



Disseminated protozoal infection in a wild feathertail glider (*Acrobates pygmaeus*) in Australia

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ABSTRACT

This is the first report of a disseminated protozoal infection in a wild feathertail glider (*Acrobates pygmaeus*) from south-eastern Australia. The glider was found dead in poor body condition. Histologically, large numbers of zoites were seen predominantly in macrophages in the liver, spleen and lung, with protozoal cysts present in the liver. Molecular and phylogenetic analyses inferred that the protozoan parasite belongs to the family Sarcocystidae and is closely related to previously identified apicomplexans found in yellow-bellied gliders (*Petaurus australis*) in Australia and southern mouse opossums (*Thylamys elegans*) in Chile.

1. Introduction

Protozoa are a large group of unicellular eukaryotes. More than 45,000 species have been described, the majority of which are free living. Of the parasitic species, many cause significant diseases in both humans and other animals (e.g., malaria, Chagas disease, leishmaniasis, trichomoniasis and coccidiosis) (Soulsby, 1982). Marsupials are also affected by infections with protozoa; arguably the most significant organism, *Toxoplasma gondii*, causes a severe, systemic disease with high mortality (Portas, 2019). A range of protozoans has been found in possums and gliders, including species of *Giardia*, *Eimeria*, *Sarcocystis*, *Klossiella* and *T. gondii* (O'Donoghue and Adlard, 2000). However, no protozoal infections have been recorded for feathertail gliders (*Acrobates pygmaeus*).

The feathertail glider is the world's smallest gliding mammal, weighing between 10 and 15 g. It is widely distributed in the forests of eastern Australia (Harris, 2015). While fleas and mites have been recorded for this species (Harris, 2015), published reports of disease are uncommon and refer predominantly to pulmonary infections, a high incidence of transponder-associated neoplasia and a single case of granulomatous pneumonia caused by *Mycobacterium avium* (Johnson and Hemsley, 2008; Tong, 2019). This is the first report of a disseminated protozoal infection caused by a novel protozoan species in a wild feathertail glider.

2. Material and methods

2.1. Necropsy

On 15 August 2019, an adult female feathertail glider was found dead on the grounds of Healesville Sanctuary (37.6816° S, 145.5299° E), a native Australian fauna park located in south-eastern Australia. The glider was taken to the Sanctuary's veterinary hospital (Australian Wildlife Health Centre) and a gross necropsy performed on the same day. The glider weighed 12 g. It was thin, with minimal fat stores, no ingesta in the stomach and no grossly detectable lesions. Tissue samples (lung, heart, liver, spleen, kidney, gastrointestinal tract, pancreas, adrenal gland and brain) were collected, placed in 10% neutral-buffered formalin, routinely processed, sectioned (3 µm), stained with haematoxylin and eosin and sections examined by light microscopy. The remains of the carcass were frozen.

2.2. DNA extraction, PCR sequencing and phylogenetic analysis

Genomic DNA was extracted from 0.25 g of liver using the PowerSoil DNA Isolation Kit (MoBio, USA), according to the manufacturer's protocol. An aliquot of this DNA was subjected to PCR, targeting a locus of the small subunit of nuclear ribosomal RNA gene (*SSU*). This PCR assay was designed to amplify a ~500 bp region of *SSU* from a broad array of

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apicomplexans (Lepore et al., 2017). Amplification was achieved using primers NTS-18S-F1 (forward: 5'-GCC ATG CAT GTC TAA GTA TAA G-'3) and NTS-18S-R1 (reverse: 5'-CCT ATC ATT CCA ATC ACT AGA AAT-'3), employing the following cycling protocol: 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 1 min (denaturation), 56 °C for 1 min (annealing) and 72 °C for 1 min (extension), with a final extension of 72 °C for 5 min. PCR was conducted in a volume of 50 µl containing GoTaq Flexi buffer (Promega, USA), 3.0 mM of MgCl₂, 200 µM of each deoxynucleotide triphosphate, 25 pmol of each primer and 1 U of GoTaq (Promega, USA) DNA polymerase. Known test-positive (*T. gondii*), test-negative and no-template controls were included in each PCR run. The PCR product was resolved in an agarose gel (1.5%) by electrophoresis (7 V/cm) using TBE buffer (65 mM Tris-HCl, 27 mM boric acid, 1 mM EDTA, pH 9; Bio-Rad, USA) to assess size with reference to the ΦX174-HaeIII (Promega, USA) marker.

