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Shedding of zoonotic pathogens and analysis of stomach contents in great cormorants (*Phalacrocorax carbo sinensis*) from Switzerland between 2007 and 2012

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Summary

208 healthy great cormorants (*Phalacrocorax carbo sinensis*) shot during 5 consecutive hunting seasons from 2007/2008 until 2011/2012 were tested for Newcastle disease virus (APMV-1), avian influenza virus (AIV), Chlamydiae, and *Salmonella* spp. In addition, stomach contents were gross macroscopically examined. None of the birds was positive for APMV1, AIV or Chlamydiae. Twice *Salmonella enterica* subsp. *enterica* serovar Typhimurium and once a rough mutant of *Salmonella* Typhimurium were found. Stomach worms were found in 199 cormorants and 12 identifiable fish species in 45 stomaches.

Keywords: great cormorant, Newcastle disease, avian influenza, Chlamydiae, *Salmonella* spp.

Ausscheidung von zoonotischen Erregern und Analyse des Mageninhaltes von Kormoranen (*Phalacrocorax carbo sinensis*) in der Schweiz zwischen 2007 und 2012

208 gesunde wildlebende Kormorane (*Phalacrocorax carbo sinensis*), geschossen in 5 aufeinander folgenden Jagdsaisons von 2007/2008 bis 2011/2012, wurden auf das Virus der Newcastle Krankheit (APMV1), das Aviäre Influenza Virus (AIV), Chlamydien und *Salmonella* spp. getestet. Alle Vögel waren negativ für APMV-1, AIV und Chlamydien. Zweimal wurde *Salmonella enterica* subsp. *enterica* serovar Typhimurium und einmal eine Rauhform von *Salmonella* Typhimurium gefunden. Zusätzlich wurde der Mageninhalt makroskopisch untersucht. 199 Kormorane hatten Magenwürmer und in 45 Mägen konnten 12 Fischarten identifiziert werden.

Schlüsselwörter: Kormoran, Newcastle Krankheit, aviäre Influenza, Chlamydien, *Salmonella* spp.

Introduction

The great cormorant *Phalacrocorax carbo sinensis* is a large black fish-eating bird. It feeds on the sea, in estuaries, freshwater lakes and rivers. In Switzerland cormorants were hunted to extinction, but since 2005 migration from the recovering northern population lead to approximately 5000–6000 winterguests on Swiss waters each year (Dollenmeier et al., 2004; Robin et al., 2010). By counting roosts it was estimated that 80 % of cormorants feed of lakes and 20 % of bodies of flowing waters (Dollenmeier et al., 2004). Since 2001 breeding takes place, with 6 small colonies of 547 breeding pairs in 2009. Established breeding pairs return to their nesting place in consecutive years (Robin et al., 2010). In Switzerland, cormorants are hunted at rivers from September throughout March, with an average of 1141 birds killed each year (Anonymous, 2010a). Post mortem examinations of great cormorants shot at the Rhine within 5 consecutive hunting seasons from 2007/2008 until 2011/2012, was performed to monitor shedding of avian paramyxovirus 1 (APMV1), the causative agent of Newcastle disease (ND), avian influenza virus (AIV) and other zoonotic agents such as Chlamydiae and *Salmonella* spp. and fish predation.

Animals, Material and Methods

208 great cormorants shot at the Rhine in the canton of Zurich from September until March 2007/2008 until 2011/2012 were delivered to the department of hunting and fisheries in Dachsen, canton of Zurich by individual hunters. The birds were stored at -20 °C until examination. About 60 % of the birds had subadult plumage, none was ringed. Body condition, inner organs and stomach contents were examined at necropsy. Identifiable fish were specified. Choanal and cloacal swabs and organ samples were immediately processed, or kept at -80 °C (RNA) or -20 °C (DNA) until further use.

DNA and RNA extraction from swabs of the choana and cloaca

RNA or DNA of pooled swabs were extracted on a Nucleid Acid Isolation System QuickGene-810 with the QuickGene RNA tissue kit SII and the QuickGene DNA tissue kit S (FujiFilm Life Science, Tokio, Japan).

