incR: a free new R package to analyse incubation behaviour

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Abstract

Incubation represents a life stage of pivotal importance for the optimal development of avian embryos. For most birds, incubation poses a trade-off between investing in self-maintenance or offspring care. Furthermore, incubation is affected by environmental temperatures and, therefore, will be likely impacted by climate change. Despite its relevance and readily available temperature logging methods, avian incubation research is hindered by recognised limitations in available software. In this paper, a new quantitative approach to analyse incubation behaviour is presented. This new approach is embedded in a free R package, `incR`. The core algorithm in `incR` is validated and it is shown that the method extracts accurate metrics of incubation behaviour (e.g. number and duration of incubation bouts). The paper also presents a suggested workflow along with detailed R code to ease the practical implementation of the new method.

Introduction

Incubation represents a crucial life stage for egg-laying vertebrates, of which birds are a paramount example. Fine control of incubation is essential and has deep ecological and evolutionary implications, notably for developing offspring but also for their parents (Conway and Martin 2000). For embryos, the thermal environment the incubating individual provides is essential for successful development. Suboptimal incubation temperatures can lead to delayed embryonic growth (Nord and Nilsson 2011), physiological changes, for example, in immunity (Ardia et al. 2010) and long-term survival consequences (Berntsen and Bech 2016). However, incubating individuals need to divide their time budget between incubation and self-maintenance (e.g. foraging) and, therefore, they allocate time to each activity according to prevalent ecological conditions (e.g. depending on ambient temperatures (Coe et al. 2015) and food availability (Londoño et al. 2008)). Despite a long standing scientific interest in incubation, we are still elucidating subtle ecological causes and consequences of this behaviour (Smith et al. 2015, Bulla et al. 2016) which may have important practical implications, for example, in the context of global climate change (Griffith et al. 2016).

The study of avian incubation is nowadays fuelled by recent technological advances (for an account of different technologies, see Smith et al. 2015). In particular, the use of iButtons® (Maxim Integrated) and probed Tinytags (Gemini Data Loggers) allows researchers to measure incubation temperature as frequently as every second for long periods of time with little logistics involved. These technologies have the potential to expand the range of species and scientific questions that researchers can address. However, the amount of data collected is usually much larger than it was traditionally (for example, via behavioural observations) and several analytical hurdles must be overcome.

Before answering questions about the biology behind an incubation pattern, the observer first needs to summarise the data, effectively reducing their dimension to a few variables that can be correlated with a set of predictors of interest. For example, number of incubation bouts and their duration are popular metrics in avian studies (Cooper and Voss 2013). The first software for the analysis of incubation temperatures was released more than 10 years ago: Rhythm (Cooper and Mills 2005). The benefits of Rhythm were instantaneous as it allowed the automated scoring of incubation (Cooper and Voss 2013, Coe et al. 2015). This software made fast and objective an otherwise time-consuming and observer-biased activity. However, in a time when incubation data collection is easier than ever before, Rhythm lacks much of the flexibility that handling big data sets require. Rhythm also has limited analytical and graphical capabilities, which are a desire when thousands of temperature records may be available. Lastly, the use of Rhythm is made difficult by its paid license under Raven. However, apart from Rhythm, no other specialised software is currently available to analyse incubation temperature data.

To overcome these difficulties, I have developed a free R package, `incR`. This package provides a suite of R functions that i) prepare and format a raw temperature time-series (via the `incRprep` and `incRenv` functions), ii) apply an automated algorithm to score incubation (`incRscan`) and iii) calculate several biologically relevant metrics of incubation (e.g. number of incubation bouts) (Figure 1). Users can apply the whole pipeline or use any of the components of `incR` separately. `incR` takes advance of the flexibility in data handling and graphical capabilities offered by R. I first explain the `incR` workflow and the automated algorithm to score incubation. Then, I use video-recordings of incubating blue (*Cyanistes caeruleus*) and great tit (*Parus major*) females along with incubation temperature data to validate the automated algorithm. I further show how `incR` can accurately calculate several metrics of incubation behaviour. Finally, I discuss the general application of this new method

and its potential limitations. A stable version of the package is available on CRAN (v 1.0.2) and a development version can be found on GitHub (v 1.0.2.9000. https://github.com/PabloCapilla/incR).

`incR` workflow

The method implemented in `incR` exploits variation in incubation and ambient temperature to calculate the presence or absence of the incubating individual in the nest. Ambient temperature data are ideally collected near the nesting site but can also be obtained from web-based sources. Code and advice to replicate the analyses presented here can be found in Appendix 1 and 2, the package documentation (https://cran.r-project.org/web/packages/incR/incR.pdf) and in the package vignette (accessible in R via: `browseVignettes("incR")`).

