Field trial of a probiotic bacteria and a chemical, chitosan, to protect bats from white-nose syndrome

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Abstract

Tools for reducing wildlife disease impacts are needed to conserve biodiversity. White-nose syndrome (WNS), caused by the fungus *Pseudogymnoascus destructans*, has caused widespread declines in North American bat populations and threatens several species with extinction. Few tools exist for managers to reduce WNS impacts. We tested the efficacy of two treatments, a probiotic bacterium, *Pseudomonas fluorescens*, and a chemical, chitosan, to reduce impacts of WNS in two simultaneous experiments conducted with caged and free-flying *Myotis lucifugus* bats at a mine in Wisconsin, USA. In the free-flying experiment, treatment with *P. fluorescens* increased apparent overwinter survival five-fold compared to the control group (from 8.4% to 46.2%) by delaying emergence of bats from the site by 30 days. Apparent overwinter survival for free-flying chitosan-treated bats was 18.0%, which did not differ significantly from control bats. In the cage experiment, chitosan-treated bats had significantly higher survival until release on March 8 (53%) than control and *P. fluorescens*-treated bats (both 27%). However, these differences were likely due to within-cage disturbance and not reduced WNS impacts, because chitosan-treated bats actually had significantly higher UV-fluorescence (a measure of disease severity), and body mass, not infection intensity, predicted mortality. Further, few of the bats released from the cage experiment were detected emerging from the mine, indicating that the survival estimates at the time of release did not carryover to overwinter survival. These results suggest that treatment of bats may reduce WNS mortality, but additional measures are needed to prevent declines.

Introduction

White-nose syndrome (WNS), caused by the fungal pathogen, *Pseudogymnoascus destructans*, has caused widespread declines in bat populations throughout eastern and midwestern North America and threatens several species with extinction [1-3]. Three species (*Myotis lucifugus*, *Myotis sodalis*, and *Perimyotis subflavus*) have declined by 70-90% across multiple states, and a fourth species, *Myotis septentrionalis*, has been extirpated from most sites within three years of WNS detection [2-4], in part, due to highly connected bat communities [5]. Although a few populations of *M. lucifugus* appear to be persisting at 10-25% of pre-WNS colony sizes, most colonies of this species have declined by >90% [4,6]. Several previously common species of hibernating bats are now relatively rare across large regions of the northeast USA [2,4,7]. Management interventions to reduce the impact of WNS on bat populations are needed to prevent further declines and restore bat populations.

Over the past seven years, several treatments for WNS have been explored and are in various stages of development, but none have been successfully tested in the field. Potential treatments to enable bats to survive hibernation have included volatile compounds released by bacteria, vaccination, chemical anti-fungals, and probiotic microbes (Table S1). The outcome of most lab and field trial studies is unclear, and there are currently no published reports of effective treatments from field trials (Table S1). Thus, at present, there are few tools for managers to reduce the impacts of WNS, and developing control options to reduce the severity of this disease among bats is a high priority [8,9].

Our goal was to determine the efficacy of two treatments, *Pseudomonas fluorescens* and chitosan, in reducing WNS mortality in a field setting. *Pseudomonas fluorescens* is a ubiquitous bacterial species complex that is used as a fungal biocontrol agent in agriculture, and has been

tested as a treatment for chytridiomycosis in amphibians [10-12]. A previous study on multiple isolates of *P. fluorescens* isolated from different species of bats showed a range of anti-P. *destructans* properties *in vitro* [13]. One strain, isolated from a hibernating *Eptesicus fuscus* in Virginia, reduced the number of lesions, and increased survival of little brown bats when applied at the time of infection in a laboratory *in vivo* trial. Chitosan is a biopolymer polysaccharide extract from crustacean shells, has powerful antimicrobial and wound-healing properties, is biodegradable and non-toxic, and is a widely used anti-fungal in agriculture [14]. Chitosan has shown promise in inhibiting growth of *P. destructans in vitro* and in reducing mortality in *in vivo* lab experiments [15].

We performed a field trial with two simultaneous experiments to balance the strengths and weaknesses of each approach. In the free-flying experiment, we treated bats, and attached an integrated passive transponder (PIT) tag to determine the date they emerged from the site. This experiment allowed bats to behave normally and roost freely throughout the site. However, there was additional uncertainty in determining the survival of free flying bats (e.g., bats could move to another site midwinter, die at the site and be eaten by predators or escape from the site by an unknown exit and not be detected by the PIT tag receiver). To balance these unknowns, we also performed an experiment with bats in cages. Placing bats in cages, as has been done in most *in vivo* experiments to date [1,16-18], prevents bats from leaving the site and provides certainty about the survival of each bat. However, caging bats may alter their behavior (e.g. bats in the same cage may be disturbed when other bats arouse from hibernation).

Results

On the day of treatment, *P. destructans* infection prevalence, fungal loads and weights of bats were very similar among treatment groups in both experiments (Figure 1). This suggests that the randomization of bats to treatment groups and experiments did not result in any initial differences. Infection prevalence and fungal loads in November were very similar to loads observed on *M. lucifugus* at other sites where the fungus has been present for at least one previous winter [19,20].

Free-flying experiment

Of the 44 bats we treated, only one bat (from the control group) appeared to have left the site due to the disturbance of being handled/treated (it was detected by the PIT tag reader on the day of treatment November 20, 2015 and never again). We detected 17 of the remaining 43 bats on the PIT tag reader between December 9, 2015 and April 17, 2016, with 6 of 7 (*P. fluorescens*), one of six (control), and two of four (chitosan) bats having left the site on or after the assumed overwinter survival date of March 7, 2016 (Figure 2). We found three additional bat carcasses inside the site (two control and one chitosan-treated bats). The fraction of bats known to be alive and detected by the PIT tag reader after March 7th (apparent overwinter survival) was 46.2% (6/13) for *P. fluorescens*-treated bats, which was significantly higher than 8.5% (1/12) for control bats (Figure 3; the remaining 7 bats had lost their PIT tag; see Methods). Apparent overwinter survival was 18.0% (2/11) for chitosan-treated bats which was not significantly different from control bats (Figure 3).

The last date a bat was detected on the PIT tag reader was significantly later for *P. fluorescens*-treated bats than control bats, and overall, was earlier for bats with higher fungal

loads in November (Figure 4). The last detection dates for chitosan-treated bats were not significantly different than untreated controls (Figure 4). We did not compare differences in fungal loads or UV-fluorescence among treatment groups in March for bats in the free-flying experiment because only three bats were found and recaptured when we visited the site on March 8. The remaining bats were likely in difficult-to-access portions of the mine.

Cage experiment

On March 8th, 2016 four of 15 (26%) bats in the *P. fluorescens* cage, eight of 15 (53%) in the chitosan group, and four of 15 (26%) bats in the control group were still alive; the others were dead. The difference between chitosan and control groups in the fraction surviving until this date was not quite significant (Figure 5; logistic regression control vs. chitosan: coef = 1.145 ± 0.78, z = 1.47, one-tailed P-value = 0.07). However, when accounting for November body mass (which didn't differ between treatment groups, but was a significant predictor of survival; Figure 6c), the difference between chitosan and control groups was significant (logistic regression (reference group: control): Intercept: 20.1±7.0; Body mass: 1.22±0.44; P = 0.0054; chitosan coeff. 1.72±0.90; one-tailed P = 0.029; *P. fluorescence* coeff. -0.40±0.97; P = 0.68). Unlike in the free-flying experiment, *P. destructans* fungal loads in November were not a significant predictor of survival (likelihood ratio test: P = 0.60). Most of the bats still alive in the cages were in very poor condition, and only three of the sixteen bats that survived to be released were subsequently detected by the PIT tag reader (two chitosan and one control bat) (Fig 5).

Secondary measures of disease severity from the cage experiment showed non-significant differences or patterns that contradicted the survival results. Fungal loads on bats in March were not significantly different among treatment groups (Figure 6a) and disease severity, as measured

by UV-fluorescence, was significantly higher for chitosan-treated bats than control bats (Figure 6b).

Discussion

White-nose syndrome has caused widespread declines in multiple species of bats throughout eastern and midwestern North America, with declines in *M. lucifugus* colonies in the first year of WNS detection averaging 79% [2,3]. Survival of untreated bats in the free-flying experiment was similarly severe, with 91% (95% CI: 62-99%) of control bats likely dying over the winter. Treatment with the probiotic, *P. fluorescens*, increased apparent overwinter survival more than five-fold by extending the last date of detection by a month into early spring. Although this effect is substantial, over half of *P. fluorescens*-treated bats still likely died from WNS over the winter. In contrast, support for a protective effect of treatment with chitosan in reducing WNS mortality was mixed. Chitosan treatment increased survival in the cage experiment until March 8th, but few of these bats were subsequently detected by the PIT tag reader emerging onto the landscape, and disease severity, as measured by UV fluorescence, was significantly higher in chitosan-treated bats.

