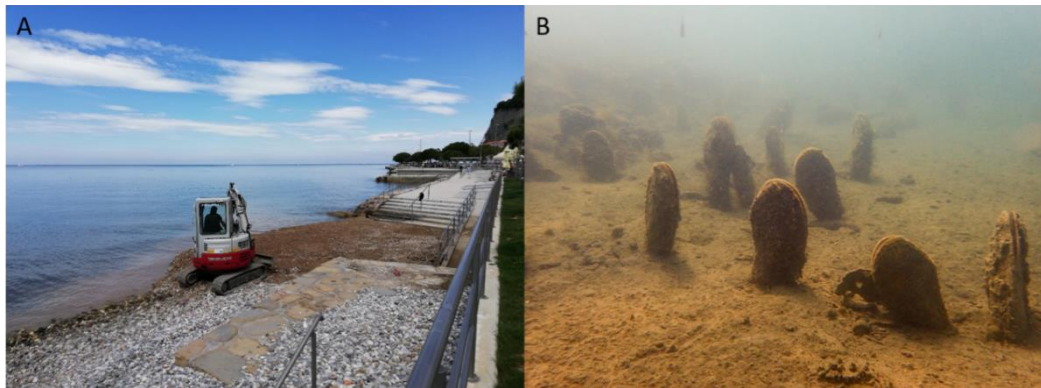


Figure 5.1: A – beach construction in front of the Marine Biology Station Piran and with that associated destruction of the mediolittoral habitat; B – beach construction which caused the collapse of the seagrass meadow that turned into a sedimentary bottom (Photos: D. Trkov).

5.2 Importance of Development of New Method to Study Fish Diet

Research on the feeding habits of fish is crucial for understanding ecological processes at an individual, population and community level (Sánchez-Hernández et al., 2013). With this purpose we developed a non-destructive (nonlethal) method to study the fish diet. Other nonlethal methods (such as the use of gastroscopies, tubes, stomach suction, stomach flushing, emetics, forceps and others) vary in the ability to remove all stomach contents. The effectiveness of other nonlethal methods for studying fish diets depends on the size, age and species of fish, and also on the size of the food particles in the stomach (Kamler & Pope, 2001). Due to the small size of clingfishes and their relatively large-sized prey, other nonlethal methods would probably be less efficient in studying this species. Based on empty defecated peritrophic membranes, it appears that 24 hours suffice for the specimens to defecate. Consequently, on the next day, the clingfishes could be released back into the sea at the place of capture. To prevent their mortality, it is important that clingfishes spend as little time as possible in stressful conditions. Most other nonlethal methods require a lot of handling, while a strong positive relationship between handling-induced mortality and water temperature was previously demonstrated by Muoneke & Childress (1994) and Wilde (1998). Different techniques for obtaining stomach contents can also cause internal injuries (*e.g.* swim bladder rupture). Stomach flushing, as one of the most efficient nonlethal methods (Kamler & Pope, 2001), can cause 60% mortality in some fish species (Hartleb & Moring, 1995). Meehan & Miller (1978) found that stomach flushing can also have a negative effect on the condition of the fish. Since the method based on faecal pellets is a non-invasive technique and requires almost no handling of fish, there is less chance of injuries and, consequently, potential fish mortality is drastically reduced. This is supported by 100% fish survival in our experiments until the release to the natural environment. The method is also suitable for fish of all sizes and was proved as effective on specimens smaller than 15 mm (*L. candolii*), while other methods (*e.g.* gastroscopes, tubes, stomach suction, stomach flushing and forceps) are unsuitable for such small specimens. Consequently, it is the only nonlethal method that can be used for very small specimens such as fish fry. Regular disinfection of all the equipment (*e.g.* chambers, aerators) after every use also proved to be very important; disinfection prevents the transmission of disease and parasites to other fish. Compared to other nonlethal methods that can be rather expensive or require

more than one handler (Kamler & Pope, 2001), the method based on faecal pellets is easy to use (it requires one handler) and rather cheap. In fact, all the required equipment cost less than 100 €. Furthermore, the equipment used for collecting faecal pellets is also suitable for transport.