Data preparation: `incRprep` and `incRenv`

Several temperature files from the same nest will be typically combined by the user before the analysis starts. This combined file should consist of two columns: date-time and temperature values. The first step in the pipeline is performed by `incRprep`, which simply prepares the dataset for other pipeline components. Then, `incRenv` can be used to assign environmental temperature to every incubation temperature observation. As explained below, environmental temperatures will be used by `incRscan` to score incubation and, therefore, `incRenv` eases this intermediate step (Figure 1).

Automated incubation scoring: `incRscan`

The algorithm implemented by `incRscan` exploits the dynamics in nest temperature that arise from the behaviour of the incubating individual and the differences between nest (i.e. temperature in the nest cup, ideally obtained by placing a temperature sensor inside an egg or

in between several eggs) and environmental temperatures (see Table 1 for definitions of terms).

Four possible situations broadly exist regarding the changes in nest temperature after the incubating individual enters (on-bout) and leaves (off-bout) the nest (see Table 1 for definitions of commonly used terms throughout the paper). These four cases are classified as follows: 1) incubation off-bout when nest temperature is high (close to incubation temperature); 2) incubation on-bout when nest temperature is high (close to incubation temperature); 3) incubation off-bout when nest temperature is low (close to environmental temperature); 4) incubation on-bout when nest temperature is low (close to environmental temperature). Cases 3 and 4 are especially sensitive to the assumption that environmental temperature is lower than incubation temperature (see Results and Discussion). The change in nest temperature that is expected after an incubation on- / off-bout differs across the four scenarios.

Assuming that environmental temperatures are lower than incubation ones, in case 1 when the incubating individual leaves the nest, there exists a marked difference between nest and environmental temperatures. Thus, a sharp drop in temperature is expected to follow. At this point, any increase in nest temperature would mean that the bird has returned to the nest (on-bout, scenario 2). In the absence of the incubating individual, nest temperatures decrease until they match environmental temperatures. If an off-bout occurs when nest temperature is close to the environmental one (scenario 3, e.g. because the incubating individual has carried out a short on-bout and then leaves the nest when temperature has not markedly increased), the decrease in nest temperature after an incubation off-bout would be small. However, in this situation, any incubation on-bout would be reflected in a large increase in nest temperature (scenario 4).

These four situations represent simplified extremes in a spectrum of possible scenarios but they illustrate the general principle. To explain the analytical approach in more practical terms, I here describe the analysis of one day of incubation (day 1), using the terminology employed in the R package (Table 1).

For every time point in the incubation time series, whether scenarios 1 and 2 or 3 and 4 (see above) are applicable is determined by calculating the difference between nest and environmental temperatures. The user has some control over this decision via the `temp.diff` argument in `incRscan`, allowing for two possibilities: i) nest temperature is higher than environmental temperature by more than `temp.diff` Celsius degrees; and, ii) nest temperature is within `temp.diff` Celsius degrees of the environmental one. Therefore, the argument `temp.diff` helps determine whether scenarios 1/2 or 3/4 are applicable for a specific time point.

Comparing the change in temperature between consecutive temperature recordings against temperature thresholds, the algorithm presented here determines whether the incubating individual is in the nest or an off-bout has occurred. Rather than having a fixed threshold for the entire analysis, a flexible threshold value is applied. Furthermore, no threshold choice is required from the user but it is calculated depending on the biology of the study species for each day of analysis. The user should decide on a time window in which the incubating individual is assumed to be in the nest. This time window is controlled by the arguments `lower.time` and `upper.time`, representing the start and end of such time window (for instance, for diurnal bird species this period can be set at night, when the incubating individual rests in the nest). Within this time window, the maximum decrease in temperature between pairs of consecutive points is set as a threshold for incubation off-bouts (hereafter, `maxDrop`) for scenario 1. Assuming that nest temperatures are above environmental values, `maxDrop` is thought to effectively represent the maximum drop in temperature associated

with periods when the incubating individual is in the nest. The threshold for incubation off-bout in situation 3 must be lower than in scenario 1 (i.e. when nest temperature is close to environmental temperature); thus, the argument `sensitivity` (taking values from 0 to 1) allows for such reduction, setting the off-bout threshold in scenario 3 as `maxDrop` *x* `sensitivity`. Similarly, `maxIncrease` is defined as the maximum increase in temperature between pairs of consecutive points and is set as a threshold for incubation on-bouts in scenario 4. Any increase in nest temperature in scenario 2 would mean an incubation on-bout (see above). Note that `maxDrop` and `maxIncrease` do not need to be chosen by the user but are reported by `incR_scan` (in a returned object named `incRscan_threshold`)

Once these thresholds are set, temperature differences between successive pairs of data points throughout the day and between `upper.time` and `lower.time` are calculated. These values are sequentially compared with the value of `maxDrop` and `maxIncrease`, following a set of conditions:

For scenario 1 and 2,

$T_i - T_{i-1} <$ `maxDrop` (A); $T_i - T_{i-1} > 0$ (B).

For scenario 3 and 4,

$T_i - T_{i-1} <$ `maxDrop` x `sensitivity` (**C**); $T_i - T_{i-1} >$ `maxIncrease` (**D**).

