

## Research article

# Seed dispersal by waterbirds: a mechanistic understanding by simulating avian digestion

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Waterbirds disperse plant species via ingestion and egestion of seeds (endozoochory). However, our understanding about the regulating effects of seed traits, underlying mechanisms and possible (co)evolutionary processes is limited by our traditional reliance on data from feeding experiments with living waterbirds. Here, we overcome these limitations by developing and applying a new bioassay that realistically simulates digestive processes for Anseriformes waterbirds. We test three hypotheses: 1) seed survival and germination are most affected by mechanical digestion in the waterbird gizzard; 2) seed size, hardness, imbibition and shape regulate seed survival; and 3) plants growing in aquatic habitats benefit most from endozoochory by waterbirds. Experiments with 28 200 seeds of 48 plant species demonstrated species-specific seed survival that was entirely determined by digestion in the avian gizzard. Intestinal digestion did not affect seed survival but affected seed establishment (germinability and germination time) for 21% of the species. Large, hard seeds survived the simulations the best, in contrast to generally higher seed survival for smaller seeds during in vivo experiments. This mechanistically explains that small seeds escape digestive processes rather than being inherently more resistant (the ‘escape mechanism’), while large seeds are retained until fully digested or regurgitated (the ‘resistance and regurgitation mechanism’). Plants growing in wetter habitats had similar seed survival, but digestive processes stimulated their germinability and accelerated their germination more than for terrestrial plants. This indicates a relative advantage of endozoochory for plant species growing in wet habitats, possibly reflecting a co-evolutionary response related to dormancy breaking by gut passage. Simulating seed gut passage using a bioassay allowed establishing mechanisms and identifying relevant seed traits involved in seed dispersal by waterbirds. This information enhances our understanding of how animal species shape plant species distributions, which is extremely relevant now that current anthropogenic pressures already severely impact plant dispersal capacities.

Keywords: Ellenberg value, endozoochory, gut passage, seed dormancy, seed traits, wetland plant



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## Introduction

Seed dispersal critically contributes to plant community assembly, regional survival, range expansion and patterns of genetic variability (Levin et al. 2003, Ozinga et al. 2009, Nilsson et al. 2010, van Leeuwen et al. 2014). The contribution of animals to plant seed dispersal has been particularly well-studied for seeds inside fleshy fruits dispersed by frugivores (Corlett 2017, Gelmi-Candusso et al. 2017, Morán-López et al. 2018, Valido and Olesen 2019). However, more than 90% of the native European flora produces inconspicuous, indehiscent seeds that are not surrounded by attractive nutritious fleshy pulp (Heleno and Vargas 2015). Many of these seeds are currently classified as being only dispersed by gravity (barochory, Julve 1998, Kleyer et al. 2008). However, they are also frequently encountered in faecal samples of ungulates, fish, reptiles and birds (Horn et al. 2011, Soons et al. 2016, Green et al. 2019, 2021, Valido and Olesen 2019, van Leeuwen et al. 2020). Hence, animal vectors typically considered herbivorous, omnivorous or even granivorous also importantly contribute to plant seed dispersal (Green et al. 2021).

The mechanisms and seed traits that are essential for effective seed dispersal via plant–frugivore interactions have been well-studied, including the identification of the underlying (co)evolutionary processes (Herrera 1984a, b, 2003, Jordano 2000, Eriksson 2016, Corlett 2017). However, (co)evolutionary processes, mechanisms and the most important seed traits for animal-mediated dispersal of plants without fleshy fruits are less clear (Albert et al. 2015a, b, Costea et al. 2019, van Leeuwen et al. 2022b). Here, we study these aspects for dispersal by waterbirds, focusing on the order Anseriformes because especially ducks, geese and swans are globally important taxonomic groups that contribute prominently to dispersal of seeds without fleshy fruit (hereafter ‘seeds’). These waterbirds disperse seeds effectively because they deliberately ingest large quantities of seeds as food, of which part is viably egested after multiple hours of retention in the digestive system (van Leeuwen et al. 2012b, Soons et al. 2016). During retention of seeds, waterbirds can move independently of the landscape structure between roosting and foraging areas on small spatio-temporal scales or over long-distances during seasonal migrations on large spatio-temporal scales (Kleyheeg et al. 2017, Martín-Vélez et al. 2021). Waterbirds can occur in high densities as a result of large population sizes and seasonal aggregations (Wetlands International 2022). Although waterbirds disperse a wide range of plants in aquatic, riparian and terrestrial habitats (Soons et al. 2016, Green et al. 2021, Almeida et al. 2022), they interact most strongly with plants more typical of aquatic habitats (Kleyheeg et al. 2017) by foraging on their leaves and seeds. This suggests a strong potential for (co)evolution between waterbirds and seed traits of plants in more aquatic habitat types, as indicated by higher Ellenberg F indicator values for preferred soil moisture (Ellenberg et al. 1992).

The most critical mechanism for successful endozoochory of plants by waterbirds is survival of gut passage by their seeds. Traditional feeding experiments with captive birds

have shown that a wide range of seeds is capable of surviving avian digestion (Charalambidou and Santamaría 2002, van Leeuwen et al. 2012b). Small, strong seeds generally survive these feeding experiments the best (Soons et al. 2008, Costea et al. 2019, Lovas-Kiss et al. 2020a). However, the data obtained from this type of experiments is generally limited: the experiments are ethically challenging, involve living animals that may be stressed by experimental handling, are limited in replicates (i.e. testing between 2 and 20 plant species per study, Soons et al. 2008, Figuerola et al. 2010), and suffer from variance due to intra-specific variation among individual animals (Pollux 2017, Zwolak et al. 2020).

A more insightful method that solves most of these limitations is to expose seeds to *in vitro* simulated conditions during avian digestion. Avian digestion consists of consecutive digestive processes, including mechanical, chemical and enzymatic digestion by grinding in the muscular gizzard, and chemical and enzymatic digestion in the intestines (Sturkie 2012). Ingested seeds therefore encounter a combination of stressors: mechanical grinding, acid conditions, high temperatures, digestive enzymes and anoxia (Martinez-Haro et al. 2009, McWhorter et al. 2009). Previous studies have exposed seeds to either chemical or mechanical simulations of digestion, for example by mechanical scarification or exposure to low pH conditions (Martinez-Haro et al. 2009, 2010, Vazacova and Munzbergova 2013, Kleyheeg et al. 2018a, Tesson et al. 2018, Carbonell et al. 2021). These experiments revealed that avian digestion is particularly effective because it combines multiple stressors in a living animal (Kleyheeg et al. 2018a). However, to date it has remained a challenge to realistically simulate all stressors combined (Moore 1998a, b).

Here, we aimed to assess whether (co-)evolutionary processes may have resulted in seed traits that increase the potential of plants to be dispersed by waterbirds, considering both seed survival and germination response after gut passage. To address our aim, we developed a new bioassay in which digestion by non-frugivorous birds can be simulated realistically and in detail to understand how digestive processes interact with seed traits. We first established the applicability of the new bioassay for studying avian digestion, and then assessed 1) how seed survival and germination are affected by the two main digestive phases in birds: mechanical, chemical and enzymatic digestion in the gizzard, and chemical and enzymatic digestion in the intestines; 2) how seed traits regulate seed survival by interacting with digestive processes; and 3) the effects of avian digestion on seeds of plant species that are characteristic for habitat types ranging from aquatic to terrestrial conditions. We hypothesized 1) seed survival and germination to be more affected by digestion in the gizzard than by digestion in the intestines, because the gizzard includes the mechanical digestive phase; 2) seed size, hardness, imbibition and shape to affect seed survival, because we expect these traits to affect the interactions between the seeds and simulated digestive processes; and 3) plants growing in more aquatic habitat types to benefit more from endozoochory by waterbirds than terrestrial plants as an indication of possible (co)evolutionary processes, because of their more frequent interactions with waterbirds.

## Material and methods

### Experimental simulation of avian digestive processes

To test how digestive processes in birds affect seeds with different functional traits, we developed a method to simulate avian digestive processes (Fig. 1). Birds digest food in complex consecutive steps that vary in intensity among species (Fig. 1a, Turk 1982, King and McLelland 1984, Sturkie 2012). We simulated the digestive system of dabbling ducks based on their best studied representative: the common generalist omnivore waterbird, the mallard *Anas platyrhynchos*. Mallard physiology is well-studied and represents a broader range of waterbird species important for zoochory (Charalambidou et al. 2003, van Leeuwen et al. 2012b, Lovas-Kiss et al. 2018). We simultaneously applied mechanical, chemical and enzymatic digestion, thereby expanding on previous studies that applied these methods in isolation (Moore et al. 1998, Martinez-Haro et al. 2009, Kleyheeg et al. 2018a). We focused on mechanical damage in the combination of the proventriculus and gizzard (the gizzard-phase), and the effects of digestive enzymes in the intestines (the intestinal-phase, Fig. 1b).

