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# DIPLOMARBEIT

Titel der Diplomarbeit

## Biocidal activity and biochemistry of *Leptodactylus pentadactylus* frog foam nests – an analysis with insights into *N*-glycosylation


Verfasserin

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# ABBREVIATIONS

| | |
|---|---|
| A | ampere (electric current) |
| ACN | acetonitrile |
| APS | ammonium persulfate |
| Asn | asparagines |
| ATCC | American Type Culture Collection |
| Bis | *N,N'*-methylenebisacrylamide |
| BCIP/NBT | 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium |
| BSA | bovine serum albumin |
| °C | degree Celsius |
| cfu | colony forming unit(s) |
| cm | centimetre |
| CNS | central nervous system |
| d | day(s) |
| Da | Dalton |
| $ddH_2O$ | double distilled water |
| DHB | 2,5-dihydroxy benzoic acid |
| DMSO | dimethyl sulfoxide |
| Dol-P | dolichol phosphate |
| Dol-P-P | dolichol pyrophosphate |
| DTT | dithiothreitol |
| Eppi(s) | Eppendorf tube(s), standard 1.5ml tube |
| ER | endoplasmic reticulum |
| EtOH | ethanol |
| FCS | (heat-inactivated) fetal calf serum |
| Fuc | fucose |
| g | gram |
| *g* | acceleration of gravity ($g$ = 9,81 m/s$^2$) |
| x *g* | multiple of *g* |
| GAE | granulomatous amoebic encephalitis |
| Gal | galactose |
| GalNAc | *N*-acetylgalactosamine |
| GDP | guanosine diphosphate |
| GlcNAc | *N*-acetlyglucosamine |
| g.u. | glucose units |
| h | hour(s) |
| HAc | acetic acid |

| | |
|---|---|
| HCl | hydrochloric acid |
| HCN | hydrogen cyanide |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HexNAc | *N*-acetylhexosamine |
| HPLC | high-performance liquid chromatography |
| ID | identification percentage |
| Ig | immunoglobulin, antibody |
| k | kilo- ($10^3$) |
| kDa | kilodalton |
| l | litre(s) |
| LB | Luria broth (medium) |
| µ | micro- ($10^{-6}$) |
| m | milli- ($10^{-3}$) |
| M | molar (mol/l) |
| mA | milliampere |
| MALDI | matrix assisted laser desorption/ionization |
| Man | mannose |
| MEM | minimum essential medium |
| MeOH | methanol |
| min | minute(s) |
| ml | milliliter |
| MS | mass spectrometry |
| MS/MS | tandem MS |
| $N_2$ | nitrogen |
| NeuA | neuraminic acid, sialic acid |
| $NH_4HCO_3$ | ammonium bicarbonate |
| NMR spectroscopy | nuclear magnetic resonance spectroscopy |
| NP | normal-phase (chromatography) |
| O/N | overnight |
| PA | 2-aminopyridine |
| PAGE | polyacrylamid gel electrophoresis |
| PBS | phosphate buffered saline |
| PNGase F | *N*-glycosidase F (= peptide *N*-glycosidase) |
| RP | reversed-phase (chromatography) |
| rpm | revolutions per minute |
| RT | room temperature |
| SA | sialic acid |
| SDS | sodium dodecyl sulphate |

| | |
|---|---|
| sec | second(s) |
| Ser | serine |
| % T | total acrylamide-bisacrylamide monomer concentration in % |
| TEMED | *N*,*N*,*N*′,*N*′-tetramethylethane-1,2-Diamine |
| TFA | trifluoroacetic acid |
| Thr | threonine |
| TOF | time of flight |
| Tris | tris (hydroxymethyl) aminomethane |
| UC | ultracentrifuge |
| UDP | uridine diphosphate |
| V | volt (electromotive force) |
| v/v | volume per volume |
| w/v | weight per volume |

# 1 INTRODUCTION

Foam nests are used by numerous animals as means to protect their eggs or juveniles against environmental challenges. Not only in invertebrates like spittle bugs, but also in bubble nests of fish, complex reproductive behaviours revolve around the building of foam nests. Moreover, recently, a biofoam has been described that ensures external fertilization and effective settlement of the larvae of marine tunicates (Castilla et al. 2007). However, among the largest foam nests are those produced by different tropical and subtropical frogs, one of which is the South American bullfrog *Leptodactylus pentadactylus.*

## 1.1 BIOFOAMS IN NATURE

The majority of fish that construct floating bubble nests live in tropical standing waters (Mol 1993) where decaying organic materials (like bacteria) and high temperatures are creating an oxygen depleted environment (Carter and Beadle 1930). In this environment, the major function of the floating bubble nest appears to be to supply oxygen to the developing eggs by lifting them above the water surface into the air while protecting them from desiccation (Hostache and Mol 1998, Mol 1993). The armoured catfish *Hoplosternum littorale* for example, a member of the South American subfamily Callichthyinae (family Callichthyidae), is actively building floating bubble nests by swimming belly-up near the water surface and by swallowing and pumping water through its gills to generate mucus. The mucus is subsequently mixed by movement of the fins with water and air bubbles ultimately resulting in a mass of foam, in which the female deposits the eggs on the next day (Andrade and Abe 1997).

As *H. littorale* shows territoriality with placing its nests in distances of approx. 10 m and guarding a smaller circle around them from both conspecific and heterospecific intruders, Hostache and Mol (1998) suggest the nests to represent means for identification of the centre of territory. Moreover, the foam may be used for synchronisation of reproductive activities, as generally several females are spawning simultaneously in one nest. Initiation of the nest building by the males the day before spawning may also stimulate the final oocyte maturation in females (Hostache and Mol 1998).

Larvae of insects, like those of the spittle bugs Cercopoidea – to which the insect of the year 2009 in Germany, Austria and Switzerland, the frog-hopper *Cercopis vulnerata* belongs (Hoch 2009) – are generating the so called “cuckoo-spit” (Šulc 1912)

predominantly in order to protect the thin-skinned larvae from desiccation and for temperature regulatory reasons (Whittaker 1970). The larvae are secreting anal fluid of excess plant sap containing mucopolysaccharides (Marshall 1966) and wax at the anal air channel. The combination is resulting in the generation of soap that is foamed with air from the anal spiracles to generate stable bubbles. These are assembled by coordinated movements of the abdomen (Šulc 1912).

Moreover, Richards and Davies (1977) suggest that the foam protects the eggs and nymphs from predacious insects and other arthropods. The foam of the larvae of *Neophilaenus* sp., for example, were described to agglutinate mouthparts of potential enemies, like ants and spiders (Hoch 2009).

Moreover, the marine invertebrate *Pyura praeputialis*, an intertidal and shallow subtidal tunicate, that can be found in Australia, Tasmania and as a non-indigenous species in Chile (Castilla and Guiñez 2000), uses foams not only to protect the fertilised brood, but rather, to initially ensure external fertilisation success during spawning, and furthermore, the survival and retention of the short lived tadpole larvae until and finally their settlement in the vicinity of the adults (Castilla et al. 2007).

The free spawning tunicate is using natural conditions of rocky shorelines and naturally turbulent aerated seawaters for the foaming of both eggs and sperm fluid into conspicuous biofoams (up to approx. 2 m height) that are preventing the larvae from being carried offshore by the currents (Castilla et al. 2007). This can be observed especially in Chile, and may be one of the mechanisms (Castilla et al. 2004, 2007) that are responsible for the still unexplained restricted distribution of the species almost exclusively along a 60–70 km coastal stretch inside the Bay of Antofagasta, as well as for its outstanding high density of settlement inside the bay (Castilla et al. 2000), while in its country of origin, Australia, the tunicate is abundant on most southeastern shores (Castilla and Guiñez 2000).

Additionally, a similar bio-foam production occurs simultaneously with massive gamet spawning of the sunstar *Heliaster helianthus* and of the chiton *Acanthopleura echinata* in coastal Chile using the same vein of mechanisms of foaming to ensure survival of the short larval periods (Castilla et al. 2007).

## 1.2 FOAM NESTS OF *LEPTODACTYLUS PENTADACTYLUS*

The huge foaming mass (2 to 7 l in volume) of *Leptodactylus pentadactylus*, the South American bullfrog, is produced by backward-and-foreward movements of the male's hind limbs, while it is sitting on the female's back during mating (amplexus). In doing so, the male mixes air and water with the egg gelatine, cloacal secretions of the female and possible mucous secretions from the body (Heyer 1969, Heyer and Rand 1977, Savage 2002). Moreover, the male secretes sperm during foam formation to ensure external fertilisation of the eggs, which are light gray and 2.9 mm in diameter. About 1,000 of them are released by the female and deposited into the foam mass by the male (Muedeking and Heyer 1976).

Mating takes place throughout the whole rainy season (May to November, in Costa Rica; for geographical distribution see chapter 1.6.2), when the breeding males are heard calling from the margins of ponds, swamps, marshes or sometimes river backwaters (Savage 2002). In the central Amazon region of Brazil, however, the males were observed to begin calling immediately after the first rains, in late September (rainy season in Amazon region of Brazil: from October to May), and to cease the behavious after two to four weeks (Hero and Galatti 1990). The calling seems to function in courthship (to attract the female) as well as in territorial spacing. After the arrival of the female, the male grasps the female under its armpits (axillary amplexus) – the male's thumb and chest spines and hypertrophied forearms help to clasp the female very tightly (Savage 2002) – and is carried from the female to an appropriate breeding site, where the foam is created in a defined series of acts, alternating with periods of rest. The nest formation activity initially starts with the male and female in a resting position. Then the pair rocks forward, the male's back is arched, and the male's legs are at right angles to the body. It may be during this position that the female prepares to release the eggs and jelly. During the next step, the male is raising the legs anterior along the sacral region. Afterwards, the legs remain relatively fixed, only the tarsi and feet are moved back and forth in lateral motions. A single feet movement takes an average of 0.40 sec, which is repeated for about 8 complete back and forth motions. One such sequence takes about 5 sec, at the end of which the legs of the male are moved to normal resting amplecting position. The kicking of the male's feet mixes air into the mucous secretions producing the foam of the nest that is sheltering the now fertilised eggs (Heyer and Rand 1977).

The nests are only rarely placed directly on the surfaces of water, usually they are produced on land near areas that will be flooded when rain sets in. Dry depressions or cavities in the ground near puddles, temporary pools or other ephemeral bodies of water are preferred places (Savage 2002). These potholes may be naturally occurring or possibly excavated by the breeding males. For successful development of the clutch, the nests at the edges of temporary standing waters have to be flooded to allow hatching larvae access to water, as they have to undergo an aquatic phase to ensure larval development and to reach metamorphosis. The tadpoles are washed out of the nest by heavy rains, into nearby bodies of water or they remain in now flooded burrows or hollows (Muedeking and Heyer 1976, Savage 2002). Thus, the development of larvae and frogs is heavily dependent on climate and rain. However, experiments by Valerio (1971) showed that tadpoles of this species are highly resistant to desiccation, and that they can exist up to seven days out of water, so even if a puddle dries up they may survive until the next rain. Additionally, foam nests function in protection of larvae from desiccation (Heyer 1969) (compare chapter 1.4).

Moreover, if rains do not flood the pothole after the larvae have hatched, and they are not washed into bigger ponds that provide the advantage of a variety of food sources, the tadpoles can also develop within the nest until metamorphosis, feeding on eggs remaining in the nest (Muedeking and Heyer 1976). Muedeking and Heyer (1976) also suggested that this form of reproduction is a kind of territoriality, ensuring strong selection against another mating pair laying their eggs in the same pothole with already present predaceous larvae.

## 1.3 OTHER FOAM NESTING FROGS

As a striking example of convergence, foam nest generaton has apparently evolved several times and occurs in (some members of) at least seven of the approximately 50 anuran families (Frost 2010) presently recognized, the Hylidae (Americas, Eurasia), the Hyperoliidae (Africa), the Leiuperidae (South America), Leptodactylidae (Americas), the Limnodynastidae (Australasia), the Microhylidae (Americas, Afro-Asia), and the Rhacophoridae (Afro-Asia) (Amiet 1974, Bastos et al. 2010, Haddad and Hödl 1997, Haddad et al. 1990, Heyer and Rand 1977, Hödl 1990, Jennions et al. 1992, Kadadevaru and Kanamadi 2000, Tyler and Davies 1979).

In all cases, the foams are generated of oviductal or other mucous secretions when the mating pair is in amplexus (Heyer 1969, Hödl 1996), but, dependent on the species, and

the species' average size, the foams are either beaten by males and/or females, either with their hind legs and/or forelegs (or by use of other mechanisms). Furthermore, the sizes (and quality) of the nests with the number of eggs, and of course, the habitats in which they are produced, are different. Figure 1 shows several nests of *Engystomops pustulosus* (Leiuperidae) in a water pond.

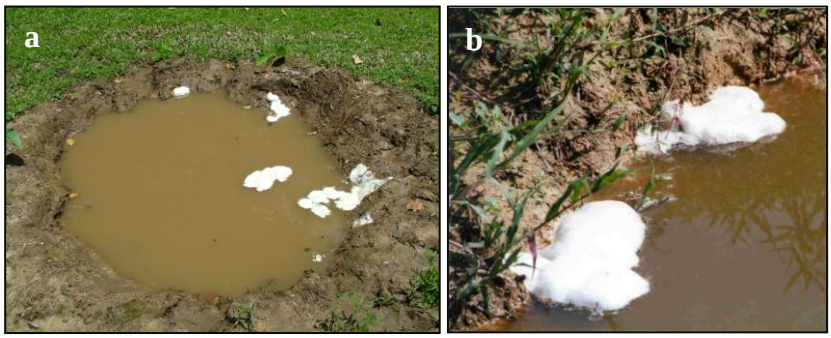



**FIGURE 1: Foam nests of *Engystomops pustulosus* in a water buffalo pond.** The pond has a size of approximately 1.5 m in diameter (a). Individual nests with approximately 10 cm in diameter (b) (Fleming et al. 2009).

In rhacophorid treefrogs, usually the female (Jennions et al. 1992) or both, female and male together (Kadadevaru and Kanamadi 2000), beat the cloacal fluids into a foamy mass with the hind legs while the female is being grasped by the male under the armpits (axillary amplexus). In the neotropical Leptodactylidae as well as Leiuperidae, the axillary-amplecting males beat the fluid with their hind legs into a foam nest of high complexicity (many small air bubbles) (Heyer and Rand 1977, Hödl 1990, 1992), while the Limnodynastidae and Microhylidae build less complex foam nests that consist of few, but large air bubbles. These nests are either produced by the females (inguinal amplexus) that use their forelegs to move water backward beneath the emerging jelly and eggs (Limnodynastidae: Tyler and Davies 1979), or by male and female together (axillary amplexus), however, in a more uncommon way, by release of air through the nostrils (Microhylidae: Haddad and Hödl 1997). Moreover, in Hylidae, the female also produces the foam not by feet movement, but by a jumping motion in order to allow the incorporation of air in the released mucus (Bastos et al. 2010).

However, foam nesting is not always an ongoing between one defined parental pair. The African rhacophorid species *Chiromantis xerampelina*, for example, is also known for occasional communal nest building by involving two or three females laying their eggs into one nest and several males that can arrive between and during the nesting sessions

(Jennions et al. 1992). A number of eggs (approx. 850) (Seymour and Loveridge 1994) is fertilised by different males – during nesting one or more unpaired males ("peripheral male") is/ are competing with the amplexing male to position his/ their cloacae against the female's back. However, the males are not involved in nest construction, which is usually done arboreally and in two to four sessions by the female (hind legs), that in between is descending to the pond to take up water for foam nesting (Jennions et al. 1992).

Peripheral males that gather around the spawning pair have also been observed, for example, in the rhacophorid foam-nesting species *Polypedates dennysi* (now *Rhacophorus dennysi*) (Pope 1931) and in *Rhacophorus schlegelii* (Fukuyama 1991).

As foam nesting frogs, are so-called semi-terrestrial frogs, with external fertilisation and egg deposition out of the water, in the nest, while the greatest part of further larval development and metamorphosis takes place in free waters after hatching and escaping of the larvae from the nest (Hödl 1996), the place of nest deposition is usually not identical to that of further larval development. According to a combination of factors, among others including oviposition and developmental site, different modes of reproduction for amphibians are distinguished – the diversity of which is much greater than that observed in any other group of vertebrates (Duellman and Trueb 1986). In a comprehensive overview of 1986, Duellman and Trueb counted 29 anuran reproductive modes, today almost 40 are known (Haddad and Prado 2005).

Foam nesting itself can be differentiated into several modes, dependent on the site of nesting and further larval development. However, usually, a general assignment of one family to one definite reproductive mode is difficult, as the diversity is more a reflection of the characteristics of the environments in which the frogs live than of the phylogenetic relationships of the families (Duellman and Trueb 1986). Moreover, some frogs may show both, a primary and an alternative secondary reproductive mode, changing between them depending on environmental conditions (Haddad and Prado 2005, Kadadevaru and Kanamadi 2000).

Even only considering foam nesting Leptodactylidae, several different modes of egg deposition can be distinguished. Leptodactylidae can deposit their eggs on the water surface, on the ground, in burrows, or in subterranean constructed chambers (Da Silva Vieira et al. 2009, Hödl 1996), from where the larvae can directly reach waters or have to be washed into waters by rain (e.g. *Leptodactylus pentadactylus*; compare chapter 1.2) (Hödl 1996, Muedeking and Heyer 1976). However, for Rhacophoridae – as most of the

species are arboreal – one mode of egg desposition in foam nests is common: the foams are usually attached to plant structures, like trees, at varying heights above temporary pools, from where the developing larvae can emerge and directly drop into the water (e.g. Kadadevaru and Kanamadi 2000). Moreover, in the family Hylidae only one of 890 species (Frost 2010), the Atlantic forest frog *Scinax rizibilis*, produces foam nests, and, thus, one reproductive modus can be described – deposition of eggs in an aquatic floating foam nest with exotrophic larvae developing in the surrounding pond (Bastos et al. 2010, Haddad and Prado 2005, Haddad et al. 1990).

Only some species of the rhacophorid genus *Philautus* (arboreal nests), the leptodactylid genus *Adenomera* (terrestrial nests), and the limnodynastid genera *Kyarranus* and *Philoria* (terrestrial nests) complete their larval development in the foam nests (Hödl 1996). Table 1 shows the reproductive modes known for foam nesting species (and only these), which is an extract of the list of reproductive modes from Duellman and Trueb (1986) updated by Haddad and Prado (2005).

**TABLE 1: Diversity of reproductive modes in foam nesting anurans (modified after Haddad and Prado 2005).** Altogether thirty-nine reproductive modes of anurans have been recorded by Haddad and Prado (2005). However, only modes concerning foam nesting taxa are mentioned in the table, while the numbering used by Haddad and Prado is maintained. Some exemplary species using the specific reproductive mode are given in brackets.

| |
|---|
| **Eggs in bubble nest (aquatic eggs)** |
| Mode 10: Bubble nest floating on the water in ponds; exotrophic tadpoles in ponds (e.g. *Chiasmocleis leucosticta;* Microhylidae; Haddad and Hödl 1997) |
| **Eggs in foam nest (aquatic eggs)** |
| Mode 11: Foam nest floating on the water in pond; exotrophic tadpoles in ponds (e.g. *Scinax rizibilis*, Hylidae; Haddad et al. 1990) |
| Mode 12: Foam nest floating on water in pond; exotrophic tadpoles in streams |
| Mode 13: Foam nest floating on water accumulated in constructed basins; exotrophic tadpoles in ponds (e.g. *Leptodactylus podicipinus*; Leptodactylidae; Prado et al. 2002) |
| Mode 14: Foam nest floating on water accumulated on the axils of terrestrial bromeliads; exotrophic tadpoles in ponds (e.g. *Physalaemus spiniger*; Leiuperidae; Haddad and Pombal 1998) |
| **Eggs in foam nest (terrestrial or arboreal eggs)** |
| Mode 28: Foam nest on the humid forest floor; subsequent to flooding, exotrophic tadpoles in ponds (e.g. *P. spiniger*; Leiuperidae; Haddad and Pombal 1998) |
| Mode 29: Foam nest with eggs and early larval stages in basins; subsequent to flooding, exotrophic tadpoles in ponds or streams |
| Mode 30: Foam nest with eggs and early larval stages in subterranean constructed nests; subsequent to flooding, exotrophic tadpoles in ponds (e.g. *Adenomera bokermanni*, Leptodactylidae) |
| Mode 31: Foam nest with eggs and early larval stages in subterranean constructed nests; subsequent to flooding, exotrophic tadpoles in streams (e.g. *L. cunicularius*, Leptodactylidae) |
| Mode 32: Foam nest in subterranean constructed chambers; endotrophic tadpoles complete development in nest (e.g. some *Adenomera* species, Leptodactylidae) |
| Mode 33: Arboreal nest; tadpoles drop into ponds or streams (e.g. *Rhacophorus malabaricus*; Rhacophoridae; Kadadevaru and Kanamadi 2000) |

## 1.4 FUNCTIONS OF FROG FOAMS

The diversity of reproductive strategies in frogs has allowed them to colonise almost all habitats including deserts as well as high mountains (Duellman and Trueb 1986). Moreover, the reproductive variation is an important precondition for the occurence of various species in the same tropical environment by use of different niches (Hödl 1996).

In this case, laying eggs in foam nests is one of several strategies that help to inhabit environments with open vegetation forms and with seasonal (or unpredictable) rainfall (Duellman and Trueb 1986, Heyer 1969). Depositing the eggs out of the water in a foam nest (on the surface, in trees, on land; compare chapter 1.3) is making egg deposition more insusceptible to variations in water availability. They can be produced, for example, at the

beginning of the rainy season, even before the water ponds that are necessary for further development of the larvae are completely formed, and they can resist drying-out of waters as they can stay (in contrast to aquatic eggs) in the nest until they are washed away by the rains (Duellman and Trueb 1986, Hissa et al. 2008).

However, a precondition for survival of the hatchlings in the semi-terrestrial nest is protection from the disadvantageous effects of drought and high temperatures that are increased in contrast to the conditions that eggs and tadpoles have to face in open waters. And as the eggs of anurans lack the protective amnion membrane and calcareous shell of birds' and reptiles' eggs, one of the main functions of the foam nest seems to be to protect the eggs from desiccation by providing a humid environment (Duellman and Trueb 1986, Heyer 1969). As a matter of fact, this was one of the first supposed roles for foam nests (Downie 1988, Heyer 1969). Hissa et al. (2008) suggested surfactant active proteins in the nests as means for desiccation protection either by reducing water evaporation or by drawing water towards the eggs and developing tadpoles.

Moreover, Hissa et al. described the foams for absorbing wave lengths of 280 nm and thus, the protection of the eggs and developing embryos against UV injury. McMahon et al. (2006) suggested the blue-coloured protein ranasmurfin, that is part of the foam nests of the tropical frog *Polypedates leucomystax* (Rhacophoridae) and that is giving them a blue/green colouration, as a possible sunscreen.

Additionally, temperature regulation is one need in arid or tropical environments to favour the development of eggs and tadpoles, as the hatching success of frog embryos – shown for the Japanese treefrog *Rhacophorus arboreus* (Kusano et al. 2006) – is very low at high temperatures (near 30°C). An examination of the thermal conditions of these arboreal foams has proved the nests to be temperature stabile by an insulation effect. The temperature at the centre of the foam mass was maintained up to 6°C cooler than the ambient temperature (> 25°C) (Kusano et al. 2006).

Moreover, analyses of the foam fluid compositions of the frogs *Engystomops pustulosus* (Leiuperidae) and *Leptodactylus vastus* (Leptodactylidae) showed a mixture of proteins and carbohydrates (Cooper et al. 2005, Hissa et al. 2008), which – besides their possible importance for cross-linking and stability of the nest (see chapter 1.5) – were assumed as providing nutrients to the developing hatchlings (Hissa et al. 2008). However, not only the foam itself, but also the non-fecundated eggs in the foam are an important food source for the tadpoles (Vinton 1951, Muedeking and Heyer 1976). *Rhacophorus arboreus* hatchlings

initially fed with foam mass proved to be at least as heavy as those fed with boiled lettuce, and thus, leading to the conclusion that the foam mass is a sufficient food source for the early development of the larvae (Kusano et al. 2006).

Moreover, as the foam is lifting the typically aquatic eggs out of the water, it is providing access to atmospheric oxygen for the embryos and the newly hatched tadpoles (Seymour and Loveridge 1994) by preventing them from sinking to waters with lower oxygen contents (Seymour and Roberts 1991). The fresh foam of *Chiromantis xerampelina* (Rhacophoridae) for example, contains 77% air, which is sufficient to supply all of the early embryos' oxygen requirements. After hatching of the larvae, however, when the oxygen demands are increasing, the oxygen uptake by the hatchlings in the wet nest becomes limited and may be stimulating their emergence from the nest. Thus, the foam seems to have an adaptive role in embryonic respiration and stimulation of hatching and leaving of the tadpoles (Seymour and Loveridge 1994).

Additionally, eggs and larvae that develop within foam nests out of the water (or on the water surface) may be protected from aquatic predators (Heyer 1969, Menin and Giaretta 2003). Moreover, the hardening of the outer surface, the high viscosity and gluing nature of the foam may also contribute to the protection of the (innermost) frog eggs from terrestrial predators (e.g. ants) (Lingnau and Di-Bernardo 2006).

However, the nests cannot totally avoid predation (e.g. Lingnau and Di-Bernardo 2006). Thus, after hatching, the larvae seem to respond to the particular situation. Departure from or staying in the foam nest seems to be a fine line between avoiding terrestrial predation and the danger of aquatic predators when leaving the nest. According to Menin and Giaretta (2003) the tadpoles of *Physalaemus cuvieri* are avoiding aquatic predators by entering the water at a later (more developed) stage, when no terrestrial threat is observed. On the other hand, the time of emergence from the nests is shorter, if the nests are infested by predators (e.g. maggots).

### 1.4.1 Biocidal activity

In the absence of eggs or developing tadpoles, the foam nests are known to stay stable for several days while resisting considerable microbial assault with no sign of bacterial or fungal degradation (Fleming et al. 2009). This is remarkable, considering the content of microorganisms in the waters with which these nests are produced. Thus, biocidal activity of biofoams has long been assumed, but has not yet been verified. In an earlier study, the foam fluid did not show acute toxicity to mice, larvicidal action against larvae or any antimicrobial activity (Hissa et al. 2008). Moreover, so far, no evidence of anti-microbial peptides such as pentadactylin of skin secretions (see chapter 1.6.5) of adult *L. pentadactylus* have been found in nest foams of frogs.

However, some components of the foam seem to fulfil the needs of offspring protection against microbiota. Recently, Rostás and Blassmann (2009) proposed that the intrinsic surfactant activity of sectretions might itself serve as a mode of defence against insect attack. On the contrary, Fleming et al. (2009) postulate that lectins, found in the foam nest of the túngara frog *Engystomops pustulosus* (formerly *Physalaemus pustulosus*) may bear the main burden of protection of the eggs from microbial colonisation. These foam nests contain a set of six predominating proteins called ranaspumines (Rsn-1 to Rsn-6) (Latin: *rana*, frog; *spuma*, froth), four of which are carbohydrate binding lectins (Rsn-3, -4, -5, -6), whose predominant role may be the defence against microbial or parasite attack (Fleming et al. 2009). Lectins are often used for primary defence of the vertebrate immune system by providing general microbe recognition components. They bind to molecular patterns of microbial surfaces, but are unable to kill without additional proteins or cells – one common feature is to induce phagocytosis by opsonization of the microbe. However, they can agglutinate microbes bearing the specific sugars that are recognized by the lectins, and thereby impede dissemination (Gupta and Surolia 2007, Van Kooyk and Rabinovich 2008). Thus, their role in frog foam nests may be to inhibit colonization of the foam by microbes and to disable nutrient transporters and cell surface receptors of invading microorganisms and pathogens (Fleming et al. 2009).

Three out of the four lectins in the nest foams of *Engystomops pustulosu*s (Rsn-3, -4 and -5) were identified by Fleming et al. (2009) for having amino acid sequences similar to the family of fucolectins (fucose-binding lectins) originally found in teleost fish (though with

additional or different sugar specificities), while the fourth (Rsn-6) falls into the class of C-type lectins that are frequently associated with galactose binding.

Moreover, as it has been shown that some lectins released by some plant seeds and tissues are (often in combination with other defensive molecules) destructive to the gut cells of certain insects, and thus, may provide protection against arthropod predators (Murdock and Shade 2002), Fleming et al. (2009) postulate that this could also be true for frog foam nests as protection against several species of insects, e.g. dipterans ("frog flies"). These are specialized in laying their larvae in the nests and, thus, frequently found on them (Menin and Giaretta 2003).

A further lectin, Rsn-1, that has been identified by Fleming et al. (2009), is structurally related to proteinase inhibitors of the cystatin class. Inhibition of proteinases that are (not only) important digestive enzymes in the midguts of insects, is another possible defence mechanism of plants against insects (Murdock and Shade 2002). Although Rsn-1 does not itself show any such activity, the natural foam fluid itself was found to exhibit potent cystatin activity. The lectins and cystatin(s) found in the nests of *E. pustulosus* may therefore act together against predation or parasitism (Fleming et al. 2009).

## 1.5 BIOCHEMICAL PROPERTIES OF FROG FOAMS

Foam nests of frogs are made by males and/ or females beating a proteinaceous fluid into bubbles. But, only a distinctive composition of components in the foam seems to fulfil the necessary requirements for foaming, as materials used for foam nest construction must be resistant to environmental and microbial challenge, while being harmless to naked sperm, eggs and developing embryos (Fleming et al. 2009). However, the production of stable foams and bubbles requires overcoming the high surface tension of water, which is usually achieved by surface-active, detergent-like compounds. As conventional detergents would by their very nature solubilize membrane proteins and lipids, leading to disruption of unprotected cell membranes at high enough concentrations (Jones 1999), the foam nests represent an interesting paradox as they harbour eggs and tadpoles without damage (Fleming et al. 2009).

Fleming et al. (2009) described a cocktail of proteins of the nest foam of the túngara frog *Engystomops pustulosus* including a probably new surfactant protein. Rsn-2 (ranaspumin) seems to satisfy the requirements of providing surface activity to allow foam nesting, while being at the same time compatible with the released gametes. This property might be

attributed to its conformation. On the one hand, the amphiphilic structure of the macromolecule (hydrophobic *N*-terminus and hydrophilic *C*-terminus) allows incorporation at the air-water interface in foam nests, while on the other hand, its size prevents the insertion into lipid bilayers (like membranes) and the subsequent disruption of the biological materials. Thus, Rsn-2 seems to be resolving the remarkable paradox of surface activity, on the one hand, and harmlessness to eggs in foam nests, on the other hand, which has been rasing questions for a long time (Fleming et al. 2009).

While the surfactant proteins (Rsn-2) perform the initial function of surface tension reduction to allow foaming, the structure may then be additionally stabilized by incorporation of lectins (Rsn-3 and Rsn-5) which have also been described for this foam (see chapter 1.4.1). The lectins have relatively hydrophobic *N*-terminal ends – highly uncommon for secreted proteins as these sequences are usually secretory signals that are removed prior to secretion –, which may serve to anchor or orient the proteins besides the surfactant proteins in the air-water interface layer. Moreover, the lectins bind and cross-link the long-chain, branched polysaccharides of the natural foam material, and thereby create a stable, water-retaining multilayer foam matrix, which explains the long-term stability of the foam (Fleming et al. 2009).

Amanzingly, such proteins seem to be present in foam nests of different frog species. The first description of ranaspumins as proteins with unusual primary structures and extraordinary surfactant properties was done by Cooper et al. (2005), who also did works on the nests of *Engystomops pustulosus*. Those authors have termed the mixture of proteins in the 10–40 kDa mass range ranaspumins and described the remarkable surfactant activity of these proteins and their contribution in cross-linking of polysaccharides into a stable multilayer foam nest. Nevertheless, the authors did not at this time associate any protein in particular with these properties, e.g. surfactant activity.

A study by Hissa and colleagues (2008) concentrated on the description of the composition and function of the foam nests of *Leptodactylus vastus* that comprise a set of different proteins with molecular masses in the range of 14 to over 97 kDa in also high concentrations. They described the foam fluid as effective in reducing the water surface tension, and connected this property to the mixture of proteins in the fluid, with one 20 kDa protein maybe representing the major function (*Lv*-ranaspumin).

McMahon and colleagues (2006) also described the foam nests of *Polypedates leucomystax* as a rich source of proteins. In detail they concentrated on the crystal structure

of a 13 kDa surfactant protein, named ranasmurfin. Since searches with the available partial amino acid sequence did not match to any known protein or structure in the databases, it has been supposed that it could also be a novel protein.

It seems that proteins are giving the special biophysical properties for foam formation and stabilization (Cooper et al. 2005). Because of the disruptive process that might occur at the air-water interface, foaming of proteins is usually avoided, in order to protect them from denaturation (Clarkson et al. 1999). Thus, the evolution of proteins and use of such proteinaceous fluids specifically adapted for the generation and stabilisation of foams poses interesting questions about their structure and characteristics (Cooper et al. 2005).

Moreover, frog foam nests appear to be a new source of surface-active compounds that could open up a huge potential for biomedical and industrial applications. New proteins like ranaspumins and ranasmurfins could be of potential use because of the long-term stability and biocompatibility of these foams (Cooper et al. 2005).

### 1.5.1 Glycoproteins/ Glycosylation

All cells and numerous macromolecules, such as proteins and lipids, in nature carry an array of covalently attached and in their structures and linkages very different sugars or sugar chains, which are generally referred to as "glycans". The glycans attached to proteins can be devided into two major classes according to the nature of the linkage of the glycan to the peptide. A carbohydrate linked to the amino-group of an asparagine residue of a polypeptide chain, is called an *N*-glycan (*N*-linked oligosaccharide), whereas *O*-glycans (*O*-linked oligosaccharide) are usually linked to a hydroxyl group of a serine or threonine residue of the peptide (Varki and Sharon 2009).

These *N*- and *O*-glycans can be abundantly found on membrane- or secretory proteins of eukaryotes, that often have one or more covalently attached carbohydrate side chains (Varki and Sharon 2009). However, in the last few years, in contrary to prior misconceptions, it has become clear that protein glycosylation is not restricted to eukaryotes only, but also occurs in prokaryotes. Additionally to the well known sugar structures of prokaryotic cell walls, they also produce glycoproteins: In Eubacteria *O*-glycans are more common, while in Archaea *N*-glycans predominate (Esko et al. 2009).