$T_i - T_{i-1}$ being the *i*th and *i-1*th temperature recording from *i*=2 to *i*=*I* (*I* being equal to the total number of daily data points evaluated). Off-bout periods are, then, defined between T*i*s satisfying A or C and the closest subsequent situation in which T*j* , when *i*<*j*, satisfies B or D. On-bout periods start after an off-bout finishes and last until A or C is fulfilled again.

To make this calculation conservative and robust against highly variable `lower.time` - `upper.time` windows, whenever |*maxDrop*| > `maxNightVariation` (in Celsius degrees) for a particular studied day, the value of `maxDrop` and `maxIncrease` of the

previous day of incubation is instead used. The result of this algorithm is a temporal sequence of 0's and 1's representing on-bouts (1's) and off-bouts (0's). Based on this sequence and using other functions within the `incR` package, biological information on inferred incubation behaviour can be calculated.

Additional functions to extract biological metrics of incubation

Regardless of whether or not `incRscan` has been used to score incubation, the `incR` package offers a suite of functions that can be applied to any binary time-series representing incubation. The current stable version (1.0.2) allows the user to calculate onset and offset of daily activity (`incRact`), percentage of daily time spent in the nest (`incRatt`), number and average duration of on/off-bouts per day as well as individual off-bout duration and timing (`incRbouts`) and nest temperature mean and variance for a customised time window (`incRt`). The implementation of these functions is straightforward as they only require a variable with binary data for on and off-bouts. These data are provided by `incRscan` under the column name `incR_score`.

Validation of `incR` using temperature and video-recording data

To show the potential of this approach to yield meaningful metrics of incubation, I assessed the performance of `incR`'s core functions. First, I carried out a sensitivity analysis in which I evaluated the accuracy of `incRscan` over simulated values for its main arguments. I then chose the optimal values for these arguments and showed that the combination of `incRscan` and other `incR` functions can yield accurate measures of incubation behaviour. I applied the whole pipeline to incubation temperatures collected using iButton® devices. For the same incubation events, I used video footage of these nests to visually score incubation and then compared these results to the automatic algorithm implemented in `incRscan`.

Field protocol and incubation data collection

Incubation temperatures were recorded during 2015 and 2016 using iButton® devices in two blue tit and six great tit clutches respectively. Blue tit data came from an urban and suburban population in Glasgow city (n = 2 clutches; 55° 52.18'N 4° 17.22'W and 55º 9'N 4º 31'W) (Pollock et al. 2017), whereas great tit incubation data were recorded in an oak forest at the Scottish Centre for Ecology and the Natural Environment (n = 2 clutches; SCENE, 56° 7.73'N 4° 36.79'W) (Pollock et al. 2017) and in a mixed forest (dominated by oak, birch and pine trees) near the Netherlands Institute for Ecology (NIOO) (n = 4; ~52º 7' N 6º 59' E) (Spoelstra et al. 2015). iButtons® were wrapped in a piece of black cloth and placed in the nest cup, above the lining materials and among the eggs. Nest temperatures were recorded by iButtons® every 2 or 3 minutes. Video cameras inside the nest-boxes were used to monitor individual females and visually score incubation (see Pollock et al. 2017 for a general explanation about video camera deployment). In total, 14 days of incubation were completely or partially monitored using both iButtons® and recording cameras. Environmental temperatures for the same period in Scotland were recorded either using iButtons® placed outside nest-boxes. For the Dutch clutches, environmental temperatures from a weather station approximately 18 Km away from the nest-box population were used. iButtons® were downloaded in the field using portable devices and a single file per nest was compiled before further analysis.

Data analysis

Using video footage, I determined whether or not the incubating female had been present in the nest at every iButton® temperature time point. After preparing incubation temperature data using `incRprep` and `incRenv`, I applied `incRscan` to score incubation and compared its results to the footage-based scoring. I tested the performance of `incRscan` to changing values of its three key arguments, i) `maxNightVariation` (testing values from 0.5 to 10 every 0.5), ii) `sensitivity` (from 0 to 1 every 0.1) and iii) `temp.diff` (from

0.5 to 10 every 0.5) (see Table 1 for definitions). When testing one argument, the others were kept to default values of 1.5, 0.15 and 3 for `maxNightVariation`, `sensitivity` and `temp.diff` respectively. `lower.time` and `upper.time` were always fixed to 10 p.m. and 3 a.m (night time). For every test, I calculated the percentage of correctly scored incubation time points. After selecting the best-performing combination of argument values (i.e. highest percentage of agreement between `incRscan` and video footage), I compared daily incubation attendance (i.e. percentage of time spent in the nest), number of daily off-bouts and mean daily off-bout duration between `incRscan`-based and video footage-based incubation scores. I present Pearson's correlations coefficients between the two metrics. `incR` functions, statistical tests and graphical illustrations (apart from the left-hand side of Figure 1) were produced in R version 3.4.1. Detailed technical guidelines to apply the `incR` workflow and the validation shown in this manuscript can be found in Appendix 1 and 2 as well as in the package´s vignette (accessible in R via: `browseVignettes("incR")`).