To simulate the gizzard-phase as calibrated on mallards, we applied mechanical (pressure, abrasion), chemical (low pH, salts) and enzymatic conditions (enzymes). Therefore, we filled a 35 ml balloon (made of > 2 mm thick natural rubber with an aperture of 10 mm) with a manually counted number

of plant seeds, grit and a solution of digestive fluids (Fig. 1b). We exposed the balloon in a rotating machine simultaneously to translational and compressive forces (following Moore et al. 1998). We filled the balloon with washed silica grit with a rough surface texture (Fleurit floors, NL) with a size range from 2 to 4 mm (Martinez-Haro et al. 2009). Grit of this size class is common in waterfowl (Mateo et al. 2000) and particularly relevant for breaking ingested seeds (Thomas et al. 1977). We filled the balloon with a counted number of seeds and 50 g of this grit, which filled the volume inside the balloon almost entirely. This process simulated avian gizzards that adjust their volume to content, and generated realistic levels of mechanical pressure (Moore et al. 1998). Mechanical movements involved 120 sideward compressions with amplitudes of 20 mm per minute, leading to six full rotations of the balloon per minute. We prepared a digestive solution for the gizzard of 1 N sodium chloride (VWR, NL) with 10 g l<sup>-1</sup> pepsin (VWR), to prepare gizzard digestive juice for mallards as described previously (Martinez-Haro et al. 2009). We regulated the fluid to a constant pH of ~2.6 via a citrate-buffer solution (89.2% 0.1 M C<sub>6</sub>H<sub>8</sub>O<sub>7</sub> and 10.8% 0.2 M Na<sub>2</sub>HPO<sub>4</sub>) to ensure a stable pH during seed breakdown and to avoid having to stop the mechanical process during digestion to add fluids. To ensure anoxic conditions in the avian gizzard the balloon was closed with a more flexible balloon filled with N<sub>2</sub>-gas.

To simulate the intestinal-phase of mallards (Fig. 1b), we assumed a dominant role of chemical and enzymatic digestion (Turk 1982, King and McLelland 1984, Sturkie 2012). Intestinal digestion was simulated in 50 ml polypropylene tubes (VWR) that were rotated in a VWR Tube Rotator to create an end-over-end tumbling motion, with 18 rotations per minute. Intestinal fluid consisted of 0.35% bile salts (Sigma-Aldrich) and 0.035% pancreatin (Alfa Aesar) in a citrate-buffer of pH 6.2 (33.8% 0.1 M C<sub>6</sub>H<sub>8</sub>O<sub>7</sub> and 66.2% 0.2 M Na<sub>2</sub>HPO<sub>4</sub>) (sensu Martinez-Haro et al. 2009). The complete experimental setup (gizzard and intestinal phases) was placed in an isolation chamber that was kept at an avian body temperature of 42°C (sensu Dawson and Whittow 2000) in the dark.

### Selected plant species

We selected 48 plant species with indehiscent seeds not surrounded by nutritious fleshy pulp that are native (or archeophytes) in the Netherlands. The plant species were selected because they represent a wide range of contrasting seed traits (e.g. variation in mass, shape; Fig. 2) and because they all grow in habitats where dabbling ducks forage; mostly along or close to shorelines of freshwater habitats. The selected plant species are typical for heterogeneous landscapes consisting of water bodies, wetlands and terrestrial habitats, with Ellenberg F-values for soil moisture ranging from 3 to 11 (obtained from Hill et al. 2004); full range of values is from 1 (extreme dryness) to 12 (submersed aquatic, Ellenberg et al. 1992). Seeds were obtained commercially from Biodivers B.V. (Oudewater, NL), who harvested the seeds in natural areas in 2017 (with all required licenses). All seeds were stored dry after harvest and placed at 4°C for stratification in

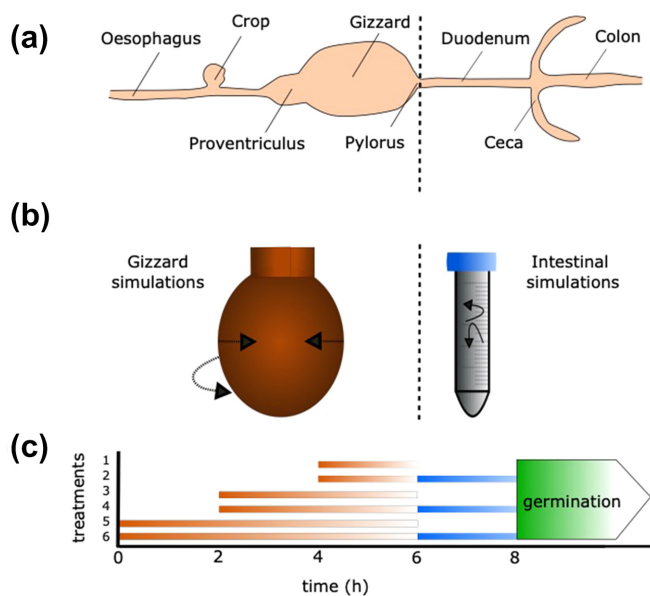


Figure 1. (a) The experimental setup consisting of a simulated gizzard phase and simulated intestinal phase. (b) The gizzard simulations were performed inside a pipetting balloon that was mechanically stimulated (more details in main text and Supporting information), and the intestinal simulations were performed in a 50 ml tube in an end-over-end shaker. (c) The variation in duration of exposure to each phase is indicated for six treatments, and ranges from 2 to 6 hours (h) of gizzard digestion with or without an additional 2 hours of intestinal digestion. After the treatments, all seeds (plus undigested controls) were set for possible germination for 60 days.