If treatment efficacy with *P. fluorescens* could be improved, *P. fluorescens* could provide a useful tool for conserving populations of *M. lucifugus* declining from WNS. One potentially important factor for future efforts with *P. fluorescens*, based on work in other systems, is whether the bacterial treatment persists or proliferates on the host species [21]. Increasing persistence and growth of *P. fluorescens* on bats by altering the dosage, or treatment frequency, or adding components to *P. fluorescens* solutions to encourage the formation of biofilms could help increase treatment efficacy [21], assuming these alterations wouldn't have more deleterious

158 side effects. Previous *in vitro* studies suggested that many different strains of *P. fluorescens* have
159 anti-*P. destructans* effects [13]. Future studies could examine alternate strains of *P. fluorescens*
160 isolated from different populations (e.g., persisting populations of *M. lucifugus*; [6]) or other
161 species of bats (the strain in this study was isolated from *E. fuscus*; [13]).

162 The effect of *P. fluorescens* or chitosan in reducing WNS impacts on other species also
163 has yet to be tested. The most important species to protect from WNS is *M. septentrionalis*,
164 which suffers nearly 100% mortality, and is on a pathway to extinction [2]. To date, no treatments
165 have been developed for, or tested on, this species in either the lab or field. This is despite *M.*
166 *septentrionalis* being the most heavily affected by WNS, with few hibernacula still containing
167 this species in the US [2,4,22].

168 In addition, our results offer potential insights for the experimental design of future field
169 treatment trials aimed at reducing WNS mortality in hibernating bats. Researchers often have to
170 choose between cage-artifacts and concerns about bats leaving the site following treatment and
171 uncertainty in the survival outcome for some free-flying bats. Our data suggest that the free-
172 flying experiment was a better experimental design, despite some challenges. Bats in this
173 experiment were able to roost and behave normally, and only one of 44 bats left the site on the
174 day of the experiment, suggesting that disturbance of handling and treatment are unlikely to
175 compromise experiments if treatment can be done quickly (treatment, weighing and banding
176 required ~1 hr. underground in this study). In addition, lower November fungal loads prolonged
177 apparent survival, as would be expected if bats were dying from WNS [22]. The main challenge of
178 the free-flying experiment was uncertainty associated with the fate of animals that were never
179 detected by the PIT tag reader but not found dead within the site. However, as noted above, the
180 extent of mortality in control bats inferred in the free-flying experiment was very similar to the

WNS declines observed in populations of *M. lucifugus* sites, supporting our assumption that most bats that were not detected by the PIT tag reader after March 7th did not survive the winter. One final challenge with mixed treatment free-flying experiments is that mixing of treatment groups (e.g., probiotic bacteria being transferred from treated to control bats) might occur through direct social interactions or indirect contact via the environment. The significant differences we observed between survival of bats in the *P. fluorescens* and control treatment groups suggest that direct or indirect contact was insufficient to transfer significant amounts of probiotic bacteria among bats.

The cage experiment suffered from several shortcomings that, in hindsight, indicate this was a problematic design. In our experiment each cage contained all the bats in each treatment, due to a limited availability of space for mounting cages to natural substrate in a predator-protected room, and to allow social bats to roost in groups. This resulted in pseudo-replication in this experiment, as in most previous laboratory studies on WNS [1,16-18]. This is particularly problematic for studies of WNS, because in small cages bats appear to disturb other bats when they arouse from hibernation [23], and increased arousal frequency is thought to be a key mechanism of WNS mortality [1,24,25]. The fact that survival in the cage experiment was correlated with initial body mass, but not fungal loads, suggests that disturbance from other bats, or an inability to move to other locations within hibernacula, was more important than WNS in determining survival in this experiment. Together, these results indicate that the ideal design for a field trial (and for WNS challenge experiments more generally) is a free-flying experiment with mixed treatment groups in each site where bats have to pass through a PIT tag antenna to leave the site or are prevented from leaving the site (e.g. by sealing entrances, which may be very difficult). Sites where dead bats are relatively easy to find and are not eaten by predators (e.g.

mice, rats, and raccoons) would reduce uncertainty in survival outcomes. If an experiment requires constraining bats within a site, one could use replicated cages (constructed of metal, as we used, to prevent mice from chewing into the cages) with a single bat in each cage to prevent cascading disturbances from infected bats, or groups of bats that are analyzed as individual data points. In addition, barriers to prevent larger predators (e.g. raccoons) from accessing the cages and eating the bats are an absolute necessity. Cages are not ideal in that they limit bats' movement within sites, but they offer higher certainty in terms of knowing the survival of each individual.

In conclusion, preventing population declines due to WNS in *M. lucifugus* and other species will likely require a combination of multiple approaches [8]. Potential strategies that could be combined with treatment include reducing the environmental reservoir of *P. destructans* [3,26], protecting and facilitating growth of populations of *M. lucifugus* that are now persisting with WNS (possibly due to resistance that limits fungal growth to moderate loads [6] or increased fat stores that allow bats to tolerate infection [27]), and improving summer and fall habitat for bats to increase reproduction and fat storage for hibernation. The latter two strategies would facilitate the evolution of resistance or tolerance which reduces the need for perpetual management action [28,29]. Finally, any strategy which slows or stops the very rapid local extirpations of *M. septentrionalis* colonies is urgently needed to prevent this species from extinction.

Methods

We performed the field trial on *M. lucifugus* bats in the winter of 2015-16 at an inactive mine in southwest Wisconsin where *P. destructans* was detected the previous winter 2014-15. The mine has one large (~3m tall by 5 m wide) entrance that was gated several years earlier, and

a single smaller entrance that was sealed with a fine mesh metal screen the year of the gating. We selected a site where *P. destructans* had been detected the previous year because lab trials with *P. fluorescens* had indicated that treating bats at the time of infection was more beneficial than treatment prior to infection [16], and previous work suggests that most bats become infected early in the second year following *P. destructans* invasion, likely due to build-up of an environmental reservoir [3]. This site had 226 *M. lucifugus* in November 2014, before *P. destructans* was detected, but the colony had declined to 82 bats by March 2015. The average temperature where *M. lucifugus* roosted was 7.0°C ± 0.4°C. We screened 55 samples for *P. fluorescens* by PCR from bats collected in the winter of 2014-15 to confirm that *P. fluorescens* naturally occurred on bats found in the site to address concerns regarding using a live bacterium as a treatment. We found DNA from *P. fluorescens* present in 20% of samples and from all four species sampled (Little brown myotis (*Myotis lucifugus*), Northern long-eared myotis (*Myotis septentrionalis*), Tri-colored bat (*Perimyotis subflavus*), and Big brown bat (*Eptesicus fuscus*)).

In September 2015 we installed a PIT-tag reader (IS1001 and HPR reader, Biomark Inc., Boise, ID) at the site entrance (Figure S1), and 3 metal screen cages (46x30x51 cm Fresh Air Screen Habitat, Zilla Products, Franklin, WI, USA) in a small chamber in the back of the mine where bats roosted in previous winters. We removed the top of each cage and mounted cages directly to the ceiling to allow bats direct access to mine substrate for roosting, and to allow for natural infection and reinfection. We also installed chicken wire with a hinged gate at the entrance of the cage room to prevent large predators (e.g. raccoons) from entering.

We briefly visited the site on Nov 16th (total time underground 14 minutes) to count the number of bats present and to assess the *P. destructans* infection status of the bats at the site. We counted approximately 95 *M. lucifugus* and sampled six of them by dipping a sterile polyester

swab in sterile water to moisten it and then rubbing the swab five times across both the forearm and muzzle of a bat [20]. We tested these samples for *P. destructans* DNA using qPCR [30], and all six samples tested positive.