5.2.1 Potential use of the method

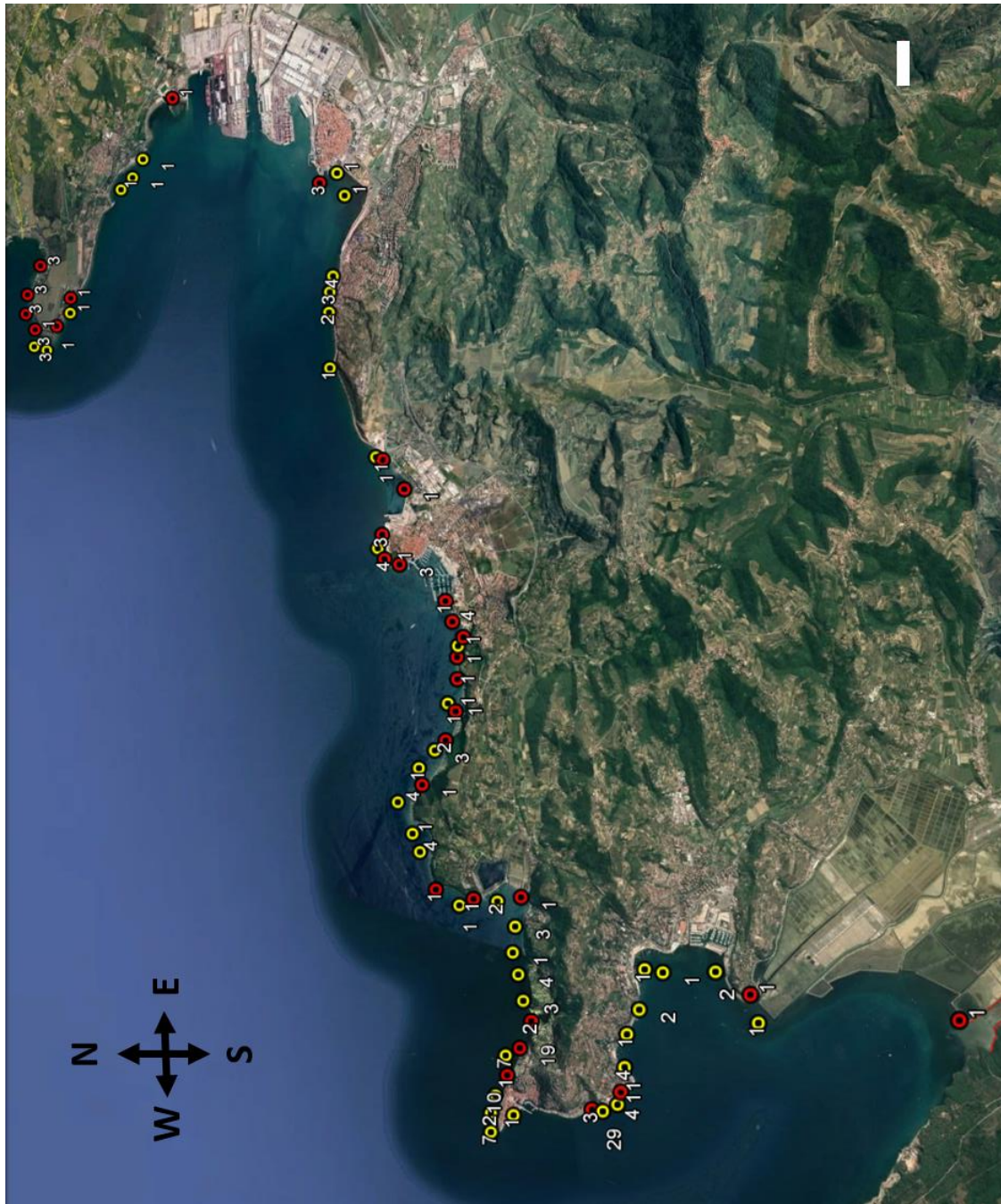
The non-destructive method has a great potential for studying the fish diet of small and less mobile coastal fish species, which can be held in small chambers. With some modifications, bigger systems for collecting faecal pellets can be built as well. This method is the most suitable for species feeding on hard-bodied prey, which is swallowed whole, have rapid digestion and poorly digest their prey. Cryptobenthic fish species are thus one of the most suitable groups on which this method could be performed. It turned out to be a very useful method for studying the feeding habits of *L. lepadogaster*, *L. candolii* and *A. incognitus*. We also tested this method on some gobies (*e.g.* *Gobius fallax* Sarato, 1889, *Gobius cruentatus* Gmelin, 1789, *Gobius cobitis* Pallas, 1814, *Gobius paganellus* Linnaeus, 1758, *Z. zebrus*), and it was proved to be as useful, since in all cases we were able to find faecal pellets with recognizable prey remains for identification. The order of gobies (Gobiiformes) is one of the most diverse orders of percomorphs and also of fish in general; they represent 5 to 10% of all teleost species (Patzner, Van Tassell, Kovačić, & Kapoor, 2011). To this end, the non-destructive method has great potential for studying their diet, especially considering the fact that gobies represent over 50% of the energy flow in coral reef habitats (Patzner et al., 2011). Furthermore, gobies represent the highest number of cryptobenthic species in the Adriatic Sea (Glavičić et al., 2016; Kovačić, 1997; Kovačić et al., 2012) and can represent more than 90% of total cryptobenthic fish abundance in certain Mediterranean habitats (Glavičić et al., 2016).

