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# *Goussia* Labbé, 1896 (Apicomplexa, Eimeriorina) in Amphibia: Diversity, Biology, Molecular Phylogeny and Comments on the Status of the Genus

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**We provide new data on morphology, host specificity, life history, pathology and phylogeny of the coccidian genus *Goussia* in European anurans. Divergence in the SSU rDNA sequences (3—4%) of three *Goussia* isolates from three anuran hosts suggests that the isolates represent three distinct species. The isolate from *Pelophylax* kl. *esculentus* was determined as *Goussia neglecta*. The isolates from *Rana dalmatina* and *Rana temporaria* are considered conspecific and are, on the basis of host specificity, light microscopical, ultrastructural, and molecular phylogenetic data, described as a new species, *Goussia noelleri*. The new *Goussia* species from *Bufo bufo* remains unnamed. During the host's metamorphosis the infection disappears, however, oocysts are retained in the liver of post-metamorphic frogs. Molecular phylogenetic analysis showed that anuran-host *Goussia* form a well-supported monophyletic clade, which together with a clade represented by piscine *Goussia metchnikovi*, constitute basal lineages of the Eimeriidae. The two lineages show polytomy, likely reflecting undersampling of the basal eimeriid taxa. *Goussia janae* represents a distinct lineage, sister to the clade containing the other eimeriorinid taxa, suggesting a paraphyly of the genus *Goussia*. The identity of *G. neglecta*, the status of the genus *Goussia*, the presence of cryptic species in anuran-host *Goussia* and their ecological peculiarities are discussed.**


**Key words:** Anura; coccidia; cryptic species; *Goussia*; phylogeny; ultrastructure.

## Introduction

Our understanding of pathogens of amphibians is of special interest in the context of global amphibian decline, frequently associated with emerging infectious diseases that cause mass die-offs and extinctions of amphibian populations (Daszak et al. 2003; Skerratt et al. 2007; Stuart et al. 2004). Coccidians represent widespread parasites of vertebrates with a potential to affect the status of host populations. Within the scope of a long-term research of amphibian parasites, we encountered a common occurrence of coccidia of the genus *Goussia* Labbé, 1896 in wild populations of anurans in Central Europe. This finding

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allowed us to conduct an extensive study of members of this elusive and overlooked coccidian genus with consequences for understanding of thus far unrecognized taxonomic diversity and phylogenetic patterns of poikilotherm coccidians in general (Dyková and Lom 1981; Jirků et al. 2002).

The genus *Goussia* was erected to accommodate piscine coccidia with oocysts possessing four dizoic sporocysts composed of two valves joined by a longitudinal suture (bivalved sporocysts). The fine, often elastic oocyst wall and the absence of an oocyst residuum are other typical features of the genus. Most species are probably homoxenous, but facultatively a heteroxenous life cycle was confirmed experimentally for *Goussia carpelli* (Steinhagen and Körting 1990).

Approximately 50 nominal species of *Goussia* have been described from freshwater or marine fish and amphibians. The generic name *Goussia* has a complicated history (Dyková and Lom 1981; Levine 1983). Although the genus name was revived by Dyková and Lom (1981), the most recent taxonomical review of the suborder Eimeriorina (Upton 2000) lists *Goussia* as a synonym of *Eimeria* without further comments. However, the generic name remains recognized as valid by some authors and new species are being continuously described assigned to it. Virtually nothing is known about the relationships of *Goussia* to other coccidian taxa except that the well-described piscine *Goussia janae* Lukeš et Dyková, 1990 is not closely related to *Eimeria* (Jirků et al. 2002; Lukeš and Starý 1992).

Among amphibian hosts, only larvae of anurans (tadpoles) were found to be parasitized by *Goussia*. Two intestinal species are known from anurans. Nöller (1920) described *Eimeria neglecta* based on oocysts recovered from tadpoles of European Ranids. In 1995, Molnár redescribed this coccidium, recognized its affinity to fish-host coccidia and emended its taxonomic status to *Goussia neglecta*. Later, Paperna et al. (1997) described *Goussia hyperolisi* from tadpoles of the reed frog *Hyperolius viridiflavus* from Kenya. Although *Goussia* infections in the anuran hosts seem to be restricted to tadpoles and the disappearance of infection during the host's metamorphosis was recorded repeatedly (Molnár 1995; Nöller 1920; Paperna et al. 1997), retention of oocysts in liver sinusoids of post-metamorphic frogs was documented only recently (Jirků and Modrý 2006).

Herein, we provide data on morphology, biology and phylogenetic affinities of anuran *Goussia*, one of which is described as a new species, and describe the relationships of European anurans with their coccidian parasites using several approaches. Small subunit ribosomal DNA (SSU rDNA)-based phylogenetic analysis of three *Goussia* species from different European anuran taxa and two fish-host *Goussia* provide novel insight into the taxonomy and phylogenetic affinities of this genus.

## Results

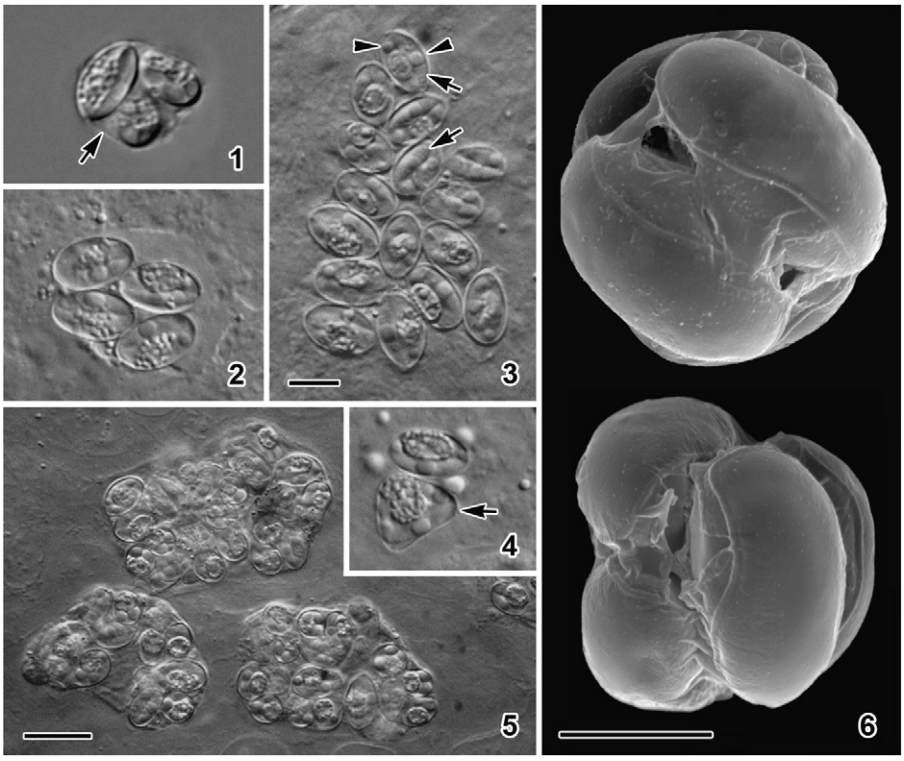
Of all examined tadpoles representing five anuran species, *Goussia* infections were recorded in tadpoles of *Rana dalmatina* (Loc1), *Rana temporaria* (Loc1, Loc2), *Pelophylax* kl. *esculentus* (Loc4) and *Bufo bufo* (Loc1). *Hyla arborea* was the only negative host. In contrast to Locality (Loc) 1 and Loc3, no infection was recorded in *B. bufo* tadpoles from Loc2. Similarly, *P.* kl. *esculentus* tadpoles were infected at Loc4, but not at Loc2. Free oocysts (Fig. 1) were detected in fresh faecal samples of wild tadpoles examined by the flotation method. Upon dissections, fully sporulated oocysts were observed either freely or enclosed within the yellow bodies in squash preparations of intestinal mucosa, intestinal contents and livers (Figs 2—5) of all tadpoles, including those appearing to be negative based on the examination of faeces by flotation.