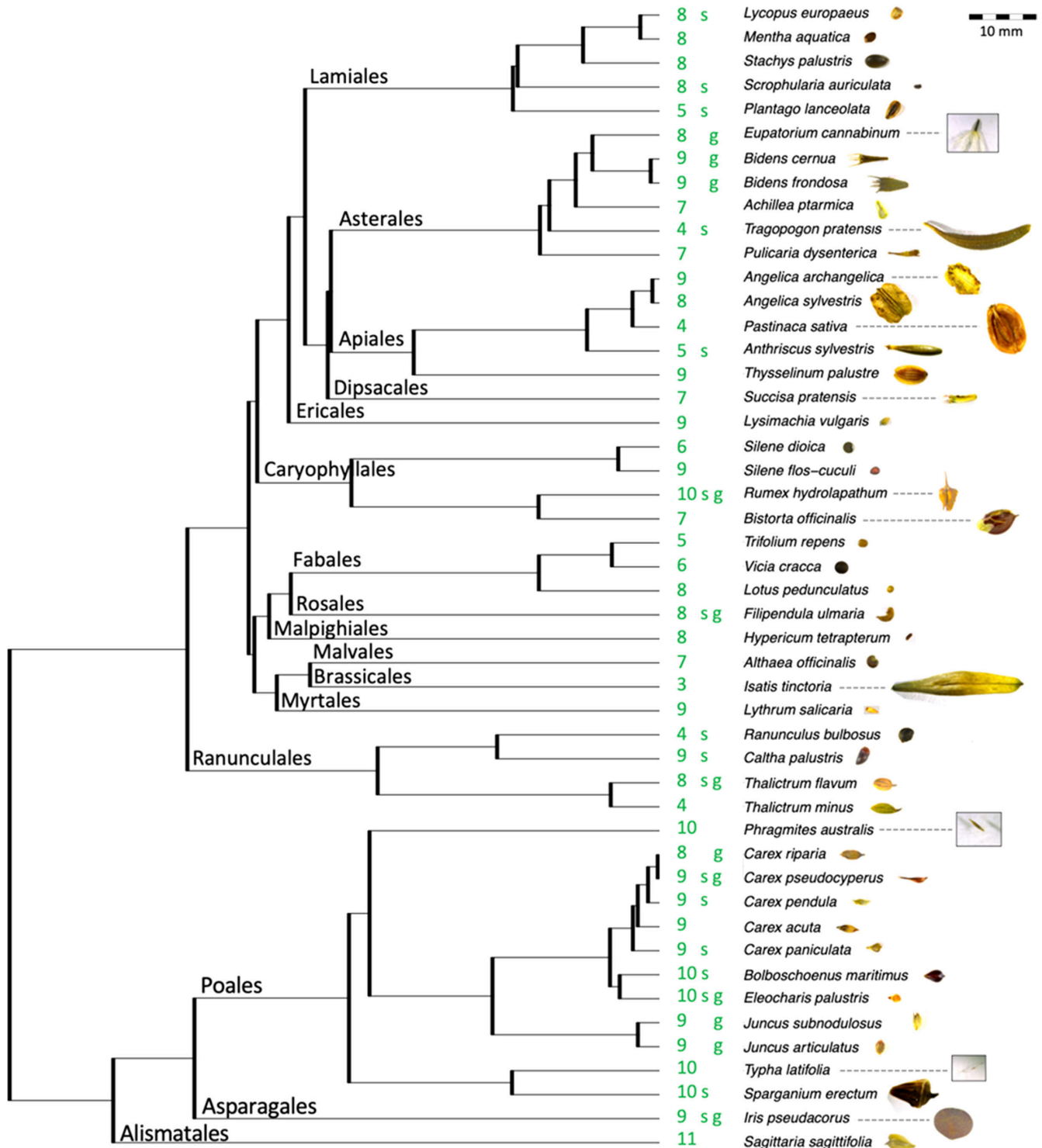


Figure 2. Phylogenetic tree of the 48 studied plant species with the names of orders and species indicated. For each species, an image of its seed is placed to the right of its name (to scale). Green numbers indicate Ellenberg F-values for each species. The 17 species for which survival was unaffected by the treatments are indicated with a green 's', and the 12 species with positive effects on germinability and/or germination time are indicated with a green 'g'. Photos by the authors.

October 2017 with the expectation of breaking physiological dormancy, until the simulation experiments were performed between March and September 2018.

Sixteen of these species overlapped with a previous study in which the same plant species were fed experimentally to captive mallards (Soons et al. 2008). In this previous in vivo study, seed survival, retention times in the digestive system and seed germination after egestion were monitored. Although the seeds for the in vivo study were obtained from different sources and years in autumn, and stratified until the following spring (shorter than in the present study), data were thus obtained for interactions of the same plant species with mallard digestive processes. This allowed us to verify and compare the new in vitro method with published in vivo data for 16 plant species during calibrations and analyses.

## Experimental procedure

Seeds of all 48 species were exposed to either simulated digestion in the gizzard-phase (mechanical treatment), the gizzard-phase followed by an intestinal-phase (full digestion), or no digestion (8 hours in tap water as control, Fig. 1c). The gizzard-phase lasted 2, 4 or 6 hours, based on probability distributions of seed egestion by Anatidae (van Leeuwen et al. 2012a, b). The intestinal-phase was standardized to 2 hours (Vispo and Karasov 1997). Combined total simulated retention times ranged from 2 to 8 hours – which covered a range of typical total digestion times in waterbird species and mallards in particular (van Leeuwen et al. 2012a, b, Kleyheeg et al. 2015, Lovas-Kiss et al. 2020a).

For each plant species we consecutively added to the artificial gizzard: 100 manually counted seeds for the target plant species (except for two large-seeded plant species *Iris pseudacorus* and *Sparganium erectum*, for which we added 50 seeds to approximate an equal volume of seeds); 50 g grit; 17 ml digestive solution. After 2, 4 or 6 hours of digestion, seeds were separated from the grit by eye while kept in 42°C digestive solution. From all seeds retrieved from the gizzard-phase, 50% continued to a 2 hour intestinal-phase, and 50% was set to germinate (Fig. 1c). All experiments were carried out in Duplo using five simulators simultaneously, with an effective  $n=50$  seeds per digestive treatment for 46 species and  $n=25$  seeds per treatment for the two larger species, *I. pseudacorus* and *S. erectum*. In total, we simulated digestion on 28 200 seeds and attempted germination on those seeds that survived the digestive treatments, plus 4750 control seeds ( $2 \times 50$  per species,  $2 \times 25$  for *I. pseudacorus*). Data were recorded on the effects of the treatments on seed survival (whether or not a digested seed was retrieved intact, i.e. all parts were present and the seed was not visibly damaged), germinability (the probability a seed germinated) and germination time (days until a seed germinated).

## Seed trait assessments

We measured the following seed traits ( $n=20$  per species): seed length, width and height (in  $\mu\text{m}$ , accuracy 0.1  $\mu\text{m}$ ) under a stereo microscope (LEICA M205 C). Seed mass was

measured (in  $\mu\text{g}$ ,  $d=0.1 \mu\text{g}$ ) using a Mettler Toledo analytical balance on dry seeds, and on seeds that had been stored dark in tap water for six weeks (hereafter 'wet seeds'), which allowed calculating the maximum water uptake of each species relative to dry seed mass ('seed imbibition'). Seed shape was calculated as a dimensionless index varying from 0 (a perfect spherical shape) to 0.2 (a slim disk or a thin needle) sensu (Bekker et al. 1998). Seed hardness was measured by subjecting seeds individually to a compressive force (increasing with  $0.1 \text{ mm s}^{-1}$ ) using an Instron 5542 Tension & Compression Tester. The Instron registered the pressure that was transmitted by a seed within the machine at 0.1 s intervals. We defined seed hardness as the flexure load (in Newton) that the seed transferred 0.1 s before the flexure load decreased by 0.1 N or more in a subsequent time step. This corresponded to the first peak load that a seed could transfer and thus endure. A decrease in transmission of the pressure was interpreted as (internal) damage to the seed and assumed to be a relative representation of seed hardness. We measured hardness of all species on both dry ( $n=10$ ) and wet seeds ( $n=10$ ).

## Germination experiments

Germinability (the proportion of germinating seeds) and germination time (the number of days before a seed germinated) were assessed for all seeds that were retrieved intact without visible damage from the treatments, plus control seeds. Seeds were placed equal distances apart (to standardize possibilities of fungi spreading) on accordingly sized petri-dishes with a double layer of moisturized Whatman No. 1 filter paper. The petri-dishes were closed with lids to limit evaporation, and placed at a light:dark regime of 16:8 hours and 24:12°C. Every two to three days seeds were counted and seeds that had germinated or were severely affected by fungi were removed. Tap water (without chloride) was added to compensate for evaporation if necessary. Seeds were monitored for 60 days after their respective treatments.

## Statistical analyses

To verify the accuracy of our simulations we compared our simulation data to previously published data on an experiment with living waterbirds. In this experiment, mallards were fed plant seeds and seed retrieval in faeces was monitored for 48 hours (for further details see Soons et al. 2008). We calibrated our in vitro approach with seed survival in vivo based on the 16 overlapping plant species between the two studies. However, this comparison required a correction for differences in methodological approaches: the in vitro simulations were performed in closed containers with controlled retention times, while during in vivo experiments seeds experienced shorter or longer retention times as they passed through the digestive system. The simulation approach did not include differential rates of seed passage through the pylorus to the intestines, which are influenced by seed size (DeVlaming and Proctor 1968, van Leeuwen et al. 2012a). Therefore, we compared the in vivo and in vitro approaches

after correcting both for effect of seed lengths. We did this by partial regression analysis for which we regressed *in vivo* survival to seed length (1), regressed *in vitro* survival to seed length (2), and then regressed the residuals from (1) against the residuals from (2). We used this method to compare the 2, 4 and 6 hour gizzard treatments to the *in vivo* data. However, because the 4 hour treatment destroyed all seeds for part of the species and did not inflict any damage to some of the other species (Results), we performed our final analyses on seed survival on the mean of the 2, 4 and 6 hour treatments as the predictor variable. This ensured we included the species-specific variation detected by the 2 and 6 hour treatments in our overall analyses.