Results and Discussion

Within nest-boxes, changing values of `maxNightVariation` did not affect the performance of `incRscan`. Similar results were found for `sensitivity` and `temp.diff`, with only one nest-box being markedly affected by changes in these arguments (Figure S1A-C). It is important to note that when `maxNightVariation` is set to a very low value (effectively not allowing for much temperature variation in the `lower.time`-`upper.time` time window) `incRscan` fails to yield any result as no temperature threshold would be available. This result can be seen in Figure S1A: when evaluating `maxNightVariation` equal to 0.5°C, data from only two out of eight nest-boxes were extracted by `incRscan`.

Consistent variation in `incRscan` best-performing argument values was found among nest-boxes (Figure 2), suggesting that differences in, for example, iButtons® deployment may be affecting the accuracy of the `incRscan` algorithm. This potential effect has been qualitatively suggested before (Smith et al. 2015) and highlights the importance of collecting high quality data in the field. However, the percentage of agreement was always high (> 80%, Figure 2). Highly consistent results were found within nest-boxes with marked among-box variation with only one exception (nest-box G178_GT, Figure 2) in which setting `maxNightVariation` to 4°C improved the percentage of agreement compared to that of the default value (3 °C). The general pattern that arises across the eight nest-boxes is that values above 1.5°C for `maxNightVariation` give the highest accuracy (90.27%. Figure S1D). Similarly, values below 0.3 for `sensitivity` (90.27%) and a `temp.diff` value of 4°C (91.16%) were found to be the most accurate argument choices (Figure S1E-F).

Given these results, I chose optimal values of 1.5°C, 0.25 and 4°C for `maxNightVariation`, `sensitivity` and `temp.diff` respectively, yielding a percentage of agreement across nest-boxes of 91.16% (maximum = 98.56%; minimum = 80.42). With these argument values, attendance calculated based on video footage and inferred by `incRscan` showed a Pearson's correlation coefficient of 0.992 ($t = 24.81$, $p < 0.0001$, 95% confidence interval = 0.971-0.998. Figure 3A). Likewise, the algorithm in `incRscan` was able to provide accurate off-bout information (Figure 3B & 3C). `incR`-estimated off-bout number and mean daily off-bout duration were highly correlated with real off-bout number and duration as extracted from video footage (for off-bout number: $r = 0.972$, $t_{10} = 13.04$, $p < 0.0001$, 95% confidence interval = 0.900-0.992. For daily mean off-bout duration: $r = 0.996$, $t_{10} = 34.69$, $p < 0.0001$, 95% confidence interval = 0.985-0.999).

These results show that the method presented here can yield accurate metrics of incubation behaviour. Based on the validation of Rhythm presented in Bueno-Enciso et al. (2017), `incR` performs better than that software and yields higher correlations between video and iButton® data (Bueno-Enciso et al. 2017) – but note the possible influence of different environmental temperatures across studies. Implemented in a much more flexible workspace, data validation and visualisation of incubation temperature data using `incR` is straightforward. For number of off-bouts, the discrepancies between `incR` and video footage seem to arise from `incR` slightly over-estimating the number of off-bouts (Figure 3B). This effect was mainly caused by data from two nest-boxes (G178_GT and GT173_GT) which were collected in the same year and location. The extent to which this result represents a consistent bias of the method or an artefact of data collection (for instance, the iButton® being placed in a suboptimal position) is unknown. However, the magnitude of this discrepancy was small (six off-bouts of maximum differences between estimates for whole days; estimated regression slope ± SE = 0.926 ± 0.071) and the error is unlikely to be differentially affected across groups of nests (e.g. experimental *versus* control in an experimental setup). Apart from the ones presented here, other metrics of incubations can be calculated using `incR` (Figure 1 and see package documentation), for which high reliability is expected given the results of this validation.

Benefits of `incR`

The benefits of `incR` are two-fold. Firstly, it represents a quantitative improvement over other methods. The results of the validation suggest that `incR` may perform better than other approaches (see validation of Rhythm in Bueno-Enciso et al. 2017). No assumptions about minimum off-bout time or off-bout temperature reductions are needed. `incRscan` uses changes between consecutive temperature points, making the detection of short off-bouts possible. The inclusion of environmental temperatures informs the analysis, allowing for off-bout detection when nest and environmental temperatures are similar. In Appendix 1 and 2, I

offer detailed instructions to reproduce the analysis presented here. More generally, using a script-based approach will improve repeatability and will ease collaboration.

Secondly, `incR` embraces the philosophy of the R project: it is completely free and is in constant improvement. Further developments in the method to score incubation could be embedded in or used jointly with `incR` to extract metrics of incubation.