Based on the weight of consumed prey and the weight of digested food residues in faecal pellets, the weight of assimilated food can also be obtained, which is a great advantage compared to other stomach content methods. This gives us a unique insight into the energy flow in certain habitats. In order to ascertain whether the method can potentially be used to study the diet of other representative species of the Mediterranean coastal fish assemblage, we preliminarily tested if we could obtain their faecal pellets in chambers and whether the prey remains are appropriate for identification. The method was proved to be useful for common fish species such as *Scorpaena porcus* (Linnaeus, 1758), *Serranus scriba* (Linnaeus, 1758), *T. tripteronotum*, *Salaria pavo* (Risso, 1810) and even on the nectobenthic herbivore *Sarpa salpa* (Linnaeus, 1758) (Figure 5.2). Due to its high survival rate, the proposed method is also suitable for studying the diet of rare and endangered fish species, and also coastal fish fauna in protected areas where traditional destructive sampling methods are not appropriate or allowed. As the method is nonlethal and relatively undemanding as regards fish handling, it is suitable for educational scopes as well.



Figure 5.2: Residues of undigested food from faecal pellets of *Sarpa salpa* (Photo: D. Trkov).

Appendix A



Number of samplings performed within different sampling locations in Slovenian sea. Yellow circles represent sampling locations where SCUBA diving equipment was used for investigation (infralittoral) and red circles represent locations sampled with snorkelling and manual collecting (mediolittoral and upper infralittoral). White bar represents 500 m.