We analysed the effects of gizzard and intestinal digestion separately and combined on seed survival, germinability and germination time. All analyses on seed survival were performed per species because of strong species-specific responses, and we therefore Bonferroni-corrected alpha-levels of 0.05 to avoid type I errors. Analyses on seed survival involved three consecutive steps: 1) for all species, we analysed whether seed survival differed from 'no loss' across all treatments via a Pearson's Chi-squared test with Yates' continuity correction. 2) For species of which part of the seeds did not survive the treatments, we tested which of the treatments significantly affected seed survival by fitting generalized linear mixed-effects models (GLMMs) with Maximum Likelihood approximation in package 'lme4' in R (Bates et al. 2015). Seed survival was modelled as binomial denominator (with logit link function), depending on time in the gizzard simulation as centred continuous explanatory variable (zero, two, four or six hours in the gizzard) and intestinal treatment (yes/no) as binomial factor. Duplicate was included as random-effects intercept. 3) For species with declining seed retrieval over time in the gizzard simulation, we attempted to fit an exponential decay function over time with a self-starting nonlinear least squares (nls) asymptotic regression model in the 'stats' package in R ([www.r-project.org](http://www.r-project.org)). We computed p-values of the exponential functions, which indicate whether or not an exponential decline was present. For those species in which an exponential decline was observed, the  $\lambda$  values (rate constants belonging to the exponential functions) indicate the strength of the exponential declines. We indicate how well the theoretical exponential curves fitted the empirical data by presenting  $R^2$ -values of linear models regressing the observed to the modelled data.

Effects of the treatments on germinability were assessed species-specifically by comparing germinability between control seeds, seeds that had experienced gizzard-simulation treatments and seeds that had experienced full digestive simulations (gizzard and intestinal digestion) with Pearson's Chi-squared tests with Yates' continuity correction. Effects of the treatments on germination time among these same three groups were assessed by ANOVAs, with gizzard treatments and intestinal treatments included as binomial factors.

Effects of seed traits on seed survival were tested with univariate models because many seed traits correlated (Supporting information), and variance inflation factors (VIF) of models

including multiple traits exceeded 5.0. We selected the following independent variables for univariate analyses: 1) seed length in mm, 2) seed mass in mg, 3) seed imbibition, 4) seed shape, 5) hardness of wet seeds, 6) hardness of dry seeds. All traits (except shape) were natural log transformed to meet model assumptions. Effects of wet and dry hardness were additionally tested using partial regression analyses in which both seed survival and hardness were corrected for effects of seed length. Effects of Ellenberg F-values were tested in identical univariate models with Ellenberg F-value as explanatory variable, and seed survival, germinability and germination time as dependent variables.

To avoid possible loss of information due to similarities among phylogenetically closely related species, all models were performed with and without corrections for phylogenetic relatedness of the 48 species. Therefore we created a phylogenetic tree (Fig. 2) of all study species based on the megaphylogeny of plants (Qian and Jin 2016) and used this to search for phylogenetic signals (i.e. the link between phylogeny and continuous trait values) with the package 'phyloSignal' (Keck et al. 2016). Significant Bloomberg's K values and/or Pagel's lambda (Pagel 1999, Blomberg et al. 2003) for several traits (Supporting information) indicated that phylogenetically more closely related species displayed similar values for some traits due to their phylogenetic proximity (Revell 2010). To avoid information loss, we fitted all models with and without corrections for phylogeny. For each dependent variable, a linear model (LM) with Gaussian error distributions was fitted, as well as a phylogenetic generalized least squares (PGLS) model with an implemented Brownian motion correlation structure based on the species phylogeny in package 'nlme' (Pinheiro et al. 2021). Normality of model residuals was assessed visually in quantile–quantile plots and via Shapiro–Wilk normality tests; and homogeneity of the residuals was assessed by plotting model residuals against the fitted values. Statistical details are presented in the figures and more details can be found in the Supporting information. All calculations were performed in R ([www.r-project.org](http://www.r-project.org)).

## Results

### Verification of the bioassay

We found strong similarities between seed survival in the bioassay and in *in vivo* experiments for the overlapping 16 plant species, conducted with waterbirds. Partial correlation tests showed a significant positive relation between seed survival *in vivo* and seed survival after *in vitro* treatments that included a 4 hour-gizzard simulation phase ( $R^2=0.34$ ,  $p=0.017$ , Fig. 3). Shorter (2 hours) or longer (6 hours) gizzard simulations showed similar relative patterns across all species (Pearson's product-moment correlations with 4 hour simulations:  $p < 0.001$ ,  $r=0.85$  and  $p < 0.001$  and  $r=0.91$ , respectively), but less reliably predicted *in vivo* digestive processes (2 hours:  $R^2=0.08$ ,  $p=0.28$ ; 6 h:  $R^2=0.21$ ,  $p=0.08$ ) as these treatments inflicted only minor damage or digested

almost all seeds, respectively. The averaged seed survival over the three durations of gizzard simulations was related to *in vivo* digestion by  $R^2 = 0.22$  with  $p = 0.06$ .

### Effects of digestion on seed survival, germinability and germination time

In total 28 200 seeds of 48 plant species were subjected to simulated digestion treatments, of which 18 448 seeds were retrieved intact (65.3% seed survival, Supporting information). This survival was entirely determined by digestion during the gizzard-phase because no seeds were digested during the subsequent intestinal digestion simulations. Seed survival was species-specific (Fig. 2, Table 1, Supporting information): for 17 species (35%) survival was unaffected by the treatments because all their seeds were retrieved intact. Particularly species in the orders of Poales, Ranunculales and Lamiales were well-represented in this category (as indicated in Fig. 2). For 11 (23%) species simulations decreased survival but longer digestion did not further decrease survival, for 15 species (31%) longer digestions continued to decrease survival, and for five species (10%) survival decreased exponentially over time.

Germinability of the seeds that survived the experimental treatments was lower than germinability of the control seeds (24.8% and 41.3%, respectively, Pearson's Chi-squared test:  $X^2 = 1616.1$ ,  $df = 1$ ,  $p < 0.001$ ). The treatments affected germinability negatively for 21 out of 48 species and positively

for four out of 48 species (Table 1, Fig. 2, Supporting information). Of all 28 200 experimentally treated seeds, 16.2% survived as well as germinated (Supporting information). The treatments accelerated germination for 10 species and delayed germination for six species compared to controls (Supporting information). Germination of plant species in the orders of Poales and Asterales was often positively affected by digestive processes (Fig. 2).

Full digestion (gizzard and intestinal treatment) did not consistently have different effects on germinability or germination time compared with partial digestion (only gizzard treatment, Supporting information). However, addition of the intestinal digestion treatment affected germinability for seven species (negatively for six species) and affected germination time for six species (of which germination was accelerated for five species) compared to only partial digestion treatments.

### Effects of seed traits on seed survival

Seed survival depended mostly on seed length and mass (Fig. 4). Despite considerable variation in survival among smaller seeds, longer and heavier seeds were generally more resistant to the treatments (Fig. 4a, b). This was independent of whether we corrected for phylogenetic relatedness among the species. Seeds of which their mass increased up to twofold due to storage in water were still retrieved in high proportions (imbibition, Fig. 4c), although survival was always lower than 100% for seeds with even stronger imbibition.

Seed hardness decreased due to six weeks of storage in water (paired t-test:  $t = -2.30$ ,  $df = 45$ ,  $p = 0.026$ ). This loss of hardness due to storage in water was weakly associated with increased imbibition ( $t = -1.78$ ,  $df = 44$ ,  $p = 0.08$ ,  $r = -0.25$ ). Stronger seeds survived digestive processes better, although this effect disappeared after correcting for phylogenetic relatedness among the studied plant species (Fig. 4d). Seed strength correlated positively with seed length (for wet seeds:  $t = 5.06$ ,  $df = 44$ ,  $p < 0.001$ ,  $r = 0.61$ ; for dry seeds:  $t = 2.88$ ,  $df = 44$ ,  $p < 0.01$ ,  $r = 0.40$ ). After correcting for this effect of seed length, seeds that were relatively hard for their length were retrieved intact more often – indicating a positive effect of relative hardness on seed survival (Fig. 4e). The trends as described for the hardness of wet seeds were similar for dry seeds (Supporting information). Seeds with more elongated shapes (like thin disks or slim needles) had higher survival than more spherical seeds (Fig. 4f), however, only after correcting for phylogenetic relationships among the tested species and not after correcting for seed mass using partial effects (Supporting information).