### Identity of Anuran Isolates

The isolate from *P.* kl. *esculentus* is treated as *G. neglecta* based on morphology and origin from the type host, and is further characterized by SSU rDNA sequence. The isolate from *B. bufo* is characterized by sporocyst morphology and partial SSU rDNA sequence and is conservatively treated as *Goussia* sp. due to the absence of ultrastructural and reliable host specificity data. Based on transmission experiments, *Goussia* from *R. dalmatina* and *R. temporaria* are considered conspecific. They are morphologically characterized using the *R. dalmatina* isolate, and according to the SSU rDNA sequence divergence, a new species is proposed, the description of which follows.

### *Goussia noelleri* sp. nov.

**Oocyst morphology:** Shape of oocysts variable, depending on position of sporocysts tightly enclosed by a fine, elastic, colourless oocyst wall




**Figures 1—6**. Light (**1—5**) and scanning electron microscopy (**6**) of oocysts and sporocysts of *Goussia noelleri* sp. nov. from *Rana dalmatina* tadpoles. **1.** Oocyst isolated from faeces by flotation showing fine elastic oocyst wall (arrow). **2.** Ruptured oocyst in squash preparation of intestinal mucosa. Halo surrounding the four sporocysts is collapsed oocyst wall. **3.** Mass of sporocysts in squash preparation of liver. Striation is only barely visible in some sporozoites (arrows). Arrowheads show the two sporozoite refractile bodies. **4.** Comparision of atypically (arrow) and typically formed sporocyst. **5.** Three yellow bodies containing oocysts in squash preparation of intestinal mucosa. **6.** Within the oocyst, sporocysts are tightly encloced by a thin oocyst wall. Note the longitudinal suture clearly visible in the sporocyst wall. Scale bar = 5 μm (**1—4, 6**); 10 μm (**5**). Nomarski interference contrast (**1—5**).

(Figs 1, 6, 7). Oocyst measurements: 10.6 (10.0—11.5) × 10.0 (8.0—11.0) μm, length—width ratio (*L/W*) 1.07 (1.00—1.25). Oocyst residuum, micropyle and polar granule absent. Sporocysts dizoic, elliptical, often assymetrical with somewhat pointed pole(s) and/or one side being slightly flattened (Figs 1—3). Occasionally, atypical triangular sporocysts were observed (Fig. 4). The sporocyst measured 7.6 (7.0—8.5) × 4.8 (4.0—5.5) μm, *L/W* 1.60 (1.40—1.78) and contained a usually compact and elliptical sporocyst residuum (4.0—6.0 × 2.5—4.0 μm) rarely scattered among sporozoites, composed of granules 0.5—1.0 μm in diameter (Figs 1—4). Sporozoites were arranged in parallel within the sporocyst partly encircling the sporocyst residuum (Fig. 7). A transversal striation was sometimes observed in sporozoites (Fig. 3). Sporozoites longer than sporocyst, arranged head to tail, each curved at opposite pole of sporocyst (Fig. 7). In the light microscope, using Nomarski

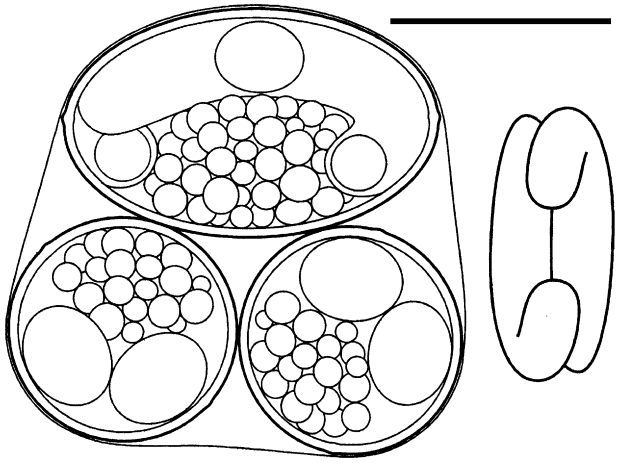


**Figure 7**. Composite line drawing of *Goussia noelleri* sp. nov. oocyst. Scheme on the right shows arrangement of sporozoites within sporocyst (not in scale). Scale bar = 5 μm.

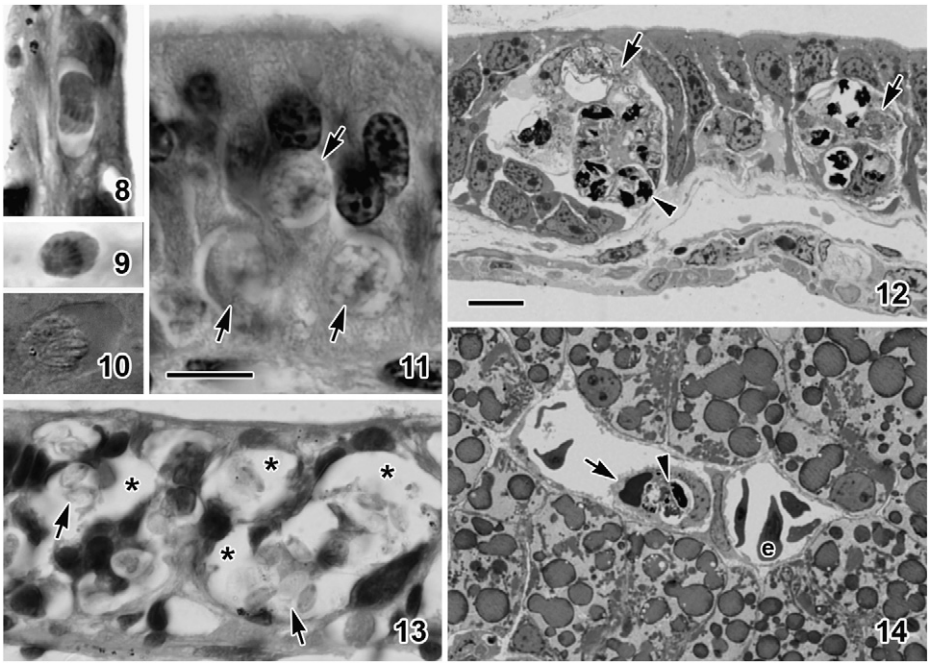
**Table 1.** Morphometrical comparision of *Goussia* spp. parasitizing tadpoles based on literature and original data. *L*/W—length/width ratio; *—type hosts and type localities; hyphens indicate missing data. Measurements are in μm.

| Species or isolate | Host(s) | Oocyst Sporocyst | Locality | Reference |
|---|---|---|---|---|
| *Goussia neglecta* (Nöller, 1920) Molnár (1995) | *Pelophylax* sp., *Rana* sp. | 9.0—10.0, spherical or irregular<br>7.0 × 3.5—4.0 | Hamburg and Thüringen, Germany | Nöller (1920) |
| *Goussia neglecta* (Nöller, 1920) Molnár (1995) | *Pelophylax* kl. *esculentus**, *Pelophylax ridibundus* | 10.6 (8.5—12.5), spherical<br>8.8 (6.5—10.2) × 4.8 (4.0—7.7), *L/W* not defined | Százhalombatta, Hungary* | Molnár (1995) |
| *Goussia neglecta* | *Pelophylax* kl. *esculentus** | —<br>9.4 (8.0—10.0) × 5.1 (5.0—5.5), *L/W* 1.82 (1.73—2.00) | Šnejdlík pond, Czech Republic | This study |
| *Goussia noelleri* sp. nov. | *Rana dalmatina** | 10.6 (10.0—11.5) × 10.0 (8.0—11.0), *L/W* 1.07 (1.00—1.25), spherical or irregular<br>7.6 (7.0—8.5) × 4.8 (4.0—5.5), *L/W* 1.60 (1.40—1.78) | Zaječí potok, Czech Republic* | This study |
| *Goussia noelleri* sp. nov. | *Rana temporaria* | —<br>7.8 (7.0—8.5) × 4.8 (4.5—5.5), *L/W* 1.63 (1.45—1.78) | Zaječí potok, Czech Republic* | This study |
| *Goussia* sp. | *Bufo bufo* | —<br>7.9 (7.0—9.0) × 4.5 (4.0—5.0), *L/W* 1.74 (1.50—2.25) | Zaječí potok, Czech Republic | This study |
| *Goussia hyperolisi* Paperna, Ogara and Schein (1997) | *Hyperolius viridiflavus** | 7.7 (7.0—9.8), spherical<br>7.2 (5.6—7.7) × 4.9 (4.2—5.6), *L/W* not defined | Sagana fish ponds, Kenya* | Paperna et al. (1997) |

interference contrast (NIC), two refractile bodies were observed. The bigger refractile body (1.5 μm in diameter) was located in the middle of the sporozoite, the smaller refractile body (1 μm in diameter) was located at the very end of its curved part (Fig. 3). A longitudinal suture was discernible in the sporocyst wall using NIC.