We returned to the site for the experimental treatment on Nov 20, 2015. We sealed off the entrance to the site (Figure S1) using fine mesh cloth to prevent bats from leaving the site during the treatment. We collected all *M. lucifugus* we could find at the site (89 bats; 23 females and 66 males) and placed them individually in paper bags and brought them to a processing station near the entrance of the site. We weighed bats to the nearest 0.1 g with an electronic scale, but we did not take a length measurement (e.g. forearm) to minimize handling time and disturbance. Recent work has shown that body mass is equally accurate in predicting fat stores (as measured by quantitative magnetic resonance) as body condition indices [31]. We sampled bats for *P. destructans* as described above and banded each bat with an aluminum band (2.9mm; Porzana Ltd., Icklesham, E. Sussex, U.K.), that had a PIT tag attached (see Supplemental Methods text).

We randomly assigned bats to each of three treatment groups: control (29), chitosan (29), and *P. fluorescens* (31). We treated each bat by spraying ~2ml of a solution containing *P. fluorescens* or chitosan solution (see Supplemental Methods text) on their wings and tail with a spray bottle (FantaSea, Blaine, WA). For control bats we replicated the handling disturbance but did not spray any liquid onto bats because both of our treatments could only be applied in liquid form and the goal of our study was to determine the effect of treatment compared to untreated bats. We split bats in each treatment group into the two experiments based on a power analysis (Figure S2) – cage (15 for each treatment group in a single cage for each treatment; 5 females and 10 males per cage) and free-flying (16, 14, and 14 bats in the *P. fluorescens*, chitosan and control groups, respectively). After treatment bats were released into cages or into the site onto a

recovery cloth ~75m away from the processing station. We removed the mesh from the site entrance so bats could freely pass through the opening surrounded by the PIT tag antenna (Figure S1). We blocked off the rest of the entrance with screening to discourage bats from attempting to leave the site without passing by the PIT tag antenna. The total time underground was 65 minutes.

We returned to the site on March 8, 2016. We removed all the bats from the cages and captured all free-flying bats we could find (some portions of the site are inaccessible). Each bat was swabbed as described above, and one wing was photographed under ultra-violet (UV) light to measure an index of disease severity [32,33]. We then released all bats into the site. We downloaded data from the PIT tag reader on July 30, 2016 to determine the dates that bats with PIT tags were detected by the PIT tag reader. We note that detection by the PIT tag reader does not indicate the direction of travel when a bat is detected by the PIT tag reader (i.e. into or out of the mine). It only indicates that the bat was alive on that date and passed near (within ~15-20 cm) the PIT tag antennae.

When processing bats from the cage experiment, we noted that some (five of 16, or 31%) of the PIT tags had become detached from the bands on the bats. As a result, we subsequently searched the site when no bats were present with a handheld PIT tag reader to determine whether free-flying had also lost their PIT tags. We found seven PIT tags that were not attached to bands, suggesting that known PIT tag loss in the free-flying group (seven of 24, or 29.2% of the bats that were never recorded on the PIT tag reader) was similar to that in the cage experiment (31%). There may have been additional bats in the free-flying group that lost their PIT tags (making our survival estimates underestimates), but there is no evidence that PIT tag loss differed by treatment group (*P. fluorescens* 4/31, chitosan 5/29; control 4/29; Fisher's exact test $P = 0.93$).

We removed the bats who had lost their PIT tags (three *P. fluorescens*, one control, three chitosan) from analyses for the free-flying group since we could not detect them on the PIT tag reader. We added a new band with a PIT tag to any bat from the cage trial that had lost its PIT tag or band.

We determined the efficacy of the treatments by comparing apparent overwinter survival of bats in the three treatments, with and without accounting for differences in initial individual fungal loads and body mass. We assumed bats that were never detected by the PIT tag reader died in the site because our reader antennae provided full coverage of the entrance and had sufficient sensitivity to detect tags on flying bats. We assumed that any bats alive and detected by the PIT tag reader on or after March 7, 2016 had survived the winter (which we term "apparent overwinter survival"). We used March 7th as a cut-off for apparent overwinter survival because after March 7, 2016 surface temperatures near the mine were consistently above 2°C (Figure 1). Bats detected by the PIT tag reader prior to March 7th could have either emerged from the site and subsequently died or successfully emigrated to another hibernacula. Alternatively bats may have been detected alive flying at the mine entrance but remained in the site and subsequently died and never detected again. In the absence of data confirming any of these bats survived the winter, we assume they either died or permanently emigrated. In March, we searched all known sites within 50 km for banded bats and did not find any. However, emigration to unknown sites may have occured. We also compared the latest date a bat was detected by the PIT tag reader between treatments as a continuous response variable of the last known date alive, while controlling for fungal loads and mass. Finally, we examined differences among treatment groups in UV fluorescence among individuals surviving the cage experiment, as an indicator of disease severity. We hypothesized that bats treated with *P. fluorescens* or

chitosan would have higher survival, have a later last detection (by the PIT tag reader) date, and lower UV fluorescence than the control group and that apparent overwinter survival would increase with initial body mass and lower initial fungal loads in both experiments.

All research was performed as described under protocol Kilp1509 approved by the University of California, Santa Cruz's IACUC committee.

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Author Contributions: AMK, JRH, KEL, JPW designed the study. AMK, JRH, KEL, JPW, HMK, JAR performed field research. KLP tested samples under JTF's supervision. AMK, JRH, KEL performed analyses and wrote manuscript. All authors reviewed and provided comments on the manuscript.

Competing interests: The author(s) declare no competing interests.

Data availability: The datasets generated during the current study are available from the corresponding author on reasonable request.

Figure Legends

Figure 1. Bat body mass (A), *P. destructans* prevalence (B), and fungal loads (C) of *M. lucifugus* in November in three treatment groups (chitosan (CH), control (CO), and *P. fluorescens* (PF). There were 14-16 bats in each of the six treatment-experiment groups. There were no significant differences among treatment groups in fungal loads, prevalence or body mass in November (likelihood ratio-tests for treatment effect in linear models of mass, generalized linear models with a binomial distribution for prevalence, and linear models for log-transformed fungal loads: all P-values > 0.57).

Figure 2. External air temperature, emergence date and fungal loads of bats in the free-flying bat experiment. The top panel shows air temperature measured at the site entrance. In the lower panel, line color indicates the *P. destructans* fungal load (in nanograms) measured on bats in November. Lines end on the last date that each bat was detected by the PIT tag reader, and dotted vertical lines show the mean emergence date for each treatment group, indicated on the Y-

axis (Pf – *P. fluorescens*, Co – Control; Ch - Chitosan). The gray vertical bar shows the date after which emerging bats were assumed to have survived.