Limitations

The capability of `incR`, or very likely of any other analytical tool to study incubation temperatures, to yield accurate results will certainly correlate with data quality. Correct logger deployment and data validation are, therefore, crucial. Two key assumptions underlie the use of the algorithm implemented by `incRscan`. First, the incubating individual is assumed to rest in the nest in the `lower.time - upper.time` time window. This assumption holds for most species in temperate and tropical zones, for which activity outside the nest is paused during night time (a reversed pattern is expected in nocturnal species). However, careful consideration of this assumption will be needed when the species of interest do not have a rhythmic incubation pattern or rhythms differ from 24 h (Bulla et al. 2016). Secondly, the accuracy of `incRscan` will also depend on the difference between incubation and environmental temperatures. Small differences between these temperatures will lead to subtle changes in temperature after the incubating individual enters and leaves the nest, affecting the detectability of such events. Further tests need to be carried out to evaluate the accuracy of `incR` in these situations. For instance, comparing the performance of `incRscan` for data collected on the same species at different latitudes (and thus with likely large or small differences between environmental and nest temperatures) might provide valuable information on the general applicability of this methodology.

Conclusions

We have developed a method that accurately extracts behavioural and temperature information from series of incubation temperature recordings. This method can potentially be used to study incubation in a broad range of species and ecological contexts and, therefore, assist the wide community of researchers studying incubation both in the wild and in the laboratory. For different species and environments, validation will be needed but we also provide detailed practical advice to carry out such validation. In order to ease its application, two appendices show in detail how researchers can easily adapt and calibrate this method to their study species.

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Conflicts of interest

PC-L declares no conflict of interest.

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Figure Captions

Figure 1. `incR` workflow and visualisation of corresponding analysis of nest temperature data at each step of the workflow. `incRprep` prepares raw data time series (1) for the pipeline and `incRenv` adds environmental temperatures (2). `incRscan` classifies data points in absence or presence of the incubating individual in the nest (3). From a sequence of 0´s and 1´s calculated by `incRscan`, `incRbouts`, `incRatt`, `incRact` and `incRt` extract information about on/off-bouts, nest attendance, start and end of activity and averaged nest temperatures for customised time windows. The green line in 2 and 3 represents environmental temperature, whereas the colour of the dots in 3 illustrates on- (light red) and off-bouts (purple).

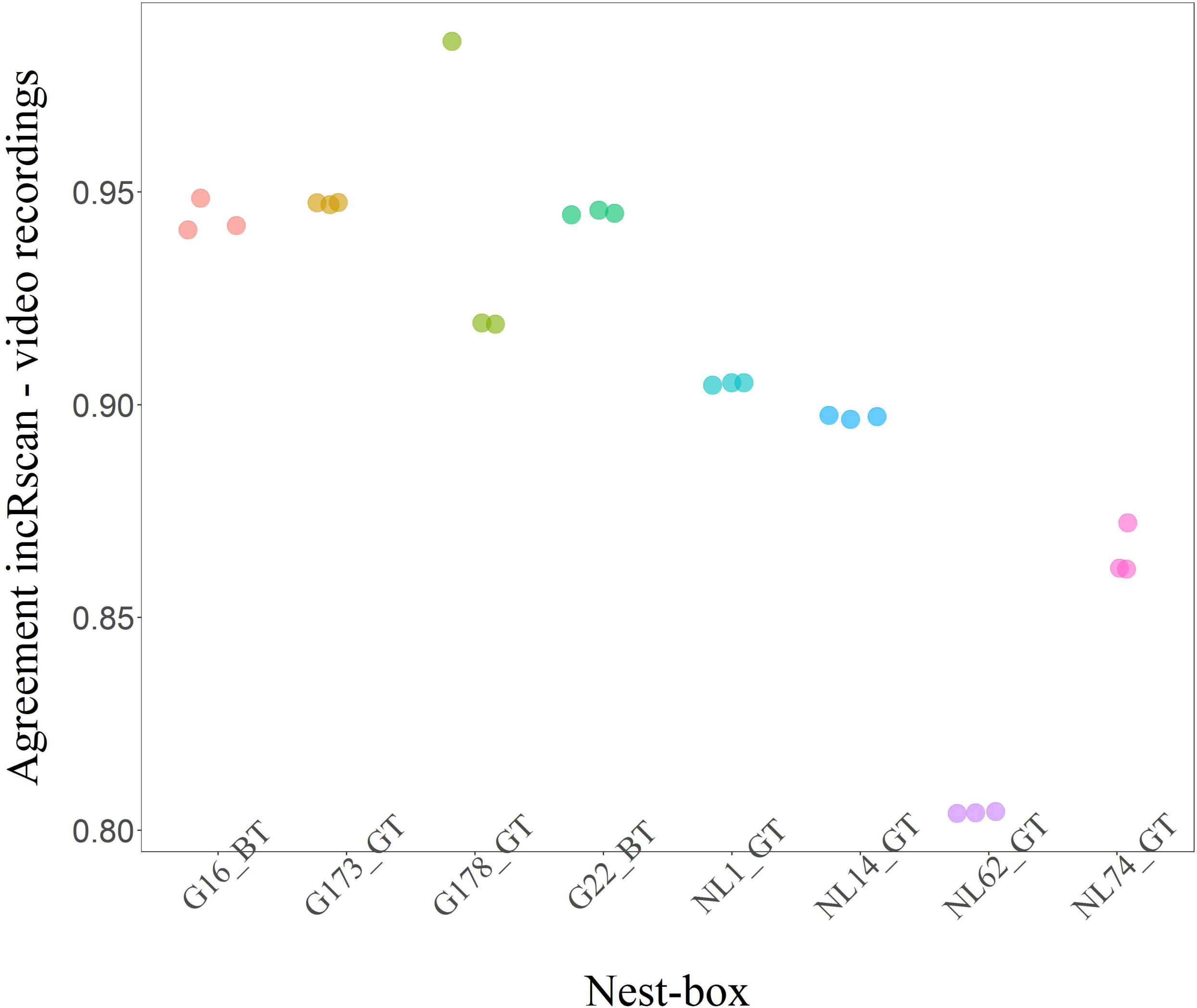
Figure 2. Percentage of agreement between `incRscan` and video-footage across eight different nest-boxes. Colour codes represent individual nest-boxes and each point within nest-box illustrates the highest percentage of agreement for each of the three simulations, testing `maxNightVariation`, `sensitivity` and `temp.diff`. Highly consistent results are found within nest-boxes with marked among-box variation – with one exception (G178_GT) in which setting `maxNightVariation` to 4°C improved the percentage of agreement compared to that of the default value (3 °C). Points are slightly offset in the x axis to aid visualisation of overlaying points.