The sporocysts of *G. noelleri* (hosts *R. temporaria* and *R. dalmatina*) and the isolate from *B. bufo* were smaller, compared to the *G. neglecta* isolate from *P.* kl. *esculentus*, but the size ranges overlapped and the isolates could not be distinguished reliably (Table 1). Morphometry of the four anuran isolates matches the original description and redescription of *G. neglecta* (Molnár 1995; Nöller 1920).

**Endogenous development:** Endogenous stages develop in the cytoplasm of enterocytes, usually in the region below the host cell nucleus (Fig. 11) throughout the entire intestine of *R. dalmatina* tadpoles. Measurements in the following text refer to the *R. dalmatina* isolate. Merogonic and gamogonic stages were randomly distributed, and were rarely observed in histological sections compared to sporogonic stages. Meronts located within a distinct parasitophorous vacuole just above the basal membrane of the enterocytes measured 6.5—8.0 × 4.5—5.5 μm and contained approximately 15 merozoites arranged in parallel (6.0—7.0 × 1.5—2.0 μm), associated with a residual body (Figs 8, 10). Mature microgamonts (5.0—9.0 μm in diameter) were irregular in shape and contained fine, spirally arranged microgametes. Spherical to subspherical macrogamonts (8.0—9.5 μm in diameter) possess a large nucleus (Fig. 11). Endogenously sporulating oocysts were observed in various stages of sporogony in histological sections, varying 6.0—9.0 μm in diameter (Fig. 12), and usually forming aggregations in the basal part of the epithelial layer. These aggregations were formed by groups of 2—6 oocysts located within the yellow bodies




**Figures 8—14**. Light microscopy of haematoxylin and eosin-stained sections (**8, 9, 11, 13**) or toluidine-stained sections (**12, 14**) of the endogenous stages of *Goussia noelleri* sp. nov. from *Rana dalmatina* tadpoles. **8.** Meront within a distinct parasitophorous vacuole. **9.** Early meront. **10.** Meront in a squash preparation of the intestinal mucosa. Note the well-visible parasitophorous vacuole. **11.** Three macrogamonts (arrows) showing typical localisation of the endogenous stages of *G. noelleri* below nuclei of the host cells. **12.** Two yellow bodies (arrows) within the host intestinal epithelium. Each yellow body contains several oocysts as indicated by the number of deeply stained sporocysts (arrowhead). Note the compressed epithelial cells surrounding the yellow bodies. **13.** Heavily infected intestinal epithelium. Note that most of the volume of the epithelial cells is replaced by the yellow bodies (*) containing oocysts (arrows). **14.** Cross section of liver sinusoid containing erythrocytes (e) and melanomacrophage (arrow) with a phagocytosed oocyst of *G. noelleri* (arrowhead). Scale bar = 10 μm (**8—11**), 10 μm (**12—14**).

(Figs 5, 12, 13). No morphological and morphometrical differences could be found between the four anuran isolates.

In both squash and histological preparations, mature oocysts were frequently observed inside melanomacrophages in the lumina of liver sinusoids (Fig. 14) of all dissected tadpoles. The oocysts are transported to this extraintestinal location by the macrophages (Jirků and Modrý 2006). In addition, coprological, squash and histological examinations of gastrointestinal tracts of frogletts of *R. dalmatina*, previously experimentally infected as tadpoles were consistently negative up to 15 months post-metamorphosis. In contrast, presence of morphologically intact oocysts was observed in squash preparations of liver of all these animals till the end of the experiment (15 months post-metamorphosis).

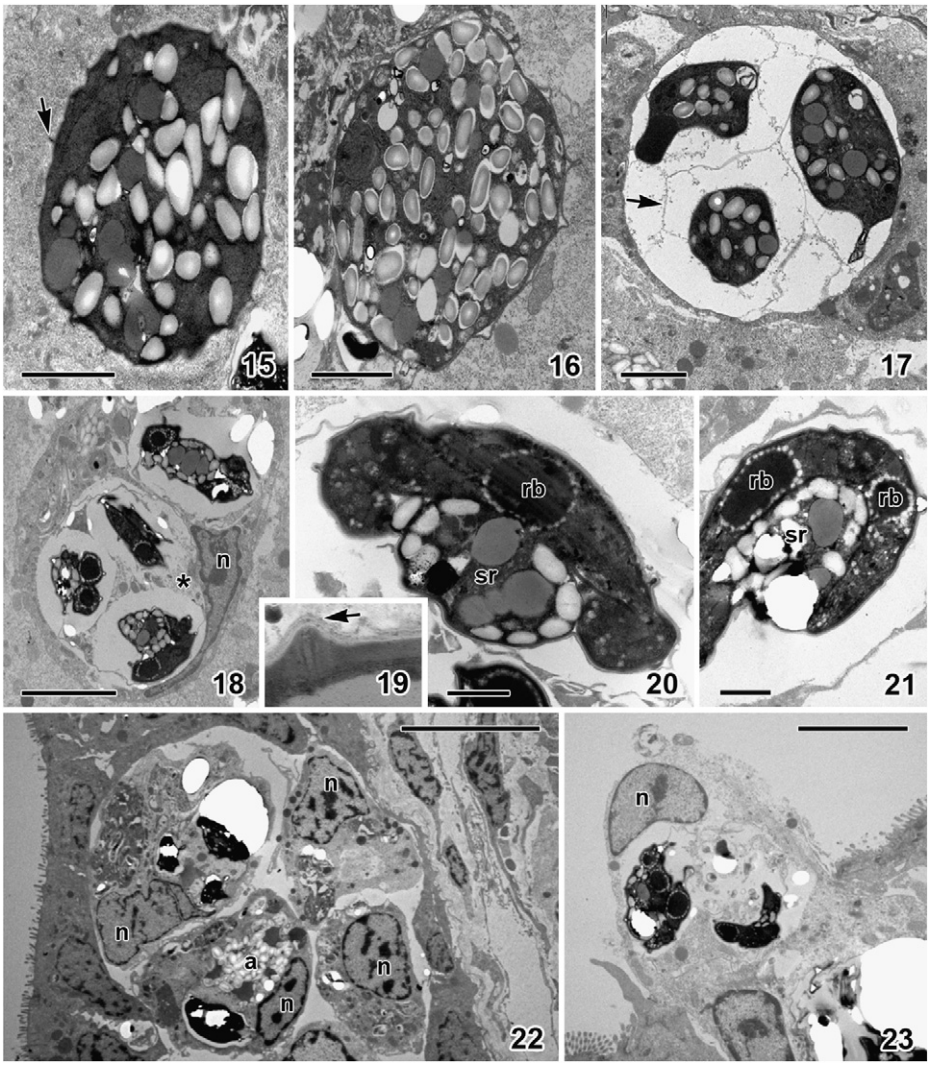
**Ultrastructure:** Macrogamonts and sporogonic stages were observed in ultrathin sections of the intestines of *R. dalmatina*. The cytoplasm of macrogamonts (7.2—8.5 × 7.2—7.8 μm) contained numerous amylopectin granules and lipid inclusions (Fig. 15), and lacked wall-forming bodies. No parasitophorous vacuole enclosed the macrogamonts, the unit membrane of which seemed to communicate directly with the host cell cytoplasm. Pellicular projections into the host cell cytoplasm could be observed on the surface of macrogamonts (Fig. 15). Zygotes or early oocysts (9.0—10.1 × 6.0—9.9 μm) (Fig. 16) differ from macrogamonts by the oocyst wall, which is in direct contact with host cell cytoplasm. No signs of an intervening membrane of the parasitophorous vacuole or an empty vacuolar space were observed. The oocyst wall is bilayered, composed of a thinner inner layer (~7 nm) and a thicker outer layer (~13 nm). In advanced oocysts, four sporoblasts bound by a unit membrane were formed, each enveloped by an additional loose membrane (Fig. 17). The space outside and inside this loose membrane(s) was filled with a sparse, foamy substance (Fig. 17). Next, advanced sporoblasts were covered by a bilayered membrane which eventually transformed into a thick sporocyst wall. The wall of fully developed sporocysts was apparently unilayered (28—30 nm thick) without any transverse striation (Fig. 19). The sporocyst wall widened up to 100 nm along the sutural line, which lacked any attached structures (Fig. 19). Sporocysts observed in SEM preparations had a smooth surface and a distinct curved longitudinal suture (Fig. 6). In mature oocysts, the space between sporocysts was filled with amorphous foamy material of variable density (Fig. 18). In all infections, aggregations of amylopectin granules representing deteriorated zygotes were observed together with mature oocysts within infected epithelial cells and yellow bodies (Fig. 22).