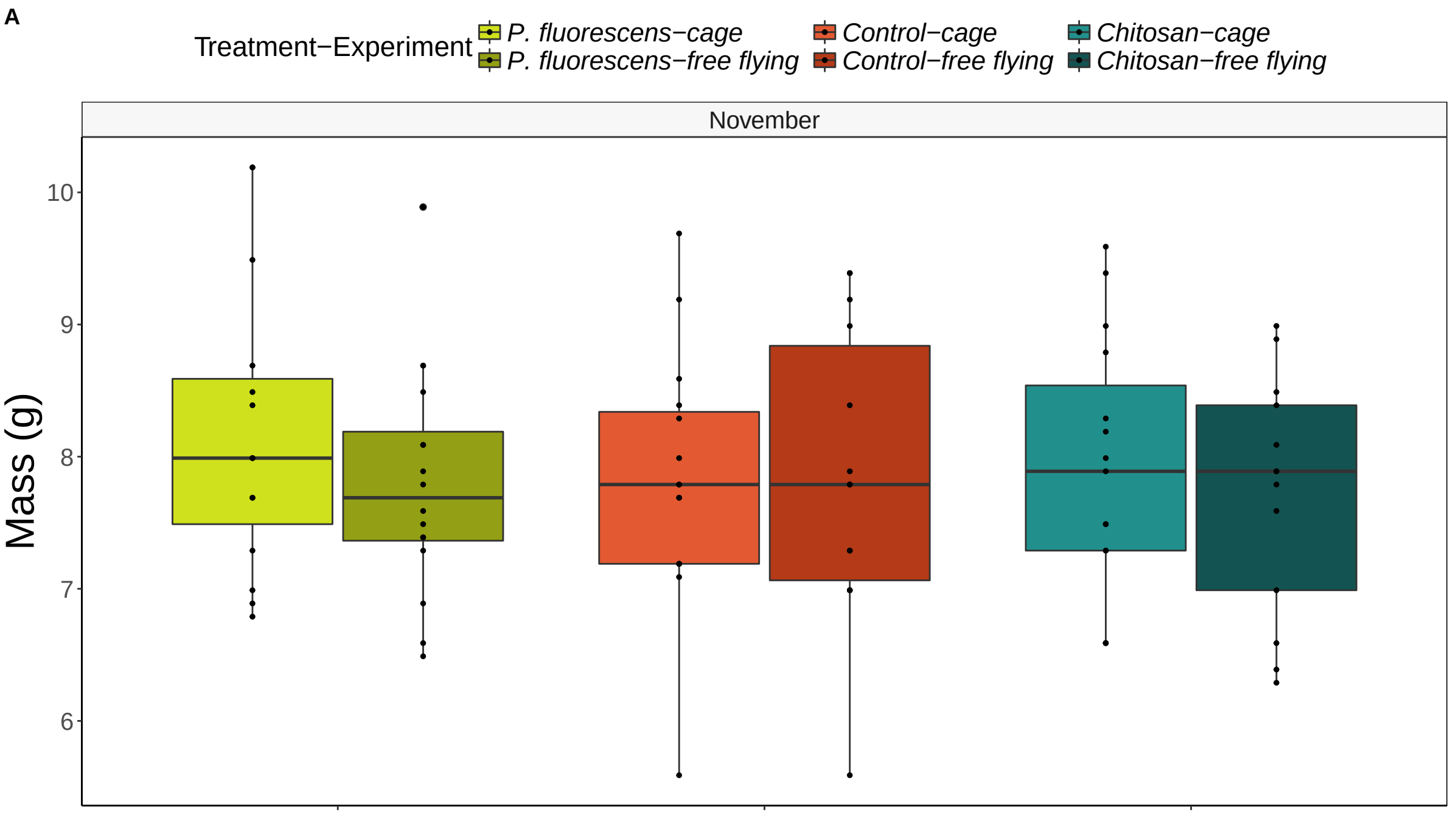
Figure 3. Apparent overwinter survival of bats in the free-flying experiment. Columns show the fraction of bats in each treatment group that were known alive and detected by the PIT tag reader on or after March 7 (with binomial 95% CI; sample sizes for each group, left to right, were 13, 12, 11; the remaining 7 bats had lost their PIT tag; see Methods). Differences among treatments were significantly different, (Fisher's exact test for all three treatments: two-tailed P = 0.012). *P. fluorescens* – treated bats had higher apparent survival than the control group (Fisher's exact test: one-tailed P = 0.046; logistic regression coef. = 2.24 ± 1.18, z = 1.896, one-tailed P-value = 0.029), but there were no significant differences between chitosan-treated and control bats (Fisher's exact test: one-tailed P = 0.47; logistic regression coef. = 0.89 ± 1.3, z = 0.69, one-tailed P-value = 0.49).

Figure 4: November *P. destructans* fungal loads and the late date of detection for individual bats in the three treatment groups (Pf – *P. fluorescens*, Co – Control; Ch - Chitosan). *P. fluorescens*-treated bats were last detected later than Control and Chitosan treated bats, and the last date of detection decreased (was earlier) with higher November *P. destructans* loads (*P. fluorescens* vs. control coef = 1.05 ± 0.57, t = -1.91, one-tailed P-value = 0.039, early winter fungal loads: coef = 0.61 ± 0.32, t = 1.84, one-tailed P-value = 0.040).

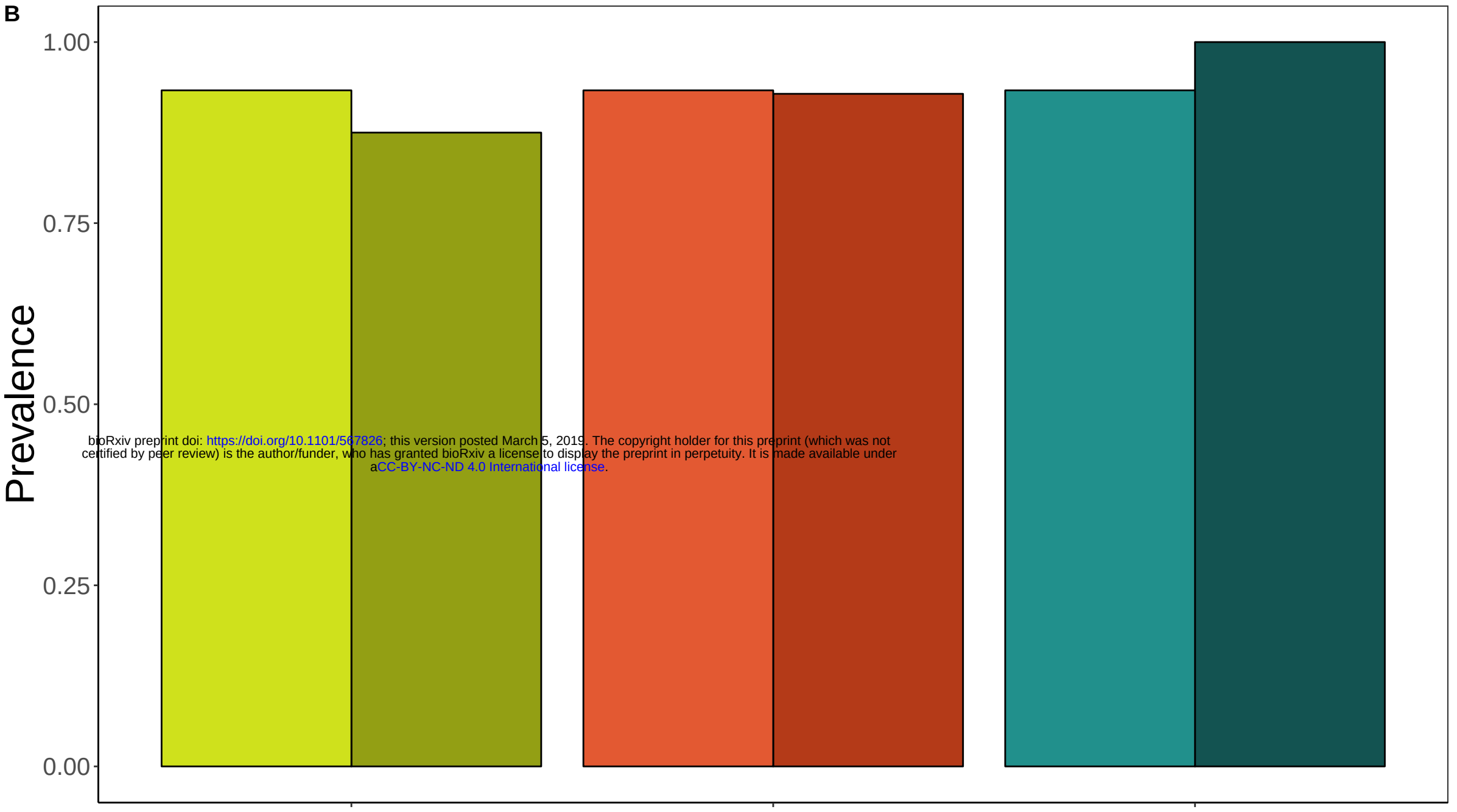
Figure 5. Survival, subsequent detection, and body mass of bats in the caged bat experiment. Each horizontal line represents a single bat, with line color indicating the body mass of that bat measured in November. Bats surviving until release March 8 (gray vertical bar) are shown by darker lines, and bats that were subsequently detected by the PIT tag reader are extended to the last date they were detected. Treatment is indicated by the first two letters of the bat identification number on the Y-axis (PF – *P. fluorescens*, CO – Control; CH - Chitosan).

Figure 6. *P. destructans* (a) fungal loads, and (b) disease severity (UV) for bats in the cage experiment that survived to March 8, and (c) differences in November mass between bats in the cage experiment that survived to March 8, or died. March fungal loads did not differ among treatment groups (likelihood ratio-tests for treatment effect in models of log-transformed fungal loads: P-value > 0.77). Disease severity (ultraviolet fluorescence) was significantly higher for chitosan-treated bats than control bats (regression on arc-sin sqrt transformed data: control vs. chitosan: coef = 0.15 ± 0.068, z = 2.273, P-value = 0.037; control vs. *P. fluorescens*: coef = 0.017 ± 0.073, z = 0.234, P-value = 0.82).