*N*- and *O*-glycans differ in size from one to more than 20 sugars. Moreover, sugar moieties of glycoproteins differ in sugar composition, the type of linkage between them, in branching pattern, acidity due to sialylation, phosphorylation, and sulfation. The sugars

present in *O*-glycans are *N*-acetylglucosamine (GlcNAc), galactose (Gal), *N*-acetylgalactosamine (GalNAc), fucose (Fuc), and sialic acid (SA), and additionally mannose (Man) in *N*-glycans (Brockhausen 1993). In contrast to nucleotides or peptides where the monomers can only undergo one type of linkage in between them, monosaccharides can theoretically form either an α or a β linkage to any one of several positions on another monosaccharide, resulting in an almost unimaginable number of possible glycans theoretically present in biological systems (Varki and Sharon 2009).

Nevertheless, due to the exquisite specificity of a variety of competing and sequentially acting glycosidases and glycosyltransferases the number of glycan structures is restricted – only about a thousand are known to occur on glycoproteins (Brockhausen 1993). Thus, in contrast to other biomolecules (peptides and nucleic acids) glycan sequences are not directly genome encoded. A few genes are dedicated to expressing the enzymes and transporters responsible for the biosynthesis and linkage of the glycans, typically as posttranslational modifications of proteins. As these glycosylation enzymes are extremely sensitive for physiological changes, the glycosylation patterns vary depending on e. g. nutrition, type and developmental status of the cell (Varki and Sharon 2009).

Eukaryotic glycoproteins (and their carbohydrate portions) are important in a number of biological processes such as fertilisation, cell adhesion, hormone action, immune recognition and receptor functions. The interactions between cells, or cells and molecules are performed via carbohydrates and carbohydrate-binding proteins and this process occurs e.g. during fertilization, development and growth. In biology and medicine, glycoproteins play an outstanding role in viral and bacterial binding and infectivity (Brockhausen 1993). In addition, diseases and metastatic cells can be defined via altered glycan metabolisms and glycan patterns – certain glycan structures are well-known markers for tumor progression (Varki et al. 2009a).

The understanding of the biological function and significance of glycosylation, however, is still very limited. Although, a little is known on cell-type, growth and disease specific differences in glycosylation, there are still many questions in dependencies of biological activities of cells and macromolecules on these alterations (Brockhausen 1993).

#### 1.5.1.1 *O*-Glycosylation

*O*-glycosylation is a common covalent post-translational modification of serine and threonine residues of glycoproteins. The α-linkage of *O*-glycans via an *N*-

acetylgalactosamine (GalNAc) as first sugar to the OH-group of serine or threonine (GalNAcα-Ser/Thr) is not only the most abundant, but probably also the most extensively studied one of the various types of *O*-glycans. The structures that can be extended by different further sugars into a variety of structural core classes (four major and four minor core subtypes are differentiated) are called mucin-type *O*-glycans and the corresponding glycoproteins are mucins. Mucins carry a large number of *O*-glycans that are clustered (closely spaced) and are found in mucous secretions and as transmembrane glycoproteins of cell surfaces with the glycans exposed to the exterior. They occur widely in mammals and other eukaryotes (e. g. fish, insects, amphibians, and nematodes), but not in lower eukaryotes such as yeast and fungi, nor in prokaryotes (Brockhausen et al. 2009).

Several other types of *O*-glycans linked to serine and threonine residues also exist, including *O*-fucose and *O*-glucose in receptors and ligands of vertebrate cells, *O*-GlcNAc (*N*-acetylglucosamine) on nuclear and cytoplasmic proteins, *O*-mannose in yeast, as well as *O*-xylose or *O*-galactose, which are examples for other reducing terminal sugars attached to serine or threonine as primary carbohydrate moieties (Brockhausen et al. 2009, Freeze and Haltiwanger 2009, Hart and Akimoto 2009).

#### 1.5.1.2 *N*-Glycosylation

The carbohydrates linked to the amino-groups of asparagines (Asn) by an *N*-glycosidic bond are termed *N*-glycans. *N*-glycans are found on soluble and membrane-bound glycoproteins and affect the proteins' properties including conformation, solubility, antigenicity, and recognition by glycan-binding proteins. Defects in *N*-glycan biosynthesis and assembly can lead to diverse, often severe human diseases.

Five different *N*-glycans have been described, of which the linkage of *N*-acetylglucosamine to asparagine (GlcNAcβ-Asn) is the most common one. Other linkages to asparagines include glucose, *N*-acetylgalactosamine and rhamnose, and in a sweet corn glycoprotein, glucose is found in *N*-linkage to arginine. Not all asparagine residues of polypeptides can accept an *N*-glycan, the minimal consensus sequence in a protein ("sequon") begins with asparagine followed by any amino acid except proline and a serine or threonine (Asn-X-Ser or Asn-X-Thr). In contrast to *O*-glycans, all eukaryotic *N*-glycans share one common pentasaccharide core region composed of three mannose (Man) and two *N*-acetylglucosamine (GlcNAc) residues (Stanley et al. 2009) (Figure 2).

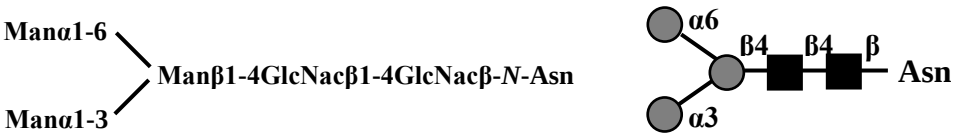



**FIGURE 2: Basic *N*-glycan core structure in eukaryotes.** Text nomenclature on the left and the corresponding symbolic nomenclature on the right, which is that recommended by the Consortium for Functional Glycomics (http://www.functionalglycomics.org): circles: hexoses; squares: *N*-actetylhexosamines; black: glucose stereochemistry; gray: mannose stereochemistry (Figure: original).

According to the attached side chains that vary in length and composition, *N*-glycans are differentiated into three major structural classes: Oligomannose- (or high-mannose-) type structures carry only mannose residues attached to the core. In hybrid-type structures the Manα1-6 arm is elongated with mannose residues and at least one *N*-acetylglucosamine residue can be found at the Manα1-3 arm, while in complex-type *N*-glycans bi-, tri-, tetra-, or penta-antennary structures are initiated by *N*-acetylglucosamine residues attached to the core (Figure 3).

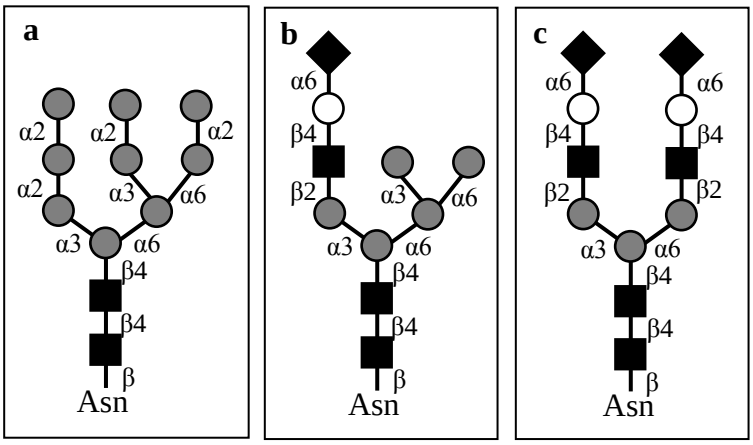



**FIGURE 3: Types of *N*-glycans**. *N*-glycans linked to asparagine (Asn) residues are of three general types in a mature glycoprotein: oligomannose (a), hybrid (b) and complex (c). Each type contains the common *N*-glycan core $Man_3GlcNAc_2Asn$. Circles: hexoses; squares: *N*-actetylhexosamines; black: glucose stereochemistry; gray: mannose stereochemistry; white galactose stereochemistry; black diamond: neuraminic acid (Figure: original).

The biosynthesis of eukaryotic *N*-glycans begins in the endoplasmic reticulum (ER) and is preserved throughout evolution and similar in lower and higher species, in all metazoans, in plants, and in yeast. However, the identity and linkage of the side chains varies depending on the activity of specific and competing glycosidases and glycosyltransferases which are exquisitely sensitive to the biochemical and physiological status of the cell in which the enzymes and the proteins that have to be modified, are located. These cellular

conditions may be altered during development, differentiation and in disease. Thus, the identities of sugars attached to a mature glycoprotein will depend on e.g. the eukaryotic species, the cell type and the developmental stage (Stanley et al. 2009).

*N*-glycosylation takes place in a series of complex pathways including lipid-linked intermediates. Dolichol phosphate (Dol-P), a lipid that is bound to the ER-membrane and comprised of approximately 20 isopren units, serves as a primary carrier to which sugars are added in a stepwise manner (Bill et al. 1998). First, GlcNAc-1-P is transferred from UDP-GlcNAc to dolichol phosphate, which is orientated to the cytoplasma, to generate dolichol pyrophosphate *N*-acetylglucosamine (Dol-P-P-GlcNAc). To this *N*-acetylglucosamine residue, another *N*-acetylglucosamine and five mannose (Man) residues are transferred from the donors UDP-GlcNAc and GDP-Man, respectively. Afterwards translocation through the membrane occurs, resulting in the sugar residues being orientated to the lumen of the ER. Subsequently, four further mannose residues from Dol-P-Man and three glucose residues (Glc) from Dol-P-Glc are attached. The tetradecasaccharide of the lipide derivative ($Glc_3$-$Man_9$-$GlcNAc_2$-P-P-Dol) is then transferred *en bloc* by oligosaccharyltransferase to the asparagine residue on the growing peptide chain (Kobata 1992, Stanley et al. 2009).

The completely translated polypeptide with the 14-sugar oligomannose glycan is then transported to the Golgi apparatus. While it is still located in the ER, trimming begins, i.e. sequential removal of sugar residues by specific glycosidases and mannosidases. In the ER three glucose residues and one mannose residue are removed, in the *cis*-Golgi, another three mannose residues are removed from the sugar chain. The translocation of the glycoprotein to the *medial*-Golgi can result in the addition of an *N*-acetylglucosamine residue to the Manα1-3 arm (giving the precursor to hybrid type glycans), leading to an steric rearrangement of the two mannose residues linked to the Manα1-6 arm which can then be removed by a Golgi mannosidase (giving the precursor to complex type glycans). The precursors can subsequently be modified by addition of specific sugars (GlcNAc, fucose, galactose, GalNAc, sialic acid). However, the modifications are performed according to different levels and acceptor specificities of the Golgi enzymes and do not follow a distinct pathway without exceptions, which is finally resulting in the high variety of species and tissue specific glycosylation patterns (Kobata 1992, Stanley et al. 2009).

#### 1.5.1.3 Glycan structures of frogs

Until now, only little is known on glycan structures of frogs in general, and even less is known on glycan structures of frog foam nests in particular. Most studies concerning frogs concentrate on the characterisation of *O*-glycans of the so-called jelly coat, a water-insoluble transparent extra-cellular matrix surrounding amphibian eggs, which is mainly composed of mucin-type glycoproteins with species-specific glycan chains (Mourad et al. 2001).

The egg jelly coats are formed by components secreted by specific glands of the oviduct and are sequentially deposited on the eggs as they are transported towards the cloaca (Strecker 1997). Interest in these jelly coats has centered in their key role in the process of fertilization, as they display the first barrier for fertilizing sperm, which have to pass through the jelly envelope before reaching the plasma membrane of the egg. The coats act in adherence of the spermatozoa to the egg surface, in prevention of polyspermy and recognition of the homologous species (Freeman 1968, Jégo et al. 1980, Katagiri 1986). Chemical analyses concerning the nature of the jelly coats have indicated the carbohydrate content of the mucin glycoproteins with approx. 80% carbohydrates (fucose is abundantly found especially in the outer layers), and 20% proteins. However, the relative amount is variable according to the species (Shimoda et al. 1994).

As foam nests are also generated from fluid released by the female (Heyer 1969), the presence of comparable *O*-glycans would be conclusive. However, *O*-glycans as well as *N*-glycans of *Leptodactylus pentadactylus* proteins as well as of its foam nest proteins still have to be investigated. To our knowledge the only description of glycans of foam nests was given shortly for *Engystomops pustulosus* (túngara frog) foam nest glycoproteins. Cooper et al. (2005) mentioned that the ranaspumines, a number of proteins in the 10–40 kDa range, are not detectably glycosylated. Fleming et al. (2009) confirmed these results for the foam nests of the túngara frog, and additionally showed by analysis of amino acid sequences of six ranaspumines that no consensus *N*-glycosylation sites occur in their sequences, although, preliminary analysis of the *N*-glycans has shown the presence of both truncated and complex-type glycans of which the most were found for having a fucosylated core. Analysis concerning the *O*-linked glycans of the foam has revealed the presence of both core-1 and core-2 structures. These are both fucosylated and sialylated (Parry et al. 2003 in Cooper et al. 2005).

## 1.6 *LEPTODACTYLUS PENTADACTYLUS*

### 1.6.1 Systematics

*Leptodactylus pentadactylus*, the smoky jungle frog or South American bullfrog, belongs to the class Amphibia and therein to the order Anura, which itself constitutes – besides the two other amphibian orders Gymnophiona (caecilians) and Caudata (salamanders) – the vast majority of living species of amphibians (32 families, ca. 372 genera, and 5227 anuran species) (Frost et al. 2006). *L. pentadactylus* is classified as a member of the family Leptodactylidae, which itself belongs to the superfamily Hyloidea of the suborder Neobatrachia ("advanced" frogs) (Frost et al. 2006).

Until recently, the family Leptodactylidae[1] Werner, 1896 (1838) has been divided into five subfamilies: One of the first classifications of the Leptodactylidae was achieved by Lynch (1971, 1973), who considered four subfamilies, based on both, synapomorphy and symplesiomorphy. Only two years later, Heyer (1975) proposed five groups that were recognized subsequently by Laurent (1986) as subfamilies, namely Ceratophryinae, Cycloramphinae, Eleutherodactylinae (the largest subfamily), Leptodactylinae, to which the genus *Leptodactylus* belongs, and Telmatobiinae.

However, recent studies found these subfamilies as partly distantly related, not clearly monophyletic or possibly polyphyletic (e. g. Faivovich et al. 2005; Figure 4). For example, Haas (2003) sampled three species of the subfamily Leptodactylinae for mostly larval, but also adult morphological characters and found the group to be poly- or paraphyletic. Moreover, Faivovich et al. (2005) discovered by comparison of multiple mitochondrial and nuclear gene loci, representatives of most genera of Leptodactylinae to be monophyletic, with the exception of *Limnomedusa* (Figure 4). Under the aspect, that several other subfamilies seemed to be nonmonophyletic as well, Frost et al. (2006) suggested to recognize the traditional subfamily Leptodactylinae (including some genera of Cycloramphinae, but without the genus *Limnomedusa*) as Leptodactylidae *sensu stricto*, a taxon that was much diminished compared with its previous namesake – from approximately 1200 to 100 species – but that was according to Frost et al. (2006) consistent with evolutionary history. The number of genera in the family Leptodactylidae, was reduced from 57 to 11 (Frost et al. 2006), and soon after, to four, by Grant et al.

[1] Nonmonophyletic taxon.

(2006), who divided the Leptodactylidae (s. str.) into the Leptodactylidae and the Leiuperidae (seven genera). Most species of the genera now belonging to the family Leptodactylidae show foam-nesting behaviour, a property that has always been and still is considered synapomorphic of the group (Frost et al. 2006).

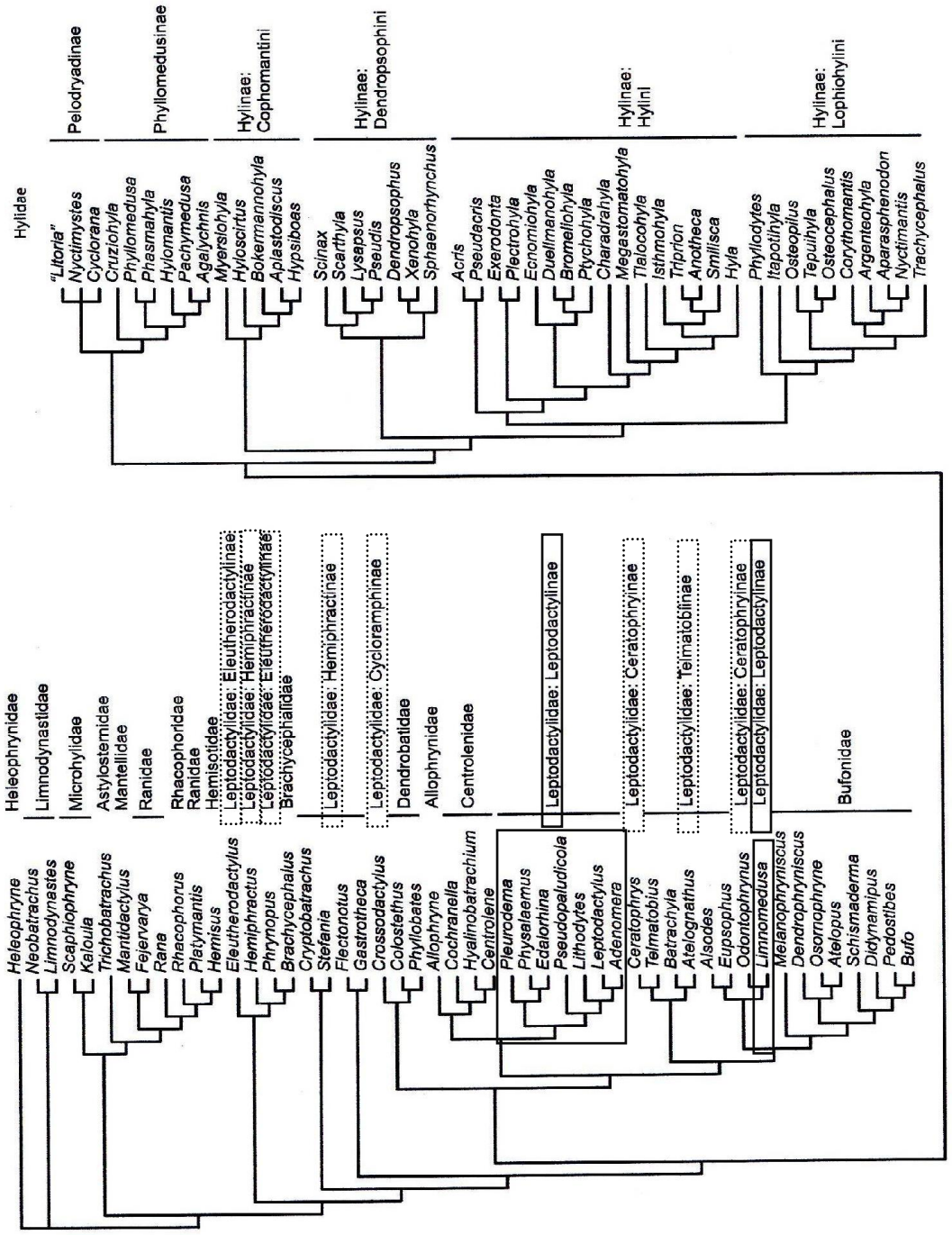



**FIGURE 4: Tree of anuran phylogenetics by Faivovich et al. (2005), from and modified by Frost et al. (2006).** The tree is based on 5.1 kb sequences from four mitochondrial (12S, 16S, $tRNA^{Val}$, cytochrome *b*) and five nuclear genes (rhodopsin, tyrosinase, RAG-1, seventh in absentia, 28S). The figure shows that several of the taxonomic groups are nonmonophyletic, particularly the leptodactylid subfamilies. Taxa referred to in the current study are highlighted.

The initial problem of classification of the family Leptodactylidae and of several subtaxa is reflected in the general problem of understanding amphibian phylogeny. The number of recognized amphibian species has increased enormously in recent years, and the understanding of the evolutionary relationships of amphibians has not kept pace with the frequency of species descriptions (Frost et al. 2006). According to Frost et al. (2006) the major progress in frog taxonomy in the 1980s and 1990s was based on a relatively small sampling of species and morphological characteristics that were all too often overly-generalised and overly-interpreted, leading to a tapestry of unresolved paraphyly and polyphyly.

The understanding of frog diversification has begun to change in the 2000s with the infusion of considerable amounts of molecular data into the discussion of phylogeny. But, although recent molecular studies have been very informative – e.g. Biju and Bossuyt (2003) suggested that Hyloidea, the superfamily to which *L. pentadactylus* belongs, is paraphyletic, and suggested the term Hyloidea *sensu stricto* for the monophyletic group within "Hyloidea" – the phylogeny of the Anura remains poorly understood. In 2006, Frost et al. provided an extensive phylogenetic analysis across all living amphibians based on molecular evidence, which led to changes in the understanding of former valid systematics, and in particular also of that of Leptodactylidae. As described above, they suggested to reduce the family Leptodactylidae to a smaller monophyletic group traditionally seen as the subfamily Leptodactylinae.

The genus *Leptodactylus* Fitzinger, 1826 is with 88 members the species-richest taxon of the Leptodactylidae (s. str.) and currently includes the genus *Leptodactylus* and the representatives of the genera *Adenomera* Steindachner, 1867, *Lithodytes* Fitzinger, 1843, and *Vanzolinius* Heyer, 1974 (Frost 2010). *Adenomera* was allocated in *Leptodactylus* after the revision of Frost et al. (2006), who on the basis of evidence presented by Heyer (1998) and Kokubum and Giaretta (2005) recognised the genus as a synonym of *Lithodytes*, and *Lithodytes* as a subgenus of *Leptodactylus*. Moreover, the genus *Vanzolinius* was regarded as rendering *Leptodactylus* paraphyletic and, thus, synonymised with *Leptodactylus* (De Sá et al. 2005, Frost et al. 2006). Nevertheless, many questions remain open.

### 1.6.2 Geographical distribution and habitat

The family Leptodactylidae is predominantly neotropical; it is distributed from the most southern parts of Texas to the South of Brazil and on certain Caribbean islands. The species *Leptodactylus pentadactylus* in particular, can be found in Central America from Honduras to the Pacific lowlands of Ecuador and in the Amazon Basin, including Southern Colombia, Eastern Ecuador, Peru, Northern Bolivia, and much of Central and Northern Brazil, with records from French Guiana (Frost 2010).

*L. pentadactylus* generally prefers moist habitats in forests or rainforests, often near swamps and slowly flowing streams (Guyer and Donnelly 2005) in the lowlands or marginally up to premontane areas (up to 1,200 m). However, it can also be found inhabiting areas in some distance from bodies of water (Savage 2002). Moreover, this frog species prefers dimly lit forests (Jaeger and Hailman 1981), where the nocturnal adults retreat into subterranean burrows, under logs, into the interstices between tree roots, or under houses to stay in hiding during the day. Juveniles, however, are active during the day and may be found on the leaf litter in dense forests (Savage 2002).

### 1.6.3 Morphology

*L. pentadactylus* is a large and long-lived frog with a potential life-span of approximaetly 15 years (Leenders 2001) (Figure 5). Adult males can reach 106 to 177 mm from snout to rump. The female is slightly larger than the male and can measure up to 118–185 mm (Savage 2002). It has a typical frog body with long, jumping hindlimbs, and shorter forelimbs. The fingers and toes on all four feet are unwebbed and long with slender tips. The genus name *Leptodactylus* that refers to these characteristics, is of Greek origin from the terms “leptos”, meaning “thin”, and “dáktylos”, meaning “finger”.

The colouration of the adults has been described as either uniform gray to reddish brown, or as spotted or barred with darker colour – particularly the limbs are often striped with dark markings (Savage 2002). The ground colour has also been described as a reticulum of dark purplish and light brown (Guyer and Donnelly 2005). The venter is dark gray with white to yellow punctuations, and the juvenile frogs are usually more brightly coloured than the adults. *L. pentadactylus* can be distinguished from other frogs by the triangular dark spotted lips, dark stripes from the nostrils to the eyes, and the presence of a pair of dorsolateral folds, that extends from the back of the head to the groin. The species has

large eyes with brown irises and large tympana with a width of one-half to two-thirds diameter of the eye (Savage 2002) (Figure 5).

Adult males have extremely muscular forearms and a black spine on each side of the chest and on the base of each thumb (= nuptial spines). These properties are thought to improve the grip of the male while sitting on the back of the female during mating (Guyer and Donnelly 2005).

**FIGURE 5:** ***Leptodactylus pentadactylus*****.**
(Photo: C. Malamud: http://www.flickr.com/photos/publicresourceorg/493822016/sizes/o/).

### 1.6.4 Feeding ecology

*Leptodactylus pentadactylus* is an opportunistic feeder. Adults eat a variety of prey including invertebrates, like arthropods, and vertebrates, like nesting birds, snakes and other frogs (Savage 2002). Tadpoles initially feed on the foam they are living in (Vinton 1951), but later will become carnivorous or cannibalistic – they might prey on eggs of their own or other species, respectively (Muedeking and Heyer 1976) – although they can also grow and survive while eating algae and plants (Vinton 1951).

### 1.6.5 Skin secretions

Adults of *Leptodactylus pentadactylus* are able to secrete immense amounts of mucus which is used as defence against predators. The mucus makes the frog slippery and difficult to hold, additionally, the skin secretions are noxious to predators and lethal to other frogs which come into contact with it (Savage 2002). Savage (2002) points out that these secretions can also cause rapid allergic responses in humans, both from direct contact or from only indirect contact, as from being in the same room when the animal is handled. The secretions can induce sneezing, swelling of the eyes and irritation of mucous

membranes. When threatened by a predator, the individuals inflate their bodies and elevate it with all four limbs, followed by repeated lifting and lowering of the body with the longitudinal axis centered on the potential predator. This has the effect of lifting the glandular back and groin above the level of the head (the hind limbs are longer), exposing the predator to the irritating amines and toxic peptides in the skin (Savage 2002).

Moreover, recent studies have shown that these skin secretions contain peptides with antimicrobial properties that are considered part of the innate immune system, as first-line defence against invading pathogens. King et al. (2005) described two antimicrobial peptides in mucous skin secretions of *L. pentadactylus*, one of which was named pentadactylin. The second one that differed from pentadactylin by eight amino acid residues was identical to fallaxin, a C-terminally α-amidated 25 amino-acid-residue that has been isolated by Rollins-Smith et al. (2005) from the skin of the Caribbean mountain chicken frog *Leptodactylus fallax*. Both peptides show growth inhibiting activity against Gram-negative bacteria. A third antimicrobial peptide of *L. pentadacylus*, leptoglycin, was described by Sousa et al. (2009).

Indeed, biocidal peptides in skin secretions of members of the genus *Leptodactylus* are common. The ocellatins from *L. ocellatus* were the first such peptides of the genus to be characterized (Nascimento et al. 2004). Laticeptin from *L. laticeps* (Conlon et al. 2006) and syphaxin of *L. syphax* (Dourado et al. 2007) are further examples. All these peptides show *in vitro* biocidal activity against potential pathogens, like different Gram-negative and/or Gram-positive bacteria.

Although the peptides of the genus *Leptodactylus* are structurally very similar (cationic, amphipathic and α-helical), the sequence similarities of skin antimicrobial peptides in the amphibian world are generally very low. However, it has been speculated that antimicrobial peptides of the skin of South American hylid and ranin frogs derive from a common 150-million-year-old ancestral precursor that existed before the radiation of the families. The diversity of these peptides (especially of their C-terminal domains) may result from repeated duplications and mutations of this precursor (Vanhoye et al. 2003).

## 1.7 TEST ORGANISMS

### 1.7.1 Protozoa

Protozoa (Greek: "proto" for first and "zoa" for "animal") are the largest single-celled, non-photosynthetic ("animal-like") eukaryotes that lack cell walls. As this description is true for a wide variety of organisms, the term "protozoa" comprises a variety of more or less (un)related groups. Protozoan pathogens cause millions of deaths, annually, and with no available vaccination they represent a significant burden on human health. Protozoa of medical importance among others include the malaria parasites, *Plasmodium* spp., *Trypanosoma* spp. causing sleeping sickness and Chagas' disease, *Leishmania* spp. causing leishmaniosis, and *Acanthamoeba* spp. causing keratitis and encephalitis. Due to the increasing problem of resistances against common pharmaceuticals, the search for new drugs, with anti-protozoal effects, is still going on. Four test-organisms – although these do not present any pathogenic threat to *L. pentadactylus* in natural habitats – were chosen to determine a potential antiprotozoal effect of the frog nest foam: *Trypanosoma cruzi*, *Leishmania donovani*, *L. infantum* and *Acanthamoeba* genotype T4.

#### 1.7.1.1 *Trypanosoma cruzi*

The genus *Trypanosoma* belongs to the Kinetoplastida, a group of single-celled flagellated protozoa with a typical elongated form. The group is characterised by the presence of a DNA-containing “organelle”, known as the kinetoplast that is located in the single large mitochondrion. The flagellum originates near the kinetoplast and emanates from a pocket in the cell membrane. Depending on the position of the kinetoplast-flagellum complex within the cell, different life cycle stages can be distinguished (Figure 6) (Cox 1993).

The genus *Trypanosoma* contains a number of morphologically undistinguishable species that cause very different diseases. *Trypanosoma brucei* is divided into three sub-species, two of them causing sleeping sickness (human African trypanosomosis) in humans – *T. b. gambiense* (West Africa) and *T. b. rhodesiense* (East Africa) –, and the third one, *T. b. brucei,* is one of several species causing animal African trypanosomosis (Nagana). *Trypanosoma cruzi*, on the other hand, is the causative agent of the Central and South American Chagas disease (American trypanosomosis), which is named after Carlos Chagas, a Brazilian doctor who first described the disease in 1909 (Barrett et al. 2003).

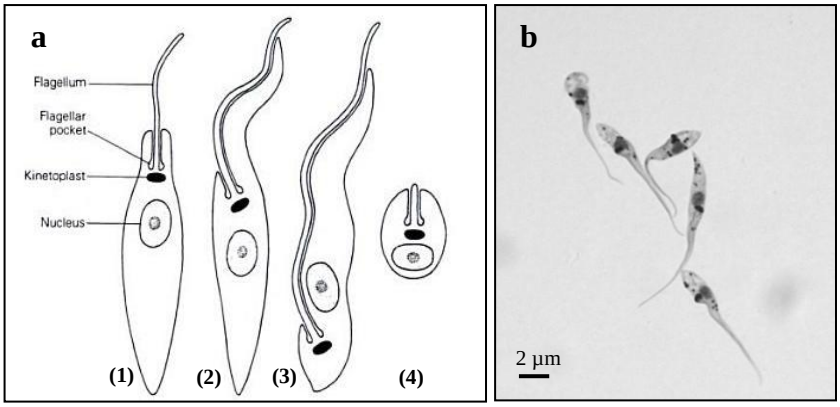



**FIGURE 6: Forms of the life cycle of a kinetoplastid flagellate (a)**. Promastigote (1), epimastigote (2), trypomastigote (3) and amastigote form (4) (Cox 1993); epimastigote *Trypanosoma cruzi* in axenic culture **(b)** (Photo: F. Astelbauer).

Infection with *T. cruzi* is commonly acquired from an infected triatomine bug (family Reduviidae) that feeds and defecates on sleeping hosts (humans or animals). Metacyclic (infectious) trypomastigotes in the feces usually enter the host if they are rubbed (during scratching of the itching wound) either into the bite, another microlesion or into mucous membranes of the conjunctiva or mouth. Moreover, infection can occur diaplacentally or from blood transfusions and organ transplants from an infected donor (Mehlhorn 2008, Walochnik and Aspöck 2010a).

In the mammalian host, the parasites enter the bloodstream, where the trypomastigotes stay during the first weeks of acute infection, before they withdraw into the tissue. Then, *T. cruzi* actively invades host cells and transforms into the amastigote form (1.5–4 µm), with no apparent flagellum. The amastigotes reproduce by binary fission before they differentiate back to bloodstream-form trypomastigotes (16–35 µm length) which then leave the cell in order to invade another one or to get taken up by a triatomine bug. Hematogenous dissemination allows the trypomastigotes to parasitize many tissues (they particularly invade muscle and ganglial cells) where replication can occur (Walochnik and Aspöck 2010a).

After the acute phase, which often passes unnoticed, or with marginal symptoms of general malaise or an oedematous swelling at the infection site (chagoma), most untreated infected persons enter into a prolonged asymptomatic form of disease (intermediate state) during which few or no parasites are found in the blood. In many individuals the parasites remain inactive in the tissue for the rest of the host's life and the larger part of infected patients is asymptomatic. However, an estimated 20–30% of the infected patients will enter the third

or chronic stage. The trypanosomes resume multiplication and the individuals will develop clinical and life-threatening manifestations. Pathology comes from the destruction of cells resulting in severe organ damages (characteristic signs are megaorgans) many years or decades after the initial infection.

As for all other protozoal infections, no vaccine is available. Nifurtimox is the drug of choice for the treatment of the disease; however, it cannot cure the damages of long-lasting *T. cruzi* infections (Barrett et al. 2003, Walochnik and Aspöck 2010a). In Central and South America where Chagas disease is endemic, an estimated 8 to 11 million people are infected with *T. cruzi* (CDC 2009), and annually 13.000–45.000 patients die from the disease. As the triatomine bug thrives under poor housing conditions (e. g. mud walls, thatched roofs), in endemic countries, people living in rural areas are at greater risk for acquiring the infection (Barrett et al. 2003, Walochnik and Aspöck 2010a).

#### 1.7.1.1 *Leishmania donovani* and *L. infantum*

The genus *Leishmania* (named after William Boog Leishman) belongs, as the genus *Trypanosoma*, to the class Kinetoplastida and is a group of obligatory parasitic, eukaryotic and unicellular organisms. Leishmanias exhibit only two different forms in their life cycles. In the arthropod vector, they are in the 10 to 20 µm long promastigote form (Figure 7), which exhibits a 10 µm long flagellum originating at the front of the cell. In the host, they transform into the intracellular amastigote form (3 to 5 µm length) with no apparent flagellum (Figure 6) (Cox 1993, Walochnik and Aspöck 2010b).