The only discernible structures in the poorly preserved sporozoites were micronemes, a bilayered pellicle and two refractile bodies. The refractile bodies were of homogenous consistence, each surrounded by a distinct layer of fine amylopectin granules (Figs 20, 21). Sporozoites partly encircled the sporocyst residuum composed of amylopectin granules and lipid inclusions with an intervening cement (Fig. 20).

**Pathology:** On the ultrastructural level, host cells of *R. dalmatina* containing the sporogonic stages demonstrated progressive cytoplasmic degradation characterized by cytoplasm homogenisation, degeneration of organelles and appearance of a multilaminated matrix. Such deteriorated host cells gradually lost integrity, often fused together and became the yellow bodies usually containing several oocysts or zygotes (Fig. 22). The yellow bodies were subsequently released from the epithelial layer into the intestinal lumen (Fig. 23).

Despite heavy infections, no inflammatory response was observed in histological sections of infected tissues, but significant histopathological changes were present during culmination of the infection, indicated by a presence of sporogonic stages. In such cases, the intestinal epithelium appeared to be disintegrated, with intervening foci of sporogonic stages associated with the yellow bodies (Figs 12, 13) or empty spaces left after their release. Most of the volume of affected enterocytes was thus occupied by sporogonic stages and the yellow bodies (Fig. 13). Despite such obvious pathology, animals which were not dissected and shed comparable quantities of oocysts as the histologically examined ones, showed no mortality and successfully completed the metamorphosis. At the level of histopathology, no differences were observed between the four isolates.

**Transmission experiments:** Most experimental trials (Table 2) suffered from the failure of negative controls, probably as a result of contamination, and cannot be classified as reliable (indicated by stars in Table 2). Of 25 trials involving European anurans, 16 resulted in infections, and only the infections of *R. dalmatina* tadpoles with isolates of *G. noelleri* originating from *R. dalmatina* and *R. temporaria* had proper negative controls and can be regarded as reliable. Tadpoles or adults of *Xenopus laevis* and *Pleurodeles waltl* never




**Figures 15—23**. Transmission electron microscopy of *Goussia noelleri* sp. nov. from *Rana dalmatina* tadpole. **15.** Macrogamont showing projections of its membrane into the host cell cytoplasm (arrow). **16.** Zygote. **17.** Immature oocyst containing four sporoblasts enveloped by loose membranes (arrow). **18.** Mature oocysts within detached host cell. Note the foamy substance filling the space between sporocysts (*) and deformed host cell nucleus (n). **19.** Cross section of the sporocyst wall thickening along the suture. The sporocyst wall is covered by bilayered oocyst wall (arrow); the electron dense layer below the sporocyst wall is the bilayered sporozoite pellicle. **20.** Longitudinal section of sporocyst showing sporocyst residuum (sr) and sporozoite with refractile body (rb) covered by a thick layer of fine amylopectin granules. **21.** Detail of a sporozoite containing two refractile bodies (rb). **22.** Cross section of a heavily infected intestinal mucosa containing group of yellow bodies with retained nuclei (n) of former host cells. Note the aggregation of amylopectin granules (a) indicating remnants of a deteriorated zygote. **23.** Yellow body protruding above the surface of epithelium just before its release into the intestinal lumen. Scale bars = 2 μm (**15—17**); 5 μm (**18, 23**); 1 μm (**20, 21**); 10 μm (**22**).

became infected. Coprological examination of all experimentally infected *R. dalmatina* tadpoles, together with histological examination of 10 of them showed disappearance of the infection from the gastrointestinal tract during metamorphosis. Fresh preparations made from these tadpoles, however, revealed oocysts in intestinal contents up to 3 days post-metamorphosis. Presence of oocysts in liver was recorded in all experimentally infected *R. dalmatina* tadpoles processed for histology before, during and after metamorphosis.

**Table 2.** Results of transmission experiments with *Goussia* isolates.

| Experimental animals | Origin of infectious material | | |
|---|---|---|---|
| | *Rana dalmatina* | *Rana temporaria* | *Bufo bufo* |
| *Rana dalmatina* | **2**/2 | **1**/2 | **1**/2* |
| *Rana temporaria* | **2**/2 | **2**/2* | 0/2* |
| *Pelophylax* kl. *esculentus* | **1**/2* | **2**/2* | **1**/1* |
| *Bufo bufo* | **2**/4* | **1**/2* | **1**/2* |
| *Hyla arborea* | 0/1 | — | — |
| *Xenopus laevis* tadpoles | 0/4 | 0/2 | 0/1 |
| *Xenopus laevis* adults | 0/4 adults | — | — |
| *Pleurodeles waltl* larvae | 0/1 | 0/1 | — |
| *Pleurodeles waltl* adults | 0/4 adults | — | — |

No. of experimental trials resulting in infections/No. of experimental trials conducted. Each trial involved 50 experimental tadpoles, except for *H. arborea* and *P. waltl* when 20 individuals were used in each trial. Stars (*) indicate trials in which negative controls became infected.

Experimentally infected *R. dalmatina* tadpoles invariably shed low numbers of oocysts 25—35 days post infection. Both fresh and histological preparations from tadpoles at the onset of oocyst shedding revealed terminating infections manifested by high numbers of sporogonic stages and significant histopathological changes of intestinal epithelia, suggesting delay of oocyst shedding, which hampered evaluation of the length of prepatent period.

**Phylogenetic analysis:** Comparison between *G. noelleri*, *Goussia* sp. from *B. bufo* and *G. neglecta* showed 96—97% similarity of SSU rDNA sequences. In our analysis (Fig. 24), three main coccidian clades — eimeriid, sarcocystid and adeleid — were recognized. Within Eimeriidae sensu lato (s.l.) (the sister clade of the sarcocystid lineage), the basal clades were invariably formed by taxa from the poikilotherm hosts, i.e. an unspecified intranuclear coccidium, *Choleoeimeria* sp., *Goussia metchnikovi*, anuran *Goussia* isolates, *Eimeria tropidura*, *Eimeria arnyi* and *E. ranae*. The only exceptions from this pattern are *Lankesterella minima* and *Caryospora bigenetica* with unstable position(s) in different analyses, both also from poikilotherms. *E. tropidura* clustered together with *Choleoeimeria* sp. and formed a well-supported lineage, which is sister to the clade comprising all Stieda body-bearing eimeriid coccidia. All *Goussia* sequences, except that of *G. janae*, appeared in all analyses at the base of the eimeriid clade. All three anuran *Goussia* isolates formed a distinct clade supported by high bootstrap values, with *G. noelleri* and *Goussia* sp. from *B. bufo* being closely related. Although the anuran *Goussia* clade together with a lineage represented by *G. metchnikovi* form a polytomy, their position at the base of the clade comprising all remaining eimeriids was stable in all performed analyses. *G. janae* represents a distinct lineage sister to the clade comprising all the other eimeriorinid coccidia (Sarcocystidae and Eimeriidae) which parasitize vertebrate hosts.

## Discussion

### Identity of *G. neglecta*

Our data showed that the concept of a single *Goussia* sp. in European anurans is unsustainable. *G. neglecta* was described from Hamburg in Germany from insufficiently determined anurans (Nöller 1920). In 1995, Molnár redescribed *G. neglecta* based on light-microscopic observations of material from *P.* kl. *esculentus* and *Pelophylax ridibundus*, without proposing a type host. Recently, Duszynski et al. (2007) justifiably proposed *P.* kl. *esculentus* as the type host of *G. neglecta*, but provided Hamburg as the type locality. This act erroneously blends together the incomplete description by Nöller (1920) with Molnár's (1995) redescription. To correct this confusion, we suggest to fully accept the redescription, and propose *P.* kl. *esculentus* as the type host, and Százhalombatta in Hungary as the type locality of *G. neglecta*.