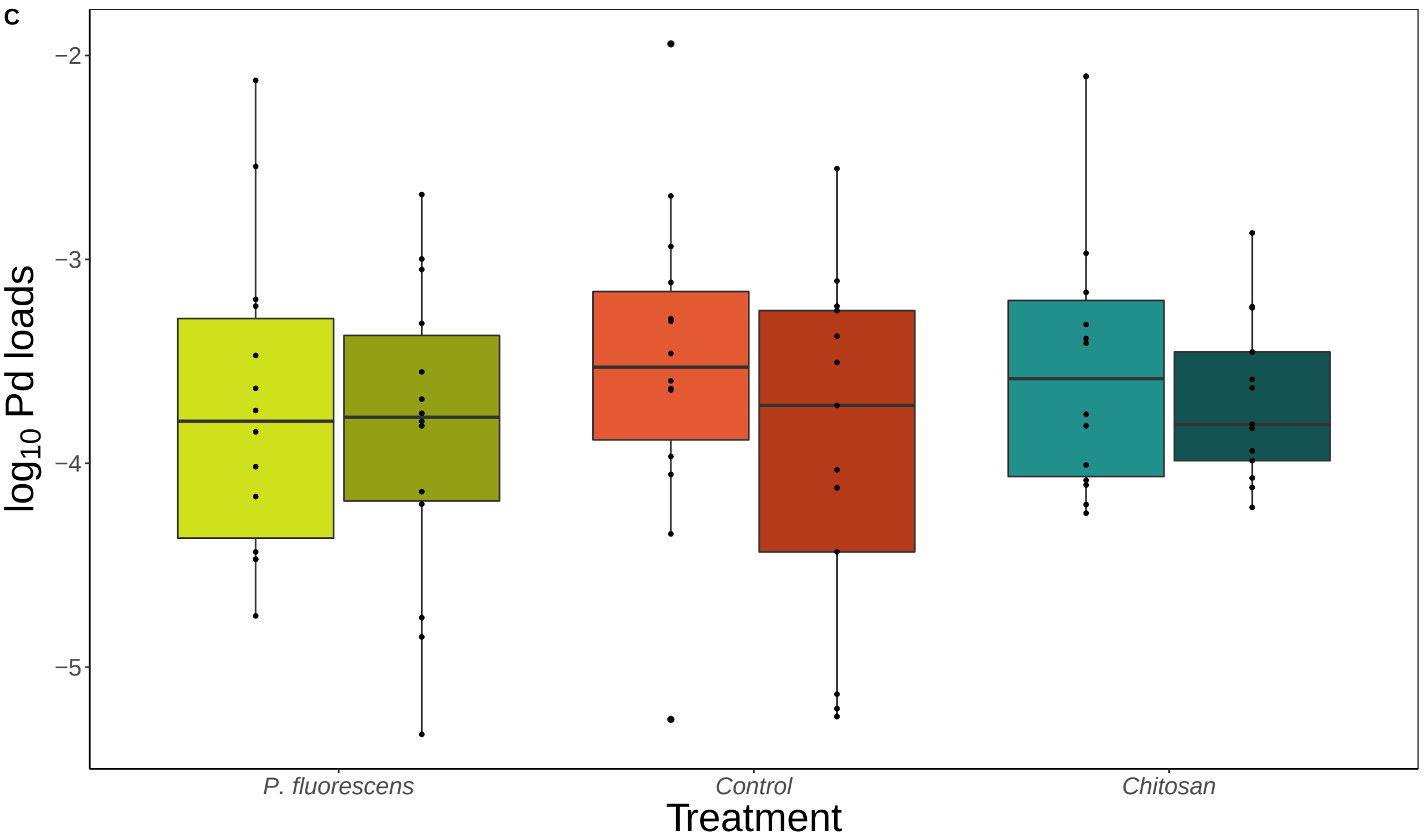
A

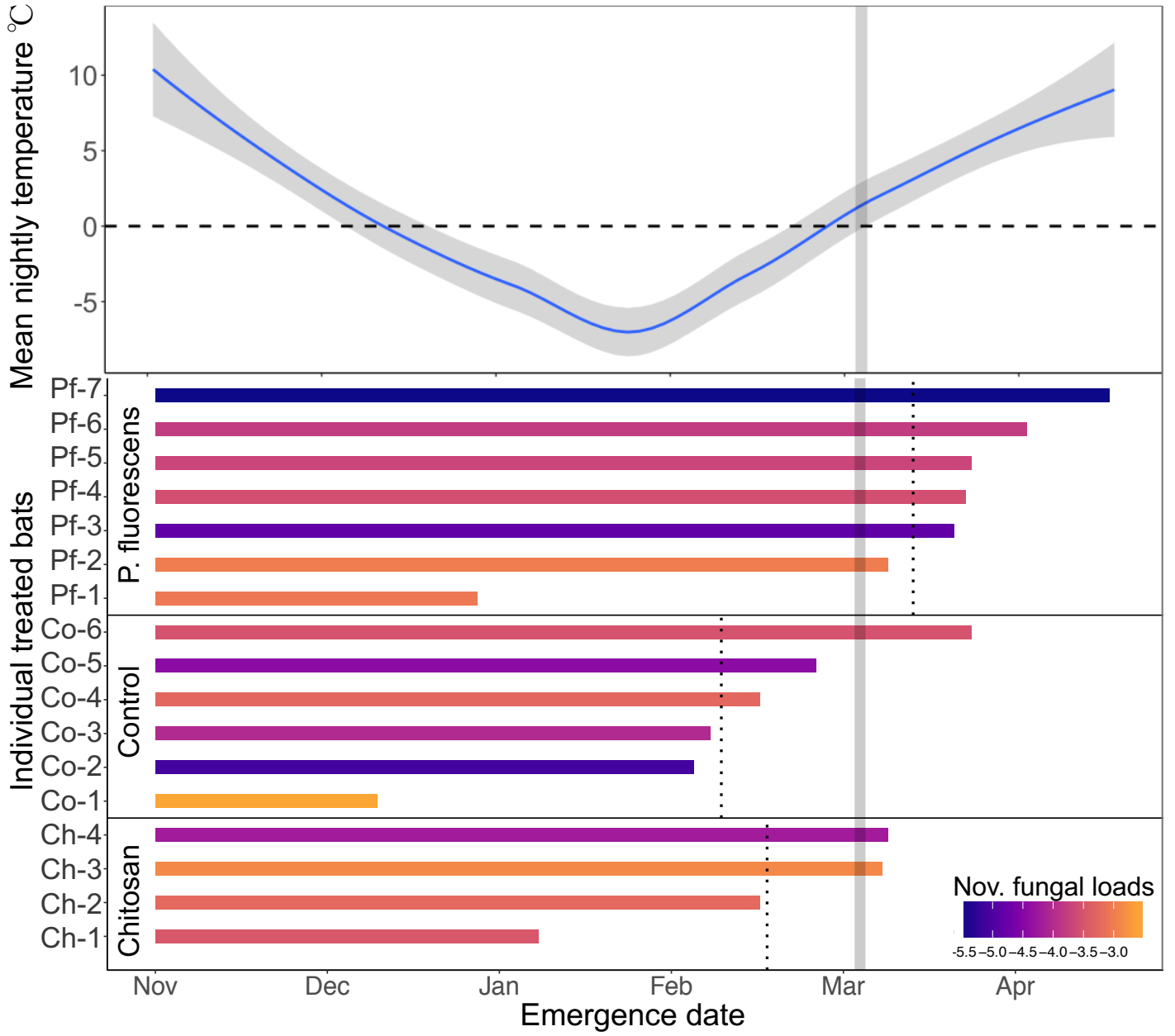


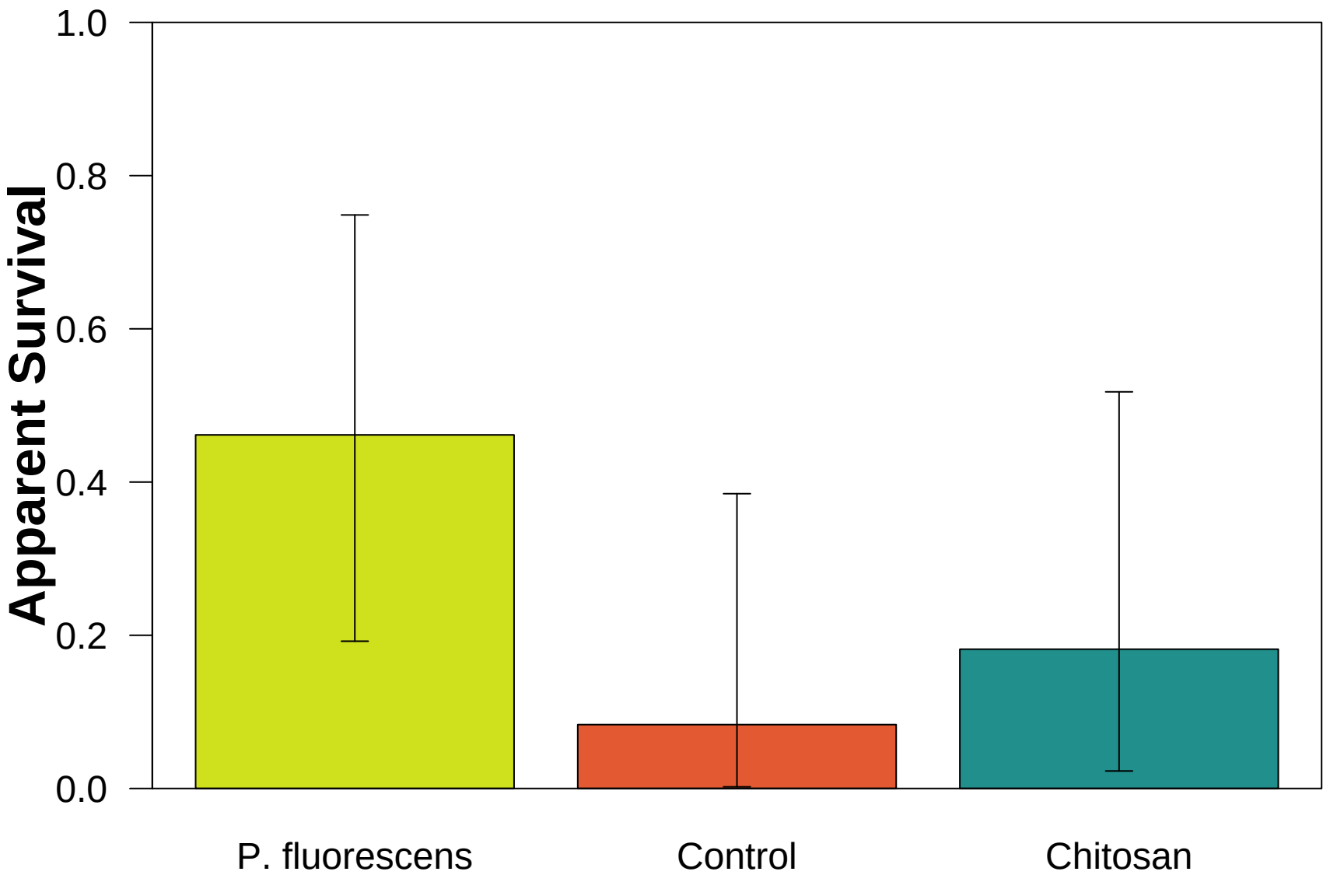
B

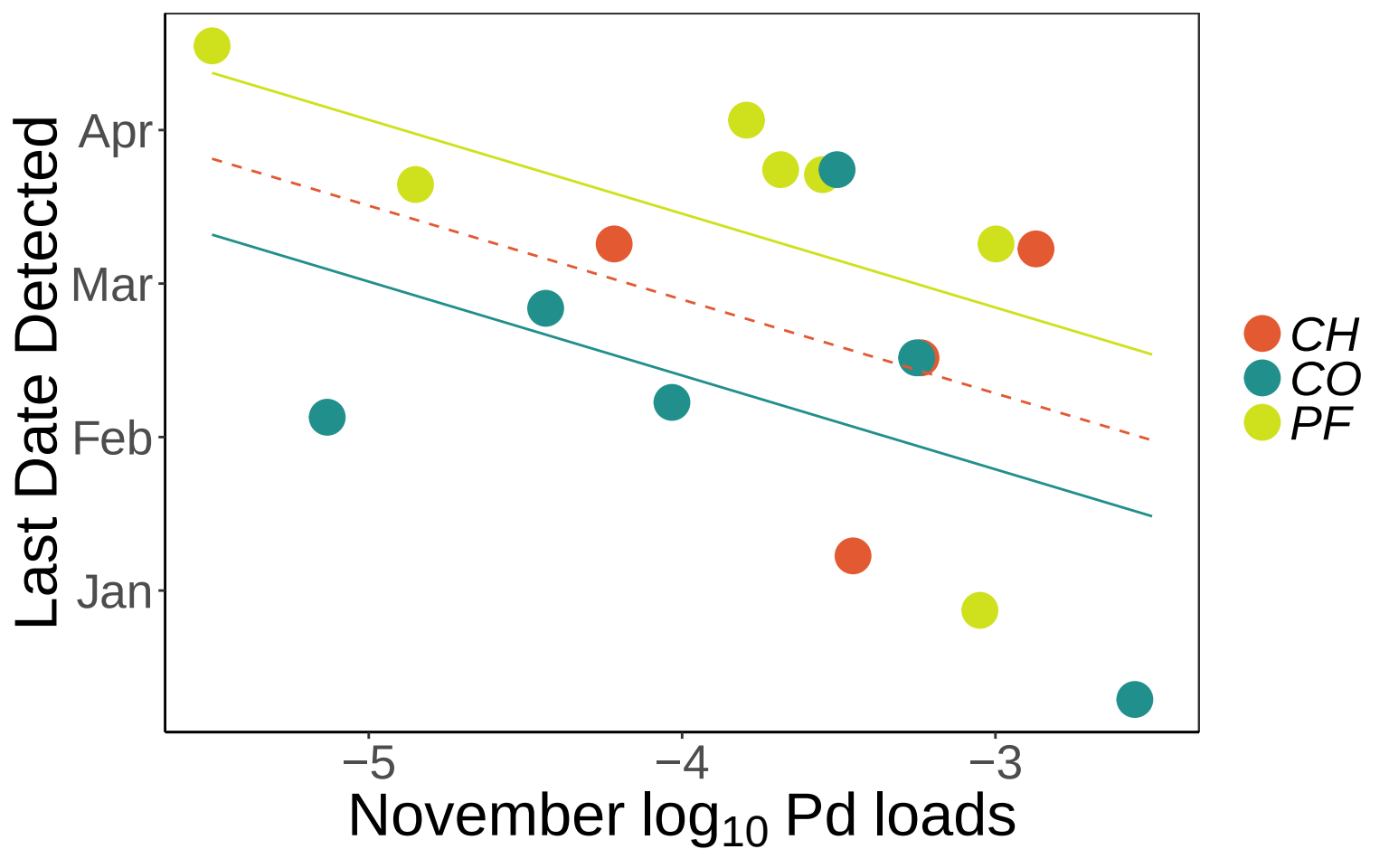


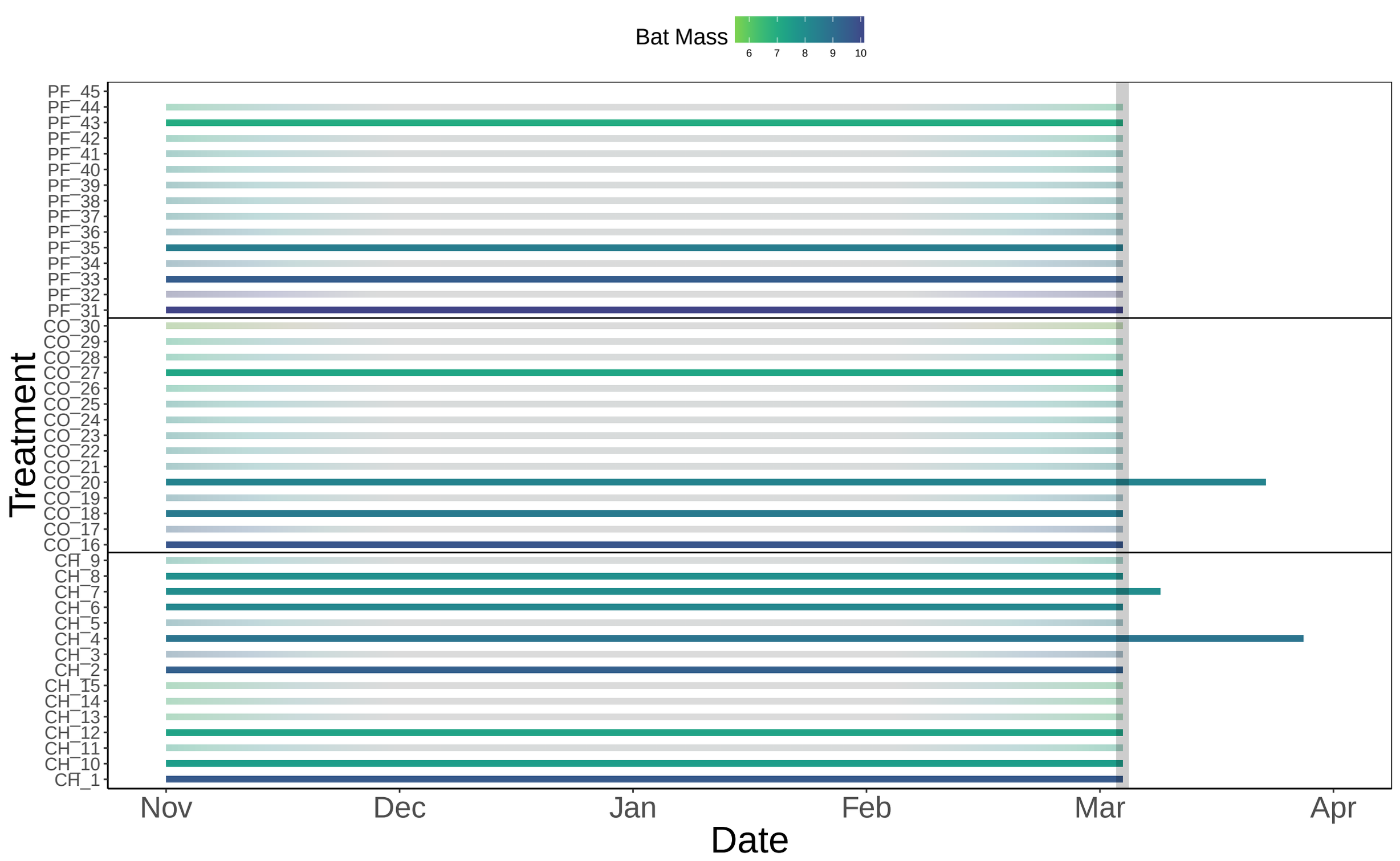
C

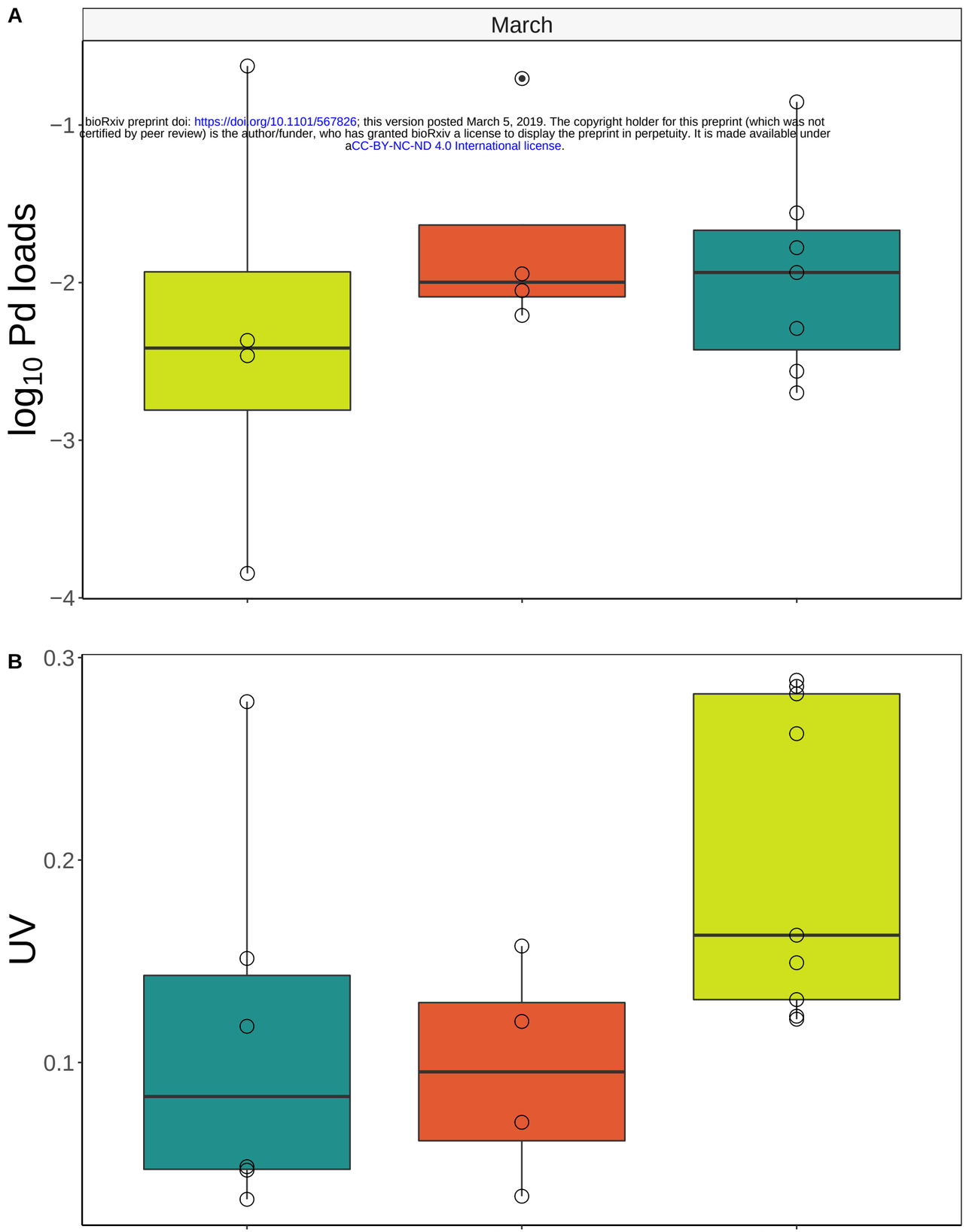


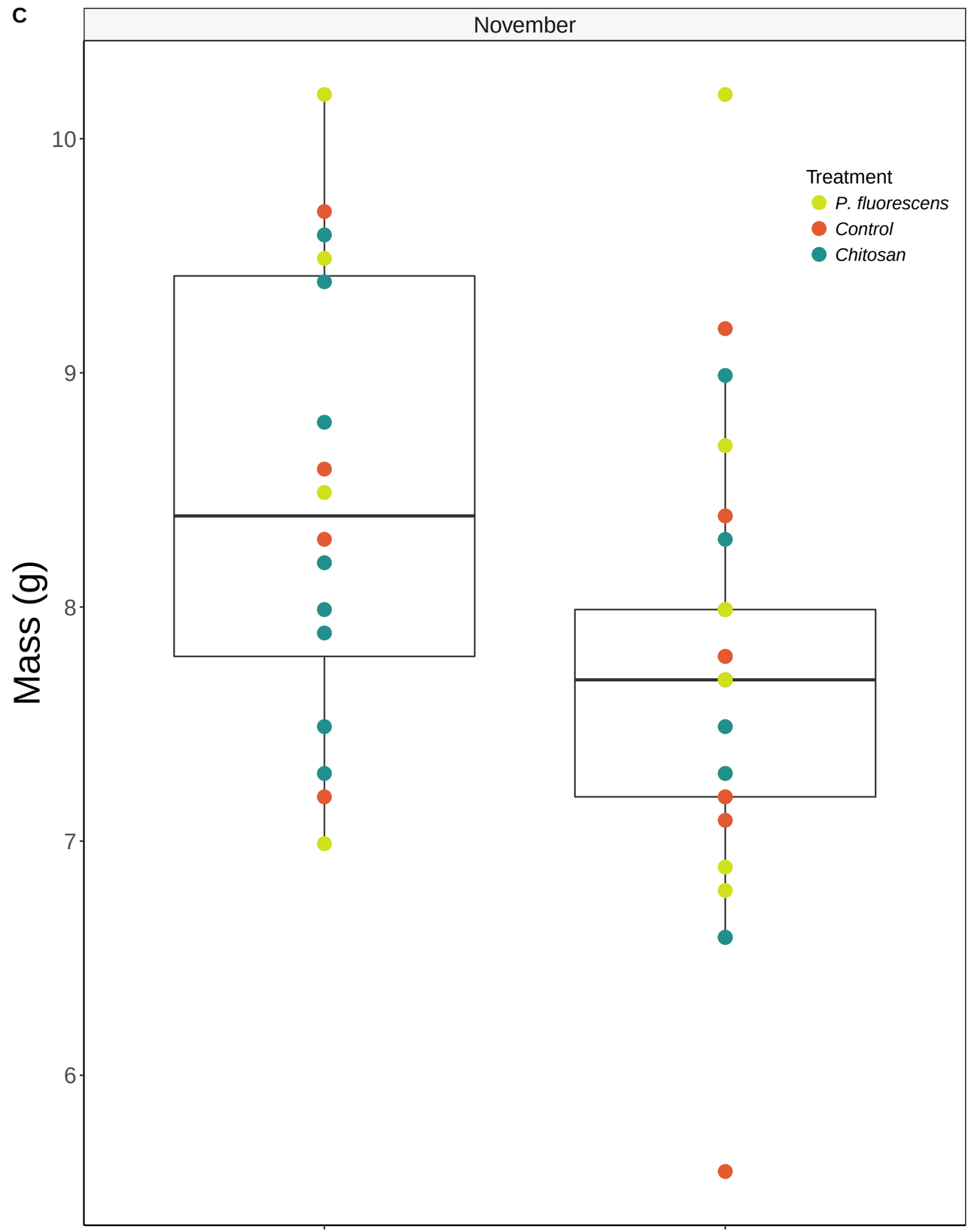

Mean nightly temperature ℃
10
5
0
-5
Individual treated bats
P. fluorescens
Pf-7
Pf-6
Pf-5
Pf-4
Pf-3
Pf-2
Pf-1
Control
Co-6
Co-5
Co-4
Co-3
Co-2
Co-1
Chitosan
Ch-4
Ch-3
Ch-2
Ch-1
Nov. fungal loads
-5.5 -5.0 -4.5 -4.0 -3.5 -3.0
Nov
Dec
Jan
Feb
Mar
Apr
Emergence date


Apparent Survival
1.0
0.8
0.6
0.4
0.2
0.0
P. fluorescens
Control
Chitosan


Last Date Detected
Apr
Mar
Feb
Jan
−5
−4
−3
November log10 Pd loads
CH
CO
PF


Bat Mass
6
7
8
9
10
PF_45
PF_44
PF_43
PF_42
PF_41
PF_40
PF_39
PF_38
PF_37
PF_36
PF_35
PF_34
PF_33
PF_32
PF_31
CO_30
CO_29
CO_28
CO_27
CO_26
CO_25
CO_24
CO_23
CO_22
CO_21
CO_20
CO_19
CO_18
CO_17
CO_16
CH_9
CH_8
CH_7
CH_6
CH_5
CH_4
CH_3
CH_2
CH_15
CH_14
CH_13
CH_12
CH_11
CH_10
CH_1
Treatment
Nov
Dec
Jan
Feb
Mar
Apr
Date





Field trial of a probiotic bacteria and a chemical, chitosan, to protect bats from white-nose syndrome

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Supplemental Tables and Figures