Real-time reverse transcriptase polymerase chain reaction (qRT-PCR) for the detection of avian paramyxovirus type 1 and avian influenza virus

Onestep qRTPCR was performed on a 7500 Fast RealTime PCR System (Applied Biosystems, Foster City, CA, USA). The conditions were set as follows: 30 min 48 °C, 10 min 95 °C, followed by 45 cycles consisting of denaturation at 95 °C for 15 s, annealing at 53 °C for 1 min and elongation at 70 °C for 1 min. Primers and probes were obtained from Microsynth AG, Balgach, Switzerland.
qRTPCR for the detection of APMV1 was done as described by Wise et al., 2004. Briefly, amplification was carried out in 25 µl reactions containing 12.5 µl "2× QuantiTect® Probe RT PCR Mastermix", 0.25 µl "QuantiTect® RT Mix" (both QIAgen, Hilden Germany), 300 nM of each forward (3'AGTGATGTGCTCGGACCTTC5') and reverse primer (3'CCTGAGGAGAGGCATTTGCTA5'), 250 nM of probe (5'YYETTCTCTAGCAGTGGGACAGCCTGCTAMRA3'), and 10 µl extracted RNA.
AIV qRTPCR was slightly modified from Spackman et al., 2002. Briefly, 25 µl reactions contained 12.5 µl "2× QuantiTect® Probe RT PCR Mastermix", 0.25 µl "QuantiTect® RT Mix" (both QIAgen), 400 nM of each forward (3'AGATGAGYCTTCTAACCGA5') and reverse primer (3'GCAAAAACATCTTCAAGTYTC5'), 100 nM of probe (5'FAMTCAGGCCCCTCAAAGCCGABHQ13'), 400 nM of each internal control (IC) primer EGFP1F (5'GACCACTACCAGCAGAACAC3') and EGFP2R (5'GAACTCCAGCAGGACCATG3'), 100 nM of IC EGFPProbe (5'HEXAGCACCCAGTCCGCCCTGAGCABHQ13') (Hoffmann et al., 2005) and 7 µl extracted RNA.

ELISA and real-time PCR (qPCR) for the detection of Chlamydiae

An antigen-ELISA (IDEIA™ PCE Chlamydia; Oxoid, Wesel, Germany) from cloacal swabs was carried out for the detection of Chlamydiae in great cormorants until 2009, according to the manufacturer's protocol. qPCRs were carried out on an 7500 Fast RealTime PCR System (Applied Biosystems) with the standard cycle protocol 2min 50 °C, 10 min 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. Primers and probes were obtained from Microsynth. Chlamydiae qPCR was done essentially as described by Ehricht et al., 2006. Briefly, 25 µl reaction contained 12.5 µl "2× TaqMan® Universal PCR Master Mix" (Applied Biosystems), 900 nM of each primer Ch23SF (5'CTGAAACCAGTAGCTTATAAGCGGT3') and Ch23SR (5'ACCTCGCCGTTTAACTTAACTCC3'), and 250 nM of probe Ch23SP (3'FAMCTCATCATGCAAAAGGCACGCCG-TAMRA5'), and 7 µl extracted DNA.

Detection of *Salmonella* spp. in organ samples

A piece of liver (2×2×1 cm) and 2 cm of the tip of one caecum of up to six (mostly three to five) cormorants were pooled. Samples were processes according to EU 2002. *Salmonella* spp. isolates were serotyped in the Institute of Veterinary Bacteriology, University of Bern.

Results

All 208 great cormorants were of good condition with unremarkable inner organs. Of the 170 cormorants examined from 2008/2009 onwards, 83 had empty stomachs, 42 had well-digested contents (fish bones or well-digested fish), and 45 cormorants contained mostly intact fish (Tab. 1). These 45 cormorants produced 88 individual fish of 12 species. The smallest was 5 cm, the largest 40 cm. 26 birds had swallowed 1 fish, 9 birds contained 2 fish, 5 birds had 3, and 5 had 4 up to 8 smaller fishes.
Weight range of cormorants was 1588 g to 3419 g (mean 2364 g, median 2345 g) for birds with empty stomachs, 1780 g to 3286 g (mean 2358 g, median 2233 g) for birds with well-digested stomach contents and 1790 g to 3320 g (mean 2408 g, median 2357 g) for birds with a recent meal. Average weight for great cormorants is approximately 2000–2500 g. Apart from a few subadults, almost all cormorants (199/208) had round pale stomach worms, mostly 13 cm of length and 23 mm width. Parasitological examination of stomach contents revealed *Ascaridida*. Intestines were not examined. All cormorants were negative for APMV1, AIV and Chlamydiae. Two pools were positive for *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium), and 1 pool was positive for *Salmonella enterica* subsp. *enterica* rough:i:1,2. The latter is an O-antigen lacking ("rough") variant of *S.* Typhimurium.