### Unmasked Diversity of Anuran *Goussia*

The distribution pattern of *Goussia* infections at different localities with similar amphibian communities

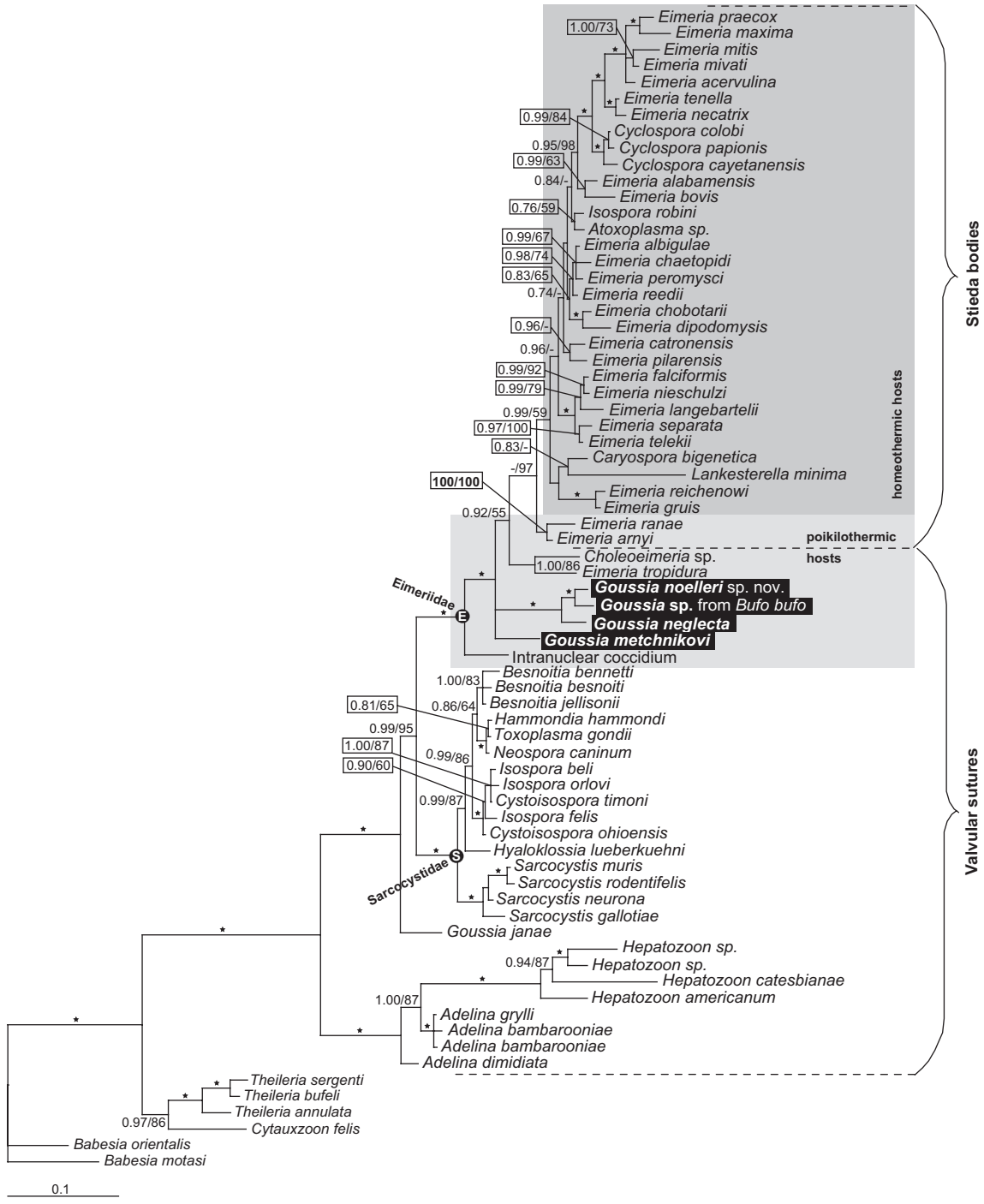



**Figure 24**. Bayesian phylogenetic tree as inferred from partial SSU rDNA sequences. Numbers above branches indicate Bayesian posterior probability/maximum likelihood boostraps/maximum parsimony bootstraps. Black stars show nodes supported by pp equal to 1.00 and both bootsraps over 90%. Brackets indicate character of excystation structures.

seems confusing. At one locality, both *Rana* and *B. bufo* tadpoles harbour *Goussia* infections, whereas at another locality, only *Rana* tadpoles are infected. High population densities of tadpoles, coupled with up to 100% prevalence and heavy infections, all resulting in high oocyst

output, necessarily lead to contamination of the aquatic environment, with oocysts being immediately infectious for new hosts due to their endogenous sporulation. In general, the tadpoles of anuran species involved in this study often occur syntopically, and it is unlikely that receptive hosts could remain uninfected in such conditions if the host range of *Goussia* found involved hosts from different genera. While the anuran *Goussia* isolates cannot be distinguished from each other and from *G. neglecta* by oocyst/sporocyst morphology, the 3—4% divergence of their SSU rDNA sequences indicates that each anuran genus hosts a distinct *Goussia* species. Infection experiments suggest that the closely related *R. dalmatina* and *R. temporaria* (Veith et al. 2003) are parasitized by *G. noelleri*. The most plausible explanation for the peculiar pattern of distribution in different localities, absence of morphological differences and sequence divergence among the anuran isolates is that three cryptic *Goussia* spp. parasitize *Rana* spp., *P.* kl. *esculentus* and *B. bufo*, respectively. This conclusion conforms with Molnár et al. (2005), who showed that morphologically indistinguishable *Goussia carpelli* isolates from different cyprinid fish represent distinct species with a narrow host specificity.

Interestingly, post mortem examination showed that the coprological results obtained by flotation do not reflect the real prevalence of *Goussia* in tapoles, possibly as a result of intermittent oocyst shedding, rendering coprology unsuitable for prevalence assessment of *Goussia* species. In agreement with others (Paperna et al. 1997) we found that *Goussia* infections are common among anuran tadpoles. Given their narrow host specificity, the diversity of anuran *Goussia* might thus be remarkable. However, the morphology of oocysts/sporocysts as well as their size ranges overlap in known anuran *Goussia* spp. and should not be considered a primary taxonomic criterion.

While oocysts/sporocysts of anuran *Goussia* are morphologically rather uniform, possibly as a consequence of being subject to similar selection forces, the situation may be different for the endogenous stages, which develop in the digestive tract of tadpoles. Different physiological environments of their hosts might therefore represent major selection mechanisms leading to species-specific morphological adaptations. Information on endogenous development should thus become regular part of future studies, that should incluce also molecular data. Following the proposed taxonomic requirements, we consider differences in the SSU rDNA sequence of the *B. bufo* isolate insufficient for the description of a new species.

## Differential Diagnosis of *G. noelleri*

At the ultrastructural level, *G. noelleri* is characterized by the absence of wall-forming bodies and parasitophorous vacuole in the gamogonic and sporogonic stages, presence of pellicular projections in macrogamonts, two refractile bodies per sporozoite, each surrounded by fine amylopectin granules, an unilayered sporocyst wall without transverse striation and a simple sporocyst suture. The lack of wall-forming bodies is a common trait of fish and amphibian coccidia (Paperna and Lainson 1995; Paperna et al. 1997), while the absence of a parasitophorous vacuole seems to be specific for *G. noelleri*. Another feature differentiating the anuran *G. hyperolisi* and *G. noelleri* from most piscine congeners is the absence of the sporocyst wall striation (Lom and Dyková 1992). Refractile bodies were observed in *G. hyperolisi*, yet in contrast to *G. noelleri*, no amylopectin granules were observed on their surface (Paperna et al. 1997). However, a layer of amylopectin granules reminiscent of that observed around refractile bodies of *G. noelleri* was described from anuran *Eimeria bufomarini* and piscine *Goussia sinensis* (Baska and Molnár 1989; Paperna and Lainson 1995).