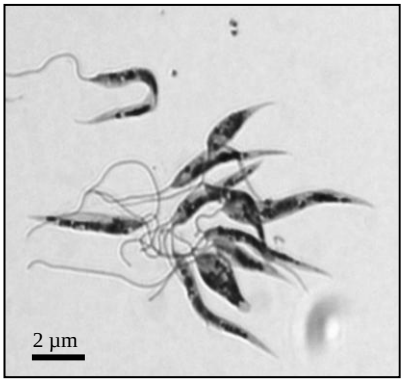



**FIGURE 7: Promastigote form of *Leishmania infantum* in axenic culture.**
(Photo: F. Astelbauer)

Sandflies of the genera *Phlebotomus* (in the Old World) or *Lutzomyia* (in the New World) transmit the parasites, segregating the infective promastigotes through their proboscis when they take a blood meal. The parasites invade mammalian macrophages by receptor-mediated endocytosis, where they transform into amastigotes that multiply by binary fission until the host cell lyses and the leishmanias are released for another round of macrophage infection. Sandflies become infected by ingesting infected cells when taking a blood meal from a human or animal host. In the gut of the vector, the amastigotes transform into promastigotes that migrate to the proboscis from where they can be transmitted to another mammal (Walochnik and Aspöck 2010b).

Infection with *Leishmania* can result in either cutaneous (CL) or visceral leishmaniosis (VL; also known as kala-azar), depending on the parasite strain but also on the vertebrate host and the vector. Moreover, there are rare forms: the anergic diffuse cutaneous leishmaniosis (ADCL) and the post kala-azar dermal leishmaniosis (PKDL). Human infections are caused by 21 of the approximately 30 known "species"[2] (strains) that are morphologically indistinguishable. *L. donovani* and *L. infantum* (both species of the so-called *L. donovani* complex, which is divided into 6 genotypes) usually cause visceral leishmaniosis, which affects the internal organs of the body, particularly the spleen and the liver (Stuart et al. 2008, Walochnik and Aspöck 2010b).

In total, about 12 million individuals (WHO 2010) are infected with *Leishmania* spp. in 88 subtropical or tropical countries that represent the major endemic areas of leishmaniosis – however, visceral leishmaniosis is also common in the Mediterranean region (Walochnik and Aspöck 2010b). The number of new cases of VL is estimated to be about 500,000 per year, while the number of new cases of CL is approximately three times higher, with an incidence of 1.5 million cases worldwide (WHO 2010). In contrast to cutaneous leishmaniosis that in some forms heals even without treatment, visceral leishmaniosis is often lethal if untreated (Walochnik and Aspöck 2010b), causing approximantely 40,000 deaths per year. More than 90 percent of all VL cases occur in India, Bangladesh, Nepal, Sudan, and Brazil (WHO 2010).

[2] Molecular biological studies have shown that some traditional species are synonyms.

### *1.7.1.2 Acanthamoeba*

*Acanthamoeba* is a genus of free-living amoebae that is ubiquitously present in the environment and is found in fresh and sea water, soil and air as well as in man-made environments and clinical settings. Acanthamoebae undergo two stages during their life cycles: an active trophozoite stage (Figure 8a) during which they constantly divide by binary fission, and a dormant cyst stage (Figure 8b-d) during which they remain inactive with little metabolic activity, but viable, for years. The trophozoites, which dominate when growth conditions are optimal, are usually flat with no specific form and a size of between 20 to 45 µm. They exhibit characteristic spine-like hyaline extensions on the cell surface, the acanthopodia. Under harsh conditions such as lack of food or desiccation, trophozoites differentiate into the persistent cysts, which are double-walled (endo- and ectocyst) and of an average size of 16 to 22 µm in diameter (Marciano-Cabral and Cabral 2003, Visvesvara et al. 2007).

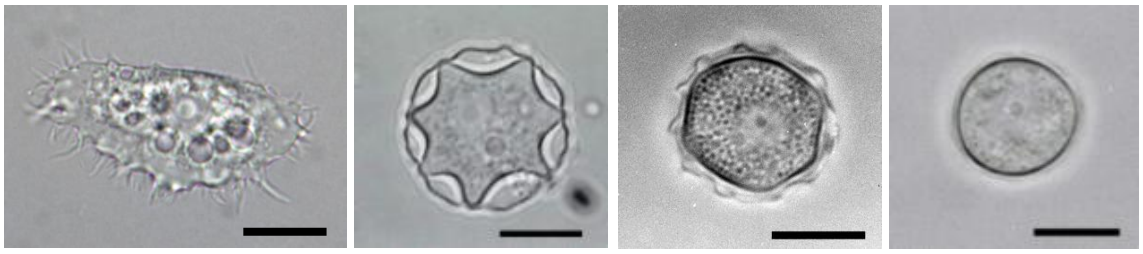


**FIGURE 8:** *Acanthamoeba* trophozoite **(a)** (Photo: original), and cysts representing morphological groups I **(b)**, II **(c)** and III **(d)** (Walochnik and Aspöck 2005). Scale bar = 10 µm.

Acanthamoebae were first described in detail by Aldo Castellani in 1930, and grouped into the genus *Acanthamoeba* by Volkonsky in 1931. For the following decades acanthamoebae were largely ignored until they were discovered as potential pathogens in the 1960s. Today acanthamoebae are recognised as the causative agents of the rare but often fatal granulomatous amoebic encephalitis (GAE), which is an infection of the brain and spinal cord that typically occurs in persons with a compromised immune system (Marciano-Cabral and Cabral 2003, Visvesvara et al. 2007). Furthermore, they are known as the causative agents of a very often seriously progressing keratitis occurring predominantly in contact lens wearers (85% of cases) (CDC 2009). *Acanthamoeba* keratitis, which develops in contrast to GAE independently of the immune status of a person, has become increasingly important within the last 20 years correlating to the growing number of contact lens users.

In *Acanthamoeba* infections, both trophozoites and cysts, can enter the body through microlesions in the eye, or in the skin or through the respiratory tract. In case of keratitis, acanthamoebae usually remain in the place of entry (the eye), while in individuals with compromised immune systems, after a cutaneous or nasopharyngeal infection amoebae are carried to other organs including the central nervous system (CNS) via the bloodstream. However, not every skin lesion causes a disseminated infection (Marciano-Cabral and Cabral 2003, Visvesvara et al. 2007).

Infections with free-living amoeba are very rare. The incidence of *Acanthamoeba* keratitis in the U.S. is approximately one to two cases per million contact lens users (CDC 2009) – today worldwide approximately 5000 cases of *Acanthamoeba* keratitis have been described. The number of systemic *Acanthamoeba* infections is with approximately 200 cases lower. However, while the prognosis for recovery of the patient from AK is good, that for GAE caused by *Acanthamoeba* is poor – less than 10 of the patients have survived (Schuster and Visvesvara 2004). Furthermore, *Acanthamoeba* can act as reservoir and vector for pathogenic bacteria, e.g. *Legionella pneumophila*, which causes the Legionnaires' disease (Rowbotham 1980). This host character of *Acanthamoeba* is supported by the ability of forming highly resistant cysts, which are able to bear bacteria for a very long time.

There are around 20 species in *Acanthamoeba*, e. g. *A. castellanii* and *A. polyphaga*, which have been described to cause disease. However, species determination in *Acanthamoeba* is difficult, as morphological species often interfere with biochemical and molecular biological classifications. Thus, acanthamoebae are nowadays differentiated according to their cyst morphology into three morphological groups (Pussard and Pons 1977) (Figure 8b-d) and additionally divided into 16 genotypes (T1–T16) based on their 18S rRNA gene sequences (Gast 2001, Hewett et al. 2003, Horn et al. 1999, Stothard et al. 1998). Morphological group II and sequence type T4 seem to be the most abundant groups not only in the environment but also in clinical specimens (Stothard et al. 1998, Walochnik et al. 2000). However, it remains unclear whether the higher frequency of sequence type T4 in clinical isolates is due to a potentially increased virulence in comparison to other genotypes, or due to their abundance in the environment. Sequence type T4 can not be generally classified as virulent, as it contains pathogenic as well as non-pathogenic members. Pathogenicity of acanthamoebae and the development of virulence traits in different strains, and under different conditions, still remain incompletely understood

(Walochnik et al. 2000). Treatment of *Acanthamoeba*-related diseases is difficult, particularly the cysts are resistant against known biocides, and can give rise to recurrence of infection once the drug level is reduced (Marciano-Cabral and Cabral 2003).

## 1.8 AIMS OF THE STUDY

The South American bullfrog *Leptodactylus pentadactylus* is a representative of frogs using foam nests in order to shelter their fertilised eggs during development until larval metamorphosis. The foam nests are unusually stable under tropical conditions and obviously protect the developing eggs against microbial degradation. However, the mechanisms ensuring long-term stability of the nests and protection of the brood are not completely understood and existing theories are contradictory.

The first aim of this study was to clarify the chemical and physiological properties of the nests. Besides analysis of solubility and the protein composition of the foam, it was one of the major aims of this study to get an impression of the specific *N*-glycan structures of the glycoproteins of the foam nest of *L. pentadactylus*. Until now, only little is known on *N*-glycan structures of frog proteins in general, and even less is known on *N*-glycosylation statuses of frog foam nest proteins, in particular.

The analysis of the potential biocidal activity of the frog foam nest fluid was another intention of the present study. Large-scale tests for analysis of the suggested antibiosis should be performed with use of a number of model organisms, including different protozoa, as well as other micoorganisms, like fungi, Gram-negative and Gram-positive bacteria. In earlier studies the foam fluid did not show any antimicrobial activity or evidence of antimicrobial peptides in the foam known from skin secretions of different members of the genus *Leptodactylus*.

Moreover, it was aimed to unravel the microbial community that is associated with the nest and its potential protective function. Therefore, cultivable bacteria should be isolated from the nest and analysed for their potential antimicrobial activities on other microorganisms, like fungi and bacteria. To our knowledge this is the first study on the importance of microbiota associated with the foam nests.

# 2 MATERIAL AND METHODS

## 2.1 FROG FOAM

Foam nests originated from *Leptodactylus pentadactylus* (smoky jungle frog or South American bullfrog), a species of the family Leptodactylidae, and were collected in cooperation with the University of Vienna at the Field Station LaGamba in the "Rainforest of the Austrians" in Costa Rica during the rainy season (July and August) following ethical and ecological guidelines; *L. pentadactylus* frogs are widespread, common, and according to the IUCN Red List of Threatened Species (2010) not currently endangered. 4 nests (including eggs) were carefully collected from the edges of temporary standing waters, placed in plastic bags, deep frozen and airmailed to the Department of Parasitology, Medical University of Vienna, Austria. For comparative studies, 2 foam nests of the frog *Polypedates leucomystax* (Rhacophoridae; arboreal nests) were collected in the aquarium of the zoological garden in Schönbrunn, Vienna, Austria. The larvae of this species had already left the nests, the foams were approximately 5 days old. Since then, the nests were stored at -80°C. All further experiments were performed at RT unless otherwise stated.

## 2.2 PURIFICATION

Prior to analysis of the foam the contained eggs (nests of *L. pentadactylus*) had to be removed. Therefore, small pieces were broken off the frozen material into a sterile petri dish and thawed at room temperature (RT). The eggs were removed manually with two heat-disinfected spattles. Due to their size of 2–3 mm in diameter the eggs were visible without microscope.

## 2.3 SOLUBILITY

Approximately 0.05 g of foam material of *L. pentadactylus* (without eggs) were weighed into standard 1.5 ml Eppendorf-tubes (Eppi) and then mixed with 100 µl double distilled water ($ddH_2O$), 100 µl of 30% ethanol (EtOH), 100 µl of 50% EtOH, 100 µl of 70% EtOH and 100 µl of 96% EtOH, respectively, in order to define the solubility. After each step the mixture was vortexed gently (2 min): As the foam was not soluble in either $ddH_2O$ or EtOH, 200 µl of methanol (MeOH) were added and the eventual solubility was tested, while vortexing up to 4 min. Finally, 100 µl of dimethyl sulfoxide (DMSO) were added,

the Eppis were once again vortexed and solubility was observed. Solubility was also tested with 200 µl of MeOH and 200 µl of DMSO only, respectively.

## 2.4 HOMOGENISATION

In order to work with and to analyse the microbicidal activity and properties of the foam, a suspension had to be made. As the foam did not dissolve in any of the above mentioned solvents (chapter 3.1), suspensions were made by using different kinds of mechanical homogenisation.

### 2.4.1 Sonicator

0.05 g of foam mass were weighed into an Eppi, then 200 µl of dd$H_2O$, of 96% EtOH, MeOH, or DMSO were added, respectively, one control without solvent was included in the setup. The Eppis were put into the sonicator (BANDELIN Sonorex RK100, BANDELIN electronic, Berlin, Germany) and left there under observation for 30 min. Afterwards another 100 µl of each solvent were added, and the samples were left in the sonicator until a homogenous solution was achieved (for another approx. 40 min), while vortexing them every now and then. The obtained extract was stored at -20°C. If the suspension was used for further testing, subsequent filtration followed (see chapter 2.4.3).

### 2.4.2 Mortar and pestle

Bigger volumes of foam (approx. 5 g) were weighed into a cooled mortar, soused with liquid nitrogen ($N_2$) and grinded as smooth as possible with the pre-cooled pestle. Subsequently, the material was weighed into Eppis or into 15 ml BD Falcon tubes (BD Biosciences, Erembodegem, Belgium), diluted with sterile dd$H_2O$ or PBS to the respective final concentration (g foam/ml) and stored at -20°C. If needed, the extract was filtered according to chapter 2.4.3.

### 2.4.3 Bead Beater

Approximately 0.5 g of foam mass were weighed into homogeniser tubes prefilled with glass beads (Precellys-Glas-Kit 0.5 mm, 2 ml volume) (Peqlab Biotechnologie GmbH, Erlangen, Germany), mixed with 900 µl of sterile dd$H_2O$ or PBS (per litre: 8.0 g NaCl, 0.20 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$, pH 7.4), homogenised in the Precellys 24 homogeniser (Peqlab Biotechnologie GmbH, Erlangen, Germany) at 6300 rpm for 10 sec,

paused for 30 sec and again homogenised for 10 sec. In order to prevent heat development during homogenisation the samples were iced before and immediately after homogenisation.

After homogenisation the supernatant was transferred to a fresh Eppi. The remaining glass balls were washed with 200 µl of $ddH_2O$ or PBS, vortexed and then the Eppis spun for 5 min at 6200 x *g* and 5°C in the Heraeus Fresco 17 ultracentrifuge (UC) (Thermo Fisher Scientific Inc., Waltham, USA). Afterwards, the supernatant was added to the rest of the sample, and the total volume spun for 30 min at 16000 x *g*, 5°C to remove any residual debris.

For filtration, the supernatant was filled up to a volume of 3 ml (as with filtration approx. 1 ml of sample volume is lost) and then sterile filtered with a Minisart® 0.2 µm filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) in order to remove all contaminations and microorganisms. When dilution of the suspension was to be avoided (when higher protein concentrations were tested), alternatively, the contents of 4 homogeniser tubes were pooled and filtered as described above.

To prevent the dilution of the foam extract and the possible loss of proteins (or other bioactive substances) during filtration, in parallel setups, the extract was not filtered, but centrifuged a second time. The procedure was followed as usual, but the supernatant of the first centrifugation was subjected to a second round of centrifugation as described above (30 min, 16000 x *g* at 5°C).

## 2.5 PROTEIN CHARACTERISATION

### 2.5.1 Protein concentration

In order to make the individual approaches comparable and as the microbicidal activity of the foam extract was supposed to be due to one protein or a composition of different proteins, the protein concentration of the foam extract had to be measured. The extracts were vortexed and then the protein concentrations of each aliquot were directly measured with the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies Inc., Wilmington, USA) at $\lambda$= 280 nm, using bovine serum albumin (BSA) as standard, and the solvent (sterile $ddH_2O$ or PBS) as background.

Simultaneously, the extinctions at λ= 260 nm and λ= 280 nm were measured, the value of the solvent was substracted and finally the protein concentration calculated after Warburg and Chrstian (1941) with the formula: P (mg protein/ ml) = 1.56 x $E_{280}$ – 0.757 x $E_{260}$.

### 2.5.2 SDS-PAGE

For analysis of contained proteins, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The employed gels were two-layer SDS-polyacrylamide gels of common format (8 x 8 cm gel with 10 wells), freshly prepared prior to use. First, the resolving gel (usually 12% T, made of 2.5 ml Lower Tris (Table 2), 4 ml 30% PAA (Table 2), 3.5 ml $ddH_2O$, 50 µl of 1% ammonium persulfate (APS) and 5 µl tetramethylethylenediamine (TEMED)) was pipetted between two glass plates cleaned with 70% EtOH (Bio-Rad Laboratories, Hercules, CA, USA), covered with a layer of isopropanol (for a planar face of the gel) and left for polymerisation at RT (ca. 40 min). Then the isopropanol was discarded, the stacking gel (4.8% T, made of 1.25 ml Upper Tris (Table 2), 0.8 ml 30% PAA, 2.9 ml $ddH_2O$, 40 µl of 1% APS and 5 µl TEMED) was poured on top of the resolving gel, a gel comb was inserted and the gel left for polymerisation for approx. 20 min. To ensure SDS-coated proteins to become concentrated at the border between the two gels the distance between the comb and the resolving gel had to be at least 0.5 mm, better 1 cm.

**TABLE 2: Composition of Lower Tris, Upper Tris and 30% (w/v) PAA.** The substances were dissolved in 180 ml $ddH_2O$, for Lower Tris the pH was adjusted to 8.8, for Upper Tris the pH was adjusted to 6.8. All three solutions were filled up with $ddH_2O$ to a volume of 200 ml. The 30% PAA solution was wrapped in foil; all solutions were stored at 4°C.

| **Lower Tris** | | **Upper Tris** | | **30 % PAA** | |
|---|---|---|---|---|---|
| **Substance** | **[g/mg/ml]** | **Substance** | **[g/mg/ml]** | **Substance** | **[g/mg/ml]** |
| Tris | 36.6 g | Tris | 12.12 g | Acrylamide | 58.4 g |
| 10% (w/v) SDS | 8 ml | 10% (w/v) SDS | 8 ml | Bis-acrylamide | 1.6 g |

Before applying the samples to the gels, they were heated at 95°C for 5 min in the presence of two-fold concentrated SDS-PAGE reducing buffer (Table 3), followed by centrifugation for 5 min at 15000 x *g* at RT. The supernatant was then subjected to electrophoresis. As a standard, Peqgold protein marker V (PEQLAB Biotechnologie GmBH, Erlangen, Deutschland; range 10–250 kDa) or PageRuler$^{TM}$ Prestained Protein Ladder (Fermentas

LIFE SCIENCES, Glen Burnie, Maryland, USA; range 10–170 kDa) (not preheated) were included.

The proteins were separated by size at 200 V and RT until the blue front reached the end of the gel (shortly before running out of the gel). Table 3 shows the composition of the running buffer used. After separation, the gel was stained with Coomassie Brilliant Blue G-250 or used for western blotting, respectively.

**TABLE 3: Composition of the SDS-PAGE reducing buffer and running buffer.** For the reducing buffer, the substances were dissolved in ddH2O, some crystals of bromophenol blue were added and the solution filled up with ddH2O to a total volume of 10 ml. 1 ml aliquots were stored at -20°C. For the 10 x running buffer the substances were dissolved in 1 litre ddH2O, for 1 x running buffer 100 ml of the 10 x buffer were diluted with ddH2O to a volume of 1 litre and the solution stored at 4°C.

| **2 x reducing buffer** | | **10 x running buffer** | |
|---|---|---|---|
| **Substance** | **[g/mg/ml]** | **Substance** | **[g/mg/ml]** |
| SDS | 200 mg | Tris | 30 g |
| Dithiothreitol (DTT) | 154 mg | Glycine | 144 g |
| Upper Tris (pH 6.8) | 5 ml | SDS | 10 g |
| Glycerol (87%) | 3.6 ml | | |

#### 2.5.2.1 Coomassie Brilliant Blue staining

After SDS-PAGE the protein bands were fixed (to prevent loss of proteins) in a non-modifying procedure with fixing solution (50% (v/v) MeOH and 7% (v/v) acetic acid (HAc)) for approx. 25 min. The gel was stained in a mixture of 3.5% (v/v) perchloric acid and 0.04% (w/v) Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, Hercules, CA, USA) for approx. 30 min and finally destained from excess material with 5 % (v/v) HAc until clear protein bands could be distinguished (approx. 20 min, as required).

## 2.6 *N*-GLYCAN ANALYSIS

### 2.6.1 Blots

Lectins and antibodies binding specifically and reversibly to mono- or oligosaccharides were used for lectin blotting in order to determine *N*-linked glycan determinants on protein surfaces. First, SDS-PAGE was performed, then the proteins were transferred from the polyacrylamide gel to a 0.2 µm nitrocellulose membrane (Whatman PROTRAN®,

Whatman GmbH, Dassel, Germany) either by tank blotting or semi-dry blotting and afterwards incubated with the specific lectins or antibodies. Finally, the glycan binding proteins were made visible with alkaline phosphatase and its substrate BCIP®/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium).

#### 2.6.1.1 Tank-blot

Before blotting, the nitrocellulose membrane, the filter papers (Mini Trans-Blot® filter paper; Bio-Rad Laboratories, Hercules, California, USA), and the fiber pads were soaked for 15 to 30 min in transfer buffer (48 mM Tris, 39 mM glycine, 20% (v/v) MeOH, 0.0375% (w/v) SDS in dd$H_2O$; pH 9.2). Afterwards the gel and the membrane were fixed between the filter papers and fiber pads in the cassette and then placed into the Mini Trans-Blot® Transfer Cell (Bio-Rad, USA) filled with transfer buffer. The transfer was performed at 350 mA for 1 h.

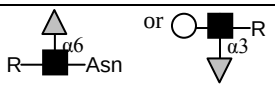
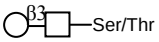
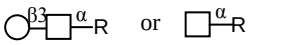
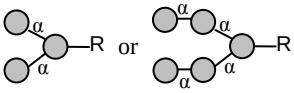
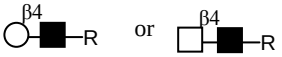
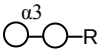
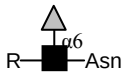
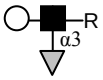
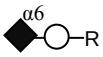
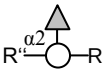
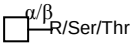
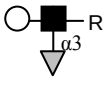
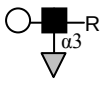
#### 2.6.1.2 Semi-dry blot

First, the acrylamide gel was equilibrated for 15 min in transfer buffer (10% (v/v) of 10x blot transfer buffer and 20% (v/v) MeOH, in dd$H_2O$; 10x blot transfer buffer: 250 mM Tris, 1.92 M glycine in dd$H_2O$). The membrane and the filter papers (Extra Thick Blot Paper Protean®; Bio-Rad Laboratories, Hercules, California, USA) were cut to gel size and pre-wetted in the buffer for 5 to 10 min. One pre-wetted filter paper, then the pre-wetted membrane, the equilibrated gel and a second pre-wetted filter paper (from bottom up) were placed on the platinum anode of the Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA). After application of the top platinum cathode the blot was run at 15 V for 25 min.

#### 2.6.1.3 Incubation with lectins and antibodies

The blotted nitrocellulose membrane was incubated in blocking buffer for 1 h: TTBS (0.1 M Tris, 0.1 M NaCl, pH 7.5 with 0.05% (v/v) Tween 20) containing 0.5% (w/v) BSA was used for blocking of the non-specific hydrophobic binding sites on the membrane. Afterwards the membrane was washed three times for 8 min with TTBS before the lectins or antibodies, diluted in blocking buffer (1:2,000), were applied to the membrane for one hour. (All steps were performed on a shaking platform.) The used lectins and their specificities can be seen in Table 4.

**TABLE 4: Origin and specificities of the used lectins (modified after Iskratsch et al. 2009).** The specificities of the lectins are the major specificities detected in the study by Iskratsch et al. (2009) and from Varki et al. (2009a). The symbolic nomenclature is that of the Consortium for Functional Glycomics (http://www.functionalglycomics.org): circles: hexoses; squares: *N*-acetylhexosamines; white: galactose stereochemistry; gray: mannose stereochemistry; black: glucose stereochemistry; gray triangle: fucose; black diamond: neuraminic acid. R indicates attachment to the remaining part of an *N*- or *O*-glycan structure, R' (in the case of RCA I) can be a hydrogen or an α2,6-linked sialic acid; R" can be any sugar residue or a hydrogen.

| | Species/ Origin | Abbr. | Major specificity | |
|---|---|---|---|---|
| | | | Name | structure |
| Lectins | *Aleuria aurantia* (Orange peel fungus) | AAL | Core α1,6-Fuc or $Le^x$ |  |
| | *Arachis hypogaea* (Peanut) | PNA | Galß1,3GalNAc-Ser/Thr |  |
| | *Artocarpus integrifolia* (Jack fruit seed) | Jacalin | (NeuA)Galβ1,3GalNac-R or α-GalNac-R |  |
| | *Canavalia ensiformis* (Jack bean) | ConA | α-Man |  |
| | *Erythrina crista-galli* (Cockspur Coral Tree) | ECL | Gal(NAc)ß1,4GlcNAc-R |  |
| | *Griffonia simplicifolia* (German: Afrikanische Schwarzbohne) | GSL I-$B_4$ | Galα1,3Gal-R |  |
| | *Helix pomatia* (Roman snail) | HPA | α-GalNAc-R/Ser/Thr |  |
| | *Lens culinaris* (Lentil) | LCA | Core α1,6-Fuc |  |
| | *Lotus tetragonolobus* (Winged pea) | LTA (LTL) | $Le^x$ |  |
| | *Lycopersicon esculentum* (Tomato) | LEA | R"[-3Galβ1-4GlcNAcβ1-]$_n$ |  |
| | *Ricinus communis* (Castor bean) | RCA I | R'-Galβ1,4GlcNAc-R |  |
| | *Sambucus nigra* (European elder) | SNA | NeuAα2,6Gal-R |  |
| | *Triticum vulgaris* (Genus: Wheat, wheat germ) | WGA | NeuAα2,3Gal-R or HexNAcβ1,4-R |  |
| | *Ulex europaeus* (Common gorse) | UEA | R"-(Fucα1,2)Gal-R |  |
| | *Vicia villosa* (Hairy vetch) | VVA $B_4$ | α/β-GalNAc-R/Ser/Thr |  |
| Antibodies | Anti-$Lewis^x$ from mouse (clone DU-HL60-3) mono-clonal anti-human (IgM) | CD15 | $Le^x$ |  |
| | Anti-$Lewis^x$ from mouse mAb (IgM), anti-human | P12 | $Le^x$ |  |

#### 2.6.1.4 Development with alkaline phosphatase and BCIP®/NBT

After incubation, the membranes were washed with TTBS to remove excess material (3 times for 8 min), followed by incubation for 1 h with alkaline phosphatase streptavidin (Vector Laboratories, Inc., Burlingame, CA, USA) (1:10,000 in blocking buffer) (in case of primary incubation with lectins). In case of incubation with the antibodies P12 (Calbiochem®, Merck Group) and CD15 (Sigma-Aldrich) the secondary antibody anti-mouse IgM conjugated with alkaline phosphatase (µ chain specific; Sigma-Aldrich) was used (1:10,000 in blocking buffer). After thoroughly washing in TTBS (3 times for 8 min) the blots were developed by adding the chromogenic substrate BCIP®/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Sigma FAST™ Buffered Substrate Tablets; 1 tablet dissolved in 10 ml water resulting in a solution of 0.15 mg/ml BCIP®, 0.3 mg/ml NBT, 100 mM Tris, pH 9.5 and 5 mM $MgCl_2$. BCIP® + Alkaline Phosphatase → BCl + $PO_4$; BCl +NBT → blue coloured product).

As the phosphatase conjugated streptavidin interacts with the biotinylated lectin, and as the secondary antibody, which is conjugated with the alkaline phosphatase, interacts with the primary antibody, respectively, the membrane stains, where lectins or primary antibodies are bound. Thus, specific glycan determinants on protein surfaces were determined. The activity of the alkaline phosphatase (the colour reaction) was stopped after 5-10 min (before building a background) with 5% (v/v) HAc.

### 2.6.2 "In-gel release method" for *N*-glycan analysis

The following protocol is an adaptation of the modified "in-gel release method" described by Rendić et al. (2007).

#### 2.6.2.1 SDS-PAGE and Coomassie staining

A sample with about 30 µg of total protein was prepared in presence of two-fold concentrated reducing buffer and later subjected to the standard SDS-PAGE procedure described above (chapter 2.5.2). However, the acrylamide concentration of the stacking gel was reduced (3.2% T), whereas that of the resolving gel was increased (17% T). Moreover, the electrophoresis was only performed until the protein standard (Fermentas PageRuler™ Prestained Protein Ladder) started to separate on the resolving gel (separation for only 3–4 mm). Then the run was stopped and the gel was stained with Coomassie Brilliant Blue G-250 following the protocol described in chapter 2.5.2.1. The stained band was excised from

the gel in the smallest size possible (i. e. no excess gel around the band, to remove contaminations) and cut into 1 x 1 mm pieces with a scalpel blade on a clean glass plate. Figure 9 shows a typical gel used in the study (Coomassie-stained). Since fingerprints and loose hair (keratins) might contaminate the sample, gloves were worn.

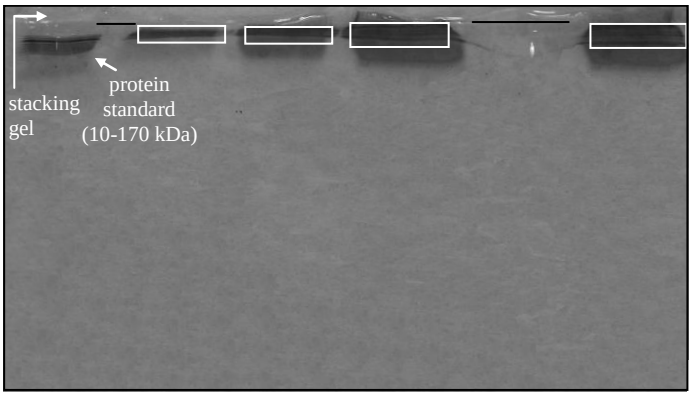



**FIGURE 9: Typical gel used in the study (Coomassie-stained).** Separation was only performed for 3–4 mm, before staining and cutting out of the protein bands. The frames show the sections of the gel that were cut out, the lines show the border between the stacking and resolving gel (Photo: original).

#### 2.6.2.2 Washing of the gel pieces

A shaking platform was used for all of the following steps (24°C). The gel pieces were washed twice with 150 µl 50% (v/v) acetonitrile (ACN) (= 3 band volumes; for dehydration of the gel pieces) for 15 min, and then for approx. 5 min with 150 µl 100% ACN. The supernatant was then removed and 90 µl of freshly prepared 0.1 M ammonium bicarbonate ($NH_4HCO_3$) were added. After 5 min, 90 µl of 100% ACN were added, the total mixed by tapping and the mixture incubated for another 15 min. Then the supernatant was removed and the gel pieces dried in a Savant SPD131DDA SpeedVac Concentrator (Thermo Fisher Scientific Inc., Waltham, USA) (for approx. 20 min), before, 150 µl of 10 mM dithiothreitol (DTT) (in 0.1M $NH_4HCO_3$) were added to the dry gel pieces. After 5 min at RT, they were incubated at 56°C for 45 min for *in situ* alkylation (i. e. to reduce, break and prevent reformation of disulphide bonds). The supernatant was then discarded and the gel pieces were incubated in the dark for another 45 min in the presence of 150 µl 55 mM iodoacetamide (in 0.1 M $NH_4HCO_3$).

Subsequently, another round of washing as described above was performed (first with 2 x 150 µl of 50% (v/v) ACN for 15 min, then with 150 µl of 100% ACN, followed by a

washing step with 90 µl of 0.1 M $NH_4HCO_3$ and 90 µl of 100% ACN for 15 min). Finally, the pieces were dried in the SpeedVac.

#### 2.6.2.3 Tryptic and *N*-glycosidase F digestion

For tryptic digestion of the proteins the dried gel pieces were incubated in a solution of 60 µl 0.1M $NH_4HCO_3$ and 2µl trypsin (sequencing grade, 200 µg/ ml; Roche Diagnostics GmbH, Mannheim, Germany) at 37°C overnight.

The next day, in order to extract the (glyco)peptides, 90 µl of 25 mM $NH_4HCO_3$ were added to the pieces. After shaking for 15 min, 90 µl of 100% ACN were added, followed by further shaking for 15 min. The supernatant was transferred to a fresh tube and the gel pieces were extracted two more times with 90 µl of 5% (v/v) formic acid for 15 min. The extracts were pooled and afterwards dried in the SpeedVac (approx. 1h). After addition of 30 µl of 0.1 M ammonium carbonate (pH 8) to the dried (glyco)peptides, the mixture was incubated for 5 min at 95°C, cooled briefly on ice and incubated in the presence of 0.5 µl *N*-glycosidase F (recombinant, 1U/µl; Roche Diagnostics GmbH, Mannheim, Germany) at 37°C for overnight.

#### 2.6.2.4 Purification of the released *N*-glycans

The next day, the sample was acidified with 40 µl of 2% (v/v) HAc and then applied to single use pipette tips packed with 15 µl Dowex® 50Wx8 (200-400 mesh; Carl Roth GmbH + Co. KG, Karlsruhe) and 10 µl of LiChroprep® RP18 (25-40 µm; Merck KGaA, Darmstadt, Germany) on top, both equilibrated with 300 µl 2% (v/v) HAc. Subsequently, the sample was applied to the column, the column washed with 2% HAc and the flow-through collected in fresh tubes. The purified *N*-glycans were then dried in the SpeedVac, resuspended in $ddH_2O$ and used for matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis.

#### 2.6.2.5 MALDI-TOF-MS analysis of *N*-glycans

The *N*-glycans were taken up in approx. 10 µl of $dH_2O$. 1 µl of the sample was applied on a MALDI steel plate which was dried under vacuum pressure. Then, the sample was overlaid with 1 µl of 2% 2,5-dihydroxybenzoic acid (DHB) in 30% (v/v) ACN with 0.01% (v/v) trifluoroacetic acid (TFA) as matrix and redried under vacuum pressure. The analysis was performed with a Bruker Ultraflex Maldi-TOF/TOF instrument, competent to perform

additional tandem mass spectrometry (MS/MS) in order to get more information on the structural identity of the *N*-glycans.