Table S1. Progress on white-nose syndrome treatments. Bolded references are from published papers and an asterisk (*) indicates a conference presentation. Full abstracts, titles and author affiliations for the conference presentations can be found at www.whitenosesyndrome.org/wns-symposia-workshops.

Treatment agent	***In vitro***	**Lab Trial**	**Field Trial**
Chitosan	[30]*	[14]*	This study
Polyethelene glycol (PEG)	[31]*	[32]*	[33]*
Propolis	**[34]**		
Pseudomonas fluorescens	**[12]**	**[15]**	This study
Rhodococcus rhodocrous DAP 96253	**[35]**	[36]*	[37]*
Trichoderma sp.	[38]*		
Turbinafine	**[39]**	**[40]**	
Vaccine		[41]*	[42]*
Valencia orange oil	**[43]**		

Figure S1. Photo of PIT tag antennae (black cable) installed at entrance of study site. The exit of the site is to the right. Shade cloth was used to prevent movement through the exit except between the boards with the attached PIT tag antenna.

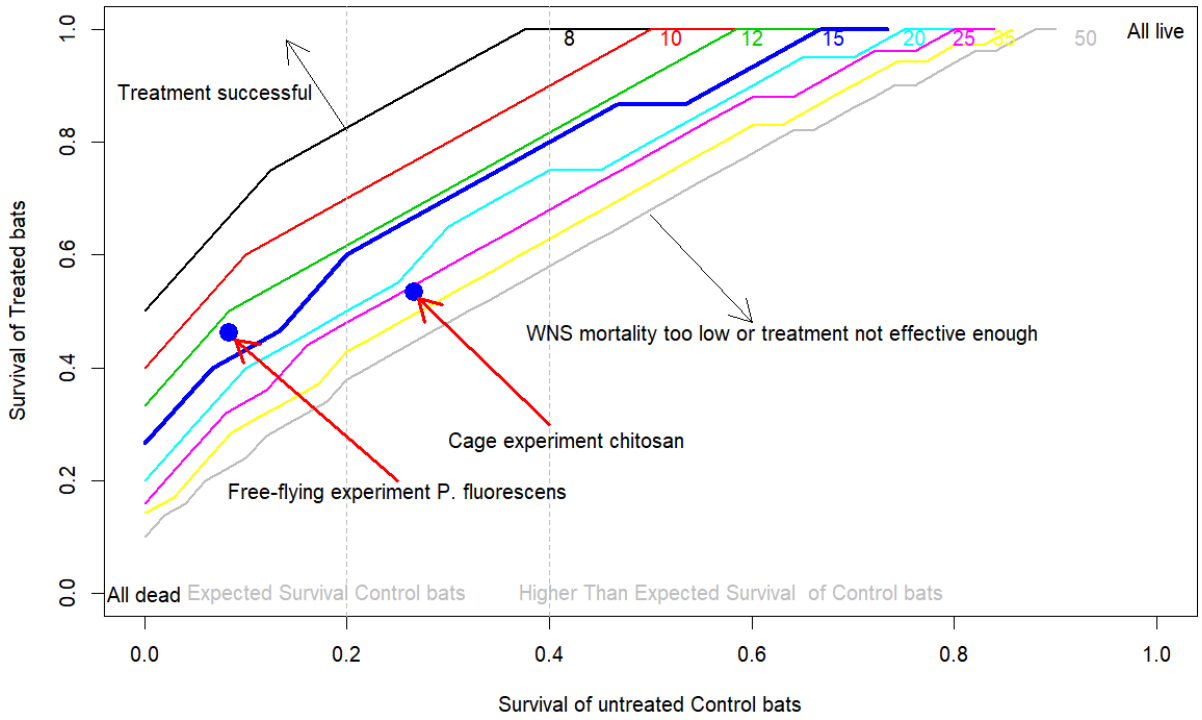

Figure S2. Power analysis used to design both experiments of the field trial. The x-axis shows survival of control (untreated bats), the y-axis shows survival of treated bats, and lines with different