**Status of the genus *Goussia*:** The solitary position of *G. janae* at the base of the eimeriorinid coccidia, along with the branching of the anuran *Goussia* spp. and the piscine *G. metchnikovi* within the Eimeriidae s.l. clade suggest that the genus *Goussia* is paraphyletic, characterized by non-unique features such as four bivalved dizoic sporocysts per oocyst. Moreover, this feature is shared with the reptile-host coccidian genera *Acroeimeria* and *Choleoeimeria* (Paperna and Landsberg 1989). Importantly, the polytomy of the anuran *Goussia* spp. and *G. metchnikovi* shows that basal eimeriid lineages are undersampled and taxonomic rearrangements using the limited available dataset would be unstable.

As there are two poorly characterized type species of the genus *Goussia* (Levine 1983), topotypic material is needed to redefine them, and re-evaluate the taxonomy of the *Goussia*-like piscine and amphibian coccidia. The genus should be redescribed by combination of oocyst/sporocyst morphological features, character of the endogenous development and phylogenetic affinity of the type species. Paperna and Landsberg (1989) erected the genera *Acroeimeria* and

*Choleoeimeria* to accommodate epicellular intestinal and biliary species parasitizing reptiles, respectively. However, both genera possess four bivalved dizoic sporocysts per oocyst, and thus fit the definition of the genus *Goussia*, if the type of the endogenous development and additional oocyst characteristics were not considered. Indeed, analyses of the available coccidian sequences confirm the position of *Choleoeimeria* as a distinct genus (Jirků et al. 2002; this study).

The genus *Goussia* s.l. currently accommodates coccidia that share oocyst/sporocyst morphology, but vary significantly in localisation and morphology of their endogenous stages (Dyková and Lom 1981, 1983). While *G. metchnikovi* is an extraintestinal species, both anuran *Goussia* spp. and *G. janae* included in our phylogenetic analysis are intestinal species. On the other hand, anuran *Goussia* spp. and *G. metchnikovi* are intracellular, whereas *G. janae* is epicellular. Although the dataset of *Goussia* s.l. available for phylogenetic analyses remains very limited, it already reveals that the genus includes several unrelated lineages. Despite its likely artificial nature, we suggest to retain the genus *Goussia* s.l. for fish- and amphibian-host coccidia possessing four bivalved dizoic sporocysts per oocyst and a fine elastic oocyst wall. This definition allows to avoid the premature introduction of new names and to distinguish them from reptile-host coccidia with similar oocyst/sporocyst characteristics, but invariably having solid non-elastic oocyst wall.

**Ecological implications for anuran *Goussia* populations:** So far, the model species *Goussia carpelli* was experimentally shown to be transmitted directly via oocysts in non-dessicating sediments (infective for up to 18 months) and via tubificid oligochaetes or chironomid larvae that serve as paratenic hosts (Steinhagen and Hespe 1998). Such patterns of transmission, however, do not seem operational for anuran coccidia for the following reasons: (i) *Goussia* oocysts do not survive even short periods in a dry environment and tadpoles often live in water bodies that dry up anually; (ii) live prey (potential paratenic host) is not consumed by tadpoles of most anurans; (iii) generations of tadpoles in anurans from temperate regions are separated by more than 6 months (absence of a suitable host) and (iv) tadpoles spend their entire larval development within isolated aquatic systems. Thus, coincidence of drought and an absence of suitable hosts might easily cause a collapse of anuran *Goussia* populations at a particular locality, with rapid (re)colonization being unlikely due to breeding site fidelity of neighbouring host populations (Duellman and Trueb 1994).

Despite these apparent constraints, G. *noelleri* was recorded at localities that periodically dry out. We propose that oocysts retained inside the metamorphed frogs (Jirků and Modrý 2006; this study) provide an alternative way for the transmission of anuran *Goussia* between consecutive tadpole populations. Anurans often hibernate in breeding water bodies, and oocysts trapped within their liver sinusoids can be released into the environment either when the host dies during hibernation and the cadaver decomposes, or is consumed by tadpoles and possibly also by scavengers or predators. Finally, the reservoir host anurans might transport the *Goussia* oocysts to a new locality when a new breeding site is colonized.

Based on the presented results, we conclude that *Goussia* spp. are diversified and common parasites of tadpoles. Importantly, our observations suggest that despite pathological processes associated with infections, *Goussia* are unlikely to have negative influence on tadpole populations of the host species involved in the study.

## Taxonomic summary

### *Goussia noelleri* sp. nov.

**Type host:** Tadpoles of agile frog *Rana dalmatina* Fitzinger in Bonaparte (Anura: Ranidae).

**Other hosts:** Tadpoles of common frog *Rana temporaria* (L.) (Anura: Ranidae).

**Type locality:** Zaječí potok, vicinity of Brno, 16°36′23″E, 49°14′15″N, Czech Republic. Both *R. dalmatina* and *R. temporaria* occur at the locality.

**Other localities:** Raduň-Zámecký rybník, vicinity of Opava, 49°53′23″N, 17°56′38″E, Czech Republic. Only *R. temporaria* occurs at the locality.

**Site of infection:** Epithelial cells of the whole intestine of tadpoles. Oocysts are present within liver sinusoids of tadpoles and metamorphosed frogs (confirmed up to 15 months post-metamorphosis).

**Prevalence:** 100% of *R. dalmatina* tadpoles at the type locality.

**Type material/hapantotype:** Histological sections of infected intestine, gold-coated oocysts for SEM, infected intestine in absolute ethanol, digital photomicrographs, and infected symbiotype *R. dalmatina* tadpole specimen in absolute ethanol deposited at the parasitological collection of the Institute of Parasitology, Biology Centre,

Academy of Sciences of the Czech Republic, České Budějovice, no. IPASCR Prot. Coll.: P-2.

**DNA sequences:** SSU rDNA available at GenBank™ under the accession number "submitted".

**Etymology:** The species is named after the German parasitologist Prof. Dr. med. vet. Wilhelm Nöller (1890—1964), who had discovered *Goussia* in anuran tadpoles for the first time, as a tribute to his significant contribution to the knowledge of protistan parasites of amphibians. Nöller's name is latinized to "Noeller" and the species name itself is created as its genitive form "*noelleri*".

**Remarks:** Up to date there are two *Goussia* spp. described from amphibians; *G. hyperolisi* from tadpoles of reed frogs *H. viridiflavus* in Kenya and *G. neglecta* from tadpoles of *Pelophylax* spp. in Europe. Morphological and morphometrical features of oocysts and sporocysts of *G. noelleri* and *G. neglecta* overlap and the two species cannot be distinguished from each other based on oocyst/sporocyst morphology (Table 1). The only differentiating trait is the divergence of SSU rDNA sequences. Sporocysts of *G. hyperolisi* (5.6—7.7 × 4.2—5.6) are somewhat smaller compared to *G. noelleri* (7.0—8.5 × 4.0—5.5). On the ultrastructural level, *G. noelleri* differs from *G. hyperolisi* by the apparent absence of a parasitophorous vacuole and presence of two refractile bodies per sporozoite, each surrounded by the layer of fine amylopectin granules.

## Methods

**Collection, handling and examination of hosts:** During 2001—2005, a total of 3703 tadpoles representing 5 anuran species were examined: *Rana temporaria* Linnaeus, 1758 ($n = 1421$), *Rana dalmatina* Fitzinger in Bonaparte, 1840 ($n = 1270$), *Pelophylax* kl. *esculentus* (Linnaeus, 1758) (formerly *Rana* kl. *esculenta*) ($n = 100$), *Bufo bufo* Linnaeus, 1758 ($n = 865$) and *Hyla arborea* Linnaeus, 1758 ($n = 47$). We use the recently established amphibian nomenclature following Frost (2007).

Upon collection, tadpoles were placed individually into 100 ml vials with dechlorinated tap water and kept in open vials for 24 h at room temperature. Faecal debris from each vial was collected after ~24 h with a Pasteur pipette. The faeces were homogenized, sieved, and 1/4—1/3 of each faecal sample was examined by a flotation method using sucrose solution (s.g. 1.3), the remaining faecal debris/oocyst suspensions being used for experimental infections (see below). Oocysts within yellow bodies were observed only in squash preparations of tadpole intestines because yellow bodies cannot be concentrated by flotation method.