### Relation of Ellenberg F-values with seed survival, germinability and germination time

Ellenberg F-value did not correlate significantly with any of the seed traits (Supporting information). The treatments had different effects on seeds of plants with different Ellenberg F-values. Seed survival (for all treatments combined) did

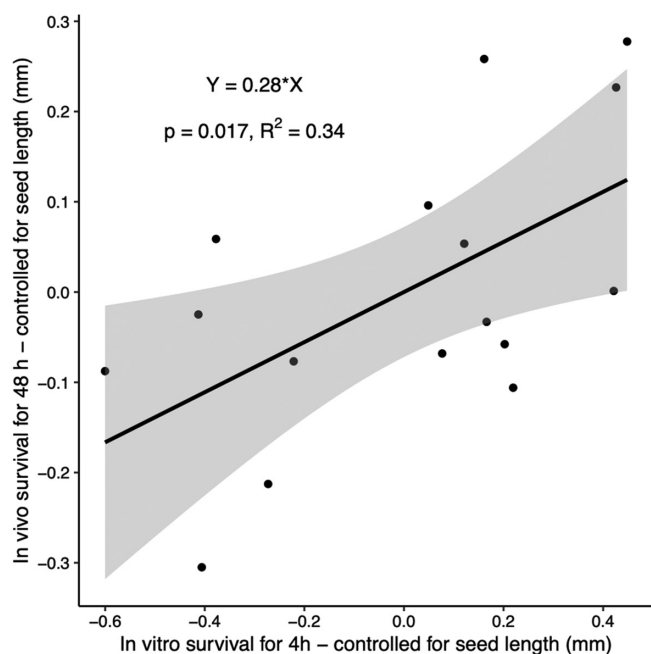


Figure 3. Partial regression plot between the proportion of seeds retrieved after 48 hours (h) during *in vivo* experiments and the proportion of seeds retrieved after 4 hours of simulated *in vitro* gizzard digestion ( $n = 16$  plant species), after pairwise controlling for the effects of seed length. The fitted line represents an ordinary least squares regression with standard error (grey area) that predicts *in vivo* survival from *in vitro* by the indicated formula.

Table 1. Effects of the treatments on seed survival (the proportion of seeds retrieved after 8 hours of complete digestion), germinability (proportion of germinating seeds) and germination time (mean number of days until germination), and trait information for the 48 plant species. Effects of the duration of digestion on seed survival are indicated by capital letters: (A) no effect; (B) damage due to digestion but no further damage due to longer digestion; (C) seed survival declined due to longer digestion; (D) seed survival declined exponentially due to longer digestion. Germinability and germination time are indicated for control seeds, after only gizzard-digestion and after full digestion; and for species with statistically significant differences between controls and treatments the direction of effects is indicated. Plant Ellenberg F-value for soil moisture, and means of seed length (mm), seed mass (mg), imbibition (times weight increase), hardness (Newton) and shape (dimensionless) are presented for all species. More details can be found in the Supporting information.

Species	Seed survival		Germinability		Germination time		Plant and seed traits										
	Seed survival	Effect simulations	Control	Gizzard	Control	Gizzard	Gizzard+intestine	Germination time	Control	Gizzard	Gizzard+intestine	Ellenberg F	Seed length	Seed mass	Imbibition	Seed hardness	Seed shape
<i>Achillea ptarmica</i>	0.39	C	0.73	0.61	0.46	0.46	0.46	0.46	7.8	9.0	8.6	7	1.9	0.2	2.0	2.7	0.11
<i>Althaea officinalis</i>	0.19	C	0.16	0.09	0.06	0.06	0.06	0.06	9.1	4.0	10.4	7	2.2	3.0	1.8	1.8	0.06
<i>Angelica archangelica</i>	0.40	D	0.01	0	0	0	0	0	54.0	0	0	9	5.7	2.7	3.6	17.6	0.08
<i>Angelica sylvestris</i>	0.78	C	0.14	0.01	0	0	0	0	31.8	36.5	0	8	5.4	2.6	2.2	8.1	0.12
<i>Anthriscus sylvestris</i>	0.99	A	0	0	0	0	0	0	0	0	0	5	7.0	5.3	1.8	11.7	0.15
<i>Bidens cernua</i>	0.80	C	0	0.32	0.08	0.08	0.08	0.08	0	28.7	21.5	9	5.1	1.1	2.0	6.8	0.15
<i>Bidens frondosa</i>	0.70	C	0.03	0.43	0.18	0.18	0.18	0.18	10.3	23.7	14.1	9	6.2	2.8	1.7	8.7	0.12
<i>Bistorta officinalis</i>	0.42	D	0	0.06	0.04	0.04	0.04	0.04	0	12.0	15.7	7	4.9	4.1	2.9	5.1	0.04
<i>Bolboschoenus maritimus</i>	1	A	0.01	0	0	0	0	0	15	0	0	10	3.2	2.4	1.3	20.5	0.09
<i>Caltha palustris</i>	0.97	A	0.15	0.01	0	0	0	0	24.5	19.0	27	9	2.6	1.4	1.9	2.7	0.09
<i>Carex acuta</i>	0.59	B	0.15	0.19	0.16	0.16	0.16	0.16	30.7	23.8	33.3	9	3.9	0.8	3.2	2.5	0.11
<i>Carex paniculata</i>	0.98	A	0.01	0	0	0	0	0	30.0	30.0	0	9	3.0	0.8	1.7	9.1	0.10
<i>Carex pendula</i>	0.97	A	0	0	0	0	0	0	0	0	0	9	2.9	0.7	2.0	6.2	0.12
<i>Carex pseudocyperus</i>	0.96	A	0.88	0.94	0.95	0.95	0.95	0.95	18.7	12.6	12.4	9	5.1	1.3	1.6	2.6	0.13
<i>Carex riparia</i>	0.70	C	0.86	0.81	0.79	0.79	0.79	0.79	21.6	17.5	16	8	3.7	0.9	3.3	3.7	0.08
<i>Eleocharis palustris</i>	0.96	A	0.12	0.43	0.42	0.42	0.42	0.42	43.8	31.5	32.1	10	1.9	0.6	1.2	16.4	0.04
<i>Eupatorium cannabinum</i>	0.14	B	0.07	0.37	0.40	0.40	0.40	0.40	41.4	23.5	22.5	8	2.5	0.3	1.6	0.8	0.14
<i>Filipendula ulmaria</i>	0.90	A	0.07	0.03	0.11	0.11	0.11	0.11	43.3	18.0	24.1	8	2.7	0.5	2.1	2.1	0.02
<i>Hypericum tetrapetrum</i>	0.05	B	0.8	0.33	0.11	0.11	0.11	0.11	17.3	15.0	16.5	8	0.7	0.0	1.3	0.2	0.07
<i>Iris pseudacorus</i>	1	A	0.82	0.14	0.15	0.15	0.15	0.15	24.1	18.8	18.5	9	5.8	69.2	1.4	341.1	0.07
<i>Isatis tinctoria</i>	0.85	C	0.61	0.32	0.53	0.53	0.53	0.53	11.8	12.6	6.7	3	16.1	4.9	4.2	5.0	0.17
<i>Juncus articulatus</i>	0.59	C	0.84	0.60	0.59	0.59	0.59	0.59	10.9	10.3	10	9	2.8	0.6	2.7	6.8	0.05
<i>Juncus subnodulosus</i>	0.43	C	0.54	0.58	0.52	0.52	0.52	0.52	11.1	10.2	9.2	9	3.0	0.6	3.0	6.8	0.05
<i>Lotus pedunculatus</i>	0.06	B	0.93	0.50	0.70	0.70	0.70	0.70	5.1	11.8	6.1	8	1.1	0.7	2.4	12.2	0.00
<i>Lycopus europaeus</i>	0.98	A	0.73	0.70	0.70	0.70	0.70	0.70	8.4	9.3	7.3	8	1.4	0.3	1.3	1.8	0.07
<i>Lysimachia vulgaris</i>	0.77	B	0.7	0.74	0.64	0.64	0.64	0.64	10.7	10.2	10.3	9	1.4	0.3	1.3	5.0	0.02
<i>Lythrum salicaria</i>	0.17	D	0.89	0.23	0.06	0.06	0.06	0.06	7.5	14.0	9.6	9	1.0	0.1	1.6	0.7	0.08
<i>Mentha aquatica</i>	0.48	B	0.48	0.44	0.42	0.42	0.42	0.42	14.2	12.8	12.4	8	1.0	0.2	1.2	1.0	0.03
<i>Pastinaca sativa</i>	0.76	B	0.03	<0.01	0.01	0.01	0.01	0.01	19.7	35.0	38.0	4	6.2	3.7	2.0	14.0	0.15
<i>Phragmites australis</i>	0.36	C	0	0	0	0	0	0	0	0	0	10	2.8	0.1	1.8	1.8	0.15
<i>Plantago lanceolata</i>	0.98	A	0.78	0	0.01	0.01	0.01	0.01	5.2	0	10.5	5	2.9	1.9	1.7	18.8	0.07
<i>Pulicaria dysenterica</i>	0.41	C	0.16	0.03	0.02	0.02	0.02	0.02	14.4	14.0	10.0	7	3.8	0.1	1.9	1.5	0.18
<i>Ranunculus bulbosus</i>	0.96	A	0.86	0.83	0.76	0.76	0.76	0.76	15.3	20.3	18.0	4	2.9	3.2	1.1	27.3	0.01
<i>Rumex hydrolapathum</i>	1	A	0.87	0.80	0.68	0.68	0.68	0.68	11.5	6.3	6.5	10	4.4	6.1	2.1	7.4	0.09
<i>Sagittaria sagittifolia</i>	0.66	B	0.35	0.10	0.11	0.11	0.11	0.11	22.1	26.3	24.1	11	4.6	1.0	2.8	2.8	0.11
<i>Scrophularia auriculata</i>	0.92	A	0.74	0.04	0.04	0.04	0.04	0.04	11.5	17.0	12.3	8	0.8	0.1	1.3	2.9	0.02
<i>Silene dioica</i>	0.59	C	0.81	0.03	0.04	0.04	0.04	0.04	9.6	24.4	26.5	6	1.2	0.7	1.3	11.6	0.00
<i>Silene flos-cuculi</i>	0.87	B	0.32	0.05	0.03	0.03	0.03	0.03	14.1	14.3	15.4	9	0.8	0.2	1.1	5.8	0.01
<i>Sparganium erectum</i>	0.98	A	0.01	0.03	0.01	0.01	0.01	0.01	23	23	38	10	7.6	29.0	2.1	41.0	0.03
<i>Stachys palustris</i>	0.20	C	0.03	0.02	0	0	0	0	23.3	0	14	8	2.3	1.5	1.6	3.3	0.02
<i>Succisa pratensis</i>	0.34	C	0.09	0.03	0	0	0	0	17.3	0	20	7	4.3	1.7	2.9	4.4	0.10
<i>Thalictrum flavum</i>	0.92	A	0.39	0.16	0.11	0.11	0.11	0.11	33.4	31.4	27.2	8	2.8	1.5	2.2	7.5	0.04
<i>Thalictrum minus</i>	0.81	B	0.25	0.01	0.01	0.01	0.01	0.01	27	47.5	21.3	4	4.0	1.7	2.0	5.3	0.09
<i>Thyselinum palustre</i>	0.83	D	0.3	0.16	0.02	0.02	0.02	0.02	23	31.3	28.5	9	4.2	2.7	1.8	6.5	0.15
<i>Tragopogon pratensis</i>	0.97	A	0.95	0.03	0.01	0.01	0.01	0.01	7	12.7	16	4	12.5	9.4	2.0	27.1	0.17
<i>Trifolium repens</i>	0.11	C	0.94	0.73	0.23	0.23	0.23	0.23	2	10.3	4	5	1.2	0.8	2.3	9.8	0.00
<i>Typha latifolia</i>	0.38	B	0.76	0.27	0.17	0.17	0.17	0.17	7.3	7	10.1	10	2.0	0.1	1.6	2.7	0.15
<i>Vicia cracca</i>	0.45	D	0.68	0.22	0.22	0.22	0.22	0.22	10.2	21.5	17.8	6	2.9	14.5	1.4	37.5	0.00