The PCR product was bi-directionally sequenced using a standard protocol (Koehler et al., 2016), and the SSU sequence obtained (451 bp; GenBank accession no. MT560683) was compared with sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov) and then aligned with reference sequences representing distinct apicomplexan species/taxa and selected outgroup taxa from this database. Sequences were compared in a pairwise manner, and sequence identities recorded using Geneious Prime v. 2020.0.5 software (www.geneious.com/). Subsequently, sequences were aligned using the program Muscle (Edgar, 2004), and alignments adjusted manually using the program Mesquite v.3.61 (Maddison and Maddison, 2015). Phylogenetic analyses of sequence data were conducted by Bayesian inference (BI) using Monte Carlo Markov Chain analysis in the program MrBayes v.3.2.6 (Ronquist et al., 2012). The likelihood parameters set for BI analyses of sequence data were based on the Akaike Information Criteria test in jModeltest v.2.1.10 (Darriba et al., 2012), with the number of substitutions (Nst) set at 6 and an invariant gamma-distribution. Posterior probability (pp) values were calculated by running 2,000,000 generations with four simultaneous tree-building chains. Trees were saved every 100th generation. At the end of each run, the standard deviation of split frequencies was <0.01, and the potential scale reduction factor approached one. A 50% majority rule consensus tree for each analysis was constructed based on the final 75% of trees generated by BI. Analyses were run three times to ensure convergence and insensitivity to priors. A phylogenetic analysis was additionally conducted using the neighbor-joining (NJ) distance method (Saitou and Nei, 1987) in the program MEGA X v.10.1.8 (Stecher et al., 2020). Evolutionary distances were computed using the 'number of differences' method (Nei and Kumar, 2000), including 'transitions and transversions' for the nucleotide data. Rates of evolution among sites were considered uniform and gaps were treated using pairwise deletion. A total of 2000 bootstrap replicates were performed and are reported as

bootstrap support percentages (bs). *Goussia* sp. were used as outgroups for the analyses.

3. Results

3.1. Histological findings

Histologically, the primary change was of a disseminated infection with protozoa whose morphology was consistent with an apicomplexan. Zoites (likely tachyzoites) were present in small clusters or were free within the sinusoids of the liver (Fig. 1) and within the interstitium of the lung (cf. Fig. 1), but such zoites were not detected in spleen sections. These organisms were ~6 µm × 2 µm, curvilinear, with tapered ends, and had a centrally placed nucleus. In numerous blood vessels, most prominent in the glomerular capillaries, basophilic bodies (1–2 µm in diameter) were present and associated with erythrocytes (possible intra-erythrocytic stage). Multifocal areas of liver with the identified zoites exhibited a dissociation of hepatic cords and degeneration and necrosis of occasional local hepatocytes. Kupffer cells were prominent, and mildly increased macrophages with lymphocytes and plasma cells were present in portal areas. The splenic red pulp contained moderately increased macrophages which frequently contained cytoplasmic zoites. Multifocal areas of the lungs had mildly increased macrophages both within the interstitium and the alveolar spaces. Along one margin of the section of lung, a focal area of the alveolar spaces contained moderate numbers of fungal stages (*Cryptococcus* sp.), also with mild associated histiocytic inflammation.

3.2. Molecular and phylogenetic results

The SSU sequence obtained from the DNA sample extracted from the liver was most similar (96% identity over 451 bp) to that representing *Hylaklossia lieberkuehni* - an isosporoid renal coccidian parasite (GenBank accession no. AF298623). When placed in a phylogenetic context (Fig. 2) with related reference sequences from GenBank and *Goussia* sp. as outgroups, the Eimeriidae and Lankestreliidae formed a well-supported clade (100 bs/1.00 pp) with the Sarcocystidae as a strongly supported (100 bs/0.99 pp) sister clade. Within the Sarcocystidae, traditionally divided into four clades (Dolezel et al., 1999; Merino et al., 2010), Clade A (Toxoplasmatinae, Cystoisosporinae and *Caryospora* spp.) was moderately supported (70 bs), while the Clades B-D (Sarcocystinae) were each supported (95 bs/0.95 pp; 91 bs/1.00 pp; 59 bs/0.95 pp, respectively) (Fig. 2). The novel apicomplexan taxon identified here in the liver forms a group between Clade A (Toxoplasmatinae, Cystoisosporinae and *Caryospora* spp.) and Clades B-D (Sarcocystinae), with moderate (72 bs) or strong (1.00 pp) support with another undescribed intraerythrocytic apicomplexan parasite found in yellow-bellied