Selected *R. dalmatina* tadpoles were pithed, dissected in 10% buffered formalin bath, various tissues examined in fresh preparations, and the gastrointestinal tract and liver were processed for histology or electron microscopy. One week after collection, non-dissected tadpoles were released at a part of the original locality from which they could not return to the original population. Specimens of gudgeon (*Gobio gobio* Linnaeus, 1758) were collected using electrofishing. Upon dissection, squash preparations of splees were examined in light microscopy and spleen infected with sporogonic stages of *Goussia metchnikovi* (Laveran, 1897) were used for DNA isolation.

**Localities:** Studies were conducted at two principal localities in the Czech Republic: Locality (Loc) 1. Zaječí [zayetchee] potok, vicinity of Brno, 16°36′23″E, 49°14′15″N, 303 m above sea level (asl.): (examined tadpoles) *R. dalmatina* $n = 1270$, *R. temporaria* $n = 667$, *H. arborea* $n = 47$, *B. bufo* $n = 420$. Loc 2. Raduň-Zámecký rybník, vicinity of Opava, 17°56′38″E, 49°53′23″N, 301 m asl.: *R. temporaria* $n = 754$, *P.* kl. *esculentus* $n = 50$, *B. bufo* $n = 396$. Additional material was collected at the following localities: Loc 3. Babí doly, vicinity of Brno, 16°36′12″E, 49°17′22″N, 390 m asl.: *B. bufo* $n = 49$; Loc 4. Šnejdlík Pond, vicinity of České Budějovice, 14°25′04″E, 49°00′20″N, 380 m asl: *P* kl. *esculentus* $n = 50$. *Gobio gobio* specimens were collected in Bohuslavice nad Vláří, 17°55′39″E, 49°05′26″N, 330 m asl.

**Microscopy:** Squash preparations of various viscera, oocysts concentrated by flotation, and histological sections were examined by light microscopy using an Olympus AX 70 microscope equipped with Nomarski interference contrast (NIC) optics. Use of NIC for examination of squash preparations proved to be necessary, as it greatly enhanced the visibility of the relatively small *Goussia* oocysts/sporocysts. In squash preparations, intact oocysts were seen rarely, as they do not withstand pressure during sample processing. For histology, tissues were fixed in 10% formalin, processed routinely, embedded in paraffin and sections were stained with haematoxylin and eosin. Measurements were obtained using a calibrated ocular micrometer on at least 30 individuals of each developmental stage.

For transmission electron microscopy (TEM), tissues from *R. dalmatina* tadpoles were fixed overnight with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), postfixed for 2 h at 4 °C in 1% osmium tetroxide, and embedded in Durcupan. Ultrathin sections were viewed in a JEOL 1010 transmission electron microscope. For scanning electron microscopy (SEM), oocysts were concentrated by flotation from faeces of *R. dalmatina* tadpoles, washed three times with tap water to remove flotation solution and stored for 2 weeks in 4% formalin. Oocysts in formalin were allowed to settle on poly-lysine coated coverslips for 20 min. Coverslips with oocysts were then fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (CB) for 30 min and washed in CB (3 × 10 min). Coverslips with oocysts were then postfixed for 30 min in 4% osmium tetroxide in CB (1:1 ratio), washed with CB (3 × 10 min), dehydrated, critical-point dried, coated with gold, and examined with a JEOL 6300 scanning electron microscope.

**Transmission experiments:** For all experiments a suspension of sieved faecal debris containing oocysts from tadpoles from Loc1 was used. The infectious material from tadpoles of particular host species were pooled and kept in dechlorinated tap water in 0.5 l containers without preservatives to avoid the intoxication of experimental animals. Every 3 days, the suspension was stirred, left to sediment and water was exchanged.

Experimental animals were kept at ~20 °C, with artificial illumination simulating the actual outdoor photoperiod. Coccidia-free tadpoles of *R. dalmatina*, *R. temporaria*, *P.* kl. *esculentus* and *B. bufo* were raised from eggs collected from Locs 1 and 2. Tadpoles of each species were kept together in

aerated dechlorinated tap water and fed *ad-libidum* with a universal granulated fish food, Lon Mix (Aqua Tropic Lonský, Prague) and chopped lettuce till the start of experimental trials. *Xenopus laevi*s (Daudin, 1802) and *Pleurodeles waltl* Michahelles, 1830 were obtained from laboratory colonies of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. Post-metamorphic juveniles and adults were kept in plastic vivaria containing wet coco substrate and plastic shelters, fed *ad-libidum* with wingless *Drosophila melanogaster* and *Gryllus assimilis* supplemented with Reptivite (Zoo Med Laboratories Inc., San Luis Obispo, CA).

In each experiment, a group of 50 tadpoles was used. After a day of starvation, each experimental group was placed into a 4 l tank and fed with a mixture of faecal debris/oocyst suspension and granulated fish food. After 12 h exposure, the tadpoles were relocated into a coccidia-free 25 l tank. As a test of the infectivity of oocysts, 50 tadpoles of *R. dalmatina*, *R. temporaria* and *B. bufo*, each in two separate experiments, were exposed to oocysts originating from wild conspecific tadpoles as positive controls. Tanks with 200—300 tadpoles of each amphibian species used in experiments were annually kept till metamorphosis as negative controls to ensure that the experimental animals were coccidia-free at the outset of the trials. All faecal sediments from tanks with tadpoles were removed using a rubber hose, concentrated by repeated sedimentation, homogenized and examined using the flotation method every second day in exposed (including positive controls) tadpoles and once weekly in negative control tadpoles.

In order to test the host specificity of the *Goussia* isolates, tadpoles of seven different anuran species were exposed to oocysts originating from naturally infected tadpoles of *R. dalmatina*, *R. temporaria* or *B. bufo* (see Table 2). In addition, four adult *X. laevis* and *P. waltl* each were orally inoculated with 0.25—0.50 ml of faecal debris/oocyst suspension originating from *R. dalmatina*.

To assess the fate of infection during and after metamorphosis, 20 experimentally infected tadpoles of *R. dalmatina* were kept beyond metamorphosis. Frogs were dissected and processed for histology at 2 week intervals for the first 2 months, at 4 week intervals for the third and fourth month, and at 12 week intervals until 15 months of age. All faeces continuously collected from vivaria with metamorphosed frogs were examined by flotation.

**DNA extraction, PCR amplification and sequencing:** Total DNA of anuran *Goussia* isolates was isolated from the mashed intestines of tadpoles heavily infected with gamogonic and sporogonic stages, as described elsewhere (Maslov et al. 1996). Total DNA of *G. metchnikovi* was isolated from mashed cysts from spleen of a gudgeon *G. gobio* containing sporogonial stages. The SSU rDNA genes from anuran isolates were amplified using universal eukaryotic primers (Medlin et al. 1988). The SSU rDNA of *G. metchnikovi* was amplified using specific primers 5′ GAAACTGCGAATGGCTCATT 3′ and 5′ CTTGCGCCTACTAGGCATTC 3′. For PCR, a program consisting of 30 cycles of 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 1 min was used. PCR products were gel-purified, cloned into the TOPO TA vector (Invitrogen), and sequenced.

**Phylogenetic analysis:** Sequences were aligned together with relevant publicly available homologues using ClustalX (Thompson et al. 1997), alignment was manually checked and gaps and ambiguously aligned regions were excluded from the analysis. Maximum parsimony (MP) trees were computed using PAUP 4b10 (Swofford 2000) with MP bootstraps computed from 1000 replicates. Maximum likelihood trees were computed with the model chosen by Modeltest 3.7 (Posada and Crandall 1998) and discrete gamma distribution in four categories and estimated proportion of variable sites as implemented in PhyML (Guidon and Gasquel 2003). ML bootstrap support was inferred from 300 replicates using the same model. The Bayesian tree and posterior probabilities were computed using MrBayes with priors, chain number, and temperature set to default. Two parallel Markov chains were run for $2 \times 10^6$ generations, every 100th tree was sampled, and first $5 \times 10^5$ generations were omitted from topology and probability reconstruction.