### 2.6.3 Preparation of 2-aminopyridine derivatised *N*-glycans

#### 2.6.3.1 Pepsin digestion

The homogenisation was performed as described in chapter 2.4.3 (with PBS), however, in order to obtain enough material for preparation, the supernatants of 20 homogeniser tubes were pooled into two 15 ml tubes. As 0.5 g of foam material were weighed into each 2 ml homogeniser tube (Peqlab, Germany), a total of 10 g foam (without eggs) was used for preparation. To remove any residual debris the two 15 ml tubes were centrifuged for 2 min at 1200 x *g*. Then the supernatant was boiled for 10 min to denature relevant glycosidases. 1.5 ml of 98 % formic acid (5% (v/v) final concentration) and 3 mg Pepsin A (in 1 ml $dH_2O$, 0.1 mg/ml final concentration; 3260 U/mg; Sigma-Aldrich Handels GmbH, Vienna, Austria) were added to the cooled extract, brought to a volume of 30 ml with $ddH_2O$ (in a 100 ml glass round-bottom flask) and incubated for proteolysis at 37°C overnight.

#### 2.6.3.2 Binding to a cation exchanger

The next day, the residual debris was removed by splitting the extract into Eppis and adjacent centrifugation at 16000 x *g* for 15 min at RT. The supernatants were taken into a beaker, the pellets were washed with 2% (v/v) HAc and centrifuged for 15 min at 16000 x *g*. After pooling the supernatants in the glass beaker, the solution was mixed with 10 ml Dowex® 50Wx8, (200-400 mesh; Roth) and left for binding for 30 min at RT. Then the gel was filled into a column (Column volume (CV): 10ml), the first residual solvent was put on the column again (to ensure the binding of the glycopeptides) and while the residual sample was being applied to the column, fractions of 50 drops (~ 2–2.5 ml) were collected. First, 2% (v/v) HAc was used for washing of the column (3 CV) and then the bound (glycol)peptides were eluted with 2 CV of 0.5 M ammonium acetate (pH 6), and their elution checked with orcinol: 2 µl of every fraction were pipetted onto a thin layer chromatography plate (TLC aluminium sheet covered with silica gel 60 $F_{254}$; Merck KGaA, Darmstadt, Germany) and, after drying, the spots were overlaid with orcinol reagent (200 mg orcinol in 100 ml 20% $H_2SO_4$) that was sprayed onto the plate. The plate was incubated for 5–10 min at 100°C, the positive fractions (bluish colour) were pooled

(approx. 15 ml) and lyophilised overnight. Usually the glycopeptides eluted in the first CV of the washing step with 0.5 M ammonium acetate (pH 6).

#### 2.6.3.3 Desalting by gel filtration

After lyophilisation, the sample (containing (glyco)peptides) was taken up in 1 ml 1% (v/v) HAc and then applied onto a Sephadex™ G-25 gel filtration column (Ø: 1.5 cm, h: 50 cm, CV= 85 ml; Amersham Pharmacia Biotech AB, Uppsala, Sweden). After sinking of the sample into the column, the Eppi was washed with another ml of 1% (v/v) HAc and the volume loaded onto the column. While washing the column with 2 CV of 1% (v/v) HAc, fractions of 120 drops (~3.5 ml) were collected. The orcinol method (like described above) was used to detect the elution of the glycopeptides. The orcinol-positive fractions were pooled and lyophilised overnight (approx. 15 ml in a glass flask).

#### 2.6.3.4 *N*-Glycosidase F digestion and purification of the *N*-glycans

The dried glycopeptides were taken up in approx. 1.2 ml dd$H_2O$ and washed from the glass flask into an Eppi. After concentration in a SpeedVac to a volume of 200 µl, 200 µl of 0.1 M ammonium carbonate, pH 8, were added and the sample boiled for 10 min to denature any residual pepsin. After cooling of the sample to RT, 3 µl of recombinant *N*-glycosidase F (1 U/µl; Roche Diagnostics GmbH, Mannheim, Germany) were added to release the *N*-glycans overnight at 37°C.

The next day, the sample was acidified with 500 µl 2% (v/v) HAc before its application onto an 8 ml (= CV) Dowex® 50Wx8 (200-400 mesh; Roth) column. Prior to application, the column was washed with 2% (v/v) HAc, then 3 CV of 2% (v/v) HAc containing the *N*-glycans, were collected in fractions of 30 drops (approx. 1.1 ml). The *N*-glycans were usually found in the first CV of the wash. Then, the peptides were eluted with 2 CV of 0.5 M ammonium acetate, pH6. The orcinol-positive fractions (*N*-glycans separated from glycopeptides) were pooled and lyophilised in a glass flask overnight.

### 2.6.3.5 Derivatisation with aminopyridine

The next day, the purified *N*-glycans were washed two times with 300 µl $ddH_2O$ from the flask into an Eppi, and lyophilised again. Then the *N*-glycans were reductively aminated with 2-aminopyridine (PA) (Sigma-Aldrich Handels GmbH, Vienna, Austria) and sodium cyanoborohydride ($NaCNBH_3$) (Sigma-Aldrich). Therefore, after boiling of the sample for 15 min with 80 µl of PA solution (100 mg PA in 76 µl HCl (conc.) and 152 µl $ddH_2O$), 4µl of $NaCNBH_3$-solution were added (10 mg $NaCNBH_3$ in 20 µl PA solution and 30 µl $ddH_2O$) and the sample incubated overnight at 90°C. In order to remove excess labelling reagent, the polysaccharides were then subjected to gel filtration with a Sephadex™ G-15 column (Ø: 1 cm, h: 50cm; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The column was washed with 1% (v/v) HAc, the sample was applied and fractions of 50 drops (~ 2–2.5 ml) were collected (with 1% (v/v) HAc as mobile phase). The elution of the PA derivatised glycans was determined by measuring the fluorescence at Ex/Em = 320/400 nm. The fractions of the first peak in the fluorescence spectrum (showing the fluorescence of PA) were pooled and lyophilised overnight. The second peak showed the elution of the excess reagent (Figure 10). The lyophilised sample was taken up in 500 µl $ddH_2O$ and purified on a Sephadex G-15 column for a second time. The fractions showing fluorescence at Ex/Em = 320/400 nm were pooled and lyophilised again. If one column was used for both filtrations, it had to be washed (to remove all PA) with 1% (v/v) HAc overnight.

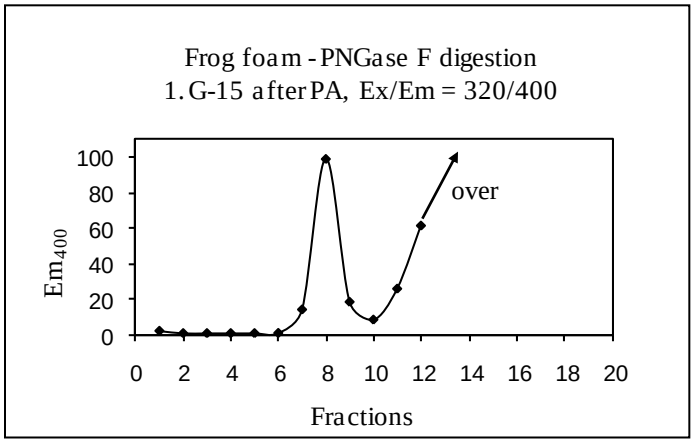



**FIGURE 10: Typical fluorescence of the fractionated sample after gel filtration.** The first peak containing PA derivatised glycans (fractions 7 to 10) was pooled, the second peak containing excess reagent was discarded. Abscissa = fractions of collected sample; ordinate = emission at 400 nm; over = too high emission, not measurable (excess reagent).

#### 2.6.3.6 Separation of pyridylaminated *N*-glycans by HPLC

##### 2.6.3.6.1 Reversed phase HPLC

The derivatised sample was taken up in 200 µl $ddH_2O$, 15 µl thereof were mixed with $ddH_2O$ to a volume of 50 µl and loaded into the HPLC-injection loop (50 µl). The glycans were separated on a Hypersil ODS C18 column (5 µm, 250 x 4 mm) using MeOH in 0.1 M ammonium acetate, pH4, (gradient of 0–9% within 30 min) as mobile phase and a flow rate of 1.5 ml/min. The derivatised glycans were detected by fluorescence (320/400 nm), every peak was collected separately into 2 ml tubes, and finally analysed by MALDI-TOF-MS (see chapter 2.6.2.5).

##### 2.6.3.6.2 Normal phase HPLC

40 µl of the sample in $ddH_2O$ were dried (to remove any water) and taken up in 20 µl 75% ACN, vortexed and centrifuged and prior to application onto the column brought to a volume of 50 µl with 75% ACN. Finally, the sample was separated on a TOSOH TSK gel Amide-80 column (4.6 x 250 mm) with a gradient starting at 71.25% ACN in 10 mM ammonium formate, pH 7, lowering to 61.75% ACN within 20 min, and to 52.25% within the next 30 min, before reversion back to 71.25% ACN within 1 min, where it was held isocratic for another 10 min. In total the run took 65 min and was performed at a flow rate of 1 ml/min and fluorescence detection at Ex/Em = 310/380 nm. As the sample was unknown, in order to save the column, a TOSOH guard column of 1 cm length and 5 µm particle size, was used upstream of the TOSOH TSKgel Amide-80 column.

Each PA fraction (i. e. every peak) was collected in a 2 ml tube, lyophilised and analysed with MALDI-TOF MS (see chapter 2.6.2.5).

## 2.7 ANALYSIS OF BIOCIDAL ACTIVITY

The biocidal activity of the foam was tested for the following microorganisms: The protozoa *Trypanosoma cruzi*, *Leishmania donovani*, *L. infantum* and *Acanthamoeba* genotype T4 were investigated for their susceptibility to the foam in microtiter plate assays, while for the bacteria *E. coli, E. hirae, M. terrae, M. avium, P. aeruginosa*, *P. mirabilis and S. aureus,* and as well as for the fungi *C. albicans*, *S. cerevisiae* and *T. mentagrophytes* agar plate diffusion assays were made. The cultures derived from the in house culture collection of the Institute of Specific Prophylaxis and Tropical Medicine of the Medical University of Vienna, Austria.

## 2.7.1 Cell culture

### 2.7.1.1 *Trypanosoma cruzi*

*Trypanosoma cruzi* strain Y 50832 (originally isolated from a Chagas patient) was cultured in sterile 12.5 $cm^2$ tissue culture flasks with plug seal caps (Becton Dickinson and Company, Franklin Lakes, USA). Therefore 8 ml LIT (ATCC 1029) liquid medium (Table 5) were inoculated with 80-100 µl of a culture, which was grown at 26°C until confluency. The culture was passaged – depending on the cell density – every 7 or 8 days.

**TABLE 5: LIT medium (ATCC 1029)**. The substances were dissolved in $ddH_2O$, and the pH adjusted to 7.2. Then, 100 ml FCS (heat-inactivated) were added. Finally the volume was adjusted to 1 litre and the medium sterile filtered.

| Substance | [g/mg/ml] | Substance | [g/mg/ml] |
|---|---|---|---|
| Liver infusion broth (Difco) | 9 g | Potassium chloride | 400 mg |
| di-Sodium hydrogen phosphate | 8 g | Sodium chloride | 1 g |
| Hemin | 10 mg | Glucose | 1 g |
| Tryptose | 5 g | | |

### 2.7.1.2 *Leishmania* spp.

*Leishmania infantum* MCAN/ES/89/IPZ 229/1/89, Zymodem MON 1 and *Leishmania donovani* MHOM/ET/67/HU3 were cultured axenically at 26°C in 8 ml MKP fluid medium (with 10 % fetal calf serum (FCS)) until confluency (Table 6). The cells were passaged every 3 to 4 days depending on cell density. Therefore, 80–100 µl of a culture were pipetted into sterile 25 $cm^2$ IWAKI tissue culture flasks with double seal caps (Asahi Glass Co., LTD., Tokyo, Japan) pre-filled with pre-warmed medium.

**TABLE 6: MKP medium (Grimm et al. 1991).** The substances mentioned were dissolved in $ddH_2O$, the pH was adjusted to 7.4, 100 ml FCS were added and the medium filled up with $ddH_2O$ to a volume of 1 litre. Afterwards it was sterile filtered.

| Substance | [g/mg/ml] | Substance | [g/mg/ml] | Substance | [g/mg/ml] |
|---|---|---|---|---|---|
| ß-Alanine | 1g | Fumaric acid $Na_2$ | 39 mg | L-Glutamic acid | 125 mg |
| L-Arginine*HCl | 270 g | NaPyruvate | 50 mg | L-Glutamine | 970 mg |
| L-Aspartic acid | 55 mg | Folic acid | 2 mg | DL-Isoleucine | 45 mg |
| L-Cystine | 15 mg | Succinic acid | 69 mg | L-Lysine*HCl | 75 mg |
| Glycine | 60 mg | Citric acid (anhyd.) | 294 mg | L-Phenylalanine | 140 mg |
| L-Histidine | 60 mg | Sodium dihydrogen phosphate dihydrate | 345 mg | Taurine | 215 mg |
| L-Leucine | 45 mg | Calcium chloride dihydrate | 75 mg | L-Tryptophan | 50 mg |
| DL-Methionine | 135 mg | Magnesium chloride hexahydrade | 1.52 g | DL-Valine | 105 mg |
| DL-Serine | 130 mg | Phenol Red | 10 mg | Guanosine | 5 mg |
| DL-Threonine | 225 mg | D(-)Fructose | 200 mg | Adenosine | 5 mg |
| L-Tyrosine | 150 mg | Sucrose | 200 mg | DL-Alanine | 645 mg |
| MEM NEAA (100x) (Sigma-Aldrich) | 5 ml | MEM vitamins (100x) (Invitrogen) | 1 ml | MEM EAGLE (Sigma-Aldrich) | 3.5 g |
| d-Biotin stock (2.5mg/100ml) | 1ml | MEM amino acids (50x) (Sigma-Aldr.) | 4 ml | Medium 199 (Invitrogen, Austria) | 1 g |
| L-Asparagine | 106 mg | L-Cystein*HCl*$H_2O$ | 45 mg | L-Malic acid | 335 mg |
| α-Ketoglutaric acid | 185 mg | p-Aminobencoic acid solution (2 mg/ml) | 0.5 ml | Sodium bicarbonate | 1.1 g |
| Potassium chloride | 1.49 g | Magnesium sulfate heptahydrate | 304 mg | L-Proline | 3.76 g |
| D(+)Glucose | 350 mg | HEPES sodium salt | 10 g | Hemin stock (2 mg/ml) | 1 ml |
| Gentamicin stock (10 mg/ml) | 1 ml | | | | |

#### 2.7.1.3 *Acanthamoeba*

*Acanthamoeba* strain DAN0012 (morphology 2, sequence type T4, clinical isolate) was cultured axenically in sterile 75 $cm^2$ IWAKI tissue culture flasks with double seal caps (Asahi Glass Co., LTD., Tokyo, Japan) in 18 ml proteose-peptone-yeast extract-glucose (PYG) medium (Table 7) at RT. For subcultivation, the acanthamoebae were resolved from the bottom of the flask by shaking. Then, 6 ml of the cell culture were pipetted into a new flask and filled with liquid medium to a volume of 18 ml. Every 3 to 4 days the old medium was replaced.

**TABLE 7: PYG medium (Visvesvara and Balamuth 1975).** The substances were dissolved in 1 litre of $ddH_2O$, the medium was sterile filtered.

| Substance | [g/mg/ml] | Substance | [g/mg/ml] |
|---|---|---|---|
| Proteose peptone | 10 g | Potassium dihydrogen phosphate | 700 mg |
| di-Sodium hydrogen phosphate | 700 mg | Sodium chloride | 5 g |
| Anitbiotic antimycotic (100x) (Invitrogen) | 10 ml | Glucose | 5 g |
| Yeast extract | 10 g | | |

#### 2.7.1.4 Bacteria and fungi

The Gram-positives *Enterococcus hirae* DSM 3320, *Staphylococcus aureus* DSM 799, the Gram-negatives *Escherichia coli* DSM 11250, *Proteus mirabilis* DSM 788 and *Pseudomonas aeruginosa* DSM 939, as well as the fungi *Candida albicans* DSM 1386, *Saccharomyces cerevisiae* DSM 70449 and *Trichophyton mentagrophytes* DSM 4870 were investigated. Furthermore the mycobacteria *Mycobacterium terrae* DSM 43227 and *M. avium* DSM 44157 were tested. The microorganisms were kept in stock cultures, and monthly prepared by streaking out frozen cultures (-80°C) onto slant agar of the appropriate medium (see below). Depending on the growth conditions (time, temperature) of the microorganisms, they were pre-cultured, before the stock was kept at 4°C in the dark: *E. hirae*, *E. coli*, *P. aeruginosa*, *P. mirabilis* and *S. aureus* were cultured on casein-peptone soy-peptone (CSA) agar and had to grow for 24 h at 36°C. *C. albicans* and *S. cerevisiae* were cultured on malt extract agar (MEA) for 48 h at 30°C, while *T. mentagrophytes* was grown for 21 d at 30°C on MEA. *M. terrae* and *M. avium* were cultured on Middlebrook 7H10 agar and had to grow for 21 d at 36°C until density. Table 8 shows the composition of the used media. Prior to use, fresh subcultures of the individual microorganisms were prepared – on agar plates of the appropriate media under the described corresponding conditions.

**TABLE 8: Media: CSA agar, MEA agar, 7H10 agar.** The substances were dissolved in dd$H_2O$, for CSA the pH was adjusted to 7.2, MEA pH 5.6 and 7H10 pH 6.6. Finally the volume was adjusted to 1 litre and the medium sterile filtered. For 7H10 the Middlebrook OADC addition was added after autoclavation and cooling of the medium to 50–55°C.

| CSA agar | | MEA agar | | 7H10 agar | |
|---|---|---|---|---|---|
| **Substance** | **[g/mg/ml]** | **Substance** | **[g/mg/ml]** | **Substance** | **[g/mg/ml]** |
| Peptone, tryptic digest of casein | 15 g | Malt extract | 30 g | Middlebrook 7H10 agar | 19 g |
| Soy peptone | 5 g | Soy peptone | 3 g | Glycerol | 5 ml |
| NaCl | 5 g | Agar | 15 g | Middlebrook OADC addition | 100 ml |
| Agar | 15 g | | | | |

### 2.7.2 Microtiter plate assays

*Trypanosoma cruzi, Leishmania donovani, L. infantum and Acanthamoeba* were tested in microtiter plate assays.

The liquid cultures of *T. cruzi* were diluted with medium to cell densities of $10^5$ cells/ml (counting with the hemocytometer; chapter 2.7.2.1). 180 µl thereof were taken and mixed in microtiter plates (Falcon® 3915, PRO-BIND™ Assay Plate, 96 wells, flat bottom, 0.4 ml; Becton Dickinson Labware, Lincoln Park, NJ, USA) with 20 µl of the homogenised and filtered foam extract in different final protein concentrations (6.25 to 125 µg protein/ml; serial dilutions with medium). Each test was performed in triplicate and repeated at least two times. The solvent used for homogenization of the foam mass was used as a negative control (20 µl) and amphotericin B (solubilized, 45% amphotericin B, 35% sodium deoxycholate; Sigma-Aldrich, Vienna, Austria) as a positive control (20 µl: final concentrations: 6.25 to 125 µg/ml). After incubation of the plates at 26°C for 48 h and 72 h, respectively (aerobic conditions, without agitation), the cells were counted with a hemocytometer (Bürker) to determine the number of motile/ viable cells/ml.

Cell densities of *L. donovani and L. infantum* were adjusted to $10^5$ cells/ml and 180 µl of the cells treated with 20 µl of the foam fluid in final protein concentrations of 6.25 to 125 µg/ml (diluted with medium). The plates were incubated at 26°C under aerobic conditions without agitation for 24 h and 48 h, respectively. The used solvent served as a negative control (20 µl) and the anti-leishmanial drug miltefosine (Formosa Laboratories, Inc., Taoyuan, Taiwan) (20 µl: final concentrations: 6.25 to 125 µg/ml) was used as a positive control.

Correspondingly, the cell density of the acanthamoebae was adjusted to $10^5$ cells/ml, then, 180 µl of the culture were treated with 20 µl of the extract in the designated final protein concentrations (6.25–100 µg/ml) and incubated for 24 h and 48 h at RT, respectively. The number of viable cells was recorded by counting the surviving cells in the Bürker hemocytomter under phase-contrast microscopy. Viability was determined by trypan blue exclusion, staining only dead cells (0.4%, prepared in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic; Sigma-Aldrich Handels GmbH, Vienna, Austria). The solvent used for homogenisation of the foam mass served as a negative control (20 µl of sterile PBS or dd$H_2O$), and miltefosine (20 µl: final concentrations: 6.25 to 100 µg/ml) as a positive control.

#### 2.7.2.1 Hemocytometer after Bürker

The Bürker hemocytometer (LO - Laboroptik GmbH, Friedrichsdorf, Germany) is divided into 9 large squares (1 $mm^2$), these being divided into 16 smaller squares (0.2 mm inner length). Four of the 16 squares were counted in diagonal and then multiplied with 4 corresponding to one large square. One large square corresponds to 0.1 µl, by multiplying with the factor 10,000 the cell count per ml could be calculated. The same procedure was performed three times, i. e. the cell number of three large squares in diagonal was determined, and afterwards the average of the three countings was calculated.

### 2.7.3 Plate diffusion assays

The bacteria *E. coli*, *E. hirae*, *P. aeruginosa*, *P. mirabilis and S. aureus*, as well as the fungi *C. albicans*, *S. cerevisiae*, *T. mentagrophytes* and the mycobacteria *M. avium* and *M. terrae* were subjected to growth inhibiton tests on agar plates.

#### 2.7.3.1 Plate diffusion assays with filter paper

Prior to use, single colonies of the subcultures (see chapter 2.7.1.4) were inoculated into 4 ml of tryptone sodium chloride solution (0.1% (w/v) tryptone, 0.85% (w/v) NaCl in dd$H_2O$) (except for fungi, which were directly taken from the plate), and finally densely plated onto fresh culture dishes with a sterile cotton swab. Three sterile filter papers (6 mm in diameter) were placed onto each freshly streaked plate. 20 µl of the homogenised sterile filtered foam extract were dropped on the filter papers in different dilutions (10 µg total protein concentration and dilutions $10^{-1}$, $10^{-2,}$ in sterile dd$H_2O$) followed by incubation of

the agar plates at 30°C or 36°C, respectively, and controlling of potential growth inhibition around the filter paper after 24 h, 48 h or 21 d, respectively, depending on the organism (see chapter 2.7.1.4).

#### 2.7.3.2 Plate diffusion assays without filter paper

As nothing was known about the chemical and physical properties of the potential biocidal agent(s) (possibly a mixture of different biocidal agents belonging to different substance classes) the plate diffusion assay was also performed without filter paper, to avoid hindering of the substance(s) in its/there diffusion onto the culture. Thus, 20 µl of the freshly prepared centrifuged and sterile filtered extract (= 6 µg protein) were pipetted directly onto the previously plated agar plates. A negative control (20 µl PBS, $ddH_2O$) and a positive control (20 µl of the disinfectant Manorapid®) were included in each setup. In order to show concentration-dependent growth inhibition, additional tests with the foam extract in higher protein concentrations and serial dilutions (17.2 µg protein/20 µl foam extract; $10^{-1}$ and $10^{-2}$) were performed for *C. albicans*, *M. avium*, *M. terrae*, and *T. mentagrophytes*. A negative control (20 µl PBS) was included.

## 2.8 MICROORGANISMS ASSOCIATED WITH THE FOAM

### 2.8.1 Isolation

After accidentally contaminating one microtiter plate with bacteria deriving from the foam, the bacteria got into our interest. To avoid contaminations, the frozen material was cut into pieces with an autoclaved saw. After each cut the saw was sterilised in the flame and small volumes from the innermost of the nest were taken with an autoclaved standard single use pipette tip or with a cooled inoculation loop sterilised in the flame. Subsequently, the samples – foam nest without eggs, and eggs only – were inoculated in 4 ml Luria broth (LB) (per liter: 5 g yeast extract, 10 g proteose peptone, 5 g NaCl, in $ddH_2O$, pH 7.0; heat-sterilised by autoclavation) and incubated for 24 h at 25°C on a shaking platform. The next day, microorganisms were transferred from the liquid cultures onto LB agar plates (LB liquid medium with 15 g bacteriological agar (OXOID LTD., Hampshire, England)), which were then incubated at RT for 2 days. Single colonies were picked and subcultured several times in order to obtain pure cultures. In total, 15 samples – foam without eggs in

14 cases, and one egg without foam – from 3 different foam nests were taken. Altogether, after subculture 25 clonal cultures of different bacteria were isolated.

### 2.8.2 Screening of bacteria for *in vitro* antibiosis

The 25 clonal cultures of different bacteria were tested for *in vitro* antibiosis. Therefore, 10 µl of bacterial suspensions, prepared under sterile conditions by scrapping single colonies from fresh agar plate cultures (grown for two days) and by adjacent inoculation into PBS ($OD_{600}$ = 0.02), were pipetted on agar plates which had been densely plated with a suspension of *C. albicans* (like described above). PBS (10 µl) was used as a negative control. The plates were incubated at 30°C for 48 h and checked for potential growth inhibition around the bacterial inoculation. Those bacterial isolates showing *in vitro* antagonism towards *C. albicans* were also tested on *M. avium*, *M. terrae* (incubation at 36°C for 21d) and *T. mentagrophytes* (21 d at 30°C). The bacterial suspensions were adjusted to $OD_{600}$= 0.002 (with dilutions: $10^{-1}$, $10^{-2}$ in PBS); 10 µl were applied onto the culture plates. PBS (10 µl) was used as a negative control.

#### 2.8.2.1 Staining of the mycelium of *T. mentagrophytes*

For microscopic analysis, mycelial samples from the periphery of the zone of inhibition of *T. mentagrophytes* and from normal growth areas were transferred directly onto coverslips by pressing the glass onto it. Scraped mycelium was stained with 1–2 drops lactophenol cotton blue (Merck KGaA, Darmstadt, Germany) and immediately examined with a phase contrast microscope (x100) (Nikon, Japan).

### 2.8.3 Characterization of the antibiotic bacteria

The bacteria showing *in vitro* biocidal effects were identified using the Analytical Profile Index (API) NE20 testing system. Strains were also identified by use of a MALDI Biotyper (Bruker Daltonics, Vienna, Austria).

#### 2.8.3.1 API

The identification of fresh single cultures of bacteria by their enzymatic specificities by API NE20 testing system was performed by members of the Institute for Hygiene and Applied Immunology, Medical University of Vienna, Austria.

#### 2.8.3.2 MALDI-Biotyper

Fresh bacterial material (LB plate culture; single colony) was scrapped into a 1.5 ml tube prefilled with 300 µl of $ddH_2O$. The suspension was washed with 900 µl of 96% EtOH and centrifuged for 2 min at maximum speed. The supernatant was discarded and the pellet dried for 2–3 min at RT to remove all EtOH. The pellet was taken up in 50 µl of 70% formic acid, then 50 µl of acetonitrile were added, the sample vortexed, centrifuged at maximum speed for 2 min, and 1 µl of the supernatant was pipetted onto the steel plate. The dried samples were covered with 1 µl Bruker $HCCA_{portioned}$ matrix (Bruker Daltonics, Vienna, Austria; dissolved in 250 µl of 50% (v/v) acetonitrile and 2.5% (v/v) trifluoroacetic acid), redried at RT and used for MALDI-TOF-MS analysis with the MALDI-Biotyper (Bruker Daltonics, Vienna, Austria). The test of each culture was performed in triplicate and the spectra analysed by use of the MALDI-Biotyper software that compares the results with the reference spectra from the database (status of June 2009) and calculates the log(score) values of the samples. The identification results can then be classified into four categories: a value of more than 2.3 indicates a highly probable species identification, a log(score) range of between ≤2.299 and ≥2.000 indicates secure genus identification with probable species identification, while a value of between ≤1.999 and ≥1.700 indicates probable genus identification and a log(score) value of ≤1.699 shows no reliable identification.

# 3 RESULTS

## 3.1 SOLUBILITY AND HOMOGENISATION

Nest foams of *Leptodactylus pentadactylus* are very stable and do not shear easily. Testing showed that the foam does not dissolve in any of the used solvents. Vortexing of the foam with 100 µl $ddH_2O$ did not dissolve the material. Moreover, the foam could not be dissolved by vortexing (up to 4 min) with any other tested solvent: neither with 30% (v/v), 50% (v/v), 70% (v/v) nor with 96% EtOH homogenous solutions could be achieved. Also, increasing the ratio of organic solvent by addition of MeOH and DMSO did not lead to solution of the foam. The foam mass could not be dissolved in 100% MeOH nor in 100% DMSO, respectively. Summing up, it could neither be dissolved in $ddH_2O$ nor in the organic solvents MeOH or DMSO by vortexing.

With the sonicator the best suspension was seen after sonication for 70 min with $ddH_2O$ in 1.5 ml tubes. With DMSO and the pure sample without solvent, a viscous suspension was also achieved, however, still with foam fragments that were not homogenised, while on the other hand, with EtOH or MeOH, fragments of the foam (possibly proteins) were precipitated after 70 min. Moreover, heat development that was observed during sonication intensified the precipitating effect of the solvents MeOH and EtOH. Heating of the material led to temperatures of approx. 40°C on the outer tube side, however, not showing the inner temperature. Because of the long duration of the process (70 min), efficient cooling of the substances was not possible, while heat development during homogenisation of the foam with the Bead Beater could efficiently be prevented by cooling the tubes on ice before and immediately after homogenisation.

Moreover, the use of the Bead Beater resulted in the generation of clear suspensions containing no residual fragments, while grinding of the frozen material with mortar and pestle did not result in homogenous suspensions. Despite long and carefully grinding of the frozen material, foam fragments were still contained in the suspension, making the following filtration with the 0.2 µm filter impossible.

The use of the three different homogenisation methods – sonicator, mortar and pestle and the Bead Beater – resulted in the same protein pattern of the extracts as can be seen in the SDS-PAGE in Figure 11. Thus, after considering the disadavantages and quality of homogenisation, the Bead Beater was used as method of choice for all further approaches.

Additionally, the quality of homogenisation with the Bead Beater did not depend on the time of homogenisation (2 x 20 sec, 1 x 20 sec, 2 x 10 sec or 1 x 10 sec). The protein pattern was the same, and the protein concentration did not show any significant difference between the different durations of homogenisation. However, as with the homogenisation for 2 x 10 sec the fewest heat development, with cooling in between, was achieved, it became the method of choice.

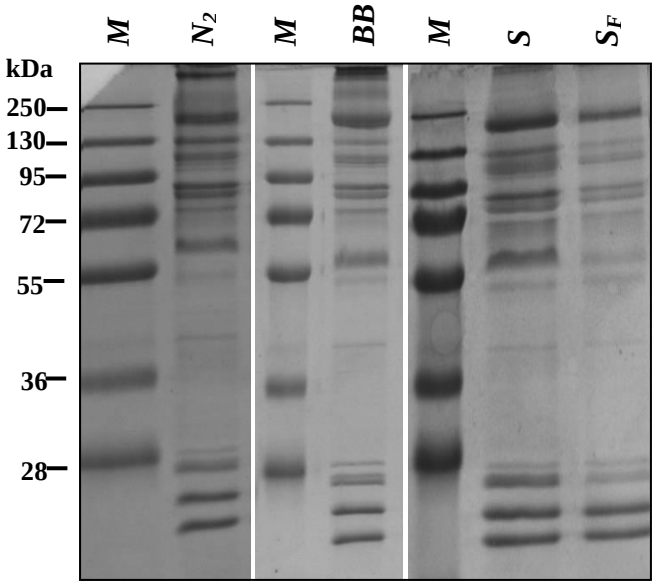



**FIGURE 11: Protein content of the foam mass after $N_2$-method, Bead Beater or sonication.** Crude extracts after grinding with mortar and pestle (**$N_2$**), homogenisation with the Bead Beater (**BB**) or with the sonicator (**S**) (dd$H_2O$ as solvent). **S** and **$S_F$:** Comparison of the protein composition of the crude extract before (left) and after filtration (right) with a 0.2 µm filter. No loss of protein bands can be observed, only loss of intensity of the protein bands in the sample before and after filtration. Coomassie Blue-stained SDS-PAGE (12% T). Molecular masses are indicated in kDa. Peqgold Protein Marker V (10–250 kDa) (**M**).

Figure 12 shows that between the singular steps of homogenisation, no difference in the protein composition can be detected: the untreated extract, whether after use of the Bead Beater, sonication or frozen grinding, showed no difference to that after centrifugation in the ultracentrifuge for 30 min (16000 x *g*), to that with two times 30 min centrifugation or to that with additional filtration. Thus, no distinctive proteins (i.e. a distinctive band, in a distinctive kDa-range) were lost in any of the steps. Only a loss of intensity was seen between the individual steps, e.g. after centrifugation and filtration (Figure 11).

## 3.2 PROTEIN CHARACTERISATION

Analysis of the protein concentration and results of SDS-PAGE showed that *Leptodactylus pentadactylus* foam nests are rich in proteins with sizes ranging from 180 to approx. 20 kDa. The pattern can be clearly distinguished from that of *Polypedates leucomystax* not showing proteins in the 20 kDa range, for example, where *L. pentadactylus* is giving strong bands in SDS-PAGE (Figure 12). Protein concentrations of the Bead Beater crude extract (centrifuged once for 30 min at 16,000 x *g*) of *Leptodactylus pentadactylus* foam were between 1.5 and 2.2 mg/ml and between 1 and 1.5 mg/ml for the additionally filtered extract, when using ~0.5 g foam material and 1100 µl liquid without further dilution prior to filtration, thus, giving a protein concentration of ~3.5–5.5 mg protein/g foam, depending on the sample. As the eggs were removed before homogenisation of the extract, the proteins of the foam did not come from disrupted eggs. Moreover, as with each approach the protein concentrations varied, the components seemed to be not regularly distributed in the foam nest.

Moreover, the proteins found in the foam nests showed stability for several weeks, without degradation or loss of protein bands in SDS-PAGE (tested after one, and three weeks) during storage at -20°C, and thawing for several times in between (Coomassie stained gels not shown).