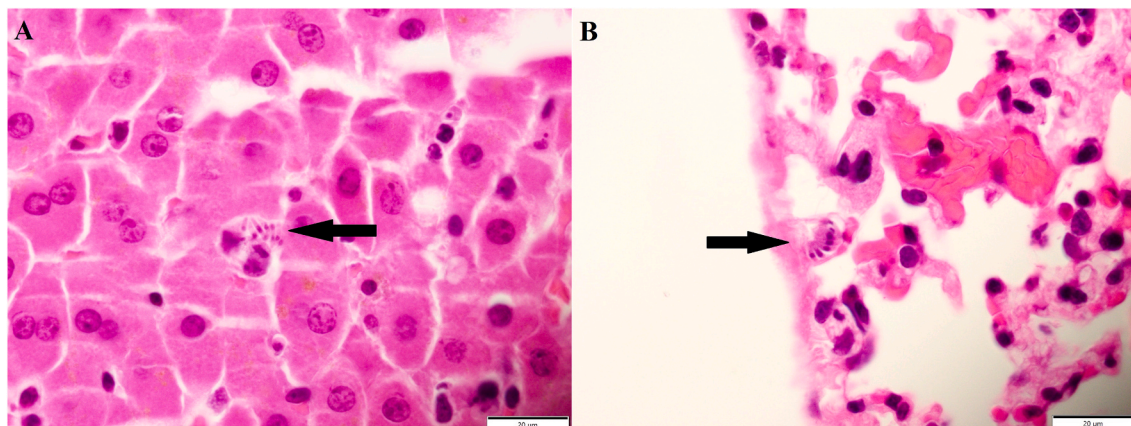


Fig. 1. Histological image of clusters of zoites (arrow) in the liver (A) and lung (B) of a feathertail glider. Haematoxylin and eosin stain; 1000-times magnification.