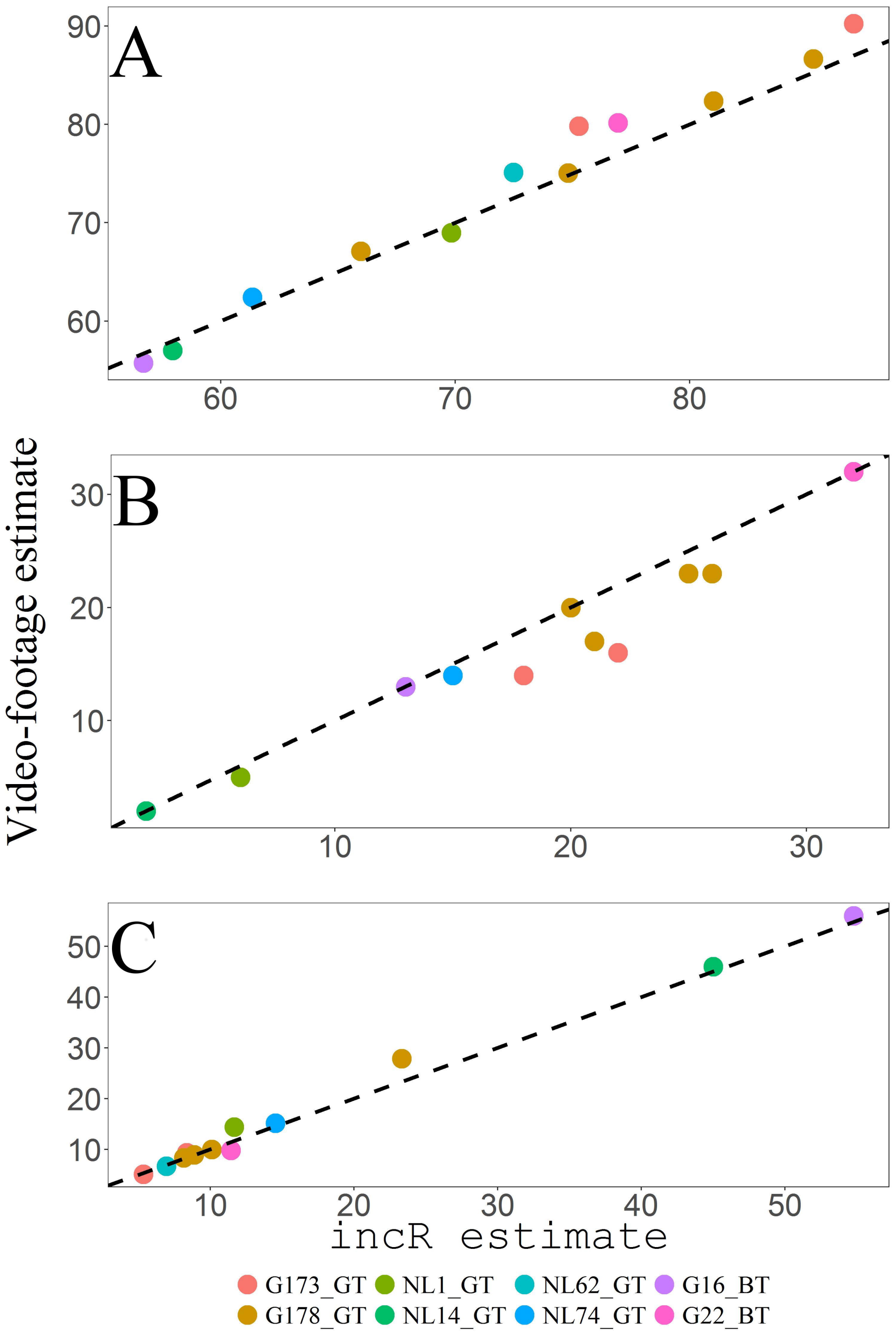
Figure 3. Correlations between video-footage and `incR` estimates of incubation attendance (percentage of daily time spent in the nest (A), number of daily off-bouts (B) and daily mean off-bout duration in minutes (C). Colour codes represent individual nest-boxes and each point illustrates one day of incubation. Dashed black line was drawn following an intercept of 0 and a slope of 1.