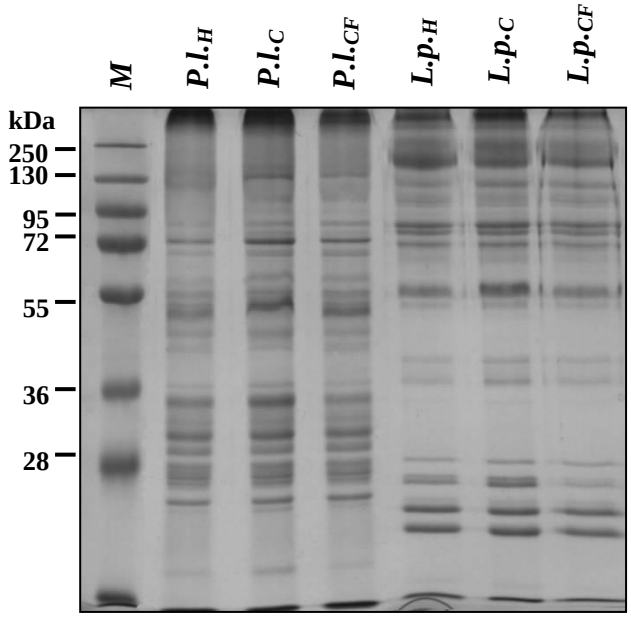



**FIGURE 12: Protein composition of the foam nests of *L. pentadactylus* (L.p.) and *Polypedates leucomystax* (P.l.).** *L. pentadactylus* and *P. leucomystax* show different proteins in their foam nests. No difference in the protein composition between the homogenised (**H**), additionally centrifuged (30 min, 16000 x *g*) (**C**) and the centrifuged and filtered extract (**CF**) can be seen. 10 µg protein per lane. Coomassie Blue-stained SDS-PAGE (12% T). Molecular masses are indicated in kDa. Peqgold Protein Marker V (10–250 kDa) (**M**).

## 3.3 GLYCOSYLATION

The initial experiments were directed at gaining an impression of the glycome of the foam nest of this organism. Both, Western blotting of proteins as well as mass spectrometry of the entire spectrum of released *N*-glycans after *N*-glycosidase F (PNGase F) digestion showed that the proteins of the foam nests of *Leptodactylus pentadactylus* are highly *N*- and also *O*-glycosylated.

### 3.3.1 *N*-Glycome

Comparison of the mass spectra of the total released unlabelled *N*-glycans obtained from different foam extracts – during "in-gel release method" of crude, centrifuged or additionally filtered extract – showed no differences in their constituents independently of prior foam treatment indicating that with centrifugation or filtration no glycans are lost (Figure 13a–c). Moreover, "the in-gel release method" gave the same glycans as the higher amount preparation with subsequent pyridylamination – the masses (*m/z* values) of unlabelled and labelled glycans were comparable with a difference in *m/z* of 78 (indicating pyridylamination) between the major species (Figure 13).

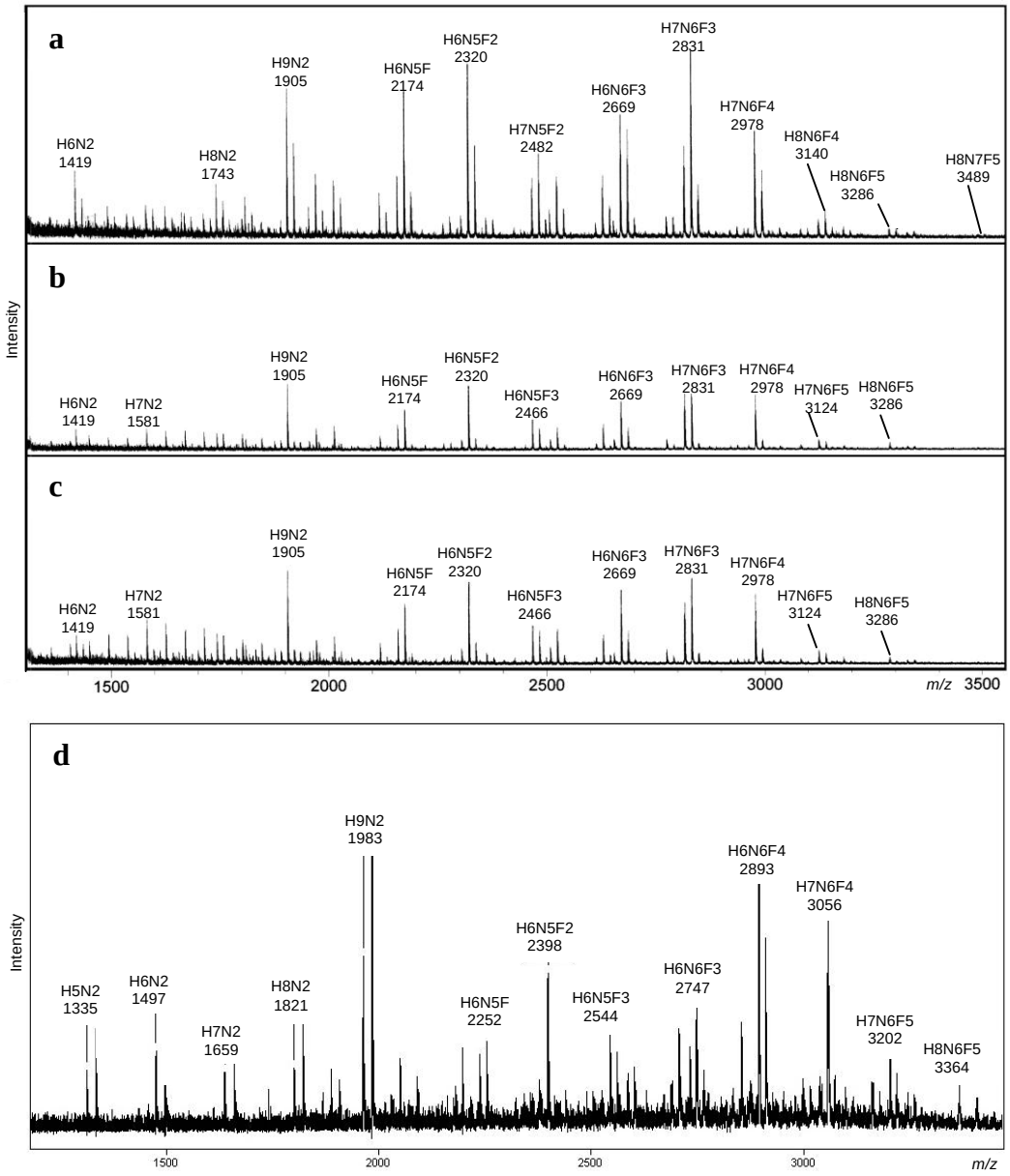



**FIGURE 13: MALDI-TOF-MS analysis of unlabelled and pyridylaminated *N*-glycans of the foam nest of *L. pentadactylus*, respectively, released from glycopeptides using *N*-glycosidase F in different methods.** Comparison of spectra of total unlabelled glycans after preparation with the "in gel-release method" of the foam in homogenised form (**a**), with subsequent centrifugation in the UC at 16000 x *g* for 30 min (**b**) and with consecutive filtration (**c**), and additionally, of total labelled glycans after high amount preparation and pyridylamination (**d**). The major species $[M+Na]^+$ are given in the form H*x*N*y*F0–5 (H...hexose; N...*N*-acetylhexosamine; F...fucose). The spectra of unlabelled *N*-glycans are comparable among themselves (**a–c**) and to those of pyridylaminated glycans (**d**) (comparison of the two spectra requires consideration of a difference of *m/z* 78 for pyridylamination for each of the species).

In total, approximately 60 *N*-glycan species could be structurally differentiated by mass spectrometry. Table 9 summarises the *N*-glycan species (*m/z* values) of the foam nest proteins purified using the different preparation and separation methods ("in-gel release method" of crude, centrifuged and filtered extracts; normal-phase HPLC, and reversed-phase HPLC of pyridylaminated glycans), as detected by mass spectrometry. The results of additional tandem mass spectrometry (MS/MS) confirmed the nature of the glycans having the predicted compositions that are given in the form H*x*N*y*F0–6, where H is hexose, N is *N*-acetylhexosamine (HexNAc) and F is fucose.

**TABLE 9: Table of masses of the major *N*-glycans released.** Major masses (*m/z* values) of unlabelled ($[M + H]^+$, $[M + Na]^+$) and pyridylaminated *N*-glycans ($[M + PA + H]^+$, $[M + PA + Na]^+$) with the predicted compositions. Glycans with these compositions were detected by MALDI-TOF-MS. Retention times in HPLC are given in terms of g.u. values, compared to the elution of an isomalto-oligosaccharide standard (3–11 g.u.). g.u. = glucose units; *= species only found once in one fraction after normal-phase HPLC.

| ***m/z*** **$[M+H]^+$** | ***m/z*** **$[M+Na]^+$** | ***m/z*** **$[M+PA+H]^+$** | ***m/z*** **$[M+PA+Na]^+$** | **Predicted composition** | **Retention in normal phase HPLC (g.u.)** |
|---|---|---|---|---|---|
| 911 | 933 | 989 | 1011 | H3N2 | 4.4 |
| 1057 | 1079 | 1135 | 1157 | H3N2F | 4.8 |
| 1072 | 1094 | 1151 | 1172 | H4N2 | 4.1 |
| 1114 | 1136 | 1192 | 1214 | H3N3 | 6.4 |
| 1235 | 1257 | 1313 | 1335 | H5N2 | 6 |
| 1317 | 1339 | 1395 | 1417 | H3N4 | 5.5 |
| 1397 | 1419 | 1475 | 1497 | H6N2 | 7 |
| 1438 | 1460 | 1516 | 1538 | H5N3 | 6.4 |
| 1479 | 1501 | 1557 | 1579 | H4N4 | 6.3 |
| 1559 | 1581 | 1637 | 1659 | H7N2 | 8.2 |
| 1641 | 1663 | 1719 | 1741 | H5N4 | 7 |
| 1721 | 1743 | 1799 | 1821 | H8N2 | 8.5 |
| 1746 | 1768 | 1824 | 1846 | H6N3F | 7.1 |
| 1787 | 1809 | 1865 | 1887 | H5N4F | 7.1 |
| 1803 | 1825 | 1881 | 1903 | H6N4 | 8 |
| 1883 | 1905 | 1961 | 1983 | H9N2 | 9.4 |
| 1933 | 1955 | 2011 | 2033 | H5N4F2 | 8 |
| 1949 | 1971 | 2027 | 2049 | H6N4F | 8.5 |
| 1990 | 2012 | 2068 | 2090 | H5N5F | 8.2 |
| 2045 | 2067 | 2123 | 2145 | H10N2 | 10 |
| 2095 | 2117 | 2173 | 2195 | H6N4F2 | 8.9 |
| 2136 | 2158 | 2214 | 2236 | H5N5F2 | 8.5 |
| 2152 | 2174 | 2230 | 2252 | H6N5F | 9 |
| 2242 | 2264 | 2320 | 2342 | H6N4F3 | 8.5 |
| 2257 | 2279 | 2335 | 2357 | H7N4F2 | 10 |
| 2282 | 2304 | 2360 | 2382 | H5N5F3 | 8.5 |
| 2298 | 2320 | 2376 | 2398 | H6N5F2 | 9.5 |
| 2339 | 2361 | 2417 | 2439 | H5N6F2 | 7.7 |
| 2403 | 2425 | 2482 | 2503 | H7N4F3 | 9.8 |
| 2429 | 2451 | 2507 | 2529 | H5N5F4 | 8 |
| 2444 | 2466 | 2522 | 2544 | H6N5F3 | 9.8 |
| 2460 | 2482 | 2538 | 2560 | H7N5F2 | 10.5 |
| 2486 | 2508 | 2564 | 2586 | H5N6F3 | 9.5 |
| 2501 | 2523 | 2579 | 2601 | H6N6F2 | 9.6 |
| 2590 | 2612 | 2668 | 2690 | H6N5F4 | 10.1 |
| 2606 | 2628 | 2684 | 2706 | H7N5F3 | 10.8 |
| 2631 | 2653 | 2709 | 2731 | H5N6F4 | 10.5 |
| 2647 | 2669 | 2725 | 2747 | H6N6F3 | 10.5 |
| 2752 | 2774 | 2830 | 2852 | H7N5F4 | 11 |
| 2779 | 2801 | 2857 | 2879 | H5N6F5 | 9.8 |
| 2793 | 2815 | 2871 | 2893 | H6N6F4 | 10.5 |
| 2809 | 2831 | 2887 | 2909 | H7N6F3 | 11 |
| 2851 | 2873 | 2929 | 2951 | H6N7F3 | 11.2 |
| 2899 | 2921 | 2977 | 2999 | H7N5F5 | 10.8 |
| 2914 | 2936 | 2992 | 3014 | H8N5F4 | 12 |
| 2940 | 2962 | 3018 | 3040 | H6N6F5 | 11 |
| 2956 | 2978 | 3034 | 3056 | H7N6F4 | 11.3 |
| 2997 | 3019 | 3075 | 3097 | H6N7F4 | 11.2 |
| 3061 | 3083 | 3139 | 3161 | H8N5F5 | 12.2 |
| 3102 | 3124 | 3180 | 3202 | H7N6F5 | 11.5 |
| 3118 | 3140 | 3196 | 3218 | H8N6F4 | 12 |
| 3143 | 3165 | 3221 | 3243 | H6N7F5 | 11 |
| 3159 | 3181 | 3237 | 3259 | H7N7F4 | 12.2 |
| 3264 | 3286 | 3342 | 3364 | H8N6F5 | 12.2 |
| 3305 | 3327 | 3383 | 3405 | H7N7F5 | 12.2 |
| 3346 | 3368 | 3424 | 3446 | H6N8F5 | 12 |
| 3467 | 3489 | 3545 | 3567 | H8N7F5 | 12 |
| 3508* | 3530* | 3586* | 3608* | H7N8F5* | 12.5* |
| 3654* | 3676* | 3732* | 3754* | H7N8F6* | 12.5* |

The results of mass spectrometry indicate that the major species are highly fucosylated, often only differing in their composition in the number of fucose residues. The glycan species with the greatest mass found abundantly in all methods, is calculated as $H_8N_7F_5$, which means the presence of 8 hexoses, 7 *N*-acetylhexosamines (HexNAc) and 5 deoxyhexoses (putatively fucoses) in the structure, however, not giving any clue to the actual identity of the structure. The variety of possible structures is high, and the indication "hexose" can still mean the presence of mannose or galactose, as well as "*N*-actetylhexosamine" can mean both stereochemistry of glucose as well as of galactose. Furthermore, calculation of the putative composition is not giving any information on the actual linkages of the several substructures in the complete glycan.

In order to analyse the masses in more detail, the pyridylaminated *N*-glycans were subjected to normal-phase and reversed phase HPLC. Figure 14 shows the chromatograms of the total pyridylaminated *N*-glycans eluting in different fractions dependent on size and polarity. After separation, the collected fractions that contain some single *N*-glycans, were analysed by mass spectrometry and additionally their glycans by MS/MS in order to get more information on some of the major species.

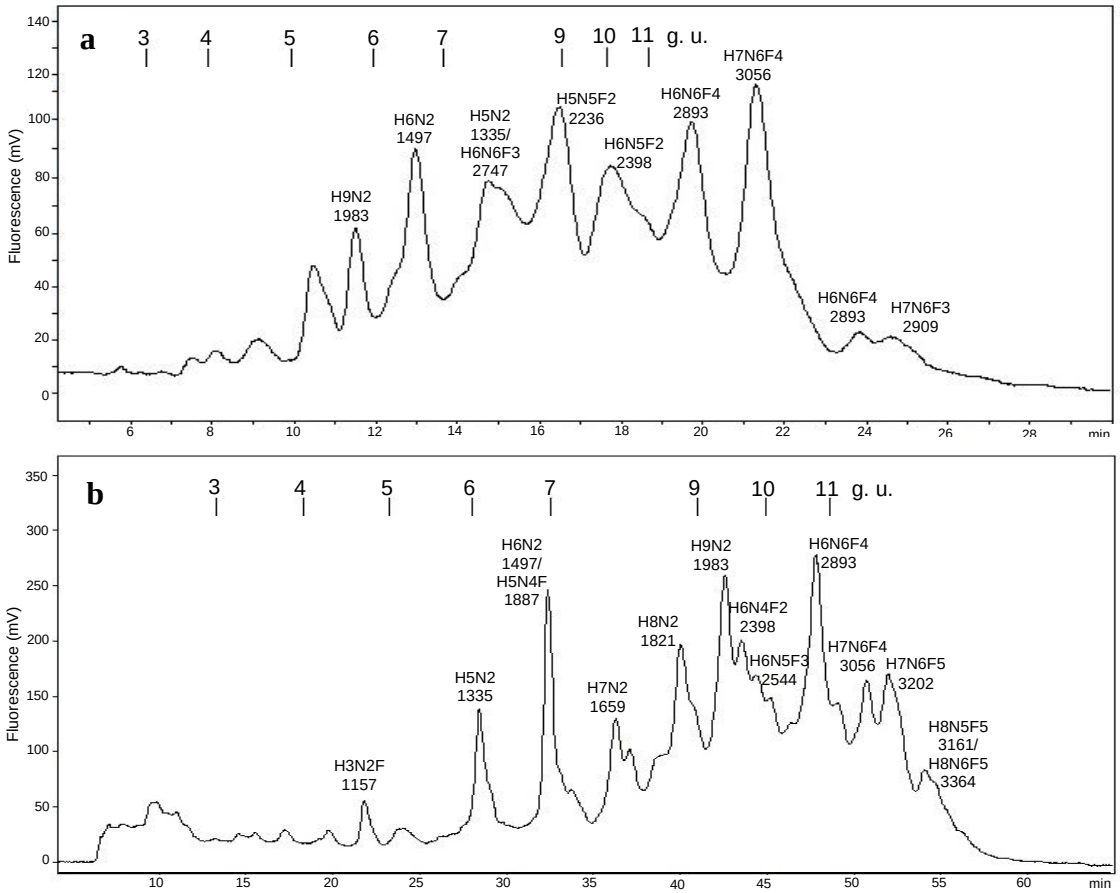



**FIGURE 14: Reversed phase (a) and normal phase (b) HPLC chromatogram of pyridylaminated *N*-glycans.** Peaks were collected and analysed by MALDI-TOF-MS (major glycan species are annotated). The masses of the *N*-glycans are given in the form H*x*N*y*F0–5 (H...hexose; N...*N*-acetylhexosamine; F...fucose). Isomalto-oligosaccharide standards were carried along and their elution positions are shown in terms of their g.u. values (glucose units).

### 3.3.1.1 Exemplary *N*-glycan species

The MS/MS spectra of the *N*-glycan species give indications to some substructures, for example to those of the masses *m/z* 2376 $[M+H]^+$ ($H_6N_5F_2$) and *m/z* 2230 $[M+H]^+$ ($H_6N_5F$) (Figure 15) only differing in their compositions by the presence of one fucose. According to the given fragments, the positions of four HexNAc (N) residues in both glycans are assumed as follows: as natural for an *N*-glycan, there may be two HexNAc residues within the glycan core. First, the fragment *m/z* 299 showed pyridylamination of the reducing-terminal HexNAc residue. The fragment *m/z* 503 was considered diagnostic for the second core HexNAc, in this case both being *N*-acetylglucosamine (GlcNAc) residues as part of the core. The other two HexNAc are indicated as "branching" modifications of the core mannose residues. One seems to be in the so-called "bisecting" position, which means it is bound to the central β1,4-linked mannose residue of the core, as indicated by the *m/z* 868 fragment (loss of *m/z* 203 was also indicative for peripheral HexNAc, putatively of a

bisecting GlcNAc). The other one is putatively linked to the α1,3-linked mannose residue. This indication is based in particular on the fragment of *m/z* 1151 suggesting a hybrid-type structure for both species, thus, only leaving the fourth HexNAc residue as antennary on the α1,3-mannose arm (putatively GlcNAc).

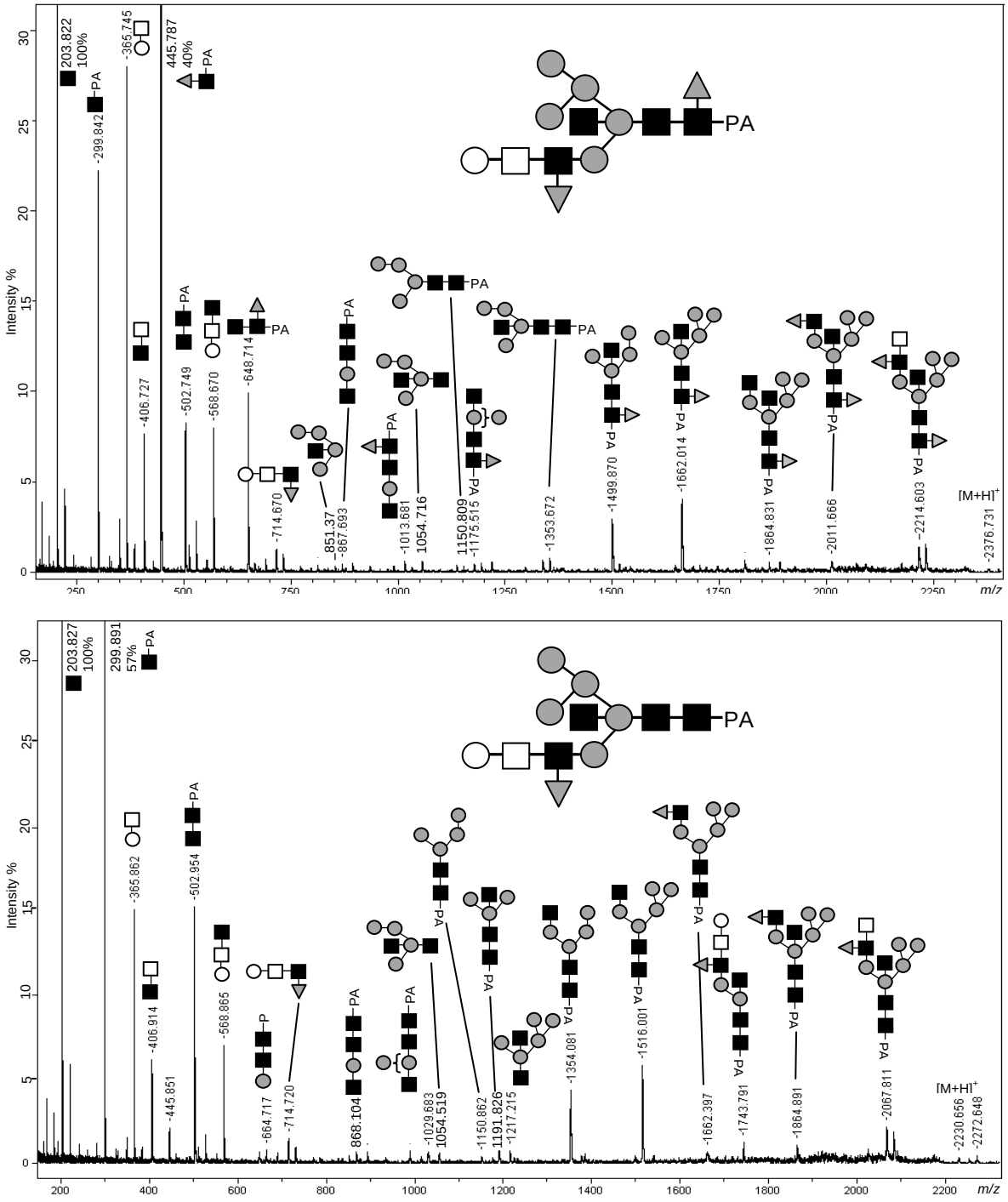



**FIGURE 15**: **MS/MS analysis of two major pyridylaminated *N*-glycans after release with PNGase F.** The postulated complete glycan structures of the masses *m/z* 2376 $[M+H]^+$ ($H_6N_5F_2$) (**a**) and *m/z* 2230 $[M+H]^+$ ($H_6N_5F$) (**b**) are given, however, the identities and linkages of the sugar residues are not fully proven. The symbols correspond to the nomenclature of the Consortium for Functional Glycomics (http://www.functionalglycomics.org): circles: hexoses; squares: *N*-actetylhexosamines; black: glucose stereochemistry; white: galactose stereochemistry (suggested); gray: mannose stereochemistry; gray triangle: fucose.

The supposed hybrid structures of both species, in which only mannose residues are attached to the Manα1-6 arm of the core (in both cases putatively 2 mannoses are attached to the arm) and one antenna is initiated by a GlcNAc residue on the Manα1-3 arm, also corresponds to the binding of the lectin ConA of the additional lectin blots (Figure 16). This lectin interacts in its highest affinity with α-mannose residues of oligomannose-type and hybrid-type *N*-glycans, and with only low affinity binds to complex-type biantennary *N*-glycans, while it fails to bind to complex-type *N*-glycans that are more highly branched.

Moreover, in case of the glycan species *m/z* 2376 $[M+H]^+$, one of the two fucoses present in the total structure of $H_6N_5F_2$ seems to be attachted to the pyridylaminated core GlcNAc, indicated by the presence of the structures *m/z* 446 and *m/z* 649, whereas these fragments were (almost) lacking from the species *m/z* 2230 $[M+H]^+$ ($H_6N_5F$). Although the linkage of the fucose to the core GlcNAs is not verified, a standard α1,6-linkage known as the main core modification in vertebrates is supposed.

However, although the composition of the structures, the presence of hybrid structures, bisecting HexNAc and one HexNAc initiating an antenna at the Manα1-3 arm (both putatively GlcNAc) in both *N*-glycan species, as well as core fucosylation of the species *m/z* 2376 $[M+H]^+$ seem to be conclusive, the postulated structures are not yet fully proven.

The actual position of the fifth HexNAc, as well as the position of the remaining hexose (and of course their identities, which are given as *N*-acetylgalactosamine and galactose, respectively) cannot unambiguously be confirmed by MS/MS. The presence of the fragments *m/z* 365, 406 and 568 were considered indicative for the presence of a peripheral Hex-HexNAc-GlcNAc residue on the Manα1-3 arm, i.e. the binding of the last hexose and the fifth HexNAc to the initiative GlcNAc of the Manα1-3 arm. Further branching of the Manα1-3 arm by the last HexNAc does not seem to be likely, as the *m/z* 406 fragment (indicating the presence of two linked HexNAc units) is abundantly present in both spectra, and could not be found with further branching. However, it is also possible that one or both of the remaining fragments are linked to the bisecting GlcNAc, which would also be conclusive with the fragments given.

Moreover, the position of the one fucose of the *m/z* 2230 $[M+H]^+$ species is not definite. It does not seem to be attached to the core as the fragments *m/z* 446 and *m/z* 649 are underrepresented. However, the actual position cannot be accurately identified based on the results of the examinations made. It seems to be attached somewhere to the Manα1-3 linked antenna.

Thus, the total structures given in figure 15 are, although being the most probable ones, however, only partially based on distinct unambiguous results of MS/MS, but rather on the knowledge of the *N*-glycan core structure and possible residues and linkages typical for vertebrates, which is still not excluding any other possibilities.

### 3.3.2 Total glycosylation

The use of lectins and antibodies for blotting was helpful to gain more insight into details of the actual structures of all glycans of the glycoproteins of the foam nest.

The used lectins and antibodies bind specifically and reversibly to specific sugar residues on glycoproteins. Table 10 summarizes the used lectins and antibodies, their binding specificities, whether they were binding to the foam nest glycoproteins or not and the possible meaning of the results for the foam nest glycan structures. In a second step, the glycoproteins were subjected to digestion with PNGase F, deglycosylating glycoproteins by cutting the *N*-linkage between the *N*-acetylglucosamine residue and the asparagine. If the digestion with PNGase F resulted in reduction of lectin/ antibody binding intensity, then the sugar residue was assumed to be on an *N*-glycan. Otherwise, some other kind of glycan (possibly an *O*-glycan) bearing this/ these sugar residue(s) could be presumed. Figure 16 shows the corresponding lectin blots.

**TABLE 10: Used lectins and antibodies and their binding to *L. pentadactylus* foam nest glycoproteins.**

| Lectin | Major specificity | | Binding | Possible meaning | PNGase F Digestion |
|---|---|---|---|---|---|
| **AAL** | Core α1,6-Fuc or $Le^x$ | Fucose | + | Presence of *N*-glycan core α1,6-Fuc, and peripheral $Le^x$-like, and α1,2-linked fucose | +/- |
| **LCA** | Core α1,6-Fuc | | + | | +/- |
| **LTA** | $Le^x$ | | + | | +/- |
| **CD15** | $Le^x$ | | -[1] | | / |
| **P12** | $Le^x$ | | - | | / |
| **UEA** | R"-(Fucα1,2)Gal-R | | + | | +/- |
| **LEA** | polyLacNAc R"[-3Galβ1,4GlcNAcβ1-]$_n$R | Galactose | + | polyLacNAc | - |
| **ECL** | Gal(NAc)ß1,4GlcNAc-R | Galactose | - | no terminal β-bound Gal or GalNAc | / |
| **PNA** | Galß1,3GalNAc-Ser/Thr | | - | | / |
| **GSL I-$B_4$** | Galα1,3Gal-R | | + | terminal α-bound Gal or GalNAc, αGalNAc-Ser/Thr | - |
| **RCA I** | R'-Galβ1,4GlcNAc-R | | +[2] | | + |
| **HPA** | α-GalNAc-R/Ser/Thr | *N*-Acetyl-galactos-amine | + | | +/- |
| **VVA $B_4$** | α/β-GalNAc-R/Ser/Thr | | +/- | | - |
| **Jac** | (NeuA)Galβ1,3GalNAc-R α-GalNAc-R | Sialic acid | +[3] | Possibly α2,3-linked, but no α2,6-linked sialic acid | + |
| **SNA** | NeuAα2,6Gal-R | | - | | / |
| **WGA** | NeuAα2,3Gal-R or HexNAcβ1,4-R | | +[4] | | +/- |
| **ConA** | α-Man | Mannose | ++ | High-mannose or hybrid type *N*-glycans | / |

(+…binding; -...not binding; /... not tested; R indicates attachment to the remaining part of an *N*- or *O*-glycan structure; R' (in the case of RCA I) can be a hydrogen or an α2,6-linked sialic acid; R" can be any sugar residue or a hydrogen.

[1] The positive control of the antibody CD15 did not show any results either, thus, the antibody seems to have lost its binding capacity.

[2] According to Iskratsch et al. (2009) RCA I also binds to Galα1,3Gal-R and NeuAα2,6Gal-R, which can explain that the non-binding of ECL is excluding the binding of RCA I to its major specificity. The binding capacity of RCA I is high, it is binding to a number of substituted Galβ1,4GlcNAc residues.

[3] As the binding of Jacalin to one of its specificities (Galβ1,3GalNac-R) can be excluded (PNA), the positive reaction seems to derive from the binding to an α-GalNac residue or to NeuAα2,3Gal-R.

[4] The binding specificity of WGA is wide spread and therefore cannot be used for definite characterisation of a specific glycan residue.

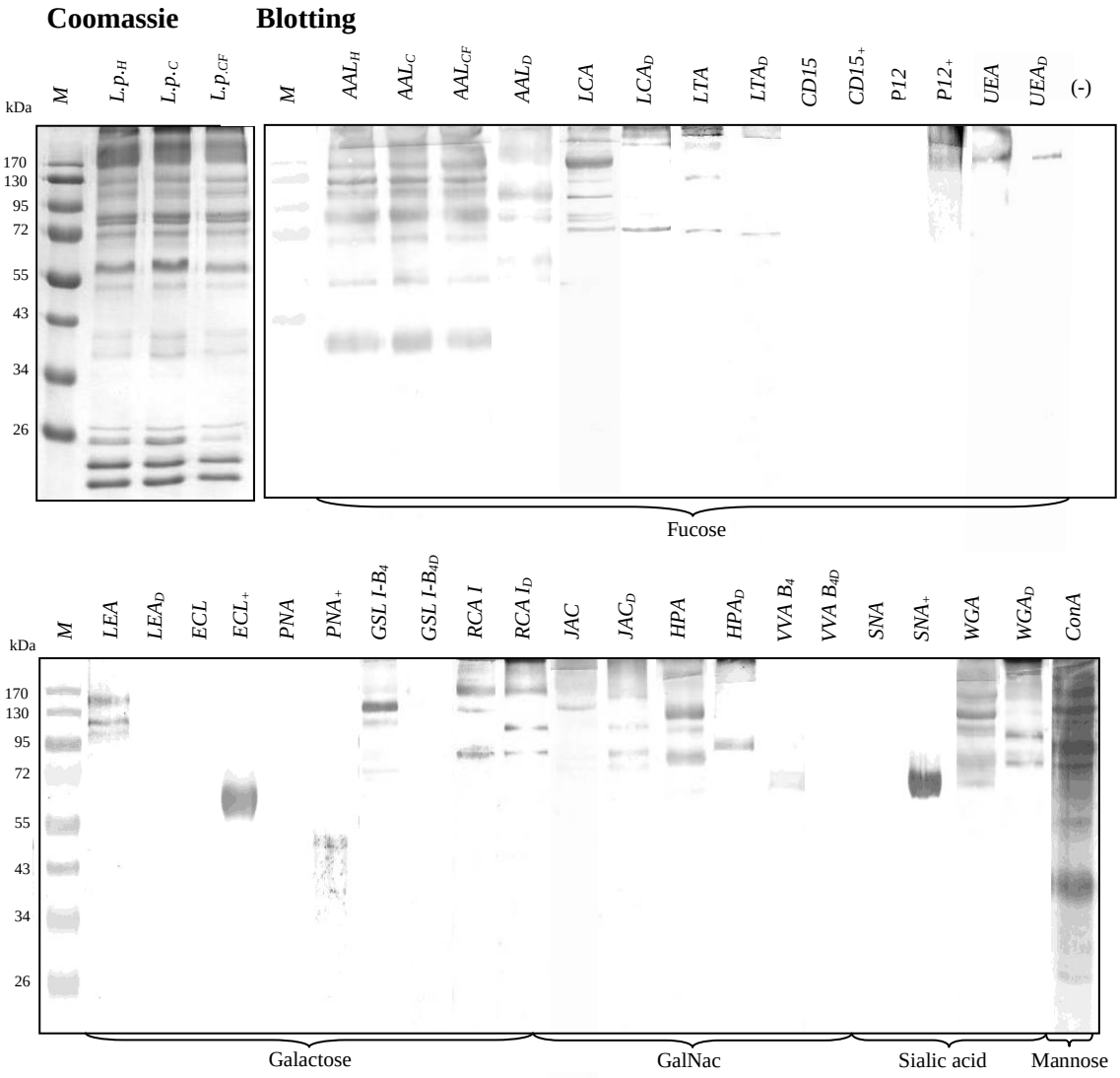



**FIGURE 16: Binding of the lectins in glycoprotein blots.** The Coomassie Blue-stained gel shows that the protein loading was approximately equal (10 µg/ lane) for the singular steps of foam preparation; the homogenised foam (*L.p.*$_{H}$), with the subsequent centrifugation at 16000 x *g* for 30 min (*L.p.*$_{C}$) and with consecutive filtration of the centrifuged extract (*L.p.*$_{CF}$). No differences in lectin binding between the singular steps (only results of AAL are shown, although the blots were performed in triplicate for each lectin). For the specificities of the used lectins, see Table 4 and 10. PageRuler$^{TM}$ Prestained protein ladder (26–170 kDa) (M); D...PNGase F digestion; +...positive control (see text), (-)...negative control (without lectin).