The following GenBank$^{TM}$ sequences were included in the SSU rDNA analysis: *Atoxoplasma* sp. AY331571, *Caryospora bigenetica* AF060975, *Choleoeimeria* sp. AY043207, *Cyclospora cayetanensis* AF111183, *Cyclospora colobi* AF111186, *Cyclospora papionis* AF111187, *Eimeria acervulina* U67115, *Eimeria alabamensis* AF291427, *Eimeria albigulae* AF307880, *Eimeria arnyi* AY613853, *Eimeria bovis* U77084, *Eimeria catronensis* AF324213, *Eimeria chaetopidi* AF339489, *Eimeria chobotarii* AF324214, *Eimeria dipodomysis* AF339490, *Eimeria falciformis* AF080614, *Eimeria langebartelii* AF311640, *Eimeria maxima* U67117, *Eimeria mitis* U40262, *Eimeria mivati* U76748, *Eimeria necatrix* U67119, *Eimeria nieschulzi* U40263, *Eimeria peromysci* AF339492, *Eimeria pilarensis* AF324215, *Eimeria praecox* U67120, *Eimeria ranae* EU717219, *Eimeria reedi* AF311642, *Eimeria separata* AF311643, *Eimeria telekii* AF246717, *Eimeria tenella* U40264, *Eimeria tropidura* AF324217, *Goussia noelleri* FJ009241; *Goussia neglecta* FJ009242; *Goussia* sp. from *Bufo bufo* FJ009243; *Goussia metchnikovi* FJ009244; Intranuclear coccidium AY728896, *Isospora robini* AF080612, *Lankesterella minima* AF080611; Sarcocystidae: *Besnoitia bennetti* AY665399, *Besnoitia besnoiti* AF109678, *Besnoitia jellisonii* AF291426, *Cystoisospora timoni* AY279205, *Cystoisospora ohioensis* AY618555, *Hammondia hammondi* AF096498, *Hyaloklossia lueberkuehni* AF298623, *Isospora beli* AF106935, *Isospora felis* L76471, *Isospora orlovi* AY365026, *Neospora caninum* U17346, *Sarcocystis gallotiae* AY015112, *Sarcocystis muris* M34846, *Sarcocystis neurona* U07812, *Sarcocystis rodentifelis* AY015111, *Toxoplasma gondii* U12138; others: *Adelina bambarooniae* AF494059, *Adelina bambarooniae* AF494058, *Adelina dimidiata* DQ096835, *Adelina grylli* DQ096836, *Babesia motasi* AY533147, *Babesia orientalis* AY596279, *Cytauxzoon felis* AY679105, *Goussia janae* AY043206, *Hepatozoon americanum* AF176836, *Hepatozoon catesbianae* AF130361, *Hepatozoon* sp. AF297085, *Hepatozoon* sp. AB181504, *Theileria annulata* AY524666, *Theileria bufeli* AY661513, *Theileria sergenti* AY661515.

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## References

**Baska F, Molnár K** (1989) Ultrastructural observations on different developmental stages of *Goussia sinensis* (Chen, 1955), a parasite of the silver carp (*Hypophthalmichthys molitrix* Valenciennes, 1844). Acta Vet Hung **37**: 81—87

**Daszak P, Cunningham AA, Hyatt AD** (2003) Infectious disease and amphibian population declines. Divers Distrib **9**: 141—150

**Duellman WE, Trueb L** (1994) Biology of Amphibians. The John Hopkins University Press, Baltimore and London pp 493—553

**Duszynski DW, Bolek MG, Upton SJ** (2007) Coccidia (Apicomplex: Eimeriidae) of amphibians of the world. Zootaxa **1667**: 3—77

**Dyková I, Lom J** (1981) Fish coccidia: critical notes on life cycles, classification and pathogenicity. J Fish Dis **4**: 487—505

**Dyková I, Lom J** (1983) Fish coccidia: An annotated list of described species. Folia Parasitol **30**: 193—208

**Frost DR** (2007) Amphibian Species of the World: an Online Reference. Version 5.0. Electronic Database accessible at: 〈http://research.amnh.org/herpetology/amphibia/index.php〉. American Museum of Natural History, New York, USA

**Guidon S, Gasquel O** (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol **52**: 696—704

**Jirků M, Modrý D** (2006) Extra-intestinal localisation of *Goussia* sp. (Apicomplexa) oocysts in *Rana dalmatina* (Anura: Ranidae), and the fate of infection after metamorphosis. Dis Aquat Org **70**: 237—241

**Jirků M, Modrý D, Šlapeta JR, Koudela B, Lukeš J** (2002) The phylogeny of *Goussia* and *Choleoeimeria* (Apicomplexa; Eimeriorina) and the evolution of excystation structures in coccidia. Protist **153**: 379—390

**Levine ND** (1983) The Genera *Barrouxia*, *Defretinella*, and *Goussia* of the coccidian family Barrouxiidae (Protozoa, Apicomplexa). J Protozool **30**: 542—547

**Lom J, Dyková I** (1992) Protozoan Parasites of Fishes. Elsevier, New York pp 99

**Lukeš J, Starý V** (1992) Ultrastructure of the life cycle stages of *Goussia janae* (Apicomplexa, Eimeriidae), with X-ray microanalysis of accompanying precipitates. Can J Zool **70**: 2382—2397

**Maslov DA, Lukeš J, Jirků M, Simpson L** (1996) Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: Implications for the evolution of parasitism in the trypanosomatid protozoa. Mol Biochem Parasitol **75**: 197—205

**Medlin L, Elwood HJ, Stickel S, Sogin ML** (1988) The characterisation of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71**: 491—499

**Molnár K** (1995) Redescription of *Goussia neglecta* n. comb. (Nöller, 1920) (Apicomplexa; Coccidia) and notes on its occurrence in the gut of tadpoles. Acta Vet Hung **43**: 269—275

**Molnár K, Ostoros G, Baska F** (2005) Cross-infection experiments confirm the host specificity of *Goussia* spp (Eimeriidae: Apicomplexa) parasitizing cyprinid fish. Acta Protozool **44**: 43—49

**Nöller W** (1920) Zur Kenntnis der Coccidien des Wasserfrosches (*Eimeria neglecta* nov. spec.) (Befruchtung und Sporogonie von *Lankesterella*). Arch Protistenkd **41**: 176—180

**Paperna I, Lainson R** (1995) Life history and ultrastructure of *Eimeria bufomarini* n.sp. (Apicomplexa: Eimeriidae) of the giant toad, *Bufo marinus* (Amphibia: Anura) from Amazonian Brazil. Parasite **2**: 141—148

**Paperna I, Landsberg JH** (1989) Description and taxonomic discussion of eimerian coccidian from African and Levantine geckoes. S Afr J Zool **24**: 345—355

**Paperna I, Ogara W, Schein M** (1997) *Goussia hyperolisi* n. sp.: a coccidian infection in reed frog *Hyperolis viridiflavus* tadpoles which expires towards metamorphosis. Dis Aquat Org **31**: 79—88

**Posada D, Crandall KA** (1998) Modeltest: testing the model of DNA substitution. Bioinformatics **14**: 817—818

**Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, Hines HB, Kenyon N** (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. Ecohealth **4**: 125—134

**Steinhagen D, Hespe K** (1998) Carp coccidiosis: longevity and transmission of *Goussia carpelli* (Apicomplexa: Coccidia) in the pond environment. Folia Parasitol **45**: 326—328

**Steinhagen D, Körting W** (1990) The role of tubificid oligochaetes in the transmission of *Goussia carpelli*. J Parasitol **76**: 104—107

**Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW** (2004) Status and trends of amphibian declines and extinctions worldwide. Science **306**: 1783—1786

**Swofford DL** (2000) PAUP* Phylogenetic Analysis Using Parsimony (*and other Methods). Sinauer Associates, Sunderland, MA

**Thompson JD, Gibson TJ, Plesniak F, Jeanmougin F, Higgins DG** (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **24**: 4876—4882

**Upton SJ** (2000) Suborder Eimeriorina Léger, 1911. In Lee JJ, Leedale GF, Bradbury P (eds) An Illustrated Guide to the Protozoa. 2nd edition. Society of Protozoologists, Lawrence, pp 318—339

**Veith M, Kosuch J, Vences M** (2003) Climatic oscillations triggered post-Messinian speciation of Western Palearctic brown frogs (Amphibia, Ranidae). Mol Phylogenet Evol **26**: 310—327