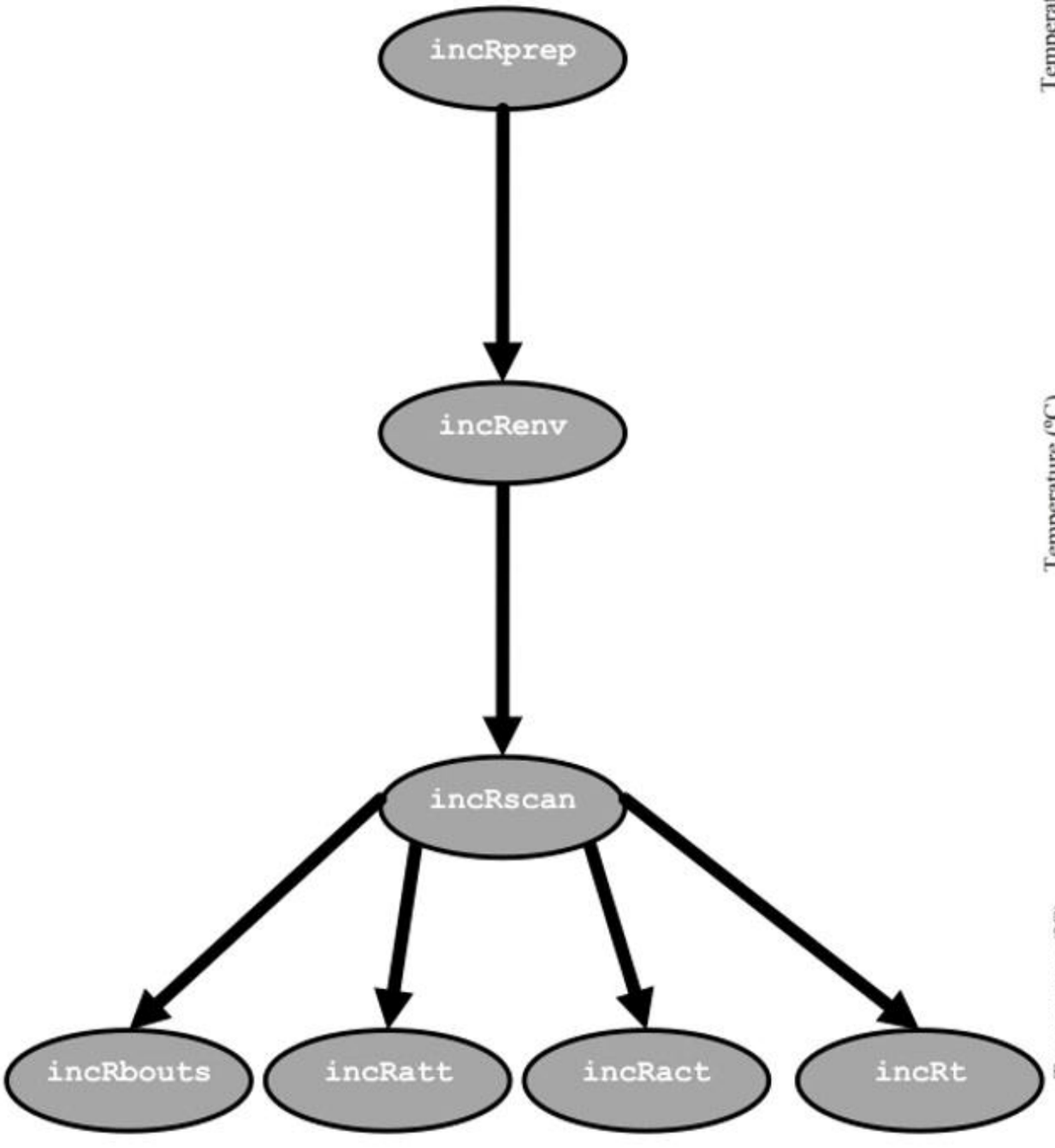
Tables

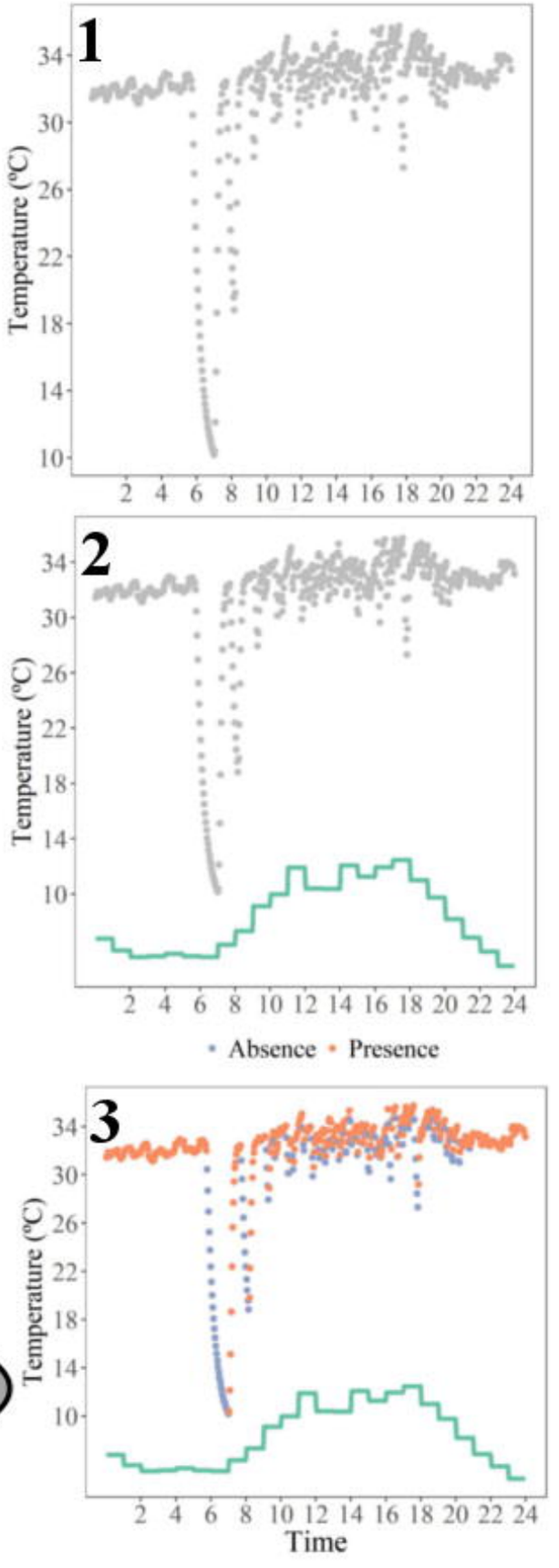
Table 1. Glossary of terms used in this manuscript.

Term	Type	Description
Incubation temperature		Maximum and stable temperature reached in the nest. Normally obtainable after a long presence of the incubating individual in the nest.
Nest temperature		Temperature inside the nest cup at any given time point. By this term I refer to a variable value that depends on whether, and for how long, the incubating individual is in or out the nest.
Environmental temperature		Air temperature outside nest.
`incR_scan`	R function	Calculates presence or absence of the incubating individual in the nest based on nest and ambient temperature variation
`temp.diff`	`incR_scan` argument	Difference allowed between nest and environmental temperatures
`lower.time`	`incR_scan` argument	Start time of a time window when the incubating individual is assumed to be in the nest
`upper.time`	`incR_scan` argument	End time of a time window when the incubating individual is assumed to be in the nest
`sensitivity`	`incR_scan` argument	Reduction in off-bout threshold when nest temperatures are close to environmental ones.
`maxNightVariation`	`incR_scan` argument	Maximum variation allowed in the `lower.time - upper.time` window. It controls for big drops in temperature within this temporal window (i.e. night-time incubation off-bouts).
`maxDrop`	Internal calculation in `incR_scan`	Maximum drop in temperature between two consecutive time points within the `lower.time – upper.time` window.
`maxIncrease`	Internal calculation in `incR_scan`	Maximum increase in temperature between two consecutive time points within the `lower.time – upper.time` window.


Agreement incRscan - video recordings
0.95
0.90
0.85
0.80
G16_BT
G173_GT
G178_GT
G22_BT
NL1_GT
NL14_GT
NL62_GT
NL74_GT
Nest-box


A
90
80
70
60
60
70
80
B
30
20
10
10
20
30
C
50
40
30
20
10
10
20
30
40
50
Video-footage estimate
incR estimate
G173_GT
NL1_GT
NL62_GT
G16_BT
G178_GT
NL14_GT
NL74_GT
G22_BT


incRprep
incRenv
incRscan
incRbouts
incRatt
incRact
incRt


1
2
3
Temperature (ºC)
Time
Absence
Presence