not differ between plants typical for dry habitats or typical for wetter habitats (Fig. 5a). Germinability and germination time varied among species and were unrelated to Ellenberg F-values, except that terrestrial plants germinated somewhat slower under the provided stratification and germination conditions (Supporting information). However, the simulation treatments increased germinability and accelerated germination significantly more for plant species more typical of aquatic habitats according to Ellenberg F-values (Fig. 5b, c, species-specific information in Table 1, Supporting information and statistical result in Supporting information).

## Discussion

We assessed interactions between digestive processes and seed traits of 48 plant species by simulating waterbird digestion. Our simulation approach extended previous work that focused on either predominantly mechanical, chemical or enzymatic digestive processes in avian digestive systems (Moore 1998a, Moore et al. 1998, Martinez-Haro et al. 2009, 2010, Kleyheeg et al. 2018a, Tesson et al. 2018). By combining multiple stressors for seeds, we developed a bioassay for wild birds previously only available for animal species such as ruminants, pigs and poultry (Stern et al. 1997, Mohamed and Chaudhry 2008, Hatew et al. 2015, Yáñez-Ruiz et al. 2016, Bryan et al. 2018). After taking into account that larger seeds generally pass digestive systems of living birds more slowly (Soons et al. 2008, van Leeuwen et al. 2012a), the simulations provide a reliable estimate of how digestive processes affect plant seed survival during endozoochory by mallards.

Simulations with a 4 hour-gizzard phase best predicted seed survival after 48 hours during in vivo experiments, suggesting that this may be close to the duration of time spent in the gizzard across seed species in (resting) live mallards (Kleyheeg et al. 2018b). Applying the bioassay for shorter and longer durations informed about dispersal probabilities of species with lower or higher survival potential (i.e. species for which no or all seeds were retrieved after 4 hours), offering potential for refined gut passage survival data. This study shows the value of simulations to reduce future experiments with live animals, perform more studies on the survival potential of seeds or other propagules that are only retrieved at very low frequencies in bird faeces in field or lab-studies (e.g. aquatic snails or fish eggs, van Leeuwen et al. 2012c, Lovas-Kiss et al. 2020b), and to refine gut passage data for seeds or other propagules that in live birds have very low or very high gut passage survival rates.

### Mechanisms regulating gut passage survival and germination

The combination of mechanical, chemical and enzymatic digestion during the simulated gizzard phase was crucial for seed survival, while no seeds were lost during the succeeding chemical and enzymatic digestion phase in the intestines. This confirms that the avian gizzard is the most important

determinant of seed survival (Swanson and Bartonek 1970, Kleyheeg et al. 2016) and ratifies using proportions of seeds extracted from intestines of birds as proxy for seed survival in faeces (Brochet et al. 2010). This confirms hypothesis 1 for seed survival. This was, however, not the case for seed germination. Adding a simulated intestinal digestion treatment to the simulations further affected seed germination for 10 of the 48 (21%) species (Supporting information). Germinability decreased due to intestinal digestion for six of these 10 species, and for five of these 10 species germination time was (also) accelerated. This implies that while seed survival rates are determined in the gizzard phase, intestinal digestion may still affect the capacity of dispersed seeds to establish. Studies aiming to predict seed dispersal capacities of waterbirds from inter- and intra-specific variation in digestive systems (Charalambidou et al. 2005, Kleyheeg et al. 2018b) therefore need to consider the physiology of both gizzards and intestines.

### The role of seed traits

We further explored how seed traits mediate the effect of these mechanisms regulating gut passage survival and germination (hypothesis 2). Our bioassay indicates that larger, stronger seeds with less permeable seed coats have the largest potential to survive digestive processes in birds. The effects of seed length and mass were most important, as they were independent of corrections for phylogenetic relatedness among the 48 tested species. Analysis of seed shape suggested higher survival for more elongated, flat, thin seeds than for more spherical seeds. However, this effect was entirely dependent on correcting for a strong phylogenetic signal in shape and can most likely be explained by the positive correlation of the shape variable with seed length. Effects of seed shape could be further explored by analysing simulation data combined with other (geometric) descriptions of seed shape based on image processing systems (Cervantes et al. 2016, Cervantes and Gómez 2019), although based on our current results we expect a stronger influence of traits other than seed shape. Seed hardness did have a clear positive effect on seed survival, but this disappeared after correcting for phylogenetic relationships. One explanation for this difference between effects of seed shape and hardness – that warrants further study – could be that seed shape mostly plays a role within plant orders (species within one order differing in seed shape and therefore differing in survival), while hardness varies more prominently among orders (some orders having structurally stronger seeds and therefore higher survival than other orders). Hardness declined due to storage in water, suggesting higher gut passage survival rates for dry seeds ingested directly from plants or soil surface in autumn than for seeds stored under moist or wet conditions until ingestion later during the winter season.