Appendix B

B.1 Location of specimens *Lepadogaster lepadogaster*

Date	Number of specimens	Location	Coordinates	
2.10.2006	3	Žusterna	45°32'49.10"S	13°42'32.85"E
12.01.2017	6	Fiesa	45°31'32.22"S	13°34'46.75"E
28.03.2017	9	Fiesa	45°31'33.40"S	13°34'45.04"E
7.04.2017	1	MBS	45°31'4.59"S	13°34'5.83"E
10.04.2017	1	MBS	45°31'4.59"S	13°34'5.83"E
11.04.2017	12	Fiesa	45°31'38.75"S	13°34'35.58"E
4.05.2017	4	Fiesa	45°31'34.06"S	13°34'43.66"E
10.05.2017	2	Fiesa	45°31'34.06"S	13°34'43.66"E
18.07.2017	1	Bernardin - arkade	45°30'55.44"S	13°34'14.82"E
1.08.2017	19	Fiesa	45°31'36.12"S	13°34'39.57"E
4.08.2017	17	Fiesa	45°31'36.12"S	13°34'39.57"E
5.08.2017	2	cesta Koper - Izola	45°32'52.58"S	13°41'24.09"E
8.08.2017	17	Fiesa	45°31'36.12"S	13°34'39.57"E
10.08.2017	18	Fiesa	45°31'34.88"S	13°34'44.10"E
14.09.2017	3	MBS	45°31'4.59"S	13°34'5.83"E
4.10.2017	17	Fiesa	45°31'36.12"S	13°34'39.57"E
9.10.2017	3	MBS	45°31'3.67"S	13°34'6.06"E
10.10.2017	4	Piran - Norik Sub	45°31'50.16"S	13°33'54.57"E
17.10.2017	7	rt Strunjan	45°32'19.15"S	13°36'18.19"E
29.01.2018	3	MBS	45°31'5.41"S	13°34'5.62"E
30.01.2018	5	Bernardin	45°31'1.66"S	13°34'6.61"E
31.01.2018	20	Fiesa	45°31'33.68"S	13°34'44.13"E
11.04.2018	5	Fiesa	45°31'38.45"S	13°34'34.30"E
17.04.2018	9	Fiesa	45°31'38.45"S	13°34'34.30"E
24.04.2018	16	Fiesa	45°31'38.45"S	13°34'34.30"E
29.05.2018	14	Fiesa	45°31'33.58"S	13°34'44.47"E
30.05.2018	5	Bernardin	45°30'59.35"S	13°34'7.87"E
4.06.2018	2	Bernardin	45°30'59.07"S	13°34'8.18"E
24.07.2018	1	Piranska cerkev	45°31'49.18"S	13°34'4.79"E
27.07.2018	2	Pozejdonka	45°32'53.65"S	13°41'42.46"E
31.08.2018	2	Molet	45°32'52.17"S	13°42'11.04"E
21.01.2019	4	Atlantida	45°32'1.44"S	13°38'46.97"E
19.02.2019	3	Belveder	45°31'57.74"S	13°38'26.31"E

22.08.2019	4	Izola - ribarnica	45°32'22.34"S	13°39'22.92"V
2.792.019	1	Strunjan	45°31'54.26"S	13°36'2.75"V

*MBS - Marine Biology Station

B.2 Location of specimens *Lepadogaster candolii*

Date	Number of specimens	Location	Coordinates	
24.08.2005	3	Fiesa	45°31'32.69"S	13°34'48.77"V
23.09.2005	1	Fiesa	45°31'33.77"S	13°34'51.45"V
27.09.2005	2	Bernardin	45°30'51.88"S	13°34'16.39"V
4.10.2005	3	Bernardin	45°31'0.04"S	13°34'6.10"V
6.10.2005	1	Bernardin	45°30'57.91"S	13°34'9.21"V
10.10.2005	1	MBS	45°31'4.31"S	13°34'5.27"V
11.10.2005	3	Bernardin	45°31'0.54"S	13°34'6.57"V
5.09.2006	2	Piran	45°31'42.48"S	13°33'55.94"V
25.09.2006	2	Bernardin	45°31'0.58"S	13°34'6.44"V
19.07.2017	1	Piranska cerkev	45°31'48.49"S	13°34'7.61"V
26.07.2017	8	Piranska cerkev	45°31'49.12"S	13°34'7.04"V
28.07.2017	1	rt Ronek	45°32'24.84"S	13°36'49.55"V
1.08.2017	1	Fiesa	45°31'36.12"S	13°34'39.57"V
10.08.2017	1	Fiesa	45°31'34.88"S	13°34'44.10"V
9.10.2017	6	MBS	45°31'3.67"S	13°34'6.06"V
10.10.2017	3	Piran - Norik Sub Mladinsko zdravilišče	45°31'50.16"S	13°33'54.57"V
12.10.2017	5	in letovišče RKS Debeli rtič	45°35'12.37"S	13°42'29.91"V
13.10.2017	15	rt Strunjan	45°32'19.15"S	13°36'18.19"V
14.10.2017	2	Fiesa	45°31'40.58"S	13°34'37.16"V
17.10.2017	8	rt Strunjan	45°32'19.15"S	13°36'18.19"V
26.10.2017	4	Piran - Norik Sub	45°31'50.28"S	13°33'49.01"V
30.05.2018	1	Bernardin	45°30'59.35"S	13°34'7.87"V
1.06.2018	1	Piran - stadion	45°31'44.18"S	13°34'23.37"V
4.06.2018	6	Bernardin	45°30'59.07"S	13°34'8.18"V
5.06.2018	3	Pacug	45°31'34.38"S	13°35'21.57"V
7.06.2018	6	Piran - obzidje	45°31'46.29"S	13°34'13.39"V
20.06.2018	2	Fiesa	45°31'36.33"S	13°34'41.54"V
12.07.2018	5	Mesečev zaliv	45°32'17.89"S	13°36'37.30"V
13.07.2018	5	Piranska cerkev	45°31'49.18"S	13°34'4.79"V
16.07.2018	1	Bele skale	45°32'9.06"S	13°37'24.86"V
19.07.2018	3	Piranska cerkev	45°31'49.12"S	13°34'6.99"V
20.07.2018	8	Pacug pod ritko	45°31'35.47"S	13°35'32.74"V
24.07.2018	1	Piranska cerkev	45°31'49.18"S	13°34'4.79"V
25.07.2018	6	Fiesa	45°31'34.60"S	13°35'1.51"V