Comparable to the results of mass spectrometry of the released *N*-glycans, there was no difference in the binding of lectins and antibodies to total glycoproteins (with all sorts of peptide glycosylation) independently of the preparation steps. This can be seen in the equal binding of AAL to the proteins of the homogenised ($AAL_H$), the additionally centrifuged ($AAL_C$), and the centrifuged and filtered extract ($AAL_{CF}$) (Figure 16). All other lectins showed the same results (data not shown).

Moreover, the blots show that with exception of the binding of ConA only peptides in a higher kDa-range than 40 kDa seem to be glycosylated. The smaller peptides in the 20–26 kDa range that are found in all of the Coomassie-stained gels were not bound by any of the used lectins, indicating that they are not glycosylated.

#### 3.3.2.1 Fucosylation

The binding of AAL, LCA, LTA and UEA demonstrates the presence of fucose in the glycans of the foam nest glycoproteins. This is also in agreement with the results of mass spectrometry and the putative compositions of the found *N*-glycan species listed in Table 9 that also indicate fucoses. The bindings of the lectins show the presence of *N*-glycan core α1,6-fucose (LCA) and peripheral α1,3-fucose in a Lewis$^x$ (Le$^x$) like linkage (Galβ1,4(Fucα1,3)GlcNAc-R) (LTA) and peripheral α1,2-fucose (R-(Fucα1,2)Gal-R) (UEA) as part of *N*- or *O*-glycans in the foam nest's glycoproteins. AAL is usually regarded as a "universal" ligand for fucosylated glycans, thus, this may explain its strong binding before and even after PNGase F digestion.

PNGase F digestion showed that the fucosylation is not only restricted to the *N*-glycan part of the glycoproteins. The only partial loss of the interaction of the three fucose binding lectins (LTA, AAL and UEA) after PNGase F digestion is indicative for the presence of both, *N*- and *O*-glycans (or other, more rare forms of peptide glycosylations), with peripheral α1,3- and α1,2-linked fucose. The loss of intensity of LCA after digestion with PNGase F is conclusive with its specificity for *N*-glycan core fucosylation.

The two antibodies CD15 and P12 that were also used to identify Le$^x$ like fucose linkages did not bind the to glycosylated peptides. As CD15 did not show any reactivity with the positive control (*Schistosoma* extract, known to carry peripheral α1,3 linked fucose in a Le$^x$ linkage), it can be assumed that the antibody has lost its binding capacity. The P12 antibody's positive control (*Schistosoma* extract), however, was bound by the antibody, which could be explained by a suggested restricted specificity of P12 to certain Lewis-type fucosylated structures only in a high density of Le$^x$ determinants or dependent on a specific underlying structure, that is possibly not given on foam nest glycoproteins.

#### 3.3.2.2 R-Galβ1,4GlcNAc residues

The binding of the tomato lectin LEA demonstrates the presence of poly-*N*-acetyllactosamine (polyLacNAc) chains, i.e. sequences of repeated Galβ1,4GlcNAcβ1,3

units. Its loss after digestion with PNGase F shows that it seems to be a moiety of the *N*-linked glycans, although they are generally known to occur on all kind of glycans. Moreover, the binding of RCA I, which is a lectin with various binding specificities, also shows the presence of Galβ1,4GlcNAc-residues, which may be part of a polyLacNac or not. RCA I binds to this kind of structure independently of its substitutes: it is known to show highest activity towards terminal Galβ1,4GlcNAc in branched *N*-glycans (but also *O*-glycans), the substitution with sialic acid α2,6-linked or of Gal α1,3-linked does not greatly affect the binding affinity. Thus, RCA I is usually not used for specific determination, as its binding capacity is high. Furthermore, the presence of the major specificity of RCA I, terminal Galβ1,4GlcNAc, is excluded by the non-binding of ECL, the major specificity of which is also towards this structure. ECL is binding primarily peripheral Galβ1,4GlcNAc (of primarily *N*-glycans but also *O*-glycans) and to a lesser extent GalNAcβ1,4GlcNAc. The lectin ECL did not show any reactivity, although the positive control (asialo-apo-transferrin) was bound by the lectin.

Thus, it can be assumed, that LacNAc or PolyLacNAc structures are present on foam nest glycoproteins. With the loss of binding of LEA after digestion, the polyLacNAc structure seems to be part of an *N*-glycan. However, it may also be *O*-linked, because of RCA I being not lost after digestion. Furthermore, it seems to be substituted due to the unbinding of ECL (peripheral Galβ1,4GlcNAc and GalNacβ1,4GlcNAc can be excluded).

#### 3.3.2.3 Galα1,3Gal and α-GalNAc residues

The terminal structures of the *N*- and *O*-glycans could be α1,3-linked Gal, which is indicated by the positive GSL I-$B_4$ binding, showing terminal Galα1,3Gal residues. Furthermore, the loss of binding after digestion with PNGase F, indicates the presence of these structures on an *N*-glycan. This is also conclusive with the binding of RCA I, that also binds Galα1,3Galβ1,4GlcNAc-R (Galα1,3LacNAc) as one of its specificities.

Another terminal structure could also be α-GalNAc, which is shown by the binding of HPA that binds α-linked GalNAc peripheral to any residue of *O*- and *N*-glycans, and also peptide-bound GalNAc (*O*-glycan). This is also corroborated by the binding of VVA $B_4$ specific for both β- and α-linked GalNAc. Since, terminal β-linked GalNAc can be excluded (ECL is not binding), the binding of both, HPA and VVA $B_4$, seems to derive from an α-linked GalNAc. As after digestion with PNGase F the binding of HPA is only partially lost, and VVA $B_4$ that was not strong anyway, had also lost its binding after

digestion, there seem to be *N*-glycans, but maybe also *O*-glycans having non-reducing terminal α-linked GalNAc residues, but also *O*-glycans, where the αGalNAc residue is bound to the peptide in an *O*-GalNAc core (Galβ1,3GalNAc-α-O-Ser/Thr or GalNAc-α-O-Ser/Thr).

The galactose binding lectin PNA did not show any reactivity with the frog nest proteins, despite the binding to the positive control (asialofetuin). PNA in its major specificity binds to peripheral Galβ1,3GalNAc, primarily of *O*-glycans (core1 and core2 *O*-glycans). Since PNA is not binding, this structure, i.e. freestanding Galβ1,3GalNAc of an *O*-glycan does not seem to be present on the foam nest glycoprotein. Thus, the binding of HPA and VVA seems to derive from the binding to Tn antigen structures (GalNAc-α-O-Ser/Thr) or to further substituted structures (see in the next chapter).

#### 3.3.2.1 Sialic acid

The *O*-GalNAc may be possibly sustituted with α2,3-linked sialic acid (= derivative of neuraminic acid; NeuA), indicated by the binding of Jac, that has the same major specificities as PNA, and was positive. The only difference in the binding of Jac is in the binding to α2,3-linked sialic acid in the linkage NeuAα2,3Galβ1,3GalNAc. This result is also confirmed by the binding of WGA, whose specificity is also for α2,3-linked sialic acid of the same structure. However, the binding specificity of WGA is wide spread, and cannot be used for definite characterisation of specific sugar residues (it is also binding to GlcNAc and GalNAc in β1,4-linkage).

On the other hand, SNA (the positive control apo-transferrin was bound), the major specificity of which is for α2,6-linked sialic acid, was negative. Thus, it can be assumed that an α2,3-linked neuraminic acid may be present, in the linkage NeuAα2,3Galβ1,3GalNAc, typical for an *O*-glycan, however, NeuAα2,6Gal can be excluded. Nevertheless, the presence of sialic acid is not verified, as the binding of Jac can also derive from αGalNAc, whose presence was indicated anyway, and that of WGA is not totally specific. As with digestion by PNGase F the binding of Jac is not lost, it really seems to derive from an *O*-glycan.

## 3.4 BIOLOGICAL ACTIVITY

The microtiter plate assays that were performed with the protozoa *T. cruzi*, *L. donovani*, *L. infantum* and *Acanthamoeba*, and the plate diffusion tests with ten different bacteria and fungi were directed at gaining an impression of the biocidity of the foam nest of *L. pentadactylus*. Treatment of the protozoal cultures and of the bacteria and fungi with the foam nest extract in different final protein concentrations showed that the foam nest had a growth inhibiting effect on four of the test organisms *in vitro*.

### 3.4.1 *Trypanosoma cruzi*

Treatment of *T. cruzi* epimastigotes with the filtered foam extract – even in the highest protein concentrations tested: 100 and 125 µg/ ml – did not result in significant reduction of cell viability compared to the negative control (PBS). Figure 17 shows the means of cell viability (in %) of *T. cruzi* after incubation with the foam extract for 48 h and 72 h, respectively. Moreover, no morphological changes of the cells could be seen in comparison to the control group. Amphotericin B, that was used as a positive control, had a 100% biocidal effect in the tested concentrations (6.25–125 µg/ml = 6.8–135 µM).

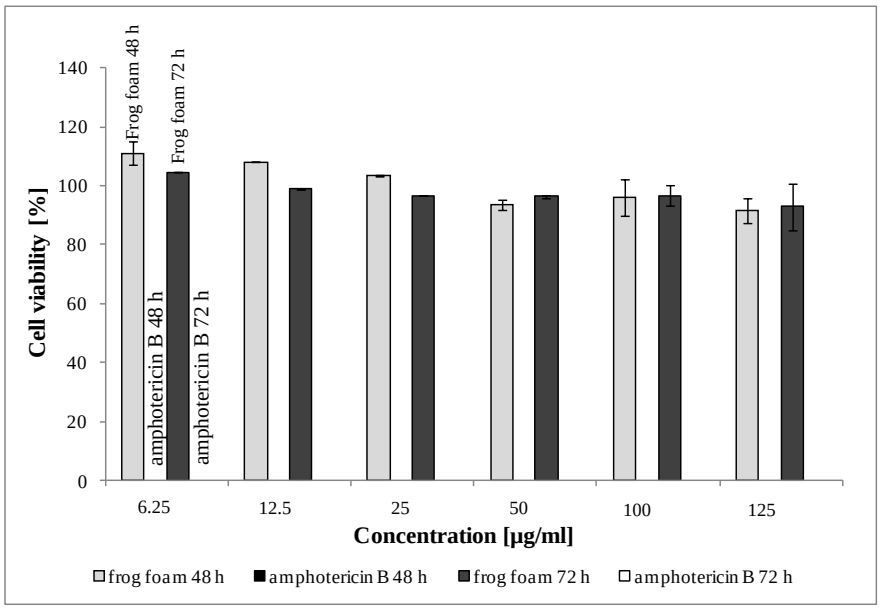



**FIGURE 17: Survival rate of *T. cruzi* after treatment with the foam for 48 h (first bars) and 72 h (third bars).** *T. cruzi* was not susceptible to the foam extract *in vitro*, no growth inhibition was seen. Each experiment was repeated at least two times in triplicate. Amphotericin B was used as positive control: treatment with final concentrations of 6.25–125 µg/ml (6.8–135 µM) for 42 h (second bars) and 72 h (forth bars), respectively.

### 3.4.2 *Leishmania* spp.

In contrast to *T. cruzi*, for both *L. donovani* as well as *L. infantum*, *in vitro* growth inhibition was observed after treatment of the cultures for 24 h and 48 h with the centrifuged and filtered foam extract in increasing protein concentrations (6.25 to 125 µg/ml). The cell viabilities (in %) of *L. donovani* and *L. infantum* are given in Figure 18.

The cells reacted to the foam extract in a concentration-dependent manner (Figure 18a): Treatment of *L. donovani* with the frog foam in the highest protein concentration (125 µg/ml) resulted in a significant reduction ($p < 0.05$) of cell viability to 17.8% after 24 h in comparison to the negative control (i.e. incubation of the culture with 20 µl PBS), while the lowest final protein concentration of 6.25 µg/ml resulted in reduction of cell viability to 90.2%.

After 48 h hours the culture recovered from treatment with the foam extract: The cell viabilities (%) were higher compared to those after treatment for 24 h. The highest concentration (125µg/ml) resulted in 48.1% cell viability compared to the control ($p < 0.05$) (Figure 18a).

*L. infantum*, in comparison to *L. donovani*, was less susceptible to the foam extract. Treatment of *L. infantum* for 24 h in the highest concentration (125 µg/ml) resulted in significant reduction of cell viability to 26.2% ($p < 0.05$), and in 61.1% cell viability after 48 h (125 µg/ml; $p < 0.05$) (Figure 18b). None of the concentrations tested resulted in total eradication of the cells, neither of *L. donovani*, nor of *L. infantum*.

Miltefosine was used as positive control (6.25–125 µg/ml = 15–307 µM). It had a 100% biocidal effect against both *L. donovani* and *L. infantum*, at concentrations of 12.5 µg/ml (= 30 µM) and 6.25 µg/ml (= 15 µM) after 24 h, respectively, and at a concentration of 6.25 µg/ml after 48 h, against both *Leishmania* strains.

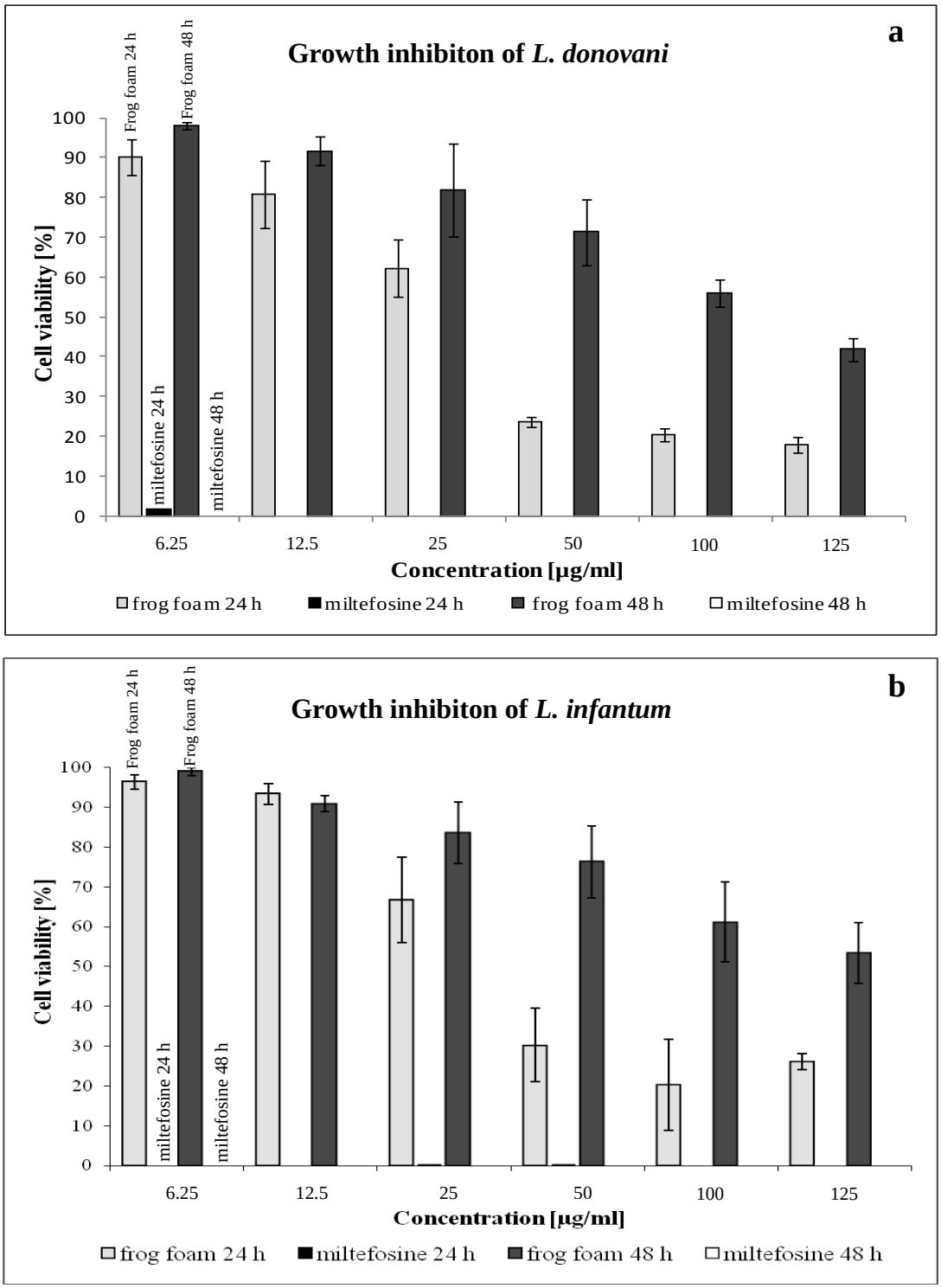



**FIGURE 18: Growth inhibition of *L. donovani* (a) and *L. infantum* (b) *in vitro*.** The results after treatment of the cells with the foam extract (final protein concentrations: 6.25–125 µg/ml) for 24 h (first bars) and 48 h (third bars) are given. Each experiment was repeated at least two times in triplicate. Miltefosine (6.25–125µg/ml) was used as a positive control (treatment for 24 h: second bars; 48 h: forth bars).

In Figure 19, the absolute cell counts of the untreated control of *L. donovani* and *L. infantum* are compared with the cell densities of the organisms after treatment with the foam extract (100 µg protein/ml) for 24 h and 48 h. In the first 24 h, the foam extract showed a cytostatic effect on both cultures at final protein concentrations of more than 100 µg/ml (only this concentration shown). This effect was lost after 48 h, when the cultures recovered from treatment with the extract. However, both, *L. donovani* and *L. infantum* still had lower cell viabilities (in %) than the control (Figure 18).

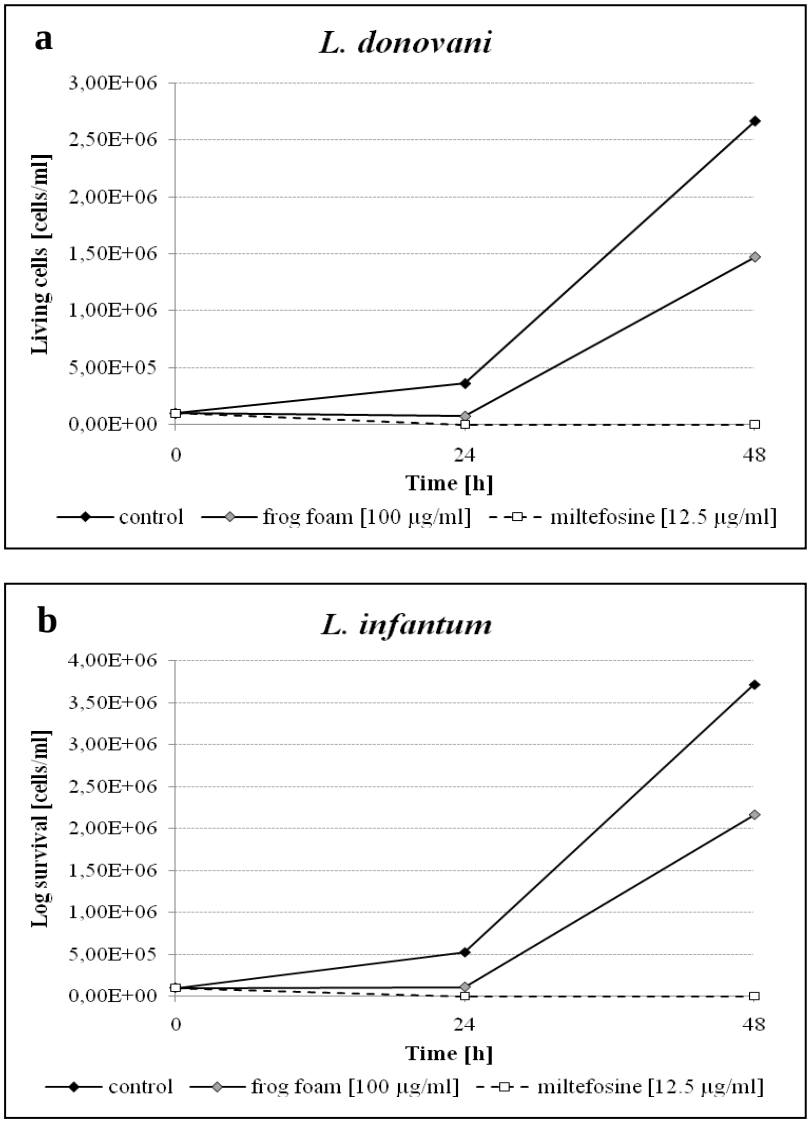



**FIGURE 19: Activity of the foam extract at a final concentration of 100 µg protein/ml on the cell counts of *L. donovani* (a) and *L. infantum* (b).** In both cases, the foam extract showed cytostatic activity in the first 24 h, which was lost in the next 24 h when the culture recovered. Miltefosin, in the concentration of 12.5 µg/ml (30 µM), showed a 100 % biocidal effect on both species after 24 h. Control...negative control (PBS).

### 3.4.3 *Acanthamoeba*

Activity against *Acanthamoeba* strain DAN0012 trophozoites was tested in microtiter plates ($10^5$ cells/ml) after 24 h and 48 h of treatment with the foam extract (centrifuged and filtered) in increasing protein concentrations (6.25–100 µg/ml). The strain was not susceptible to the foam extract, i.e growth was not inhibited and no cytotoxicity compared to the negative control was observed (Figure 20). Moreover, at a low protein concentration (6.25 µg/ml), the extract even seemed to stimulate *Acanthamoeba* growth to 106.9 % and 117.9 % compared to the control after 24 h and 48 h, respectively, however, this was not statistically significant.

Treatment with miltefosine at a concentration of 50 µg/ml (= 120 µM) resulted in a 100% reduction of the trophozoites within 48 h and a concentration of 100 µg/ml (= 240 µM) was enough to eradicate the cells within 24 h.

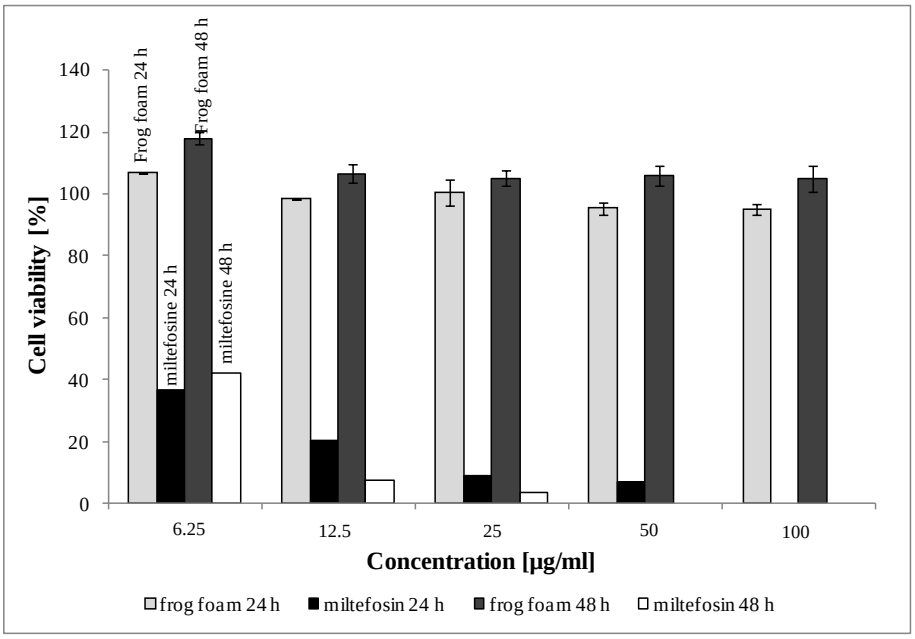



**FIGURE 20: Percent growth of *Acanthamoeba* DAN0012 after treatment with the foam extract *in vitro*.** The cells were incubated with the foam in various protein concentrations (6.25–100 µg/ml) for 24 h (first bars) and 48 h (third bars), respectively. Each experiment was repeated at least two times in triplicate. As positive control the culture was incubated with the drug miltefosine (6.25–100µg/ml) for 24 h (second bars) and 48 h (forth bars), respectively.

### 3.4.4 Bacteria and fungi

The frog foam extract showed no biocidal activity on the bacteria *Enterococcus hirae, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa,* and *Staphylococcus aureus,* nor on the fungi *Candida albicans, Saccharomyces cerevisiae* and *Trichophyton mentagrophytes.* Only the mycobacteria *Mycobacterium avium* and *M. terrae* were susceptible, however, with only minor growth inhibition.

After incubation of the microorganisms with the centrifuged and filtered foam extract on agar plates no *in vitro* inhibition of growth could be seen after 24 h for *Enterococcus hirae, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus aureus,* in both tests with and without filter paper in the designated protein concentrations (10 µg/20 µl with two dilutions, and 6 µg/20 µl, respectively) (Figure 21; not all data shown). In both tests, the growth of *Candida albicans* and *Saccharomyces cerevisiae* was evaluated after 48 h (on 30°C) of incubation in the presence of the foam extract and again no inhibition zones were observed (data not shown). Although *Trichophyton mentagrophytes* needs 21 d (at 30°C) for generation of a dense lawn, the incubation was stopped in the first test with filter paper after 48 h, as the fungus had already produced a fine (even though not dense) lawn with no detectable growth inhibition in the presence of the foam extract. Longer incubation for 21 d in the test without filter paper did also not result in any growth inhibition by the foam nest extract. However, the extract in this setup was lower concentrated (6 µg/20µl) (data not shown).

As the two strains of the genus *Mycobacterium* are so-called slow growers (mycobacteria that require more than 7 days to form colonies visible to the bare eye on culture plates), the control was performed after incubation of the cells with the foam extract for 21 d at 36°C, in both test setups. *M. avium* and *M. terrae* both showed susceptibility to the foam material in all tests – with and without filter paper – expressed in weak zonal growth inhibition around the paper (Figure 21) and in the region, where the extract had been dropped on, respectively (Figure 22).

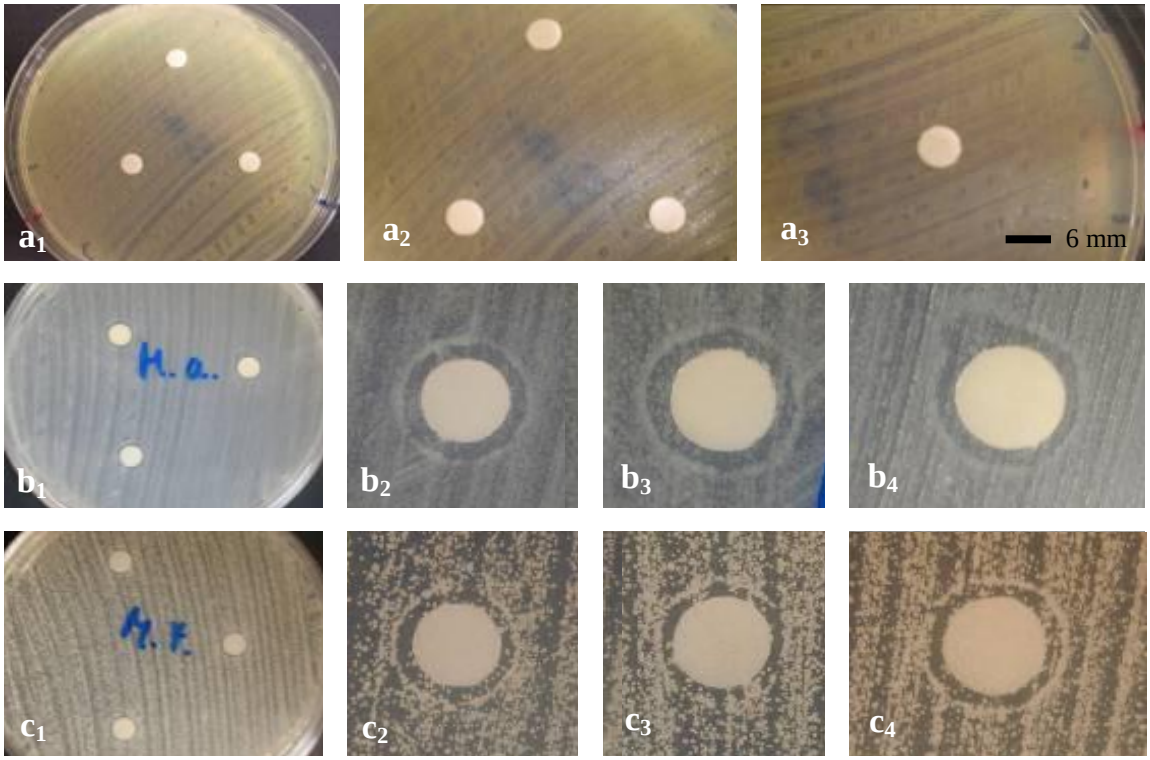



**FIGURE 21: Plate diffusion test with filter paper**. Examples of the investigations made: *Staphylococcus aureus* after 24 h of incubation with the foam extract in all three dilutions on filter paper (**$a_1$ and $a_2$**): left: concentrated (10 µg total protein); right: dilution $10^{-1}$; up: dilution $10^{-2}$. No growth inhibiton can be seen, even in the highest concentration (**$a_3$**). Negative results on other microorganisms are not shown. *Mycobacterium avium* (**b**) and *M. terrae* (**c**) after incubation for 21 d at 36°C in the presence of three dilutions of the foam nest extract on filter paper. **$b_1$ and $c_1$:** bottom: concentrated (10 µg protein/20 µl); top left: $10^{-1}$; top right: $10^{-2}$. **$b_2$-$b_4$ and $c_2$-$c_4$:** from left to right: concentrated sample, $10^{-1}$ and $10^{-2}$ dilutions.

However, the growth inhibition zone of *M. avium* and *M. terrae* in the test with the filter paper was equal in all three dilution steps (concentrated: 10 µg protein/20 µl, and dilutions $10^{-1}$, $10^{-2}$), i.e. inhibition was independent of the protein concentrations of the foam extract. Unfortunatelly, no negative control ($H_2O$) was carried along, thus, it cannot be excluded that the inhibition zone was caused by diffusion of liquid from the filter paper onto the plate.

Nevertheless, inhibition of growth of the two strains of mycobacteria, was also seen after treatment with the foam nest extract (6 µg/ 20 µl) directly dropped on, and incubation of the plates for the appropriate time. In this setup negative controls were carried along (both $H_2O$ and PBS), and they did not affect bacterial growth (Figure 22). Additionally, a positive control (20 µl of the disinfectant Manorapid®) was included that resulted in the generation of an inhibition zone of 1.8 cm in size (data not shown).

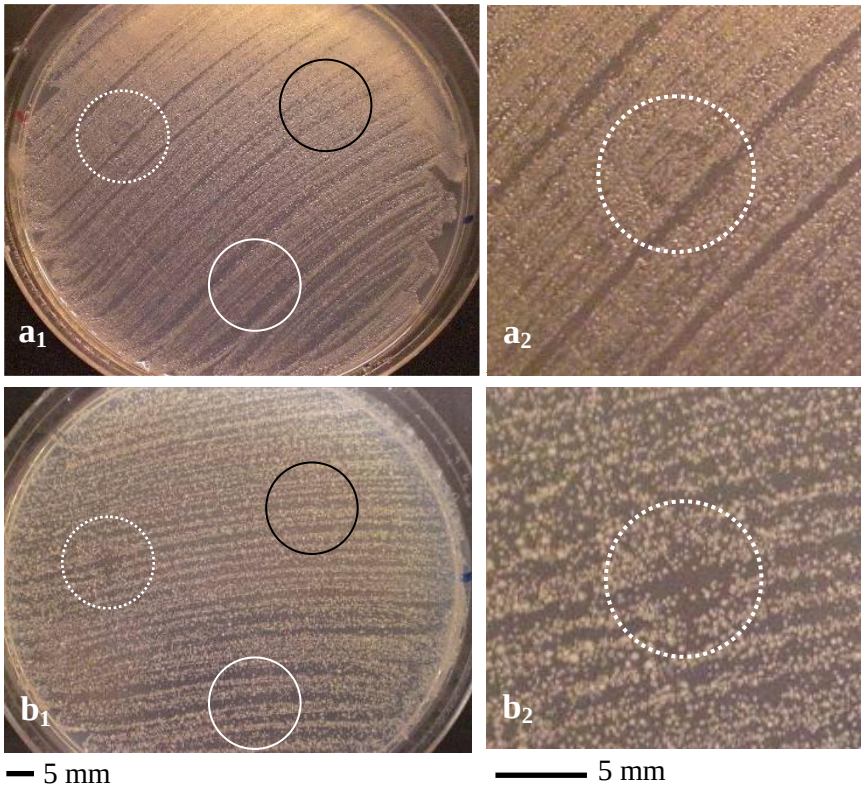



**FIGURE 22: Agar-plate growth inhibition test on *M. avium* (a) and *M. terrae* (b) without filter paper.** The plates were incubated for 21 d at 36°C in the presence of the foam extract (6 µg protein/ 20 µl). **$a_1$, $b_1$:** Overall view of the plates with a short inhibition of growth in the presence of foam material (left) and with the negative controls PBS (right) and $H_2O$ (bottom). **$a_2$, $b_2$:** enlarged views of the inhibition zones in $a_1$ and $b_1$.