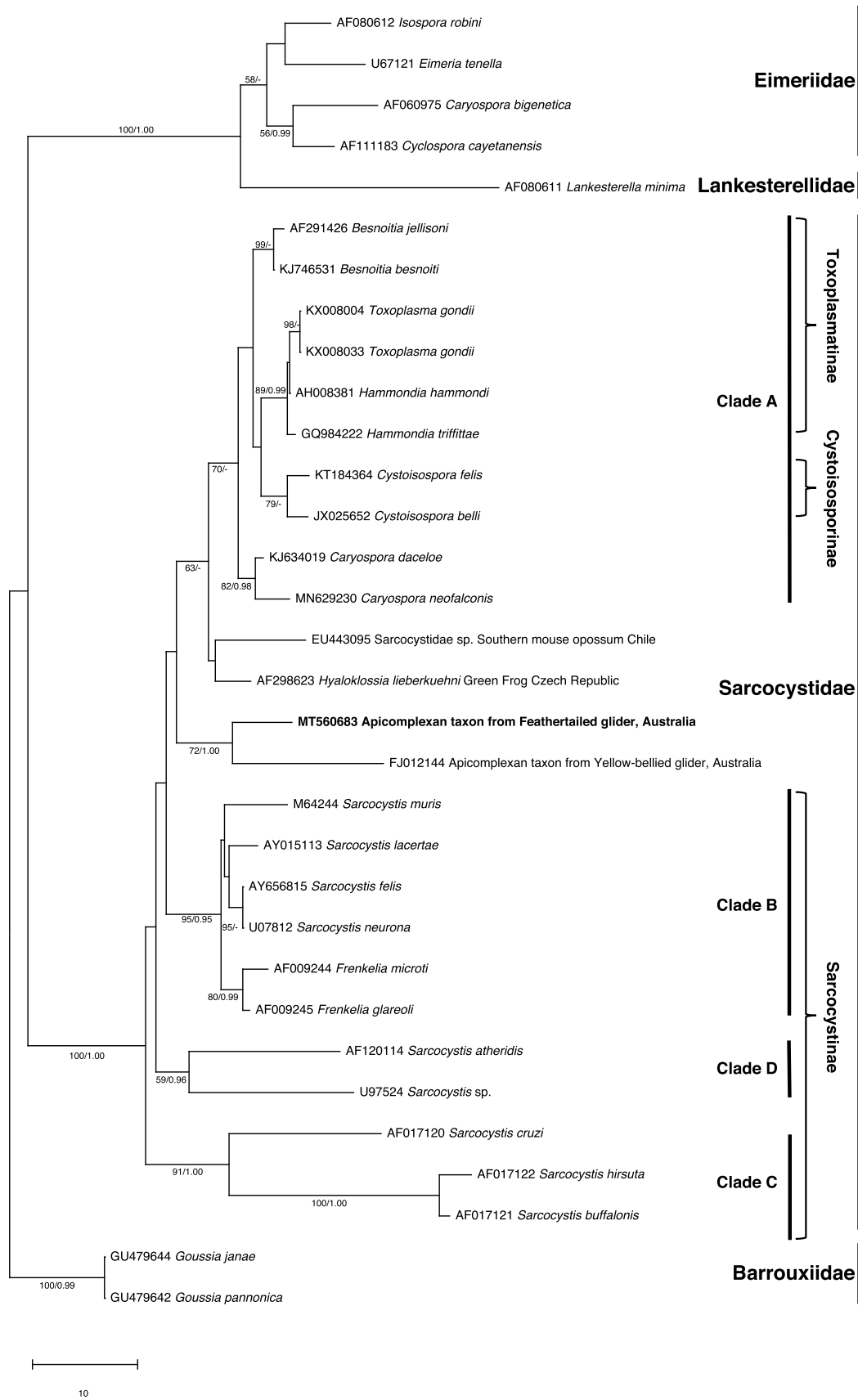


Fig. 2. Relationship of the novel apicomplexan taxon (in bold) from the liver of the feathertail glider with representative sequences from members of the Eimeriidae, Lankesterellidae and the Sarcocystidae, established based on a phylogenetic analysis of small subunit of nuclear ribosomal RNA gene (SSU) sequence data employing distance and Bayesian methods. Branch supports include neighbor-joining bootstrap percentages followed by Bayesian posterior probabilities. Members of the genus *Goussia* were used as outgroups.

gliders (*Petaurus australis*) from Queensland, Australia (Zhu et al., 2009). Adjacent are two ‘unsupported’ sequences, one (GenBank accession no. AF298623) from *H. lieberkuehni* from a green frog (*Pelophylax esculentus*) from the Czech Republic (Modry et al., 2001) and another (accession no. EU443095) from an undescribed intraerythrocytic apicomplexan from the Chilean southern mouse opossum (*Thylamys elegans*) (Merino et al., 2008) (cf. Fig. 2). The SSU sequence obtained here from the glider (accession no. MT560683) had the highest identity (94.43%) to the latter sequence (EU443095) from the opossum, followed by slightly lower identities with those of *Caryospora daceloe* (94.19%) from a kookaburra and of a novel apicomplexan taxon (94.00%) from the yellow-bellied glider (Supplementary material S1).

4. Discussion

This is the first report of a disseminated infection in a wild feathertail glider by a microorganism in the family Sarcocystidae. Phylogenetic analysis revealed that this novel apicomplexan does not group with strong statistical support with the subfamily Toxoplasmatinae, Cystoisosporinae or Sarcocystinae (Fig. 2). In the following, we discuss the characteristics of each subfamily.

Sarcocystinae, which includes species of *Sarcocystis* and *Frenkelia*, form compartmentalised cysts and have a two-host life cycle involving a carnivorous definitive host and an intermediate host, such as a herbivorous mammal or bird, and their oocysts sporulate within the gut of the definitive host (Mugridge et al., 2000; Levine, 2018). The definitive host sheds oocysts in the faeces, which are then inadvertently consumed by the intermediate host. After consumption, sporocysts (containing sporozoites) are released. The infective sporozoites undergo multiple generations of merogony before forming tissue cysts containing bradyzoites (Soulsby, 1982; Gardiner et al., 1998), predominantly in skeletal muscle (Dubey et al., 2016). *Sarcocystis* spp. infections usually cause no clinical signs in the definitive host and, in the majority of cases, are mostly asymptomatic in the intermediate host, muscle cysts being found incidentally at necropsy (Ladds, 2009).

Sarcocystids have been observed in a large number of Australian native mammals which, like the feathertail glider, likely act as intermediate hosts. These include native rodents, macropods, dasyurids, bandicoots and possums (Munday et al., 1978; Ladds, 2009). They have also been identified in South American marsupials, such as opossums (Smith and Frenkel, 1995; Gardiner et al., 1998; Torres-Castro et al., 2016) and mouse opossums (Shaw and Lainson, 1969; Dubey and Odening, 2001; Dubey et al., 2016), but have not been previously recognised in feathertail gliders. There is little published information on the effect that *Sarcocystis* spp. infections have on wild intermediate host populations (Obendorf and Munday, 1983; Obendorf et al., 1996; Donahoe et al., 2015; Hillman et al., 2016). Data from wild mule deer (*Odocoileus hemionus*) found that infections affected fawn growth and predisposed them to predation (Dubey and Odening, 2001).