colors indicate sample sizes of bats in each treatment group that separate significantly different outcomes (above the line) from non-significant differences when results are analyzed with a fisher's exact test. The two blue circles show the outcomes of the two experiments (note that the sample size in the study was ~15 (14-16) – the bold blue line).

Supplemental Text

Methods

Measurement of fluorescence on bat wings under ultraviolet light

We took pictures of bats wings using a digital camera, approximately 15 cm above the wing under illumination with an UV light. We quantified the fraction of bat's wings (the area of the plagiopatagium proximal to the fifth digit, and below the radius) that fluoresced orange under ultra-violet light using Adobe photoshop, as the number of orange pixels divided by the total number of pixels in the photos of bats' wings.

PIT tag attachment to bands

We attached a PIT tag (12mm; Biomark Inc., Boise, ID) to the lip of each aluminum band using super glue (Loctite super glue gel control; Henkel corporation, Rock Hill, CT, USA). The lip of the band was abraded using 100 grit sand paper and the PIT tags were chemically etched using commercially available glass etching cream (Armour etch; Armour products, Hawthorne, NJ, USA) to provide maximum adhesion between the band and the PIT tag. We glued PIT tags to bands rather than gluing them directly to bat's backs to minimize disturbance and time underground (30-60 sec. per bat for glue to dry).

Preparation of treatment solutions

The two treatment solutions were prepared ahead of the visit. The *P. fluorescens* solution was prepared by plating bacteria from frozen stock on sabouraud dextrose agar (SDA). Colonies were allowed to grow for one day at room temperature then suspended in a 10X phosphate buffer (PBS) and glycerol solution, by flooding the plate. The solution was homogenized and serial

dilutions were performed using an aliquot of the prepared solution under the same culturing conditions and the remaining liquid was frozen at 20°C. After determining the concentration from the serial dilution plates using colony-forming units (CFU), the remaining frozen liquid was diluted to $1x10^8$ CFU's. The bacterial solution was shipped overnight on ice and was applied to bats the following day to minimize CFU loss. Chitosan was diluted 1:10 from stock using acetic acid and water.