26.07.2018	2	Salinera	45°31'49.12"S	13°34'7.04"V
27.07.2018	2	Pozejdonka	45°32'53.65"S	13°41'42.46"V
31.07.2018	7	med Fieso in Pacugom	45°31'36.66"S	13°35'13.20"V
31.08.2018	9	Molet	45°32'52.17"S	13°42'11.04"V
4.09.2018	7	med Fieso in Pacugom	45°31'35.56"S	13°35'14.68"V
5.09.2018	5	Pozejdonka	45°32'54.03"S	13°41'48.89"V
13.09.2018	3	Pozejdonka	45°32'53.22"S	13°41'56.95"V
14.09.2018	10	Debeli rtič - greben med sipinami	45°35'27.28"S	13°42'10.18"V
19.09.2018	3	Debeli rtič - svetilnik	45°35'28.26"S	13°41'58.76"V
20.02.2019	4	Debeli rtič	45°35'36.97"S	13°42'41.76"V
19.07.2019	4	Pacug	45°31'35.22"S	13°35'19.71"V

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B.3 Location of specimens *Apletodon incognitus*

Date	Number of specimens	Location	Coordinates	
20.08.2001	1	Strunjan	45°31'41.44"S	13°36'0.60"V
10.11.2005	2	plaža za News cafe	45°30'53.12"S	13°34'30.84"V
14.09.2006	2	MBS	45°31'4.38"S	13°34'5.43"V
25.09.2006	1	Bernardin	45°31'0.25"S	13°34'6.17"V
09.10.2006	1	Avtokamp Lucija	45°30'13.85"S	13°35'27.33"V
14.03.2007	1	MBS	45°31'3.92"S	13°34'4.56"V
15.03.2007	9	MBS	45°31'3.92"S	13°34'4.56"V
29.03.2007	1	MBS	45°31'3.92"S	13°34'4.56"V
03.04.2007	1	MBS	45°31'3.92"S	13°34'4.56"V
06.04.2007	10	MBS	45°31'3.92"S	13°34'4.56"V
13.04.2007	15	MBS	45°31'3.92"S	13°34'4.56"V
17.04.2007	7	MBS	45°31'3.92"S	13°34'4.56"V
26.04.2007	14	MBS	45°31'3.92"S	13°34'4.56"V
12.05.2007	6	MBS	45°31'3.92"S	13°34'4.56"V
17.05.2007	10	MBS	45°31'3.92"S	13°34'4.56"V
23.05.2007	9	MBS	45°31'3.92"S	13°34'4.56"V
30.05.2007	16	MBS	45°31'3.92"S	13°34'4.56"V
11.06.2007	5	MBS	45°31'3.92"S	13°34'4.56"V
19.06.2007	8	MBS	45°31'3.92"S	13°34'4.56"V
19.06.2007	8	Upravna stavba Droge	45°30'45.18"S	13°35'3.94"V
09.07.2007	5	MBS	45°31'3.92"S	13°34'4.56"V
13.07.2007	12	Salinera	45°31'36.12"S	13°35'49.81"V