Toxoplasmatinae, which includes species of *Toxoplasma*, *Hammondia* and *Besnoitia* have a similar heteroxenous life cycle to sarcocystids, but often have complex transmission patterns and their oocysts sporulate in the environment, outside of the definitive host animal (Ogedengbe et al., 2016; Levine, 2018). Toxoplasmid are found in a range of Australian animals, most notably *T. gondii* which, due to the introduction of cats, has been well documented from Australian marsupials (Duszynski, 2015). *Besnoitia besnoiti* and *B. wallacei* have been recorded in cattle and rodents, respectively (Mason, 1980; Nasir et al., 2012) and a *Besnoitia* species was associated with haemorrhagic rhinitis in a western grey kangaroo (*Macropus fuliginosus*) (Ladds, 2009).

Cystoisospora spp. have been found in cats, dingoes, dogs, humans and pigs in Australia (O’Donoghue and Adlard, 2000). Both *Caryospora daceloe*, from a laughing kookaburra (*Dacelo novaeguineae*) in Australia (Yang et al., 2014), and *Caryospora neofalconis*, from a falcon in Saudi Arabia (GenBank accession no. MN629230, unpublished), might represent novel members of the Toxoplasmatinae, as the majority of

Caryospora spp. fall within the Eimeriidae (Yang et al., 2014).

Regardless of which family the novel apicomplexan belongs to, the glider is most likely an intermediate host in a predator–prey heteroxenous life cycle. The list of possible definitive hosts for the feathertail glider parasite is a long one (Harris, 2015) and includes avian predators such as owls, kookaburras, ravens and currawongs, snakes, monitor lizards, dasyurids, introduced foxes and cats and even ghost bats (*Macroderma gigas*). Feathertail gliders consume nectar, pollen and insects (Harris, 2015) and could become infected by ingesting food or water that is contaminated with the definitive host’s faeces. Alternatively, invertebrates, such as cockroaches, flies and earthworms can act as paratenic hosts for the organism and the glider may have been secondarily infected by consuming an infected arthropod (Bettiol et al., 2000; Dubey, 2010).

Phylogenetically this protozoan falls within the Sarcocystidae, between the Toxoplasmatinae and Cystoisosporinae clade and the Sarcocystinae clade (Fig. 2), and is most closely related to the other apicomplexans that fall between these two clades (Fig. 2; Supplementary Table 1) including *H. lieberkuehni* from a European frog, two apicomplexan taxa found in yellow-bellied gliders from Queensland and southern mouse opossums from South America (Merino et al., 2008; Zhu et al., 2009) and two *Caryospora* spp. (Yang et al., 2014). Recent molecular study has shown that the close relatedness of the yellow-bellied glider parasite and the mouse opossum parasite indicate that they share a common ancestor and have likely co-evolved with their respective vertebrate hosts (Merino et al., 2010). This may also be the case in the current study, assuming the feathertail glider is a usual or accidental intermediate host for this particular apicomplexan. Australian marsupials diverged from their South American counterparts ~45–50 million years ago (Nilsson et al., 2004), but still retain similar parasites.

While these parasites appear to be genetically similar, their morphology cannot be compared because the other two were only identified from blood smears (Merino et al., 2008; Zhu et al., 2009). No blood was available from the feathertail glider for examination, as it was found dead. However, parasite stages were visible histologically in blood vessels and macrophages, making it likely that they would also have been present in blood smears. Future studies should examine blood from wild feathertail gliders to determine whether the parasite can be found in blood smears. Its genetic identity could then be compared with the taxon found in the current study and those reported in the previously published works.

It appears that disease is more likely to occur in an aberrant intermediate host and can include clinical signs such as anaemia, haemorrhage, emaciation, central nervous system (CNS) signs, hepatitis, abortion or death (Dubey and Odening, 2001; Dubey et al., 2016). An example of this occurred when *S. falcatula*, which is not pathogenic for its cowbird and grackle intermediate hosts, caused the deaths of a large number of psittacines in an outdoor breeding facility in the USA (Clubb and Frenkel, 1992). Given the somewhat atypical appearance of the tissue cysts in the liver of this feathertail glider and apparent severity of disease, it is possible that the glider may also have been an aberrant host for this particular parasite.

As only one feathertail glider affected by this parasite has been identified, it is not possible to determine the significance, if any, of this parasite for the feathertail glider population. This case is also complicated by the fact that the glider had a concurrent *Cryptococcus* sp. infection. While cryptococcosis in feathertail gliders has been reported previously (Krockenberger et al., 2005; Johnson, 2008), no mention was made of the types of lesions or any potential predisposing factors. It is possible that the *Cryptococcus* sp. infection exacerbated the effects of the protozoal infection or vice versa. Further monitoring of the wild population will be necessary to assess whether either of these agents could constitute an emerging disease threat in feathertail gliders.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijppaw.2020.07.012>.

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