Seed size is one of the best known traits that predicts seed gut passage survival during in vivo studies, where survival rates are higher for smaller seeds (Figuerola and Green

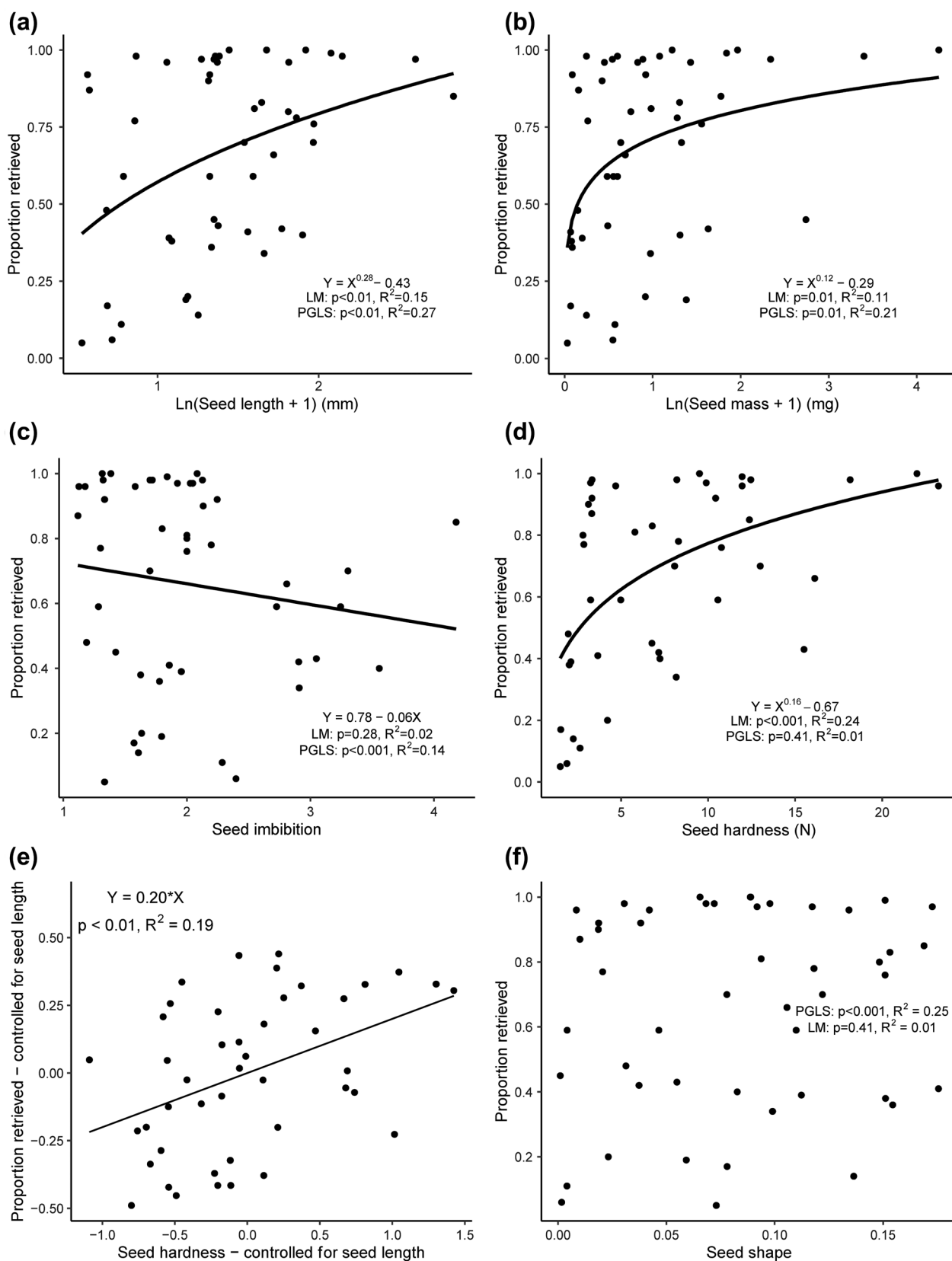


Figure 4. Effects of seed traits on the proportion of intact seeds retrieved (seed survival) during all simulated digestion treatments. Statistical results are indicated in the panels with corrections for phylogenetic relatedness among the 48 species (phylogenetic generalized linear

Figure 4. Continued

models, PGLS) and without (linear models, LM). (a and b) Seed survival increased with seed length and seed mass, with and without correcting for phylogenetic relationships. (c) Seeds with impermeable seeds coats (low imbibition) were retrieved in high proportions. However, there was strong variation among seeds with only little imbibition, and patterns were only statistically significant after correcting for phylogenetic relationships. (d) Hardness of wet seeds positively increased seed retrieval, but significance was lost after correcting for phylogenetic relations. (e) Partial regression plot between seed survival and wet seed hardness, pairwise controlled for the effect of seed length. The fitted line represents a reduced major axis regression to account for the errors in both x and y variables. Survival was higher for seeds that were relatively strong for their length. (f) Seed survival depended only on seed shape after correcting for phylogenetic relationships among the species. Results for panels (d) and (e) are visualized for hardness of seeds after storage in water (wet seeds), but similar results were found for hardness of dry seeds (Supporting information). Statistical details can be found in the Supporting information.

2002, Soons et al. 2008, van Leeuwen et al. 2012a, Reynolds and Cumming 2016, Lovas-Kiss et al. 2020a). However, it remained unclear whether smaller seeds were more capable of resisting digestive processes due to for instance allometric scaling of hardness (Fricke and Wright 2016), or that higher survival was primarily caused by escaping digestive processes (van Leeuwen et al. 2012b). By controlling seed exposure times to digestive processes in our simulations, we found that longer, heavier and stronger seeds were more capable of resisting digestive processes than smaller, lighter and weaker seeds. Thereby, these results reveal that smaller seeds are better at escaping rather than resisting digestive processes.

The mechanism that small seeds survive gut passage by escaping digestive processes is in line with at least three other observations. First, smaller seeds generally have shorter retention times (within six to eight hours, van Leeuwen et al. 2012a, Kleyheeg et al. 2015) than larger seeds, likely because the pylorus valve that determines whether food is transferred to the intestines (Ferrando et al. 1987) only allows smaller items to pass into the intestines. This confirms more rapid gut passage of egested small seeds and slower passage of larger seeds, which may only accidentally pass or first need to break down into smaller parts. Second, the low proportion of small seeds that is excreted after long retention (Viana et al. 2016, Lovas-Kiss et al. 2019), has most likely been retained in the digestive system beyond the gizzard-phase, e.g. in the intestines or caeca (as suggested for plankton, Malone 1965). Third, endozoochory by waterbirds is facilitated by the birds optimizing their energy intake per unit of time, as opposed to per unit of food ingested (van Leeuwen et al. 2012b). Hence, processing time is optimized such that part of all ingested food passes the digestive system intact or partly digested, which facilitates escape of digestion.

The seeds that do not escape digestion but are still dispersed by endozoochory must be very strong (such as *I. pseudacorus* or *S. erectum*) and capable of resisting digestion for the durations that they are typically retained in living birds. These large, strong, sometimes more elongated seeds will have difficulty to pass the size-selectivity of the pylorus valve (Ferrando et al. 1987). If they remain intact for too long in the gizzard, such seeds are more likely to be expelled via regurgitation in pellets (Kleyheeg and van Leeuwen 2015, Green et al. 2019). Combined, these observations predict endozoochory via escape of the digestive process to be the key long-distance dispersal (LDD) mechanism for small seeds that are impermeable to water (the ‘escape mechanism’), and

regurgitation to be the key long-distance dispersal mechanism for (very) large and strong seeds (the ‘resistance and regurgitation mechanism’).

### The potential for co-evolution

We identified multiple seed traits that are important for endozoochory by waterbirds. However, the capacity of each trait to predict seed survival by itself remained low. Rather, seed survival of waterbird digestive processes depends on a suite of traits, which contrasts to dispersal of fleshy fruits by frugivorous birds: in these plant–disperser interactions, fleshy pulp is the key trait that has specifically evolved multiple times to reward animals for their dispersal function (Eriksson 2016). All traits we identified as relevant for dispersal by the largely granivorous or omnivorous waterbirds are likely exaptations rather than adaptations: they are not exerting strong enough directional selective pressures because they are also highly important for other life history parameters of plants. The evolution of seed size, hardness and imbibition is constrained by many selection pressures of equal, similar or higher importance than dispersal by birds (Westoby et al. 1992, Leishman et al. 2000). Furthermore, we identified multiple mechanisms that enhance endozoochory by waterbirds, which require contradictory traits (the escape mechanism for small seeds, the resistance and regurgitation mechanism for large seeds). Hence, it is difficult to define one specific seed morphology as ‘the dispersal syndrome for endozoochory’ (Costea et al. 2019, Green et al. 2021).