23.07.2007	14	plaža za News cafe	45°30'53.01"S	13°34'29.10"V
08.08.2007	2	MBS	45°31'3.92"S	13°34'4.56"V
29.07.2008	1	MBS	45°31'3.92"S	13°34'4.56"V
21.08.2008	1	MBS	45°31'3.92"S	13°34'4.56"V
28.08.2008	1	MBS	45°31'3.92"S	13°34'4.56"V
24.10.2008	7	MBS	45°31'3.92"S	13°34'4.56"V
19.06.2009	1	Grand hotel Emona	45°30'57.50"S	13°34'9.86"V
4.07.2011	2	MBS	45°31'3.92"S	13°34'4.56"V
19.05.2014	2	MBS	45°31'3.92"S	13°34'4.56"V
12.10.2016	4	Ankaran	45°34'27.24"S	13°44'6.48"V
19.05.2017	5	Portorož	45°30'42.44"S	13°35'28.83"V
7.06.2017	2	MBS	45°31'3.49"S	13°34'5.85"V
21.07.2017	2	MBS	45°31'3.49"S	13°34'5.85"V
		Mladinsko		
12.10.2017	3	zdravilišče in letovišče RKS	45°35'12.37"S1	13°42'29.91"V
		Debeli rtič		
4.07.2018	1	ustje Jernejevega kanala	45°30'2.62"S	13°35'9.76"V
10.07.2018	2	Bele skale	45°32'7.79"S	13°37'31.28"V
12.07.2018	1	Mesečev zaliv	45°32'17.89"S	13°36'37.30"V
22.08.2018	3	desno od kampa Jadranke	45°32'33.55"S	13°40'31.05"V
13.09.2018	1	Pozejdonka	45°32'53.22"S	13°41'56.95"V
27.09.2018	1	Debeli rtič	45°35'28.14"S	13°42'1.32"V
3.07.2019	1	Bele skale	45°32'10.94"S	13°37'26.74"V
8.07.2019	12	Debeli rtič	45°35'30.89"S	13°42'4.65"V
11.07.2019	9	Debeli rtič	45°35'30.89"S	13°42'4.65"V

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Journal Articles Related to the Dissertation

- Trkov, D., & Lipej, L. (2019). A non-destructive method for assessing the feeding habits of coastal fish. *Mediterranean Marine Science*, 20(2), 453–459. <https://doi.org/10.12681/mms.20234>
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Biography

The author of this thesis Domen Trkov was born on 12 January 1990 in Ljubljana. In 2009, he successfully finished the upper secondary school at the Biotechnical Educational Centre Ljubljana. He continued his first-cycle studies at the Biodiversity programme at UP FAMNIT (Faculty of Mathematics, Natural Sciences and Information Technologies) in Koper. On 11 September 2012, he graduated with a graduation thesis entitled: Mitochondrial markers as a tool for species determination. Domen continued his second-cycle studies at the Nature Conservation programme at the same faculty. He obtained his Master's degree on 27 October 2014 with a master's thesis entitled: Intraspecific and interspecific interactions in the selection of nesting niches of mediolittoral blennies (Blenniidae) in the Gulf Trieste. After graduating, Domen started teaching as an assistant of Marine Ecology at the Faculty of Natural Sciences and Mathematics, University of Maribor and Marine Biodiversity at UP FAMNIT (Faculty of Mathematics, Natural Sciences and Information Technologies). He continued his doctoral studies at the Jožef Stefan International Postgraduate School and in October 2015 he started working as a young researcher at the National Institute of Biology, Marine Biology Station Piran. As part of his PhD, he started the study of fish ecology and their ecological role in the environment. At the same time, he also studies the ecology of sea slugs and bio-globalization in the Slovenian part of the Adriatic. Domen is the co-author of the scientific monograph: "Polži zaškrgarji slovenskega morja" and various publications in scientific magazines.