In order to show concentration-dependent growth inhibition, additionally to the previous test without paper that was performed for all microorganisms, the experiment was repeated with higher concentrations (17.2 µg protein/20 µl) and serial dilutions ($10^{-1}$ and $10^{-2}$) for the positive reacting (inhibited) microorganisms, i.e. for both strains of mycobacteria, and for two further test organisms that had not been susceptible to the foam extract before, *C. albicans* and *T. mentagrophytes*. Once again, *C. albicans* and *T. mentagrophytes* did not show any susceptibility – even in the higher concentrations (data not shown) –, while for both mycobacteria, a concentration-dependent inhibition manner was observed. Total protein contents of 1.7 µg and 17.2 µg (per 20 µl) were enough to inhibit *M. avium* and *M. terrae*, respectively (Figure 23). In comparison to the previous results of the first test without filter paper (Figure 22), it can be assumed that concentrations of more than 6 µg protein (per 20 µl) on freshly streaked agar plates are enough to inhibit growth of *M. terrae*.

Moreover, in comparison to the first results of the test with filter paper (Figure 21), where for both, *M. avium* and *M. terrae*, concentrations of 10 µg/20 µl, 1 µg/µl and 0.1 µg/20 µl

yielded the same inhibition zones, in this test, a concentration of 1.7 µg/20 µl only inhibited *M. avium* while a concentration of 0.17 µg/20 µl did not yield any inhibition of growth at all.

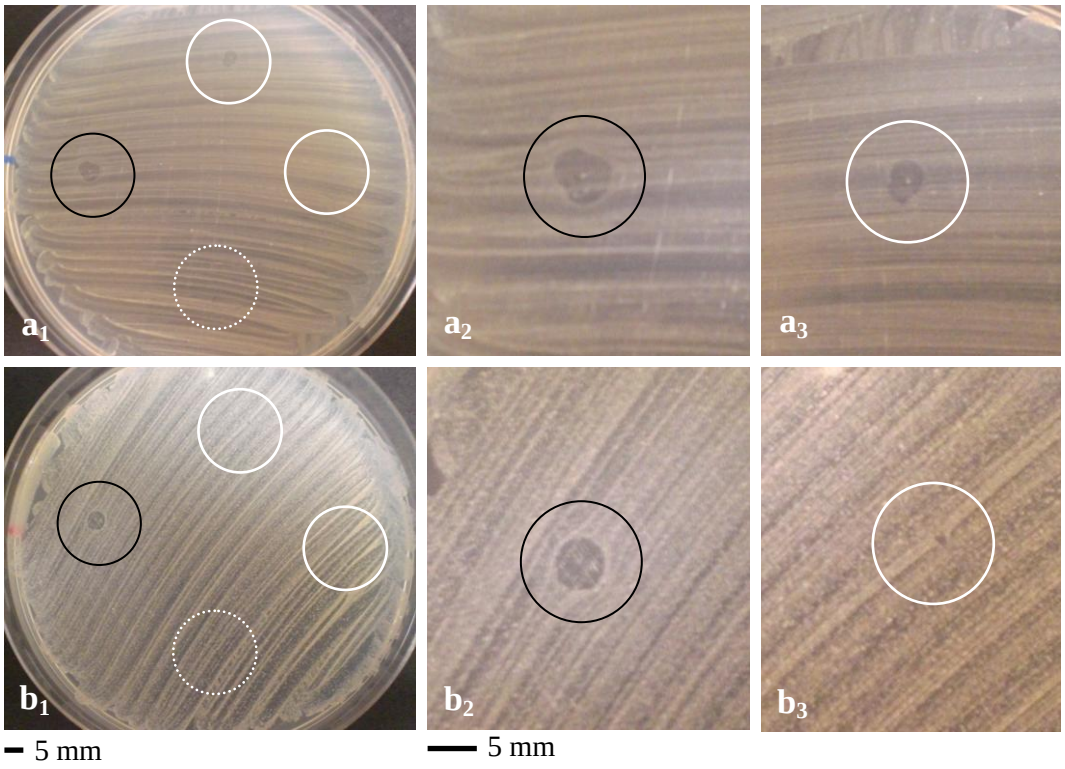



**FIGURE 23: Growth inbiting effect of the centrifuged and sterile filtered foam nest extact on *M. avium* (a) and *M. terrae* (b)**. The different protein concentrations were pipetted in clockwise rotation starting from the black labelling: 17.2 µg protein; 1.72 µg; 0.17 µg (per 20 µl); PBS as negative control (**$a_1$ and $b_1$**). Enlargement of $a_1$ and $b_1$ shows inhibition of growth by the foam extract in the highest protein concentration on both strains (**$a_2$ and $b_2$**) and in the first dilution (**$a_3$ and $b_3$**) on *M. avium* only.

Again, the negative control (PBS) did not have any influence on the growth of the mycobacteria (and on all other tested bacteria and fungi), thus, it can be assumed, that the inhibition of these bacteria was due to the presence of growth inhibiting agent(s) in the foam nest. The component(s) was/ were interacting with both mycobacteria – and with only these test organisms. Table 11 gives a summary of the ten microorganisms tested and the observations made.

**TABLE 11: Overview of the results of the growth inhibition tests on bacteria and fungi.** -…no growth inhibition; +…weak growth inhibition; /... not tested; (-) negative control.

| | Test organism | Nutritional agar/ temp./ duration of incubation | Foam extract [µg protein/ 20 µl] | | | | | | | (-) (PBS/ $H_2O$) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | With filter paper | | | Without filter paper | | | | |
| | | | 10 µg | 1 µg | 0.1 µg | 6 µg | 17.2 | 1.72 | 0.17 | |
| *Bacteria* | *Enterococcus hirae* DSM 3320 | CSA/ 36°C/ 24 h | - | - | - | - | / | / | / | - |
| | *Escherichia coli* DSM 11250 | CSA/ 36°C/ 24 h | - | - | - | - | / | / | / | - |
| | *Proteus mirabilis* DSM 788 | CSA/ 36°C/ 24 h | - | - | - | - | / | / | / | - |
| | *Pseudomonas aeruginosa* DSM 939 | CSA/ 36°C/ 24 h | - | - | - | - | / | / | / | - |
| | *Staphylococcus aureus* DSM 799 | CSA/ 36°C/ 24 h | - | - | - | - | / | / | / | - |
| | *Mycobacterium avium* DSM 44157 | 7H10-Agar/ 36°C 21 d | + | + | + | + | + | + | - | - |
| | *Mycobacterium terrae* DSM 43227 | 7H10-Agar/36°C 21d | + | + | + | + | + | - | - | - |
| *Fungi* | *Candida albicans* DSM 1386 | MEA/ 30°C/ 48 h | - | - | - | - | - | - | - | - |
| | *Saccharomyces cerevisiae* DSM 70449 | MEA/ 30°C/ 48 h | - | - | - | - | / | / | / | - |
| | *Trichophyton mentagrophytes* DSM 4870 | MEA/ 30°C/ 21 d | - | - | - | - | - | - | - | - |

## 3.5 MICROORGANISMS ASSOCIATED WITH THE FOAM

After inoculation of small volumes from the innermost of the foam into liquid LB medium all 15 samples (one egg and 14 samples of foam without eggs) revealed bacteria. Subsequent cultivation on LB agar, separation of clones distinguishable by the naked eye and several subcultures yielded 25 pure clonal cultures of bacteria from the three foam nests. However, these do not necessarily represent 25 different bacterial species and they need not represent all bacteria present in the foam nests.

The bacteria that could be identified were predominantly Gram-negative bacilli. The characterisation of seven random samples of the 25 cultures by API testing revealed the genera *Pseudomonas* (Gammaproteobacteria), *Aeromonas* (Gammaproteobacteria), *Pantoea* (Enterobacteria) and *Klebsiella* (Enterobacteria) in four cultural isolates of the first nest and the presence of *Ralstonia* (Betaproteobacteria) and *Pseudomonas* in the second nest (2 clonal cultures analysed). The one sample analysed from the third nest was also identified as belonging to the genus *Pseudomonas*. As the volumes were taken from the innermost of the nests and under sterile conditions, it can be assumed that these bacteria naturally occurred in the nests.

### 3.5.1 Bacteria with *in vitro* antibiosis

In the test for *in vitro* antibiosis towards *Candida albicans* three of the 25 isolated clonal cultures showed weak antagonism by growth inhibition around the bacterial inoculums (Figure 24). These were identified by the API system and in parallel by using a MALDI-Biotyper.

The three isolates exhibiting weak antifungal activity against *C. albicans* derived from two different frog nests. Two strains (strain 1 and 2) had been isolated from the inoculum of one egg and the third (strain 3) from another nest and an inoculum of foam without eggs. Strain 1 was identified by API testing system with 60.9% ID (identification percentage; probability of species identification) as *Pseudomonas fluorescens* or with 38.8% ID as *Pseudomonas putida*. The results given by the MALDI-Biotyper confirmed the genus as *Pseudomonas* with "probable genus identification" according to the pattern matching with the reference spectra from the database (status of June 2009).

Strain 2 was identified by its enzymatic activities as *Ralstonia pickettii* (64.6% ID) or *Alcaligenes xylosoxidans* with 28.2% ID, while the MALDI-Biotyper indicated with "secure genus identification and probable species identification" *Pseudomonas monteilii*.

The third strain was identified as *Pseudomonas fluorescens* with 99.4 % ID in its analytical profile. Results of mass spectrometry did also reveal the genus *Pseudomonas*, however with "no reliable identification".

Thus, at least two of three strains (the results of strain 2 are contradictory) that showed antifungal activity belonged to the genus *Pseudomonas*. According to the results of API testing, isolate 1 (when regarding the highest probability) and 3 were identified in both cases as *Pseudomonas fluorescens*.

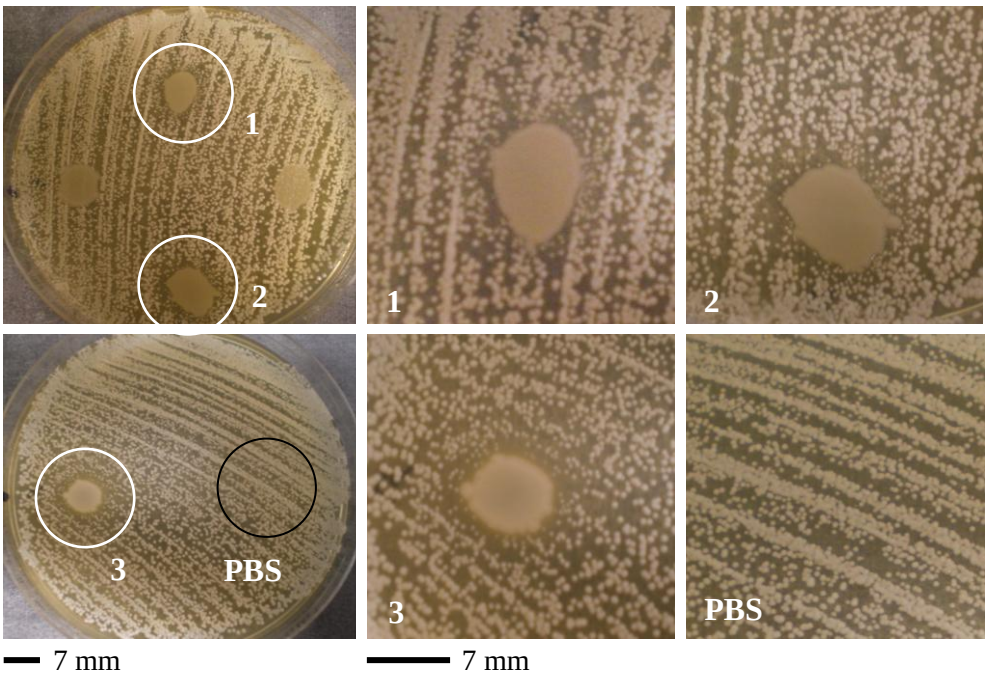



**FIGURE 24**: **Inhibition of growth of *C. albicans* by three of the 25 isolates.** The bacterial clonal cultures were numbered as 1, 2 and 3. Later characterization by API testing and a MALDI-Biotyper identified the strains as *Pseudomonas fluorescens* (**1**), *Ralstonia pickettii* (**2**) and *Pseudomonas fluorescens* (**3**) (see text). PBS was used as a negative control.

While all three strains showed weak growth inhibiton on *C. albicans* (initial $OD_{600}$ = 0.02), not all three were biocidal to the other tested organisms. Strain 1 (putatively *Pseudomonas fluorescens*) only showed antibiosis against *M. avium*, while it did not inhibit *M. terrae* and *T. mentagrophytes* in their growth (data not shown). However, growth inhibition was the same in all bacterial dilutions (OD = 0.02, dilutions $10^{-1}$ and $10^{-2}$ at initial inoculation) with no difference in the size of the inhibiton zone after 21 d. Unfortunately, growth of *M. avium* was weak – the mycobacteria did not form a dense lawn after plating and incubation for 21 d – thus, the growth inhibition zone is hardly recognizable in Figure 25a, however, could be clearly observed in the orginal. The negative control PBS did not show any inhibition.

For strain 2 (putatively *Ralstonia pickettii*), besides the low growth inhibiting activity against *C. albicans*, no further activity was observed. Neither *M. avium*, nor *M. terrae* and *T. mentagrophytes* were inhibited in their growth around the bacterial inoculation (data not shown).

Isolate 3 (putatively *Pseudomonas fluorescens*) on the other hand showed growth inhibition on *T. mentagrophytes* (Figure 25b), while the strain did not inhibit one of the mycobacteria in their growth (data not shown). Growth inhibition was independent of the

initial density of the bacterial inoculation (OD = 0.002, and $10^{-1}$, $10^{-2}$ dilutions) – no difference in the size of the inhibition zone was observed. Staining of the mycelium of *T. mentagrophytes* and microscopic analysis showed deformed mycelia in the growth inhibition region (Figure 25$b_2$–$b_3$). The negative control PBS did not influence the fungal growth.

The growth inhibiting effects of the three strains are summarised together with the results of both identification methods in Table 12.

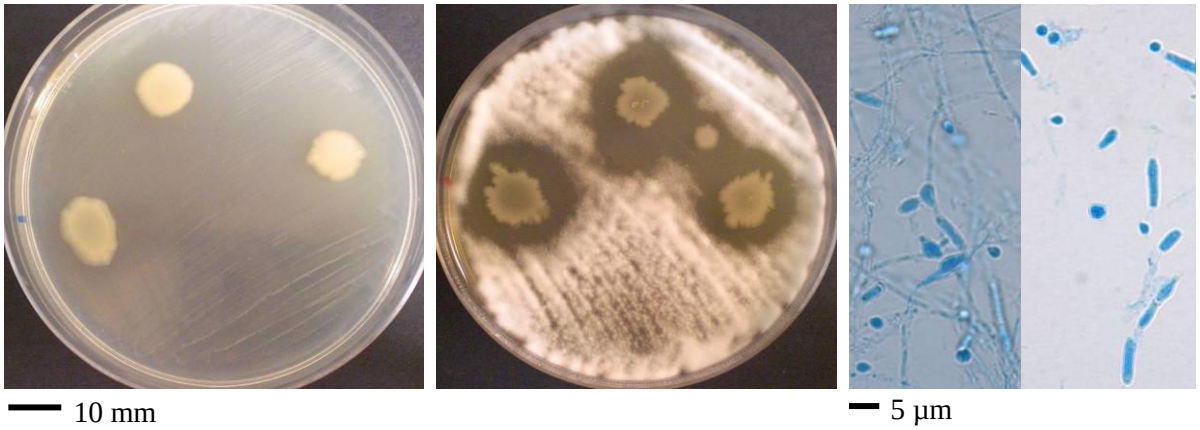



**FIGURE 25: Growth inhibition of *Mycobacterium avium* (a) and *Trichophyton. mentagrophytes* (b) by strain 1 (putatively *Pseudomonas fluorescens*) and strain 3 (putatively *Pseudomonas fluorescens*), respectively.** Three bacterial dilutions were applied onto the plates in clockwise rotation (initial $OD_{600}$= 0.002, $10^{-1}$, $10^{-2}$). **$b_2$–$b_3$)** Lactophenol cotton blue-stained normal and deformed mycelia of *T. mentagrophytes* in region of normal growth and region of growth inhibition, respectively. Black circle: PBS as control.

**TABLE 12: Isolated bacteria and their growth inhibition on the test organisms.** Bacteria showing growth inhibition on other microorganisms were identified via API (percentage of reliability in brackets) and by a MALDI-Biotyper: (++) reliable genus identification, probable species identification; (+) probable genus identification; (-) not reliable identification. Growth inhibition: (-) no growth inhibition and (+) growth inhibition.

| **Isolated bacteria** | | | **Growth inhibition of test organisms** | | | |
|---|---|---|---|---|---|---|
| **No.** | **API** | **MALDI** | ***C. albicans*** | ***M. avium*** | ***M. terrae*** | ***T. mentagrophytes*** |
| 1 | ***Pseudomonas fluorescens*** (60.9% ID)/ ***P. putida*** (38.8% ID) | ***Pseudomonas*** *putida* (+)/ *P. monteilii* (+) | + | ++ | - | - |
| 2 | ***Ralstonia pickettii*** (64.6% ID)/ ***Alcaligenes xylosoxidans*** (28.2% ID) | ***Pseudomonas*** *monteilii* (++) | + | - | - | - |
| 3 | ***Pseudomonas fluorescens*** (99.4% ID) | ***Pseudomonas*** (-) | + | - | - | ++ |

# 4 DISCUSSION

## 4.1 PROTEINS

Foam nests of *Engystomops pustulosus* and *Leptodactylus vastus* were described for containing low protein concentrations of approx. 1 mg/ml fluid of their foam nests (Cooper et al. 2005, Hissa et al. 2008). The protein concentrations observed for *L. pentadactylus* foam nests in the present study were with approx. ~12 mg/ml foam fluid (when 1 g of homogenised foam gives approx. 300 µl fluid and a protein concentration of ~3.5–5.5 mg/g foam was measured) significantly higher than that of these both organisms.

The mixture of proteins in the foam nest of *E. pustulosus* named ranaspumins is in the 10–40 kDa mass range (Cooper et al. 2005, Fleming et al. 2009), and its profile in electrophoresis can be clearly distinguished from that of the foam nest of *L. pentadactylus* showing proteins in the 20–180 kDa range in the present study. While the amino acid sequences of ranaspumins have been described for not having direct or close matches to other proteins (Fleming et al. 2009), the foam proteins of *L. pentadactylus* are similar – at least in their profile in SDS-PAGE – to that of another leptodactylid: The foam fluid of *L. vastus* comprises several proteins with molecular masses in the range from 14 to over 97 kDa (Hissa et al. 2008).

Moreover, the proteins of the nests of *E. pustulosus* (ranaspumins) and of *L. vastus* (Lv-ranaspumins) have been described particularly for their surfactant activities (Cooper et al. 2005, Fleming et al. 2009, Hissa et al. 2008), and those of *E. pustulosus* additionally for their carbohydrate binding and proteinase inhibition activities (Fleming et al. 2009). These functions are on one hand necessary for production, cross-linking and stability of the foams, but on the other hand have also been associated with protection against predation and parasitism (Cooper et al. 2005, Fleming et al. 2009, Hissa et al. 2008). The properties of the proteins of the *L. pentadactylus* foam nests, like surfactant activity, amino acid sequence, relatedness to other such proteins or their possible association to biocidity of the foam, however, still have to be investigated.

## 4.2 GLYCOSYLATION

Although proteins of frog foams have come into interest of scientists, because of their surfactant activity (those of the frog foams of *Engystomops pustulosus*, for example, are well known), only little is known about the glycan parts of these proteins.

In the current study, the presence of almost 60 structurally different *N*-linked carbohydrate chains on the frog nest peptides of *L. pentadactylus* could be shown, the natures of which were determined in their composition of hexoses, *N*-acetylhexoamines and fucoses. Moreover, indications to further glycans, most putatively *O*-glycans, were given by the binding of the lectins to foam nest proteins even after digestion with PNGase F. To the best of our knowledge, the description of glycans from the foam nests of *L. pentadactylus* is the first of this kind, although the actual sugar identities and linkages in between them were not fully proven.

Moreover, the glycosylation of the peptides of *L. pentadactylus* was observed to be almost restricted to the proteins in higher kDa ranges (40–170 kDa), which could be verified for being consistently glycosylated by the binding of different lectins. Foam peptides in small kDa ranges (20–26 kDa) did not show (with exception of the binding of ConA) any glycosylation in the blots.

Until now, only for the túngara frog *Engystomops pustulosus*, foam nest glycoproteins have been described shortly, namely as containing both, core-1 and core-2 *O*-glycans, and truncated and complex-type *N*-glycans (Parry et al. 2003 in Cooper et al. 2005). In contrast to these results, further studies of Cooper et al. (2005) and Fleming et al. (2009) that concentrated on the foam nest proteins (ranaspumines) of *E. pustulosus* (proteins in the 10–40 kDa mass ranges) showed that these are not detectably glycosylated. Moreover, analysis of the amino acid sequences of the six ranaspumines revealed no consensus *N*-glycosylation sites (Fleming et al. 2009).

### 4.2.1 Oligomannose-, hybrid- and complex-type *N*-glycans

Analysis of the *N*-linked carbohydrates of the foam nests of *E. pustulosus* has shown complex-type glycans, of which the majority had a fucosylated core (Parry et al. 2003 in Cooper et al. 2005). This is also true for the proteins of the foam nest of *L. pentadactylus*, the *N*-glycans of which according to the results of mass spectrometry (MS/MS) also show complex-type structures. The presence of poly-*N*-acetyllactosamine residues on *N*-glycans

(shown by the lectin LEA that was no longer binding after PNGase F digestion) is also an indication for the presence of complex type *N*-glycans, as these preferentially occur on multi-antennary structures (Stanley and Cummings 2009). However, hybrid-type and oligomannose-type structures also seem to be present as shown by the binding of the lectin ConA that recognises α-mannose residues of high-mannose- and hybrid-type structures of *N*-glycans, and with only low affinity binds to complex-type biantennary *N*-glycans, while it fails to bind to complex-type *N*-glycans that are more highly branched (Cummings and Etzler 2009). Moreover, mass spectrometry not only of the two exemplary *N*-glycan species that display hybrid-type structures, but of several *N*-glycan species confirms the presence of oligomannose- and hybrid-type, and, thus, the presence of all three major types of *N*-glycans in the foam nests of *L. pentadactylus*.

### 4.2.2 *N*-Glycans with bisecting modifications

The two hybrid-type exemplary *N*-glycan species (Figure 15) showed bisecting *N*-acetylhexosamine residues attached to the β-mannose of the core. Although the bisecting *N*-acetylhexosamine, as well as the other branching HexNAc residue, could not be identified by mass spectrometry, they were assumed as being *N*-acetylglucosamines, which are the general initiating residues of *N*-glycan antennae. In the two given *N*-glycan species, the bisecting GlcNAc were found to be present in hybrid structures, which is conclusive with the literature, as the bisecting *N*-acetylglucosamine is added by *N*-acetylglucosaminyltransferase III (GlcNAcT-III) to hybrid- and (biantennary) complex-type *N*-glycans only (Stanley et al. 2009).

Bisecting *N*-acetylglucosamine residues as demonstrated in the current study for the first time in frog nests, are common modifications in vertebrates, which in comparison to invertebrates and plants generally possess more complex *N*-glycans (Varki et al. 2009b). They have so far been described, for example, for *N*-glycans of human serum immunoglobulin G (IgG) (Takegawa et al. 2005), but to our knowledge not yet explicitely for frogs, although the gene encoding GlcNAcT-III is present in amphibian genomes, like in that of *Xenopus laevis* (NCBI GenBank 2010). That these modifications have not been described earlier seems to be due rather to the fact that they had not been searched for than that they are not present.

The described linkages of the further residues on the Manα1,3 arm (Hex-HexNAc-GlcNAc) of the two given hybrid-type species could not unambiguously be identified, as

the MS/MS fragments gave several options. In contrast to the suggested structure, it could be possible that the terminal hexose of the Manα1,3 arm is bound to the bisecting *N*-acetylglucosamine, which would not only also be conclusive with the fragments (and binding of the lectins) given, but also with the recently proposed quite common modification of (hybrid) *N*-glycans containing a galactosylated bisecting GlcNAc residue (Harvey et al. 2008). Moreover, the identities of the residues which are suggested as galactose and *N*-acetylgalactosamine are not proved. They are based on known linkages between GalNAc and GlcNAc, for example in (poly-)LacdiNAc sequences (repeated GalNAcβ1,4GlcNAcβ1,3 units) of oviductal mucins of the newt *Triturus alpestris* (Florea et al. 2006) and on the interactions of the galactose binding lectins. Thus, the given total structures are only partially based on distinct unambiguous results. Particularly the linkages and identities of their terminal residues are only probable suggestions that do not exclude other possibilities.

### 4.2.3 High fucosylation

Tandem mass spectrometry of *N*-glycans showed a number of fucose residues positioned in the periphery of the glycans. For example, the *N*-glycan species with the greatest masses found abundantly in the foam nest were calculated as having four to five deoxyhexoses (putatively fucoses) in their compositions. Moreover, the interaction of each of the fucose-binding lectins tested (AAL, LCA, LTA and UEA) demonstrated the presence of fucose residues on both *N*- and *O*-glycans of the foam nest glycoproteins. This is also in agreement with Parry et al. 2003 in the description of frog foam nest proteins of the túngara frog *Engystomops pustulosus* by Cooper et al. (2005) who detected both *N*- and *O*-glycans with fucosylation. In general, fucose residues are common moieties of amphibian *O*-glycans of egg jelly coat mucins and of mucins of oviductal secretions of frogs. They can constitute up to 10% of various amphibian egg jelly coats that have an average total carbohydrate content of 35–50% of the mass of the crude material. The jelly coats that are deposited around the amphibian eggs are secreted by the female's tubular gland cells lining the oviduct (Strecker 1997). Thus, the presence of several fucose residues in the foam nest glycoproteins of *Leptodactylus pentadactylus* is conclusive, as the foams are also generated of oviductal (and other mucous) secretions released by the female (Heyer 1969).

### 4.2.4 *N*-Glycans with core fucosylation

In case of the *N*-glycans, fucose was part of the core of the majority of the analysed glycan species, which was shown by tandem mass spectrometry (not only of the two exemplary species). Although the linkage of the fucose to the core could not be verified by mass spectrometry, the binding of the lectin LCA shows an α1,6-linkage of fucose to the *N*-acetylglucosamine adjacent to asparagine in the *N*-glycan core. That kind of core fucosylation is typical for vertebrates, whereas in invertebrates and plants the fucose can also be/ is only added in α1,3-linkage (Stanley et al. 2009). This result is also in agreement with the analysis of the *N*-glycans of the nest of *E. pustulosus* that has shown *N*-linked glycans, with the majority also having a fucosylated core (Parry et al. 2003 in Cooper et al. 2005).

Moreover, the presence of bisecting *N*-acetylglucosamine in combination with core fucosylation was shown (for example for the *N*-glycan species *m/z* 2376 $[M+H]^+$) which is a rather unusual modification, as the insertion of a bisecting *N*-acetylglucosamine inhibits the activity of the α1,6-fucosyltransferase transferring fucose to the core *N*-acetylglucosamine (Schachter et al. 1983). Thus, the presence of such structures is only possible when GlcNAcT-III, the enzyme that adds the bisecting *N*-acetylglucosamine, operates after the addition of the core fucose. The presence of both structures – bisected hybrids with and without core fucosylation (*m/z* 2230, *m/z* 2376 $[M+H]^+$) – in the *N*-glycome of *L. pentadactylus* foam nests demonstrates the variability of the compositions of glycan structures dependent on the presence and activity of competing glycosyltransferases. As the intensity of the species with core fucosylation (*m/z* 2376 $[M+H]^+$) in the total *N*-glycome spectrum of *L. pentadactylus* (Figure 13) was higher than that of the one without (*m/z* 2230 $[M+H]^+$), this might be indicative for a higher level or activity of fucosyltransferase than GlcNAcT-III in the cells secreting the foam extract.

### 4.2.5 Peripheral fucosylation (A, B, H and $Le^x$, $Le^y$ determinants)

The lectin blots indicate both, the presence of peripheral α1,3-linked fucose in a $Lewis^x$ (Galβ1,4(Fucα1,3)GlcNAc-R) linkage and α1,2-linked fucose (R-(Fucα1,2)Gal-R), on both, *O*- and *N*-glycans, of the frog foam nest glycoproteins. In accordance with the results of other lectin blots, these fucose residues may be part of a LacNAc or polyLacNAc structure (repeated Galβ1,4GlcNAcβ1,3 units), whose presence was shown by the binding of the lectin LEA. Moreover, Galα1,3Gal-R as well as α-GalNAc structures seem to be

present, which are also residues, that are known to occur in combination with fucose in a number of well known structures.

For example, the antigens of the ABO blood group system would be conclusive with the results of the lectin blots. In humans and other higher organisms, polyLacNAc chains (repeated Galβ1,4GlcNAcβ1,3 units) are subject to glycosylations that are tissue-specific and form the ABO blood group antigens. During biosynthesis of the ABO antigens, fucose is α1,2-linked by an α1,2-fucosyltransferase to the terminal galactose of the polyLacNac units in order to form the H (O) antigen as a precursor for the A and B antigens. In case of A antigen, *N*-acetylgalactosamine and in case of B antigen, galactose is added in α1,3-linkage to the terminal galactose of the precursor (Fucα1,2)GalβGlcNAc-R in order to form GalNAcα1,3(Fucα1,2)GalβGlcNAc-R and Galα1,3(Fucα1,2)GalβGlcNAc-R structures, respectively (Stanley and Cummings 2009).

ABO blood group determinants are common structures in *O*-glycans of amphibian egg jelly coat mucins. The egg jelly coat mucins of the frog *Rana dalmatina*, for example, have been shown for carrying α1,2-linked fucose in the structure Fucα1,2Galβ1,3GalNAc-α-O-Ser/Thr (H antigen) (and expansions of this structure) (Florea et al. 2002). Mourad et al. (2001) also described oviductal mucins of the frog *Rana ridibunda* that contain a series of carbohydrate chains with inner blood group B determinants in a Galα1,3(Fucα1,2)Galβ1,3GalNAc-α-O-Ser/Thr sequence, itself extended with an additional β1,2-linked galactose unit to the terminal α-galactose.

However, the blots also indicate the presence of α1,3-linked fucose, which would fit the presence of Lewis$^x$ (Le$^x$) antigens with the structure Galβ1,4(Fucα1,3)GlcNAcβ1,3-R. This residue could also be part of the polyLacNAc structure, as it constitutes the same background (repeated Galβ1,4GlcNAcβ1,3 units) as the ABO blood group antigens, however, with different fucosylation – in α1,3-linkage at the *N*-acetylglucosamine residue rather than in α1,2-linkage to the galactose residue. However, as both, the presence of Fucα1,2Gal-R and Fucα1,3GlcNAc-R, was verified (shown by the lectins UEA and LTA) another structure is more probable: Le$^y$ antigen. This determinant shows adjacent α1,2- and α1,3-linked fucose residues in the structure (Fucα1,2)Galβ1,4(Fucα1,3)GlcNAcβ1,3-R. *O*-linked glycans from the jelly coat eggs of the newt *Pleurodeles waltlii*, for example, were characterised with Le$^x$, Le$^y$ and A Le$^y$ antigen determinants (the expansion of the Le$^y$ with an additional GalNAcα1,3-R giving the structure GalNAcα1,3(Fucα1,2)Galβ1,4(Fucα1,3)GlcNAcβ1,3-R) (Strecker et al. 1992). An

expansion of $Le^y$ with α1,3-linked galactose to form B $Le^y$, could also be present in the foam nest glycans of *L. pentadactylus*.

In fact, numerous described structures would fit to the given results of *L. pentadactylus* foam nest glycans, however, none of them was unambiguously confirmed by the lectin blots or *N*-glycan tandem mass spectrometry. The presence of Fucα1,2Gal-R and Fucα1,3GlcNAc-R residues were confirmed for the *N*- and *O*-glycan species, the actual linkages of the singular moieties in the total structures, however, still have to be clarified.

### 4.2.6 Sialic acid

The binding of Jac lectin in combination with the results of the other lectins, that exclude several of the binding specificities of Jac, may possibly indicate the presence of α2,3-linked sialic acid (= substituted derivatives of neuraminic acid; NeuA). As after PNGase F digestion the binding of Jac is not lost, the putative α2,3-linked sialic acid could be part of the residue NeuAα2,3Galβ1,3GalNAc-α-O-Ser/Thr of an *O*-glycan.

The presence of sialic acid would not be surprising, as in vertebrates sialic acids are frequently found as "capping" residues of *N*- and *O*-glycan chains (and glycolipids) (Varke et al. 2009b). Accordingly, they have been described to be part of *O*-linked glycan chains from egg jelly coats of the newt *Pleurodeles waltlii*, that contain Kdn (2-Keto-3-deoxynononic acid) – that is in comparison to *N*-acetylneuraminic acid (Neu5Ac) the more unusual sialic acid – in an α2,6-linkage (Strecker et al. 1992). This α2,6-linkage can be excluded in the current case because of the lectin SNA that did not bind. The presence of neuraminic acid (and several derivatives) has also been described for the egg jelly coats of the newt *Triturus alpestris* (Florea et al. 2006) and of the frog *Bufo viridis* (Coppin et al. 2001). Moreover, analysis of the foam *O*-linked glycans of the frog *Engystomops pustulosus* has revealed both core-1 and core-2 structures to be present in the nest. These show both fucosylation and sialylation (Parry et al. 2003 in Cooper et al. 2005).

However, the presence of sialic acid in this study was not absolutely verified, as the binding specificities of the two lectins Jac and WGA are wide spread, and none of the given *N*-glycans showed the presence of sialic acid in mass spectrometry.

### 4.2.7 Conclusion

Summing up, the presence of *N*-glycans and putatively *O*-glycans were shown in the foam nest glycoproteins of *L. pentadactylus* for the first time, however, according to the results of lectin blots and mass spectrometry, the variety of the possible linkages of the sugar residues in the individual glycans is still high. And although some sugar moieties seem to be verified, none of the structures could be totally clarified. The actual identities and linkages of the sugar residues of the *N*-glycans are still open to further methods like enzymatic or methylation assays, or to the use of nuclear magnetic resonance (NMR) spectroscopic methods.