Discussion

Examined great cormorants were culled at the Rhine to reduce numbers and to check stomach contents (Swiss cormorant management plan (Rippmann et al., 2005)). All fish species found (Tab. 1) are also permitted to be fished by law (Kirchhofer et al., 2007; Anonymous, 2010b). Yet, this is only a descriptive snapshot of stomach contents. Cormorants were unsystematically shot throughout the season. 50 % had empty stomaches, and only macroscopically identifiable fish were analysed. Thus, pellet analysis,

Table 1: Detection of avian influenza virus, avian paramyxovirus 1, Chlamydiae and *Salmonella* spp. and gross macroscopic analysis of stomach contents in great cormorants (*Phalacrocorax carbo sinensis*), shot in Switzerland from 2007 until 2012.

hunting season	2007/2008	2008/2009	2009/2010	2010/2011	2011/2012	total
cormorants examined	38	56	53	30	31	208
pathogen detection in cormorants						
avian influenza virus PCR positive	0	0	0	0	0	0/208
avian paramyxovirus 1 PCR positive	n.d.	0	0	0	0	0/170
Chlamydiae antigen-ELISA positive	0	0	n.d.	n.d.	n.d.	0/94
Chlamydiae PCR positive	0[a]	n.d.	0	0	n.d.	0/121
pools of organ samples *Salmonella* spp. positive	2/8[b]	0/22	1/11[c]	0/11	0/11	3/63
stomach content of cormorants						
stomach worms	38	48	53	30	30	199/208
empty (no food)	n.d.	34	23	13	13	83/170
well-digested content	n.d.	12	15	7	8	42/170
identified fish	n.d.	10	15	10	10	45/170
number of fishes per stomach	n.d.					
1		7	12	4	3	26
2		2	0	2	5	9
3		1	1	2	1	5
5 to 8		0	2	2	1	5
number of identifiable fishes found in stomachs/ with IUCN[d] status	n.d.	14	27	24	22	87
common rudd (*Scardinius erythrophthalmus*)/LC		7	0	0	1	8
grayling (*Thymallus thymallus*)/VU		2	7	0	0	9
European perch (*Perca fluviatilis*)/LC		2	9	10	12	33
crucian carp (*Carassius carassius*)/n.l.		1	0	0	0	1
brown trout (*Salmo trutta fario*)/NT		2	0	0	0	2
rainbow trout (*Oncorhynchus mykiss*) n.l.		0	0	1	0	1
European chub (*Leuciscus cephalus*)/LC		0	5	1	1	7
common roach (*Rutilus rutilus*)/LC		0	3	8	7	18
common barbel (*Barbus barbus*) NT		0	1	2	1	4
burbot (*Lota lota*) LC		0	1	0	0	1
northern pike (*Esox lucius*) LC		0	0	2	0	2
sunfishes (*Centrarchidae*) n.l.		0	1	0	0	1

n.d. = not done
[a] published in Zweifel et al., 2009
[b] twice *Salmonella* Typhimurium
[c] *S. enterica* subsp. *enterica* rough:i:1,2
[d] International Union for Conservation of Nature, VU = vulnerable species, NT = near threatened species, LC = least concern, n.l. = not listed, (Kirchhofer et al., 2007; *Anonymous* 2010b).

where a bird population can be monitored over time, and seasonal variation in foraging in the same birds can be detected (Keller, 1998), is a more holistic approach. Apart from a few subadults, almost all cormorants (169/177) had stomach worms.

Cormorants are also under scrutiny for being a possible source of Newcastle disease virus. ND in cormorants is occurring worldwide (Kuiken, 1999). In the course of massive outbreaks with a hugh number of fatalities due to ND (Kuiken, 1999), some birds were found to asymp-

tomatically excrete APMV1 (Kuiken et al., 1998). The Swiss outbreaks in Safnern (1995) and Marin (2011) occurred in the vicinity of lakes and rivers. The causative agent was found to be APMV1 genotype VI (lineage 4d) in Safnern and genotype VII (lineage 5b) in Marin (Lomniczi et al., 1998; Aldous et al., 2003, Anonymous, 2011). As in both cases viruses deriving from cormorants were found to cluster in the same branches of a genetic tree as the outbreak isolates, it was speculated that wild birds and especially comorants could be the source (Aldous et al., 2007; Anonymous, 2011). Reintroduction by migratory birds was suspected in a few outbreaks along European coastlines, e. g. in Denmark 1996 (Jørgensen et al., 1999). The only confirmed direct ND correlation was described by Macpherson (1956), where offal from cormorants shot for human consumption was fed to chickens. Thus, while there is clearly an epidemiological link between cormorant isolates and outbreaks in commercial fowl, it is still controversial whether APMV1 is endemic in cormorants or a temporary pathogen. While antibodies against APMV1 were found in 10/53 cormorants by haemagglutination inhibition test in France (Artois et al., 2002), shedding of APMV1 could neither be demonstrated by Camenisch et al. (2008), nor by the current study in Switzerland. Although cormorants are mobile and use small and big water bodies during migration and their stay in winter and thus in theory may spread or help spreading ND, cormorants do not appear to be a major source of ND in Switzerland. A direct link between cormorants and domestic outbreaks like in Marin could therefore not be affirmed and remains truly speculative.