Yet, our findings indicated there is potential for co-evolution. We hypothesized that signals of co-evolution would be strongest in plant species more typical of aquatic habitat types, for which waterbirds are effective seed dispersers (Kleyheeg et al. 2017). We therefore expected seeds from plant species of wetter habitats to be better capable of resisting digestive processes and germinate better after gut passage, possibly related to the seed traits identified as important for endozoochory. Although seeds from different habitats survived equally well and none of the seed traits correlated to Ellenberg F-values, germination of more aquatic plants was significantly more stimulated (higher germinability and faster germinating) by gut passage than germination of more terrestrial plants. This indicates that gut passage leads to stronger breaking of seed dormancy (Baskin and Baskin 1998) for plants more typical of aquatic habitats, which could be a co-evolutionary response in line with hypothesis 3: seeds

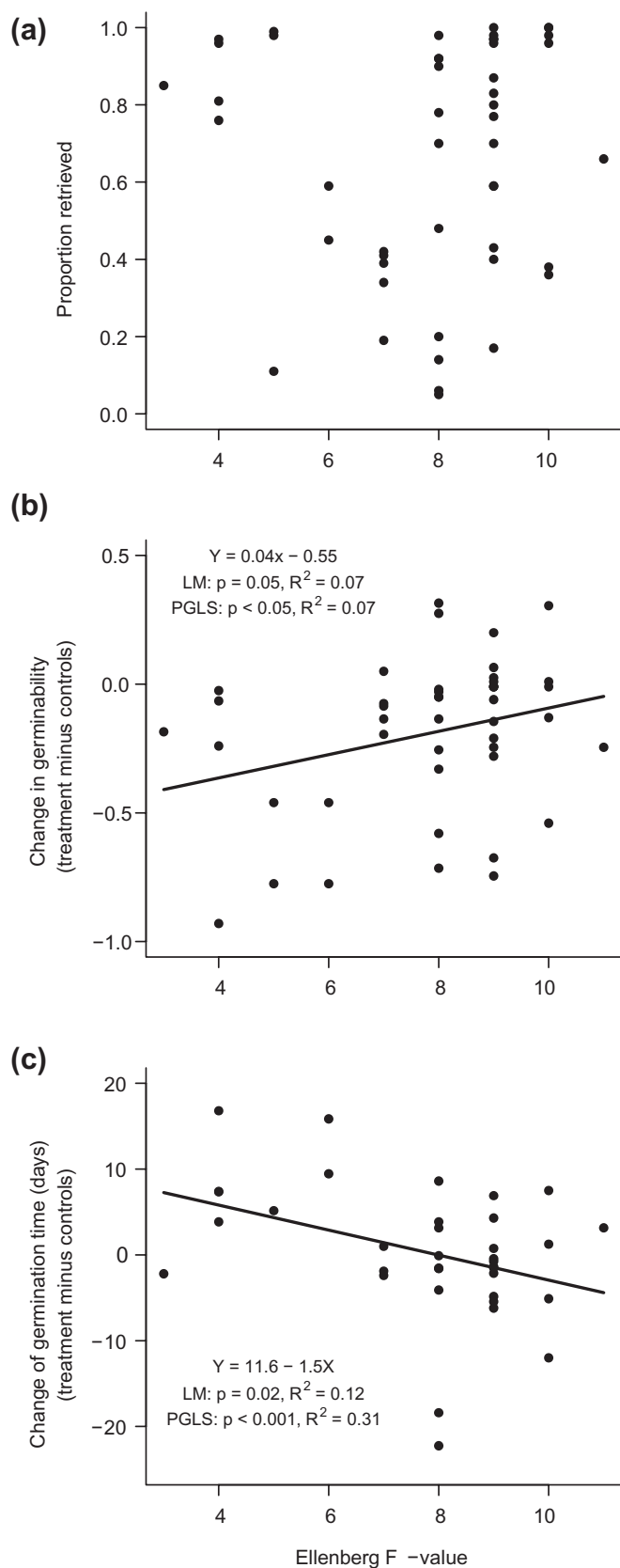


Figure 5. Effects of the treatments on mean seed survival, germinability and germination time in relation to Ellenberg F-values of the

of more aquatic plants could remain dormant while ‘waiting for’ waterbird dispersal, with dormancy breaking during gut passage so that dispersed seeds germinate. This provides new perspectives on the observations that gut passage is generally only considered to break dormancy of water-impermeable seeds with physical dormancy (Soltani 2018), and suggests it can also be important for dormancy-breaking of water-permeable seeds of more aquatic plants with physiological dormancy such as included in this study.

## Outlook

We have shown how simulations of seed gut passage using a bioassay can significantly advance our understanding of endozoochory by studying the survival and germination of seeds under controlled conditions. Such simulations provide standardized alternatives to animal experiments which overcome the limitations typical for such experiments, and could be applied to any plant species. This methodology provides an excellent opportunity to further study the interactions between gut passage, seed traits and factors driving seed dormancy and germination in plants, which can help to compile data for large numbers of plant species to advance existing plant databases (Hintze et al. 2013, Green et al. 2021). If estimations on the endozoochory potential of a wide variety of plant species become available in databases this could subsequently be linked to species distributions by means of geographical modelling. Among testable hypotheses are for instance whether plant species with the best surviving seeds are most common along migratory flyways or most widespread throughout suitable climatic regions (for an example of such an approach see Brochet et al. 2009).

Future extensions of the bioassay for more realism can include the addition of the rapidly developing field of microbial digestion (Grond et al. 2018), adjusting the protocol to include bird species-specific variation in digestive physiology, or testing the impact of the type of food ingested along with seeds (van Leeuwen et al. 2012c). Recalibration

of 48 plant species. (a) Seed survival did not differ between plants typical for dry habitats (low Ellenberg F-value) or typical for wetter habitats (higher Ellenberg F-value). (b) Germinability relative to control seeds (more positive values indicate higher germinability than controls) in relation to Ellenberg F-values, showing a more positive effect on germinability for plant species with a higher Ellenberg F-value. (c) Change in germination time relative to control seeds (more negative values indicate faster germination due to treatments) in relation to Ellenberg F-values, showing a negative effect of Ellenberg F-value. Note: The main effects of Ellenberg F-value on germinability and germination time of control seeds and digested seed are shown in the Supporting information. Species-specific information can be found in Table 1, Supporting information, and statistical details in the Supporting information. Ellenberg F-value showed a strong phylogenetic signal, of which details can be found in the Supporting information. Ellenberg F-value did not correlate to any of the seeds traits (Supporting information).

of the methods for other bird orders than Anseriformes – such as Charadriiformes, Suliformes or Pelecaniformes – would be a valuable next step. Many species in these orders are difficult to use in feeding experiments, yet sufficient knowledge on their digestive physiology is available for calibrating simulations. This would facilitate comparative studies among the most important seed dispersing avian orders, which is an important next step in dispersal ecology (van Leeuwen et al. 2022b).

Generating more detailed data on endozoochory by herbivorous, omnivorous and/or granivorous animals is important for our understanding of how animal species shape plant species distributions (Viana et al. 2012, Viana 2017, Kleyheeg et al. 2019). This is currently a topic of extreme relevance, as anthropogenic pressures resulting in defaunation are already impacting plant dispersal capacities (Fricke et al. 2022). It is important to know when and where this defaunation is causing loss of unique plant–animal interactions, in which systems waterbirds of different orders may be complementary in their dispersal services to plants, and which plant species may be least affected because they also disperse their seeds via other mechanisms (other animals, anemochory, hydrochory). Increasing our knowledge on plant dispersal capacities can importantly identify the most vulnerable species or those non-native species with the greatest potential to become invasive.

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## Author contributions

**Casper H. A. van Leeuwen:** Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (lead); Investigation (lead); Methodology (lead); Writing – original draft (lead); Writing – review and editing (lead). **Merel B. Soons:** Investigation (supporting); Methodology (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Laura G. V. T. I. Vandionant:** Investigation (equal); Methodology (supporting); Writing – review and editing (supporting). **Andy J. Green:** Formal analysis (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **E. S. Bakker:** Funding acquisition (supporting); Methodology (supporting); Project administration (supporting); Writing – original draft (supporting); Writing – review and editing (supporting).

## Transparent peer review

The peer review history for this article is available at <https://publons.com/publon/10.1111/ecog.06470>.

## Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.41ns1rnj2> (van Leeuwen et al. 2022a).

## Supporting information

The Supporting information associated with this article is available with the online version.

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