Moreover, comparision with known structures of amphibians is difficult, as most studies concentrate on the characterisation of *O*-glycans of mucin-type glycoproteins of egg jelly coats or of oviductal secretions of amphibian species that are additionally only distantly related to *L. pentadactylus*, rather than on *N*-glycans of foam nest components. And although glycoenzymes catalyzing the binding of sugar residues in the periphery of *O*- and *N*-glycans can be the same, their number is high, resulting in a variety of possible glycan structures (Varki et al. 2009a). Thus, it would not be surprising, if the structures given for *L. pentadactylus* were novel structures and would not fit to structures known of any other amphibian. The description of glycans of *L. pentadactylus* foam nests in the present study not only contributes to the knowledge of foam nest components, but is further expanding the knowledge of *N*-glycan structures in the amphibian world in general.

Moreover, the functions of glycans in foam nests still have to be investigated. Fleming et al. (2009) described that four of the six frog foam proteins, named ranaspumines, of the túngara frog *Engystomops pustulosus* (Leiuperidae) are lectins, which themselves show carbohydrate binding specificities. As the foam nests of this species contain similar quantities of proteins and carbohydrates in the non-water mass of the nest (Cooper et al. 2005), the lectins may bind to the sugars and participate in a cross-linked matrix that stabilizes the structure of the foam and thus, greatly extends the foam's lifetime (Fleming et al. 2009). The described glycans of the foam nest glycoproteins, as part of the total carbohydrate content of the foam, may contribute to such a cross-linked matrix by interaction of the glycans with the lectins.

Furthermore, as with preparation and treatment of the foam nest, none of the glycans were lost, and the biocidal activity was still present, both, the protein as well as the glycan part,

of the foam nest proteins could contribute to biocidity. Tests with the foam extract without any glycan (possibly released with PNGase F) would be of further interest.

## 4.3 BIOLOGICAL ACTIVITY

Biocidal activity of biofoams has long been anticipated, but has not yet been proven. In this study, the foam fluid showed *in vitro* cytostatic effects against *Leishmania donovani* and *L. infantum*, while *T. cruzi* and *Acanthamoeba* were not susceptible to the foam extract. However, apparently, the biocidal agent(s) were used up within 48 h and the culture (*Leishmania* spp.) recovered from treatment with the extract.

Moreover, growth inhibition was also observed for the two strains of mycobacteria (*M. avium* and *M. terrae*), while the foam did not have a growth inhibitory effect on the bacteria *E. hirae*, *E. coli*, *P. mirabilis*, *P. aeruginosa* and *S. aureus* and the fungi *C. albicans*, *S. cerevisiae* and *T. mentagrophytes*. Although, the first test with filter paper on *M. avium* and *M. terrae* may have been false positive, as it gave the same results of growth inhibition in three serial dilutions, two further tests without filter paper, confirmed the results of the presence of growth inhibiting agent(s) in the foam nest interacting with both mycobacterial strains.

Thus, the diverse protozoan, bacterial and fungal species appear to react to the foam extract differently. The actual mechanism of action may affect some pathway that these organisms have in common. In comparable tests with the foam nest fluid of *Leptodactylus vastus* in similar protein concentrations (15 µg protein) on several bacterial species, two of which were, like in the present study, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, also no biocidal activity of the foam was observed. Tests including a number of fungi also showed no antimicrobial activity (Hissa et al. 2008). Thus, *L. vastus* possibly does not confer biocidity to its foam nests, or possibly not against those model organisms tested in the study of Hissa et al. 2008. According to our knowledge, tests on *M. avium* and *M. terrae*, as well as on *Leishmania donovani* and *L. infantum*, have not been performed before. The results, showing growth inhibition for both protozoa and mycobacteria are novel and contrary to the investigations of the foam of *L. vastus*, that did not show any activity of the foam nest in the chemical defense against the (tested) microbiota.

### 4.3.1 Identity of the inhibiting agents

The fact that setups were calculated in µg protein/ml in order to ensure comparability and that the *Leishmania* spp. and mycobacteria reacted in a protein concentration-dependent manner, does not necessarily indicate that the biocidity is conferred by one or more peptides, as still nothing is known about the actual identity of the biocidal agent(s). Different biocidal agents belonging to different substance classes may be present, and as the foam extract was not further purified into its ingredients, the protein as well as the carbohydrate moiety could be responsible for the observed biocidal activity.

However, a number of proteins showing biocidal properties have been described in different amphibians. Even the skin secretions of *L. pentadactylus* contain peptides with antimicrobial properties that are considered as first line defense against invading pathogens as part of the innate immune system. King et al. (2005) described two antimicrobial peptides in mucous skin secretions of *L. pentadactylus*, one of which they named pentadactylin. Pentadactylin inhibited amongst others with low potencies the growth of the Gram-negatives *E. coli* and *P. aeruginosa*, as well as of the Gram-positive bacterium *S. aureus*, which, however, were not susceptible to the foam nest extract in the present study. The second one that differed from pentadactylin by eight amino acid residues was identical to fallaxin, a C-terminally α-amidated 25 amino-acid-residue from the skin of the Caribbean mountain chicken frog *Leptodactylus fallax,* characterised by Rollins-Smith et al. (2005). The peptide inhibited the growth of the Gram-negative bacteria *E. coli*, *P. aeruginosa*, *P. mirabilis*, but was not active against the yeast *C. albicans* and, in contrast to leptodactylin, not active against *S. aureus*. As fallaxin is highly abundant in the skin secretions of *L. fallax*, the authors suggested that, despite the low potency against the tesed microorganisms, its concentration in the skin may exceed the minimal inhibitory concentrations for many microorganisms and pathogens the animal may have to face in nature (Rollins-Smith et al. 2005). This may also be true for the foam nests of *L. pentadactylus*, as a protein concentration of ~3.5 mg/g foam was calculated and, for example, 1.7 µg total protein already inhibited growth of *M. avium*. A third antimicrobial peptide of *L. pentadacylus* skin secretions, leptoglycin, was described by Sousa et al. (2009) that also showed *in vitro* inhibiting activity against Gram-negative bacteria.

Indeed, biocidal peptides in skin secretions of members of the genus *Leptodactylus* are common. The ocellatins from *L. ocellatus* were the first such peptides of the genus to be

characterized (Nascimento et al. 2004). Laticeptin from *L. laticeps* (Conlon et al. 2006) and syphaxin of *L. syphax* (Dourado et al. 2007) are further examples. All these peptides show *in vitro* biocidal activity against potential pathogens, like different Gram-negative and/or Gram-positive bacteria.

However, not only the actual identity but also the mechanisms of action of the inhibiting ingredients still remain to be characterised. The biological activity could in fact also be associated with some recognized defence compounds such as enzymes or lectins commonly detected in secretions released by vertebrates. Fleming et al. 2009 postulated foam nests of *Engystompos pustulosus* to be involved in the protection of the eggs not by use of antimicrobial peptides, but by that of a combination of different proteins (like proteinase inhibitors) and lectins, that are known to agglutinate microbes as general recognition components in the immune system of vertebrates. They agglutinate microbes and thereby impede dissemination in different plants and animals. Fleming et al. (2009) suggested the protection of the eggs by inhibition of mibrobial colonization of the foam by lectins as the main defence mechanisms, rather than the actual killing of the invading microbes by the presence of biocidal substances.

### 4.3.2 Conclusion

The foam nests do play a significant role for the protection of the developing eggs and tadpoles, as the nests remain stable for several days without fungal or bacterial degradation, although the frogs construct their nests using pond water that is heavily contaminated with microbes. However, the mechanisms of action are still unclear. As *in vitro* growth inhibition of microorganisms by the foam extract was observed in the current study, some biocidal agents seem to be present, on the one hand. On the other hand, the given results do not exclude other possible defence mechanisms of the nest, as surface activity and the agglutinating activity of lectins. In fact, there may be a combination of several defence strategies that together achieve microbial defence of the nest and of the contained eggs.

## 4.4 MICROORGANISMS ASSOCIATED WITH THE FOAM

Although biocidity of the foam nest on both protozoa and bacteria was shown, the nests do not totally prevent the colonization of the foam with microorganisms, which could be demonstrated by the inoculation of 15 samples of foam and eggs into liquid medium, and

by the subsequent growth of bacteria from each inoculum. The foam nests of *Leptodactylus vastus* have also been found to harbour a bacterial community that accounted for 3.0–4.0 ($10^7$) cfu/ml foam fluid (Hissa et al. 2008) and consisted of predominantly Gram-positive bacilli, while in the present study only Gram-negatives were found. However, the detection of only Gram-negatives does not exclude the presence of other bacteria as only seven random samples of the 25 isolates were identified, which themselves only represent the bacteria that could be grown on LB (liquid or agar) at 25°C without taking into account the needs of more specialised bacterial strains that might be present in the foam nest environment.

Three of seven clonal cultures were identified as representatives of the genus *Pseudomonas*. They derived from three distinctive nests, thus, in every tested nest, the genus *Pseudomonas* was prevalent. Pseudomonads are Gram-negative rod shaped and flagellated bacteria. They can be found, for example, as plant growth-promoting bacteria on plant surfaces and inside plant tissues, where they can function e.g. by suppression of pathogenic microorganisms or by inducing the plant to better defend itself, a phenomenon termed "induced systemic resistance" (Bakker et al. 2007, Haas and Keel 2003, Kruijt et al. 2009). Others, however, may also be pathogenic and cause disease. *Pseudomonas syringae* for example is a common plant pathogen (Scholz-Schroeder et al. 2003). All pseudomonads have in common that they are ubiquitously found in soil and water. *Pseudomonas aeruginosa*, that can cause disease in animals and humans, has been described to occur ubiquitously in natural waters such as lakes and streams and also in man-made environments (Mena and Gerba 2009). Thus, the presence of the genus in the foam nest of *L. pentadactylus* is not surprising, however, described here for the first time.

### 4.4.1 Bacterial strains provide *in vitro* biocidal activity

Three of the 25 different strains isolated showed biocidal activity against *C. albicans* in dense plated cultures. These three bacteria could be identified with high reliability as *Pseudomonas fluorescens* (strain 1), *Ralstonia pickettii* (strain 2) and *Pseudomonas fluorescens* (strain 3). While strain 2 (*Ralstonia pickettii*) only showed *in vitro* antagonism towards *C. albicans*, the *Pseudomonas fluorescens* strains additionally induced growth inhibition of *M. avium* (strain 1) and of *T. mentagrophytes* (strain 3). Even though the two pseudomonads were identified as belonging to the same species, they clearly exhibited different biochemical properties.

Antagonistic activities of different strains of pseudomonads have been reported from roots of plants where they show biocidity against soil-borne plant pathogens (parasitic nematodes, bacterial and fungal pathogens) (Haas and Keel 2003). Such activities have been associated with the production of several secondary metabolites, of which especially the phenolic compound 2,4-diacetylphloroglucinol (2,4-DAPG) has received particular attention because of its production by a wide range of pseudomonads – also by *Pseudomonas fluorescens* – and its broad spectrum antifungal as well as antibacterial activities (Ramette et al. 2006, Schnider-Keel et al. 2000, Velusamy and Gnanamanickam 2003). Moreover, it inhibits the growth of nematodes of potato (Cronin et al. 1997). In 2003, Haas and Keel have reviewed six classes of compounds that include the most important and well-characterised antibiotics released by *Pseudomonas* spp. Additionally to phloroglucinols, they include hydrogen cyanide (HCN), lipopeptides, phenazines, pyoluteorin and pyrrolnitrin. All these six compounds inhibit phytopathogenic microorganisms *in vitro*.

Moreover, recently, Péchy-Tarr et al. (2008) discovered that two strains of *P. fluorescens* have potential insecticidal activity by producing the large protein toxin Fit (*P. fluorescens* insecticidal toxin), whose gene, when expressed in a non-toxic test organism (*Escherichia coli*), was sufficient to render the bacterium toxic to two insect hosts.

Also, the species *Ralstonia pickettii*, – a species that has formerly been accommodated in the genus *Pseudomonas* (Anzai et al. 2000) and that is common in soil and water and can also be found as nosocomial pathogen in humans (Ryan et al. 2006) – has been identified recently for showing *in vitro* antagonistic activities. In a study of Baschien et al. (2009) concerning the interactions between fungi and hyphosphere bacteria (bacteria associated with the hyphae of fungi) living in the same environment of decomposing leaves, *R. pickettii* was found to induce growth inhibition of the fungus *Cladosporium herbarum* on leaf extract agar plates. However, the mechanisms of action of *R. pickettii* were not identified. Resource or interference competition was suggested, rather than the production of interfering allelochemicals, the presence of which could not be determined by Baschien et al. (2009). Comparable mechanisms may also be present in the bacteria that showed biocidity in the present study, as the biochemical nature of the modes of action neither of *R. pickettii* nor of *P. fluorescens* was elucidated. It is possible that a complex mechanism

is responsible for the inhibition of the test organisms *in vitro*. Further investigation on the actual biocidal mechanisms would be of interest.

Summing up, the identification of strains of *Pseudomonas* and *Ralstonia* that exhibit antagonistic activities is not totally new. However, they have been identified in frog foam of *L. pentadacytlus* for the first time.

### 4.4.2 Relevance for the foam nests

The functions of the growth inhibiting bacteria in the foam nests of *L. pentadactylus* are not entirely clear. We propose that the foam nests may have mutualistic associations with bacteria, the biocidity of which is possibly explaining (parts of) the biocidal activity of the foam nest described before.

Not only symbiotic interactions between *Pseudomonas* spp. and plants are well known, like described above, also mutualistic associations between bacteria and metazoans are common. McFall-Ngai (1999) described the interactions between squids and marine luminous bacteria of the genus *Vibrio* – that are important for the function of the squid light organ – as model of a stable association between animals and bacteria. Another example are *Philanthus* solitary hunting wasps, the females of which cultivate antifungal bacteria in specialised antennal glands. Prior to oviposition, the females apply the bacteria to brood cells in order to protect the cocoon and larvae from fungal growth (Kaltenpoth et al. 2005).

Such a mechanism may also be present during oviposition of *L. pentadactylus*. The fact that two of the three bacterial strains that caused growth inhibiton of *C. albicans, M. avium* and *T. mentagrophytes* were isolated from the inoculum of one single egg and thus, in the direct proximity of what should be actually protected in the nest, could be an indication of the presence of symbiotic bacteria in the nest. The bacteria could be applied to the eggs during passage through the oviduct of the female. However, to the best of our knowledge, the investigations of symbiotic bacteria in the oviduct of amphibians and in the foam nest of frogs have not been performed.

Possibly, the active bacteria found in the nest may also derive from the skin of the female or male during foaming. In 2007, Lauer et al. proposed amphibians for having mutualistic associations with bacteria of the skin microflora. They described the presence of resident bacteria in the skin of the salamander *Plethodon cinereus* that can inhibit pathogenic fungi found in the moist habitats these amphibians inhabit. Some of the strong antifungal

bacteria identified by Lauer et al. were related to the genus *Pseudomonas*, the genus to which at least also two of the three bacterial strains that exhibited antibiosis against other microorganisms in the current study were allocated.

Brucker et al. (2008) even see the antifungal activities of bacteria on the skin of amphibians in a wide ecological context. The presence of bacterial symbionts on the skin of amphibians inhibits the growth of the fungal pathogen *Batrachochytrium dendrobatidis*, a pathogen that causes a disease that is linked to the decline of many frog populations especially in the tropics. According to Brucker et al., some species resist the disease despite infection with the pathogen due to cutaneous antifungal symbionts that are more or less effective depending on the individual species.

### 4.4.3 Conclusion

The growth inhibiting bacteria of the *L. pentadactylus* foam nests may have particular importance for the biocontrol of (pathogenic) microorganisms in the foam nests, as such interactions are known from diverse species and organism groups. However, in the present study it could not be entirely clarified, whether the isolation of bacteria showing in vitro growth inhibition was only random, or whether they fulfill a distinct function in the foam nests. Further research on bacterial-foam interactions could clarify, whether such bacterial species also occur in the oviduct or on the skin of leptodactylid frogs, where they might have further commensal or mutalistic relevance. Moreover, the actual biochemical nature of the modes of action of the growth inhibiting bacteria and whether they suffice to defend the eggs or the total nest in the environment would be of further interest.

Moreover, as two of three active bacteria were isolated from the inoculum of an egg further studies could be directed at inoculating eggs (as only one single egg was inoculated), in order to determine whether the bacteria are particularly associated with the eggs, and whether there are also other bacteria than in the present study.

# 5 GLOSSARY

| | |
|---|---|
| Acanthopodia | Cytoskeletal structures of *Acanthamoeba* that produde from the main body of the cell and possess hyaline cytoplasma |
| Aerobic | Term for the presence/ use of oxygen; aerobic organisms use oxygen for cellular respiration |
| Allelochemical | Messengers that allow interactions and communication between organisms of different species (interspecific) |
| Amastigote | Round or oval-shaped form of Kinetoplastida with no apparent flagellum |
| Amplexus | During mating the male is sitting on the back of the female, holding the female in the armpits (axillary) or around the waste (inguinal) bringing his cloaca into close proximity with that of the female |
| Axenic | A culture is axenic, if it only contains one organism without contaminations e.g. *Leishmania* sp. in liquid culture |
| Carnivore | Animals that obtain their energy and nutrients from casual or exclusive feeding on animal tissue |
| Chagoma | Primary symptom of Chagas disease; an oedematous swelling at the site where *Trypanosoma cruzi* entered the body |
| *cis*-Golgi | Part of the Golgi apparatus that is orientated to the endoplasmic reticulum and receives vesicles from that |
| Conjunctiva | Mucous membrane of the eye |
| Conspecific | Animals are called conspecific, if they belong to the same species |
| Convergent evolution | Different unrelated taxa have acquired characteristics that serve the same functions (because of the use of e.g. the same niches) but that had evolved independently in each taxon. |
| Cyst | Persistent life cycle stage of different protozoa with little metabolic activity, e. g. in *Acanthamoeba* |
| Ectocyst | Outer wall of a cyst |
| Endocyst | Inner wall of a cyst |
| Endophyte | Endosymbiont, often a bacterium or fungus that lives within a plant; they are often symbiotic and provide benefits for the plants |
| Endoplasmic reticulum | Organelle of eukaryotes that can be differentiated into two parts: the rough endoplasmic reticulum harbours the ribosomes that are necessary for the synthesis of peptids. The smooth endoplasmic |

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| | reticulumn functions in different metabolic steps, e.g. synthesis of lipids and steroids. |
| Epimastigote | Spindle-shaped flagellate in which the kinetoplast is located between the nucleus and the anterior end |
| Eukaryote | Cell that contains a true DNA containing nucleus covered by a nuclear envelope; most eukaryotes contain further organelles like mitochondria, chloroplasts, Golgi-apparatus |
| Exotroph (or exotrophic) | Larva that feeds on various materials not parentally derived, or on trophic eggs provided by the mother |
| Family | Taxonomic category of related organisms, ranking below an order and above a genus |
| Genome | Entirety of an organism's genetic information; it is encoded in deoxyribonucleic acid (DNA), or for many viruses, in ribonucleic acid (RNA) |
| Genus (pl. genera) | Taxonomic category of related organisms ranking below a family and above a species |
| Glycan | Sugar chains (oligo- or polysaccharides) covalently attached to proteins or lipids that often function in providing cell-cell interactions during important biological activities |
| Golgi apparatus | Organelle found in eukaryotic cells that plays important roles in processing and packaging of cellular macromolecules (especially proteins) |
| Granulomatous amoebic encephalitis | Infectious disease of the central nervous system caused by *Acanthamoeba* |
| Heterospecific | Animals are heterospecific, if they belong to different species |
| Host | An organism that harbours another organism (like a parasite) on or in itself |
| Hyaline | Transparent cytoplasma, which excludes various vacuoles and particles that are normally present in the interior of the cell |
| Hypertrophy | The enlargement of a particular organ or tissue, due to the enlargement of the cells of the tissue (in contrast to hyperplasia, where the number of cells increases without changes in their size) |
| Hypha | A long, branching filamentous structure of a fungus, which is the main vegetative growth form; the totality is termed mycelium |
| Intraspecific | A term that is involving the members of one species; occurring within a species |
| Keratitis | Inflammation of the cornea of the eye |

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| Kinetoplast | DNA-containing organelle in the single large mitochondrion of single-celled flagellated protozoa (Kinetoplastida) that contains many copies of the mitochondrial genome |
| Lectin | Lectins are sugar-binding proteins or glycoproteins that are ubiquitous in nature and are binding specifically and reversibly to specific sugar residues that are part of glycoproteins or glycolipids. They play a role in biological recognition phenomena involving cells and proteins, and are able to agglutinate certain animal cells |
| Mass spectrometry | An analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, to elucidate the composition of a sample or the chemical structures of molecule, in this case of glycans |
| *Medial*-Golgi | Middle section of the Golgi apparatus. |
| Microlesion | A very small, minute injury of some tissue. |
| Monophyletic | A group containing a hypothetical common ancestor and all its descendants; characterized by the possession of synapomorphies (see synapomorphy; paraphyletic; polyphyletic) |
| Mucin | Form of a glycoprotein that usually carries a large number of *O*-glycans that are linked via *N*-acetylgalactosamines to the OH-groups of serines or threonines. Mucins are often found in mucous secretions of various eukaryotes |
| Mycelium | The total of the vegetative part of a fungus, the summary of several filamentous cells, the hyphae. |
| Neotropic | Biogeographical term for the ecozone that includes Central and South America, the lowlands of Mexico, the Caribbeans and South Florida |
| Neuraminic acid | Neuraminic acid is a 9-carbon monosaccharide, that does not occur naturally, but many of its derivatives are found widely distributed in animal tissues and in bacteria, especially in glycoproteins. The *N*- or *O*-substituted derivatives of neuraminic acid are known as sialic acid |
| Non-indigenous | A species that is living outside of its natural distributional area and has been introduced by humans accidentally or intentionally into its new environment. |
| Nuptial spine | Modified skin tissue of male frogs that can be found, e.g. on the chest or thumbs, and assist them to grasp the females during mating. The spines may also have a role in combats between males |

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| Opsonization | Process in which a pathogen (e.g. bacterium) is marked by antibodies or lectins for phagocytosis by phagocytes that destroy the invading microorganism. |
| Paraphyletic | In cladistics, a group is paraphyletic, if its members have a direct common ancestor, however, the group does not contain all taxa of descendants of this ancestor. |
| Parasite | An organism that lives at the expense of a host organism. The parasite can live inside (endoparasite) or on the surface (ectoparasite) of the host. |
| Pathogen | Biological agents, like viruses, bacteria, helminths, protozoa etc., that cause diseases to their hosts. |
| Pathogenicity | The ability of a pathogen to cause an infectious disease in another organism. |
| Phylogenetics | The study of the relatedness of different groups of organisms (e.g. of genera, species), that is based mainly on molecular sequencing and morphological similarities of the organisms |
| Polyphyletic | In cladistics, a group is polyphyletic, if it does not have a direct common ancestor. |
| Promastigote | Slender form of Kinetoplastida in which the kinetoplast and the flagellar basal body are located at the anterior end of the cell |
| Proteinase | Also termed protease or peptidase; enzymes that break peptide bonds between amino acids by hydrolysis, a process called proteolysis. They naturally occur in all animals and are important in a number of physiological reactions, like digestion. |
| Protozoa | Single-celled, non-photosynthetic, heterotrophic eukaryotes. |
| Rhizoplane | The part of a plant's root that lies at the surface of the soil, where many microorganisms adhere to it |
| Sialic acid | Compare “neuraminic acid”; Derivatives of neuraminic acid are collectively known as sialic acid. The predominant form in mammalian cells is *N*-acetylneuraminic acid (Neu5Ac). |
| Spiracle | Small holes on the surfaces of many animals (in that case arthropods) that are usually associated with the respiratory system. |
| Surfactant | The term stands for “surface active agent”. Surfactants function in reduction of the surface tension of liquids or lowering of the interfacial tension between two liquids or between a liquid and a solid. Proteins and lipids that function as surfactants both have a hydrophobic and hydrophilic region. |

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|---|---|
| Symplesiomorphy | In cladistics, a characteristic that is primitive and shared between two or more taxa, and shared with a common ancestor. The possession of a symplesiomorph character is not evidence that the taxa in question are closely related. For example, several mammals possess a spinal column, however, this character cannot be used to describe the relatedness of the subgroups of mammals. |
| Synapomorphy | In cladistics, traits that have originated with the last common ancestor of the taxa under consideration. The characteristics are shared by two or more taxa and by their most recent common ancestor, whose ancestor in turn does not possess the trait. For example, the presence of a spinal column in mammals is a new (apomorph) characateristic in comparison to their common ancestor. |
| Tandem mass spectrometry (MS/MS) | Some kind of mass spectrometry that involves multiple steps of mass spectrometry, with some form of fragmentation occurring in between the stages, in order to get more information on the composition and structure of usually one molecule. |
| Trophozoite | The actively feeding and dividing stage of protozoa. |
| Trypomastigote | The kinetoplast of this form of flagellate is situated at the posterior end of the cell. The flagellum emanates from the flagellar pocket and runs to the anterior end along an undulating membrane. |
| Tympanum | An external hearing organ in frogs and toads, located just behind the eye. It transmits sound waves to the amphibian's inner ear, while protecting it from water and other foreign objects. |
| Vector | In medicine, a vector is a carrier of a pathogen, like a parasite. The vector of *Plasmodium* spp. (parasites causing malaria) is a mosquito of the genus *Anopheles*. |
| Virulence | The relative ability of an organism (pathogen) to cause a disease; not the actual ability to cause disease, but rather, the degree of pathogenicity of an organism. |

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# 7 APPENDIX

## 7.1 ABSTRACT

Foam nesting is one of the numerous strategies evolved in tropical frogs to protect their eggs and developing tadpoles against environmental challenge. *Leptodactylus pentadactylus* – the smoky jungle frog of Central and South America – produces voluminous foam nests containing fertilized eggs, lying in the jungle for several days without degradation by fungi or other pathogens. However, the mechanisms ensuring long-term stability of the nests and protection of the brood are not completely understood and suggestions are contradictory.

In the first line, it was aimed to clarify the chemical properties of the foam nest material and to get an impression of the specific glycan structures, especially of the *N*-glycans of the glycoproteins of the foam nest of *L. pentadactylus.* Another prior intention of the present study was the analysis of a potential biocidal activity of the frog foam nest. Finally, it was aimed to unravel the microbial community that is associated with the nest and its potential protective function.

The glycans were analysed by lectin blots followed by analysis with alkaline phosphatase, and by glycan preparation followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis.

For testing the biocidal potential of the foam *Trypanosoma cruzi*, *Leishmania donovani*, *L. infantum* and *Acanthamoeba* were investigated for their susceptibility to the homogenized and filtered foam extract in different concentrations in microtiter plates. Moreover, the bacteria *Enterococcus hirae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Mycobacterium avium, M. terrae* and *Staphylococcus aureus*, as well as the fungi *Candida albicans*, *Saccharomyces cerevisiae* and *Trichophyton mentagrophytes* were investigated for their susceptibility in agar-plate diffusion assays with foam material.

Moreover, bacteria were isolated from the inside of the nest by inoculation of small volumes of foam pieces into liquid medium. Several isolated bacterial strains were tested for *in vitro* antagonism towards *C. albicans*, and those showing biocidal effects, were tested additionally against *Mycobacterium avium*, *M. terrae* and *Trichophyton mentagrophytes*. The bacteria were finally classified via their enzymatic activities and by mass spectrometry (MALDI-TOF-MS).

In total, approximately 60 *N*-glycan species could be structurally differentiated. The presence of both, hybrid- and complex-type *N*-glycan structures was indicated, as well as the presence of other glycans (putatively *O*-glycans) within the foam nest glycoproteins.

Moreover, biocidity of the foam nest was revealed. Treatment of *L. infantum* and *L. donovani* with the foam resulted in growth inhibition. *T. cruzi* and *Acanthamoeba* were not susceptible to the foam material. Furthermore, there was no microbicidal activity on most bacteria and fungi; weak growth inhibition was observed for *M. avium* and *M. terrae*.

Finally, 25 different bacterial strains were isolated from the inside of the nest, three of which also showed biocidal effects on *Candida albicans*. These bacteria were classified with high reliability as strains of *Ralstonia pickettii* and (in two cases) *Pseudomonas fluorescens*. One *P. fluorescens* strain additionally showed a biocidal effect on *M. avium* and the other one on *T. mentagrophytes*.

The presence of *N*-glycans in the foam material of *L. pentadacytlus* was shown for the first time and is a general contribution to the knowledge of *N*-glycans in the amphibian world, as until now in particular *O*-glycans of amphibians have been studied. Moreover, antimicrobial activities of foam nests have long been assumed, however, could here be shown for the first time, although the nests do not seem to totally inhibit bacterial growth. In fact, they harbour different kinds of bacterial species, some of which themselves cause growth inhibition of other microorganisms. *P. fluorescens* is known to show antimicrobial activities, however, in the current study it was detected in the foam nests of *L. pentadactylus* for the first time. A possible connection between the biocidity of the nest and the antagonistic microorganisms in the nests is suggested.

## 7.2 ZUSAMMENFASSUNG

Die Bildung von Schaumnestern während der Paarungszeit ist eine von zahlreichen Strategien, die von tropischen Fröschen zum Schutz der Nachkommenschaft vor schädlichen Umwelteinflüssen genutzt werden. *Leptodactylus pentadactylus* – der Südamerikanische Ochsenfrosch, welcher in Mittel- und Südamerika beheimatet ist – legt die befruchteten Eier in Schaumnester ab, die bis zum Schlüpfen der Kaulquappen in tropischen Gewässern liegen, ohne von Pilzen oder anderen Pathogenen überwuchert zu werden.

In vorliegender Arbeit sollten zunächst die chemischen Eigenschaften des Schaummaterials untersucht werden. Eines der primären Ziele bestand darin, einen Eindruck von den

spezifischen Glykanstrukturen der Glykoproteine der Nester von *L. pentadactylus* zu gewinnen, wobei vor allem auf die Charakterisierung der *N*-Glykane Wert gelegt wurde. Zusätzlich war die Untersuchung der potentiellen bioziden Aktivität des Schaumes ein wichtiges Ziel dieser Studie. Darüber hinaus, sollten außerdem die Mikroorganismen, welche mit dem Schaum assoziiert sind sowie deren mögliche schützende Funktion im Schaum, untersucht werden.

Die Analyse der Glykane erfolgte mit Hilfe von Lektin-blots und Detektion mittels alkalischer Phosphatase. Weiters wurde eine Glykanpräparation mit anschließender MALDI-TOF (matrix-assisted laser desorption/ ionization time-of-flight) Massenspektrometrie-Analyse durchgeführt. Zur Untersuchung der bioziden Wirkung des Schaumes wurden Versuche an *Trypanosoma cruzi*, *Leishmania infantum, L. donovani* und *Acanthamoeba* in Mikrotiterplatten durchgeführt, in denen die Parasiten mit dem homogenisierten und filtrierten Schaum (in verschiedenen Konzentrationen) behandelt wurden. Die Wirkung des Schaumes gegen die Bakterien *Enterococcus hirae, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Mycobacterium avium, M. terrae* und *Staphylococcus aureus*, wie auch gegen die Pilze *Candida albicans*, *Saccharomyces cerevisiae* und *Trichophyton mentagrophytes* erfolgte in Kulturplatten, auf die das Schaumextrakt aufgetropft wurde.

Zur Isolation von Bakterien aus dem Inneren des Nestes, wurden kleine Stücke des Schaumes in Flüssigmedium inokuliert. Sämtliche isolierte Stämme wurden auf *in vitro* Antagonismus gegen *C. albicans*, und jene die biozide Effekte zeigten auch zusätzlich gegen *Mycobacterium avium, M. terrae* und *Trichophyton mentagrophytes* getested. Die Bakterien wurden schließlich über ihre enzymatischen Aktivitäten und mittels Massenspektrometrie identifiziert.

Insgesamt konnten etwa 60 *N*-Glykane strukturell differenziert werden. Darüber hinaus konnte das Vorliegen von hybriden und komplexen *N*-Glykanen sowie das Vorhandensein weiterer Glykane, vermutlich *O*-Glykane, in den Glykoproteinen des Schaumnestes nachgewiesen werden.

Weiters konnte auch die antimikrobielle Wirksamkeit des Schaumnestes gezeigt werden. Für *L. infantum* und *L. donovani* zeigte sich durch die Behandlung mit dem Schaum eine deutliche Wachstumshemmung. *T. cruzi* und *Acanthamoeba* hingegen zeigten keine Reaktion. Die meisten Bakterien und Pilzen wurden durch die Substanz nicht inhibiert,

jedoch konnte eine schwache Wachstumshemmung bei *M. avium* und *M. terrae* beobachtet werden.

Von den 25 Bakterienstämmen, die insgesamt aus dem Nest isoliert werden konnten, zeigten drei biozide Effekte auf *Candida albicans*. Diese Bakterien wurden mit großer Verlässlichkeit als Vertreter der Arten *Ralstonia pickettii* und *Pseudomonas fluorescens* identifiziert. Ein *P. fluorescens*-Stamm zeigte eine zusätzliche biozide Aktivität gegen *M. avium*, der andere gegen *T. mentagrophytes*.

Die vorliegende Arbeit konnte zum ersten Mal die Anwesenheit von *N*-Glykanen im Schaummaterial von Fröschen zeigen und stellt einen allgemeinen Beitrag zur Kenntnis der *N*-Glykane bei Amphibien dar. Der Schwerpunkt bisheriger Studien lag vor allem bei der Untersuchung von *O*-Glykanen. Auch die Biozidität der Schaumnester wurde schon seit Langem diskutiert, konnte jedoch in der vorliegenden Studie zum ersten Mal tatsächlich nachgewiesen werden, auch wenn das Wachstum von Bakterien offenbar nicht vollständig gehemmt wird. Tatsächlich wird der Schaum sogar von einer Reihe von Bakterien bevölkert, von denen einige wachstumshemmend bei bestimmten anderen Mikroorganismen wirken. *P. fluorescens* ist für seine antimikrobiellen Aktivitäten bekannt, die Art konnte jedoch zum ersten Mal im Froschschaum von *L. pentadactylus* detektiert werden. Ein möglicher Zusammenhang zwischen der Biozidität des Nestes und den antagonistischen Mikroorganismen wird vermutet.

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## 7.4 CURRICULUM VITAE

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| Since 2005 | Study of Genetics and Microbiology at the University of Vienna (focus on Immunology) |
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