Avian influenza (AI) was extensively monitored in wild birds after the emergence of the highly pathogenic avian influenza H5N1 in Europe 2005. After the detection of 32 H5N1 positive samples in Switzerland in 2006, a follow-up examination from 2006–2008 found 84 AIV positive swabs out of 2108 samples from healthy wild birds, but almost all low pathogenic strains (Baumer et al., 2010). In total, 106 cormorants tested during various studies from 2003–2008 were negative for AIV (NRGK, unpublished data). Likewise Artois et al. (2002) could not demonstrate antibodies against AIV in great cormorants in France. Reports of influenza A virus isolation in cormorants are scarce. In Germany, only 18 AIV could be isolated from 4500 cormorants (Süss et al., 1994). All cormorants in the present study were negative. Hence cormorants do not seem to play a role as reservoir in AI epidemiology.

The Chlamydiae taxonomy has been changed to a great extent. Therefore it is not easy to compare different studies using different tests. In a review on the occurrence of *Chlamydophila* spp. (syn. *Chlamydia* spp.) in birds, serum antibodies have been detected in a few studies by complement fixation test (CFT) in *Phalacrocorax* spp. (Kaleta and Taday, 2003) In Switzerland *Chlamydia psittaci* was found in feral pigeons by qPCR, but not in water birds (Zweifel et al., 2009). All cormorants tested in the same study as well as in the present survey were negative by PCR or ELISA (Tab. 1). Positive results from earlier studies can be explained by the higher sensitivity and lower specificity of tests used (Kaleta and Taday, 2003). Further, CFT may detect low levels of antibodies circulating following a past infection, while no shedding of the agent can be traced.

Salmonella spp. are often reported in healthy free-ranging birds (Refsum et al., 2002), including cormorants (Dobbin et al., 2005). While healthy birds seem to be able to cope with *Salmonella* spp. colonisation, diseased or stressed birds may die. Indeed, wild double-crested cormorant chicks lived untroubled with their *Salmonella* spp. infection, while captured chicks from the same colony often succumbed to salmonellosis in captivity (Dobbin et al., 2005). *S.* Typhimurium is the most consistently isolated serovar reported in birds (Dobbin et al., 2005; Refsum et al., 2002). The three isolates from this study were also *S.* Typhimurium, whereof one was an O-antigen "rough" type.

Conclusion

Shedding of AIV, NDV or Chlamydiae in cormorants could not be detected. Only few were *Salmonella* spp. positive. The birds were in good health, although almost all contained stomachs worms. The overall risk emanating from cormorants concerning the infectious agents tested is considered to be low and thus pathogen spreading is deemed an invalid argument to promote higher culling rates. No fish species listed as endangered was found in stomach contents of any cormorant, however grayling, listed as vulnerable because of combined human and avian predation, were found.

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Excrétion d'agents zoonotiques et analyse du contenu stomacal de cormorans (*Phalacrocorax carbo sinensis*) en Suisse entre 2007 et 2012

208 cormorans sauvages en bonne santé (*Phalacrocorax carbo sinensis*), tirés au cours de 5 années de chasse consécutives, de 2007/2008 à 2011/2012, ont été testés quant au virus de la maladie de Newcastle (APMV1), au virus de l'influenza aviaire (AIV), aux chlamydias et aux *Salmonella* spp. Tous les oiseaux étaient négatifs en ce qui concerne APMV-1, AIV et chlamydias. On a isolé deux fois *Salmonella enterica* subsp. *enterica* serovar Typhimurium et une fois une forme de base de *Salmonella* Typhimurium. En outre on a examiné macroscopiquement le contenu stomacal. 199 cormorans étaient atteints de vers gastriques et on a pu identifier, dans 45 estomacs, 12 sortes de poissons différents.

L'eliminazione di agenti patogeni e analisi del contenuto dello stomaco dei cormorani (*Phalacrocorax carbo sinensis*) in Svizzera tra il 2007 e il 2012

Sono stati controllati 208 cormorani selvatici e sani (*Phalacrocorax carbo sinensis*), durante 5 stagioni di caccia consecutive dal 2007/2008 fino al 2011/2012, per il virus della malattia di Newcastle (APMV-1), per il virus dell'influenza aviaria (AIV), per le clamidie e per la *Salmonella* spp. Tutti gli uccelli sono risultati negativi all'APMV-1, all'AIV e alle clamidie. Sono state rilevate per due volte la *Salmonella enterica* subsp. *enterica* serovar Typhimurium e una volta una forma grezza di *Salmonella* Typhimurium. Inoltre, il contenuto dello stomaco è stato esaminato macroscopicamente. In 199 cormorani si sono riscontrati vermi dello stomaco e in 45 stomaci sono stati identificate 12 specie di pesci.

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